



Genetic control oContrôle génétique de la sensibilité au virus Zika chez la souris à l'aide de lignées du Collaborative Cross

Caroline Manet

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par

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Genetic control of susceptibility to Zika virus in the mouse using strains from the Collaborative Cross

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Abstract

Zika virus (ZIKV) is a mosquito-transmitted flavivirus responsible for worldwide epidemics and constitutes a major public health threat. The majority of ZIKV infections in humans are either asymptomatic or result in a mild febrile illness. However, some patients develop a more severe, sometimes life-threatening, form of the disease. Recent evidence showed that ZIKV infection can trigger Guillain-Barré syndrome and encephalitis in adults, as well as congenital malformations such as microcephaly. The severity of ZIKV disease in humans depends on many factors, likely including host genetic determinants.

We investigated how genome-wide variants could impact the susceptibility to ZIKV infection in mice. To this end, we used mouse strains of the Collaborative Cross (CC), a new genetic reference population encompassing a genetic diversity as broad as that of human populations.

First, we described that the susceptibility of *Ifnar1* (receptor to type I interferon) knockout mice is largely influenced by their genetic background. We then showed that the genetic diversity of CC mice, which IFNAR was blocked by anti-IFNAR antibody, expressed phenotypes ranging from complete resistance to severe symptoms and death with large variations in the peak and rate of decrease of plasma viral load, in brain viral load, in brain histopathology and in viral replication rate in infected cells. Differences of susceptibility between CC strains were correlated between Zika, Dengue and West Nile viruses. We identified highly susceptible and resistant CC strains as new models to investigate the mechanisms of human ZIKV disease and other flavivirus infections. Genetic analyses revealed that phenotypic variations were driven by multiple genes with small effects, reflecting the complexity of ZIKV disease susceptibility in human population. Notably, our results also ruled out a role of the *Oas1b* gene in the susceptibility to ZIKV.

In a second part, we searched for genes which modify the susceptibility of *Ifnar1* knockout mice in an F2 cross between C57BL/6J and 129S2/SvPas mice harboring the mutation. Genetic analysis revealed two Quantitative Trait Locus (QTL) controlling either the peak viremia or the mouse survival. Although these QTLs critical intervals contained hundreds of genes, data mining led us to identify a few candidate causal genes.

Then, we investigated how host genetic factors influence viral replication in infected cells using Mouse Embryonic Fibroblasts (MEFs) derived from a series of CC strains with contrasted phenotypes observed in response to ZIKV infection *in vivo*. MEFs from CC071 strain displayed unique features of increased viral replication rate in late infection. Using transcriptomic analysis, we demonstrated that the phenotype of CC071 infected MEFs resulted from a delayed induction of the type I interferon (IFN) response. Genetic analyses ruled out single gene deficiencies but rather suggested combined effects of multiple factors in the type I IFN induction signaling pathway.

Finally, we characterized the ZIKV-induced type I IFN response in MEFs and primary neurons derived from C57BL/6J mouse strain. Primary neurons were less capable than MEFs to control the viral replication due to a delayed IFN response. We later showed that host genetic factors also play a critical role in this context as ZIKV-infected CC071 primary neurons displayed an extreme phenotype compared to neurons from strains that are more resistant.

Altogether, our work has unraveled the role of host genes in the pathogeny of ZIKV infection and illustrates the potential of CC mouse strains for genetic studies and as new models of infectious diseases. Extensive analysis of CC strains with extreme phenotypes help us elucidate how genetic variants affect susceptibility as well as immune responses to flaviviral infection and will provide deeper understanding of the pathophysiology of human ZIKV disease.

Keywords: Zika virus, Mouse, Susceptibility to infectious diseases, Genetics of complex traits, Collaborative Cross, Innate immunity

Résumé

Zika est un Flavivirus émergent transmis à l'Homme par piqûre de moustique. Il a récemment été à l'origine de plusieurs épidémies d'envergure mondiale et représente une menace pour la santé publique. L'infection Zika est souvent asymptomatique ou engendre un syndrome grippal bénin. Cependant, des complications sévères ont été associées au virus Zika, telles qu'un syndrome de Guillain-Barré ou des encéphalites chez l'adulte, ainsi que des malformations congénitales comme la microcéphalie. De nombreux facteurs sont susceptibles d'influencer la sensibilité d'un individu au virus Zika, y compris les variants génétiques de l'hôte.

Nous avons étudié le rôle des facteurs génétiques de l'hôte dans sa sensibilité à l'infection par le virus Zika. Pour cela, nous avons utilisé des lignées de souris du Collaborative Cross (CC), une population génétique de référence caractérisée par une diversité génétique aussi vaste que celle des populations humaines.

Nous avons d'abord montré que le fond génétique de souris déficientes pour le gène du récepteur à l'interféron de type I (*Ifnar1*) joue un rôle drastique dans leur sensibilité au virus Zika. La diversité génétique des souris CC, préalablement traitées par un anticorps bloquant le récepteur IFNAR, s'exprime par des phénotypes allant d'une résistance complète jusqu'à des formes sévères de la maladie. L'influence des facteurs génétiques de l'hôte s'exerce sur de nombreux paramètres tels que la virémie, la charge virale et les lésions pathologiques dans le cerveau, et enfin le taux de réplication dans les cellules infectées. Les différences de sensibilité entre lignées CC s'avèrent corrélées entre les Flavivirus Zika, Dengue et West-Nile. Nos analyses génétiques ont montré que de multiples gènes à effets faibles sous-tendent ces variations phénotypiques, reflétant la complexité de la sensibilité au virus Zika dans les populations humaines, et permettent d'exclure un rôle majeur du facteur de résistance *Oas1b*.

Nous avons ensuite cherché des gènes agissant comme modificateurs de la sensibilité chez des souris déficientes pour le gène *Ifnar1* dans un croisement F2 entre des souris C57BL/6J et 129S2/SvPas portant la mutation. L'analyse génétique a permis l'identification de deux QTLs (Quantitative Trait Locus), l'un contrôlant le pic de virémie et l'autre la survie. Une étude bio-informatique nous a permis d'identifier quelques gènes candidats.

Nous avons également étudié comment les facteurs génétiques de l'hôte impactent la réplication virale dans des fibroblastes embryonnaires murins (MEFs) dérivés d'une série de lignées de souris présentant des phénotypes contrastés en réponse à l'infection Zika. Nous avons identifié une augmentation de la réplication virale tardive dans les MEFs de la lignée CC071, résultant d'un retard à l'activation de la réponse interféron (IFN). Des analyses génétique et transcriptomique ont exclus des déficiences causées par des gènes uniques et ont favorisé l'hypothèse d'une combinatoire de gènes exerçant des effets faibles dans la voie d'induction de la réponse IFN.

Pour finir, nous avons caractérisé la réponse IFN induite par le virus Zika dans des neurones primaires murins. Cette étude a montré que la capacité des neurones primaires à limiter la réplication virale est moindre que celle des MEFs en raison d'un retard à l'induction de la réponse IFN. Enfin, les facteurs génétiques de l'hôte exercent un rôle critique dans ce contexte puisque les neurones primaires de CC071 présentent un phénotype extrême par comparaison avec des lignées plus résistantes.

Notre travail a mis en évidence le rôle des facteurs génétiques de l'hôte dans la pathogénie de l'infection Zika et illustre le potentiel des souris CC dans des études génétiques aussi bien qu'en tant que nouveaux modèles d'infection. Une analyse poussée des lignées aux phénotypes extrêmes permettra d'élucider les mécanismes génétiques de la sensibilité au virus Zika et améliorera notre compréhension de la maladie chez l'Homme.

Mots-clés: Virus Zika, Souris, Sensibilité aux maladies infectieuses, Génétique des caractères complexes, Collaborative Cross, Immunité innée

Résumé substantiel

Zika est un virus émergent désormais bien connu. Après avoir circulé de façon inaperçue pendant 70 ans, il s'est très rapidement propagé dans le monde au cours des dix dernières années, affectant des millions de personnes.

Contexte scientifique et problématique

Le virus Zika appartient à la famille des Flaviviridae, genre Flavivirus, et est étroitement apparenté à d'autres Flavivirus pathogènes pour l'Homme, tels que les virus de la Fièvre Jaune, du West-Nile, de l'encéphalite Japonaise, ou encore de la Dengue. Tous ces virus ont en commun d'être transmis à l'Homme par piqûre de moustiques et appartiennent à ce que l'on appelle les arbovirus, c'est-à-dire les virus transmis par des insectes vecteurs. Le génome des Flavivirus est constitué d'un ARN positif simple brin qui code pour une unique polyprotéine, qui est ensuite clivée en trois protéines structurales : capsid, membrane et enveloppe, qui constituent le squelette du virus ; et en sept protéines non-structurales qui assurent la réplication du génome et l'assemblage des virions. Les particules des Flavivirus sont de petite taille et enveloppées d'une membrane lipidique, elles peuvent exister sous plusieurs états différents : particules immatures ou particules matures qui sont infectieuses. Le cycle infectieux des Flavivirus commence par une phase d'attachement à la cellule pendant laquelle le virus peut se lier à une variété de récepteurs cellulaires. En effet, il n'existe pas un unique récepteur spécifique aux Flavivirus et plusieurs familles ont été impliquées telles que les récepteurs TAM (TYRO, AXL, MER) ou TIM, les récepteurs au Mannose ou encore des lectines de type C. L'entrée du virus dans la cellule se fait par endocytose et l'ARN viral est ensuite libéré dans le cytoplasme. Suivent ensuite les étapes de réplication du génome et de traduction des protéines virales au niveau du réticulum endoplasmique, puis de nouvelles particules sont assemblées, et subissent des étapes de maturation avant d'être sécrétées hors de la cellule par exocytose.

Chez l'Homme, de nombreuses infections par des Flavivirus sont considérées comme des pathologies émergentes ou ré-émergentes, qui peuvent être définies comme des maladies dont l'incidence a beaucoup augmenté au cours des deux dernières décennies. C'est le cas de la Fièvre Jaune qui était historiquement endémique sur le continent Africain mais dont l'incidence a fortement diminué après le développement du vaccin atténué 17D dans les années 1930. Cependant, on assiste régulièrement à des récurrences de la maladie, en Afrique, mais aussi au Brésil depuis 2016. Un autre exemple bien connu est celui de la Dengue qui est actuellement endémique dans toutes les régions tropicales et subtropicales et dont l'incidence a augmenté de manière exponentielle ces dernières décennies et atteint aujourd'hui plus de 200 millions de personnes chaque année. Les virus de la Fièvre Jaune et de la Dengue circulent sous forme de cycle enzootique sylvestre entre des moustiques du genre *Aedes* et des primates. Les moustiques *Aedes aegypti* et *albopictus* en sont les principaux vecteurs, ce sont des moustiques anthropophiles qui peuvent donc transmettre ces virus aux

populations humaines. De plus, les moustiques *Aedes albopictus*, ou moustiques tigre, peuvent coloniser les environnements urbains et entretenir la circulation de ces virus au sein de la population humaine. Le virus Zika est également transmis à l'Homme par des moustiques *Aedes* et suit un cycle de transmission similaire à celui de la Dengue et de la Fièvre Jaune.

Le virus Zika a été identifié pour la première fois en 1947 en Ouganda. Pendant plusieurs dizaines d'années, seuls quelques cas sporadiques de maladie ont été rapportés en Afrique, puis en Asie. Ce n'est qu'en 2007 qu'a été décrite la première épidémie importante dans les îles de Yap, en Micronésie dans le Pacifique. D'autres épidémies ont suivi, en 2013 et 2014 en Polynésie Française, puis le dernier épisode majeur qui s'est déroulé entre 2015 et 2017 en Amérique du Sud et en Amérique Centrale. Des études phylogénétiques des souches virales ont permis d'établir l'existence de deux lignages distincts : le lignage Africain et le lignage Asiatique, auquel appartiennent les souches épidémiques. L'augmentation de l'incidence des cas d'infection par le virus Zika a permis de mettre en évidence de nouveaux syndromes cliniques. En Polynésie Française, une augmentation du nombre de cas de syndrome de Guillain-Barré, une paralysie flasque ascendante, a été associée avec les cas d'infection par Zika. Pendant l'épidémie Brésilienne, plusieurs dizaines de milliers de cas de microcéphalie ont été décrits chez des nouveau-nés infectés par Zika *in utero*. Les études épidémiologiques ont également décrit de nouveaux modes de transmission du virus : par voie sexuelle, materno-fœtale ou encore par contact avec les fluides corporels. Chez l'adulte, l'infection est asymptomatique dans 80% des cas et responsable, dans le cas contraire, d'un épisode fébrile de type syndrome grippal. Cependant, dans une plus faible proportion des cas, l'infection par le virus Zika peut engendrer des complications neurologiques sévères comme un syndrome de Guillain-Barré mais aussi des encéphalites ou bien des paralysies des nerfs crâniens. De plus, le virus Zika peut être transmis de la mère au fœtus pendant la grossesse, avec un taux de transmission verticale estimé entre 20 et 30%. L'infection peut engendrer une mortalité embryonnaire ou bien un syndrome congénital de sévérité variable associant des défauts de développement du cerveau, du tube neural, des structures oculaires etc. Cependant, la majorité des nouveau-nés infectés *in utero* sont asymptomatiques à la naissance, mais des conséquences à long terme commencent à être décrites telles que des troubles de l'apprentissage, du sommeil ou de l'alimentation.

Plusieurs facteurs peuvent expliquer la variabilité des présentations cliniques, tels que la souche et la dose virale, la voie de transmission, le stade de la grossesse au moment de l'infection ou encore les facteurs génétiques de l'hôte. En effet, de nombreuses études ont pu montrer que les facteurs génétiques de l'hôte ont une influence forte sur la sensibilité d'un individu à une maladie infectieuse. Ces variants génétiques peuvent notamment moduler la morbidité et la sévérité clinique d'une infection ou encore la réponse à un traitement. Nous avons récemment publié une revue dans le journal *Mammalian Genome* intitulée « Host genetic control of mosquito-borne Flavivirus infections », qui dresse un bilan des études menées chez la souris et chez l'Homme sur le contrôle génétique des infections par les Flavivirus. Parmi les exemples bien connus, on peut citer celui du gène *Oas1b*, qui a été identifié par des études génétiques chez la souris comme un gène de sensibilité à l'infection par le virus du West-Nile. Un variant du gène homologue humain a, par la suite, été associé à la forme sévère de la Dengue.

Chez l'Homme, plusieurs stratégies permettent d'identifier des variants génétiques qui contrôlent la sensibilité à une maladie infectieuse telles que des études d'association à l'échelle du génome entier, de type GWAS (Genome-wide association study), ou bien des études à l'échelle d'un gène donné, de type étude de gène candidat. Les études GWAS recherchent, sans hypothèse préalable, une association statistique entre l'ensemble des variants du génome et le phénotype de milliers d'individus, cas et contrôles. Malgré certains succès, les études génétiques humaines sont compliquées par de nombreux facteurs. Elles nécessitent souvent un très grand nombre d'individus, et la définition précise des cas et des contrôles peut être difficile, notamment si le spectre clinique de la maladie est très hétérogène. De plus, de nombreux paramètres ne peuvent pas être contrôlés, tels que la souche virale ou la dose infectieuse.

Les études génétiques humaines peuvent donc être complétées par des études génétiques chez la souris qui présentent de nombreux avantages : elles disposent d'un environnement bien contrôlé ainsi que de conditions expérimentales standardisées. Elles permettent de mesurer une grande variété de phénotypes et d'aller jusqu'à la validation fonctionnelle du variant génétique. Comme chez l'Homme, les études génétiques chez la souris peuvent se faire à l'échelle d'un gène donné ou à l'échelle du génome entier. Pour les études de génétique inverse, la souris est un très bon outil puisqu'on dispose de très nombreuses lignées portant des mutations, spontanées ou induites, dans des gènes spécifiques, et qui permettent d'étudier les fonctions de ces gènes. Les lignées consanguines, nombreuses et diverses, permettent également de réaliser des études de génétique directe, de type GWAS ou analyse QTL (Quantitative trait locus). L'approche classique pour les analyses QTL chez la souris est basée sur l'utilisation de croisements entre deux lignées consanguines qui diffèrent pour le phénotype d'intérêt. Les individus de génération F1 sont ensuite croisés entre eux pour aboutir à une génération F2 d'individus recombinants. La ségrégation génétique au sein de cette population peut ensuite être analysée par une étude d'association statistique entre phénotype et génotype et aboutit à la cartographie génétique des QTLs. Une approche alternative consiste à utiliser des lignées recombinantes consanguines. Ces lignées sont obtenues par un croisement F2 puis par des croisements frère-sœur sur plusieurs générations jusqu'à la mise à l'état homozygote à tous les locus du génome. L'utilisation de lignées recombinantes consanguines permet d'atteindre une résolution de cartographie génétique plus fine en raison du plus grand nombre d'évènements de recombinaison. En revanche, ces deux approches peuvent souffrir d'un manque de diversité génétique. En effet, si les lignées parentales partagent certaines régions de leur génome, celles-ci seront exclues de l'analyse en raison de l'absence de polymorphisme génétique. De nouvelles populations de souris ont donc été créées afin d'augmenter cette diversité génétique, notamment le Collaborative Cross (CC).

Les lignées de souris du CC constituent une population génétique de référence caractérisée par une diversité génétique aussi vaste que celle des populations humaines. Ces lignées ont été obtenues à partir d'un croisement multi-parental entre cinq lignées de laboratoire et trois lignées de souris sauvages, permettant de mimer la diversité génétique de la population humaine. Le CC combine donc les avantages des lignées recombinantes consanguines avec une extrême diversité génétique. Les souris CC représentent donc un outil pertinent pour étudier l'influence des facteurs génétiques de l'hôte sur la sensibilité au virus Zika.

Cependant, les premières études expérimentales sur le virus Zika ont montré que les souris de laboratoire sont naturellement résistantes à l'infection. La réponse interféron (IFN) de type 1 joue un rôle crucial dans la réponse immunitaire précoce et constitue la première ligne de défense de l'hôte contre les virus. Des études récentes ont montré que chez l'Homme, la protéine NS5 du virus Zika est capable de se lier à STAT2, un effecteur essentiel de la voie IFN, et de déclencher sa dégradation. La réponse IFN est ainsi inhibée par le virus, qui peut alors se répliquer dans les cellules. En revanche, chez la souris, STAT2 n'est pas dégradé par le virus, la réponse IFN reste fonctionnelle et empêche la réplication virale. C'est pourquoi, les études sur le virus Zika ont utilisé des souris qui présentent une déficience de cette voie IFN, qu'elle soit constitutive chez les souris *Ifnar1^{-/-}*, qui portent une délétion du gène du récepteur à l'IFN de type 1, ou bien inductible et transitoire chez des souris traitées à l'aide d'un anticorps bloquant ce récepteur.

Dans ce travail de thèse, nous avons décidé de combiner ces modèles avec les souris du CC pour répondre à la problématique de notre étude. Plus spécifiquement, les objectifs de mon projet étaient donc de caractériser l'influence des facteurs génétiques de l'hôte sur la sensibilité au virus Zika à l'aide de souris génétiquement variées, et d'identifier des gènes ou mécanismes qui contrôlent ces différences. Mon projet s'est articulé en quatre grandes parties : (1) la première a porté sur la caractérisation du contrôle génétique de la sensibilité au virus Zika chez des souris du CC et a fait l'objet d'un article publié dans le *Journal of Virology* ; (2) dans la deuxième partie, nous avons réalisé une étude génétique de la sensibilité au virus Zika chez des souris *Ifnar1^{-/-}* ; (3) la troisième partie a porté sur l'identification des mécanismes moléculaires contrôlant la réplication du virus dans des fibroblastes embryonnaires et fera l'objet d'une seconde publication dans les prochains mois ; (4) et enfin dans la quatrième partie, nous avons caractérisé la réponse IFN induite par le virus Zika dans des neurones primaires murins.

(1) Caractérisation du contrôle génétique de la sensibilité au virus Zika chez des souris CC

Les souris *Ifnar1^{-/-}* font partie des modèles très utilisés pour étudier Zika, nous nous sommes donc d'abord intéressés à l'influence du fond génétique de ces souris sur leur sensibilité à l'infection. Les souris *Ifnar1^{-/-}* existent sous deux fonds génétiques différents, soit C57BL/6J soit 129S2/SvPas, nommées ci-après *Ifnar-B6* et *Ifnar-129*. Après infection avec le virus Zika, les souris *Ifnar-B6* développent des symptômes de plus en plus sévères jusqu'à atteindre 100% de mortalité 7 jours après infection alors que plus de 80% des souris *Ifnar-129* survivent à l'infection. Ces résultats ont donc apporté une première preuve solide de l'influence des facteurs génétiques de l'hôte sur la sensibilité des souris à l'infection par Zika. Afin d'explorer l'influence d'une diversité génétique plus grande et mimant celle de la population humaine, nous avons poursuivi notre étude en utilisant des souris CC.

Voici le schéma expérimental que nous avons utilisé : les souris CC ont été traitées avec l'anticorps bloquant le récepteur IFNAR, puis infectées le lendemain avec une souche asiatique du virus Zika provenant d'un isolat clinique de Guyane Française de 2015. Plusieurs paramètres ont été mesurés après l'infection, tels que la sévérité des symptômes, la virémie et les lésions pathologiques dans le cerveau. Nous avons d'abord confirmé que l'anticorps anti-IFNAR est requis pour la

réplication du virus *in vivo*. En effet, les souris traitées développent une virémie soutenue et prolongée alors qu'elle n'est que faible et transitoire chez les souris non traitées. Nous avons par ailleurs validé l'efficacité de l'anticorps dans plusieurs fonds génétiques et montré que le sexe des souris n'affecte pas leur sensibilité au virus. Enfin, nous avons mesuré la cinétique de virémie chez des souris de plusieurs lignées différentes et nous avons établi que le pic de virémie est atteint deux jours après l'infection, indépendamment du fond génétique des souris.

Dans cette étude, 35 lignées CC ont été infectées avec le virus Zika. Des signes cliniques ont été observés dans trois lignées, notamment la CC071 dont la majorité des souris sont mortes entre 7 et 9 jours après l'infection. Ces résultats ont donc apporté une preuve supplémentaire que le fond génétique des souris contrôle la sévérité clinique et la mortalité liée à l'infection par Zika. Nous avons également mesuré la charge virale dans le sang deux jours après l'infection, soit au pic de virémie, et nous avons mis en évidence de très grandes variations entre lignées, responsables d'un très fort effet du fond génétique, avec une héritabilité de 86%. Nous avons mesuré la virémie six jours après l'infection et observé de nouveau de grandes différences entre lignées. Par contre, nous n'avons observé qu'une corrélation modérée de ces deux paramètres, indiquant que la valeur au pic de virémie n'est pas totalement prédictive de la virémie six jours après l'infection. Enfin, pour avoir une estimation plus précise de l'élimination du virus de la circulation sanguine, nous avons utilisé la différence logarithmique des virémies à jours 2 et 6. Nous avons mesuré une gamme phénotypique large et observé que certaines lignées, comme la CC026, éliminent jusqu'à 1 000 fois plus de virus que d'autres lignées dans un même laps de temps.

Afin de mieux comprendre le contrôle génétique de la sensibilité au virus Zika, nous avons réalisé une analyse QTL pour le pic de virémie et pour l'élimination du virus. Nous avons utilisé le logiciel R/qtl2 et déterminé les seuils de significativité à partir de 1 000 permutations. Notre analyse n'a pas révélé de QTL dont le LOD score atteint le seuil de significativité. Or, une équipe a récemment montré qu'avec 35 lignées CC et 5 souris par lignée, la puissance de détection d'un QTL expliquant 30% de la variation phénotypique atteint presque 80%. Ainsi, nos résultats semblent écarter un manque de puissance statistique et suggérer un contrôle polygénique complexe de la sensibilité au virus Zika.

Nous avons poursuivi notre étude par une analyse phénotypique détaillée sur quelques lignées aux phénotypes contrastés. Nous avons choisi la CC001 parmi les plus résistantes, et la CC071 qui est la lignée la plus sensible. Nous avons parfois inclus la CC005 qui présente une virémie élevée mais pas de symptômes. Nous avons d'abord cherché à établir si les différences de sensibilité observées entre lignées pourraient être conservées après infection par une souche Zika du lignage Africain. La lignée CC001 s'est avérée complètement résistante à l'infection par la souche Africaine, avec une absence de symptômes et de mortalité ainsi qu'une virémie faible, contrairement aux souris CC071 qui ont présenté une virémie élevée et des signes cliniques sévères aboutissant à un taux de mortalité de 100%. Nous avons également cherché à établir si ces différences seraient conservées après infection par différents Flavivirus, comme les virus de la Dengue et West-Nile. Le virus de la Dengue n'est pas hautement pathogène chez la souris, nous avons donc étudié les différences de virémie entre ces lignées. Les résultats obtenus ont montré une corrélation étroite avec l'infection par Zika puisque les souris CC071 ont développé une virémie significativement plus élevée que celle des souris CC001.

Quant au virus West-Nile, le gène *Oas1b* constitue un facteur de restriction majeur de l'infection, or les trois lignées CC sélectionnées sont toutes déficientes pour *Oas1b*, ce qui les rend naturellement sensibles à l'infection. Après infection par le virus West-Nile, nous avons observé que les souris CC071 mourraient significativement plus rapidement que les CC001 et les CC005. Dans l'ensemble, ces résultats ont permis d'établir que les différences de sensibilité observées entre lignées sont conservées après infection par les virus Zika, Dengue et West-Nile.

Comme le virus Zika est neurotrope, nous avons ensuite caractérisé les conséquences pathologiques de l'infection dans le cerveau des trois lignées. Les souris CC005 et CC071, qui ont une virémie élevée, présentent également une charge virale dans le cerveau élevée six jours après infection. Nous avons identifié des différences notables de lésions histopathologiques entre lignées CC. En effet, aucune lésion n'a été détectée dans le cerveau des CC001 de même qu'aucun signe de neuroinflammation observé grâce à un marquage Iba1 normal des cellules microgliales. Au contraire, des lésions d'encéphalite subaigüe ont été détectées chez les CC005 et les CC071 avec une augmentation du marquage Iba1. La nature et la sévérité des lésions cérébrales peuvent dépendre de plusieurs paramètres, tels que la charge virale circulante, la capacité du virus à traverser la barrière hémato-encéphalique, ainsi que la permissivité des cellules neurales. Pour évaluer la différence de sensibilité des cellules du cerveau entre lignées, nous avons infecté les souris par voie intracérébrale afin d'injecter le virus directement dans le cerveau. Nous avons mesuré des différences de charge virale dans le cerveau similaires à celles de l'infection systémique et noté des différences lésionnelles marquées entre lignées. Nous avons observé chez les souris CC071 des lésions de lepto-méningo-encéphalite sévères, caractérisées par des infiltrations de cellules inflammatoires sous les méninges, par une gliose majeure ainsi que par une activation marquée des cellules microgliales. Au contraire, les souris CC001 n'ont présenté qu'une gliose mineure et une neuroinflammation modérée. Dans l'ensemble, ces résultats ont montré que la diversité génétique des souris CC contrôle la pathologie dans le cerveau et indiqué des différences de permissivité à l'infection entre lignées.

Pour confirmer ces dernières données, nous avons mesuré la production de particules virales dans des fibroblastes embryonnaires dérivés des lignées CC001 et CC071. Après infection *in vitro* par le virus Zika, nous avons noté que les cellules de CC071 produisent une quantité croissante de particules virales entre 24 et 72 heures après infection, contrairement aux cellules de CC001. Des expériences préliminaires montrent des résultats similaires dans d'autres types cellulaires tels que des macrophages péritonéaux et des neurones primaires. Ces résultats ont montré que le taux de réplication virale élevé chez les CC071 pourrait expliquer en partie son phénotype sensible.

En conclusion de cette première partie, nous avons montré que la diversité génétique des souris CC : contrôle la gravité de la maladie, la virémie et la pathologie dans le cerveau ; montre que les différences de sensibilité entre lignées sont conservées entre Flavivirus ; permet d'identifier des lignées résistantes et sensibles qui peuvent servir de nouveaux modèles d'étude du virus Zika ; révèle des dé-corrélations phénotypiques et enfin suggère un contrôle polygénique complexe de la sensibilité au virus Zika.

(2) Etude génétique de la sensibilité au virus Zika chez des souris *Ifnar1*^{-/-}

Dans cette deuxième partie, nous avons cherché à identifier des gènes modificateurs du phénotype de sensibilité au virus Zika des souris *Ifnar1*^{-/-}. Pour cela, nous avons généré un croisement F2 entre les lignées *Ifnar-B6* et *Ifnar-129*. Nous avons infecté environ 200 souris F2 puis nous avons mesuré la sévérité clinique de l'infection à l'aide d'un score clinique calculé entre les jours 6 et 14 après infection. La majorité des souris ont présenté des signes cliniques d'intensité modérée alors que des symptômes plus sévères ont été observés chez un nombre plus faible d'individus, avec pour conséquence un taux de mortalité global de 18%. La majorité des souris sensibles sont mortes entre 6 et 8 jours après infection. Nous avons mesuré la virémie deux et six jours après infection et observé une distribution continue des valeurs de la virémie au pic et de l'élimination du virus. Nous avons ensuite choisi 94 individus en vue du génotypage, nous avons sélectionné toutes les souris sensibles, c'est-à-dire morte après l'infection, ainsi que les souris présentant des valeurs phénotypiques extrêmes, hautes ou basses, pour la virémie au pic et pour l'élimination du virus. Ces 94 souris ont été génotypées à l'échelle du génome entier avec le panel MiniMUGA. Au final, plus de 2 700 marqueurs informatifs et répartis sur l'ensemble des chromosomes ont été retenus. Nous avons pu identifier deux principaux QTLs, le premier situé sur le chromosome 12 est associé à la survie des animaux. Pour ce QTL, l'allèle B6 est associé à une sensibilité significativement plus importante et semble agir sur un mode récessif. Le deuxième QTL, associé à la virémie au pic, est situé sur le chromosome 5. Les intervalles de confiance de ces deux QTLs couvrent des régions génomiques assez grandes qui contiennent par conséquent plusieurs centaines de gènes. L'étude génétique doit donc être poursuivie par des analyses complémentaires afin de réduire la liste des gènes candidats.

Génotyper des individus supplémentaires pour des marqueurs aux bornes des QTLs pourrait dans un premier temps permettre d'améliorer la résolution de cartographie et de réduire l'intervalle de confiance des QTLs. Les gènes situés dans des régions génomiques identiques par origine entre les deux lignées parentales peuvent aussi être exclus. De plus, des gènes candidats peuvent être sélectionnés selon leurs fonctions biologiques ou leurs phénotypes. Par exemple, en choisissant un critère phénotypique en lien avec la physiologie du système immunitaire, nous avons pu présélectionner 22 gènes candidats pour le QTL de survie et 48 pour le QTL de virémie. L'analyse des variants de séquence de ces gènes pourrait permettre d'affiner encore la sélection. Enfin, un test de complémentation quantitatif pourrait apporter une validation expérimentale de l'effet d'un gène candidat.

(3) Etude de la réplication du virus Zika dans des fibroblastes embryonnaires de souris CC

Cette troisième partie a porté sur l'identification de mécanismes moléculaires contrôlant la réplication du virus Zika dans des fibroblastes embryonnaires issus de souris CC. Pour rappel, nous avons montré dans la première partie que les fibroblastes de CC071 produisent des quantités croissantes de virus entre 24 et 72 heures après infection. Nous avons testé dix lignées CC afin d'évaluer l'influence du fond génétique des cellules sur la réplication du virus et nous avons observé

des différences significatives de titre viral entre lignées cellulaires. Par contre, aucune des lignées testée n'a montré une cinétique de réplication virale similaire à celle des CC071 caractérisée par une augmentation croissante de titre viral. Nous avons donc cherché à comprendre les mécanismes cellulaires et moléculaires qui sont à l'origine du phénotype unique des CC071 et nous avons donc poursuivi notre étude en restreignant la comparaison aux cellules de souris CC071, CC001 et B6. Nous avons d'abord cherché à identifier plus précisément l'étape du cycle viral qui est impactée. Nous avons mesuré la capacité d'attachement du virus à la membrane cellulaire ainsi que la phase d'internalisation du virus dans la cellule, et nous n'avons pas observé de différence significative entre les cellules de CC071 et les autres lignées. En revanche, nous avons mesuré un nombre de cellules infectées et un taux de réplication du génome viral élevés chez CC071 à des temps tardifs, 48 et 72 heures après infection. Ces résultats ont donc suggéré un défaut de contrôle de la réplication virale par les cellules de CC071.

Comme la réponse IFN de type I constitue l'une des premières lignes de défense en cas d'infection virale, nous avons mesuré par PCR quantitative (qPCR) l'expression du gène *Ifnb1* après infection par le virus Zika. Alors que l'expression du gène *Ifnb1* augmente très fortement dès 24 heures post-infection dans les cellules de CC001 et B6, elle est retardée chez CC071. Pour avoir une vision plus complète de cette réponse IFN et des événements précoces qui contrôlent la réplication virale, nous avons réalisé une analyse transcriptomique globale sur ces cellules. Nous avons pour cela utilisé des triplicatas biologiques des trois lignées cellulaires, infectés ou non avec le virus Zika et à trois temps précoces : 16, 24 et 32 heures après infection. Nous avons pu noter que des centaines de gènes sont surexprimés chez CC001 dès 16 heures et jusqu'à 32 heures après l'infection, alors que seuls quelques gènes sont surexprimés chez CC071, seulement à partir de 32 heures après infection. Nous nous sommes plus précisément intéressés à un groupe de gènes fortement surexprimés chez CC001 et B6 et nous avons alors identifié de nombreux gènes impliqués dans la réponse immunitaire innée, la réponse IFN, la production de cytokines ou bien encore les réponses de défense contre les virus. En ce qui concerne la réponse IFN, l'analyse transcriptomique a confirmé les résultats précédemment obtenus par qPCR avec une absence d'expression du gène *Ifnb1* à des temps précoces chez CC071. Nous avons observé par ailleurs une forte augmentation de l'expression de certains gènes stimulés par l'IFN (ISGs) comme *Rsad2* ou *Ifit3*, excepté dans les cellules de CC071. Ce défaut d'induction des ISGs chez CC071 pourrait être lié directement au défaut d'induction de l'IFN ou bien à un défaut de réponse à la stimulation par l'IFN. Pour répondre à cette question, nous avons évalué la réponse des cellules de CC071 à la stimulation directe par l'IFN et nous avons pu montrer que le niveau d'expression des ISGs *Isg15* et *Ifitm3* est similaire dans les trois lignées suite au traitement IFN. Ces données ont permis d'établir que les cellules de CC071 sont capables de répondre à l'IFN et que le défaut réside, par conséquent, dans la cascade d'induction de l'IFN. Pour déterminer quels éléments pourraient être responsables du phénotype de CC071, nous avons comparé l'origine parentale d'une dizaine de gènes de cette cascade entre CC071 et dix autres lignées CC. Pour chaque gène, nous avons pu identifier au moins une autre lignée CC portant le même haplotype que CC071. Pourtant, aucune de ces lignées n'est caractérisée par un phénotype similaire à CC071. Ainsi, cette analyse nous a permis d'écarter une déficience dans un gène unique et d'envisager plutôt des interactions défectueuses entre plusieurs gènes.

En conclusion, cette troisième partie nous a permis de montrer que dans les fibroblastes de CC071 : la réplication du virus Zika n'est pas contrôlée, l'expression du gène *Ifnb1* et des ISGs est retardée après l'infection mais la réponse à la stimulation par l'IFN est normale. Afin de mieux caractériser les mécanismes moléculaires sous-jacents, plusieurs expériences sont actuellement menées pour répondre aux questions suivantes : (1) Les voies de signalisation TLR3 et RIG-I, intermédiaires dans la cascade d'induction de l'IFN, sont-elles fonctionnelles chez CC071 ? (2) Quels éléments peuvent faire défaut dans ces cascades ? Nous évaluerons pour cela l'effet d'agonistes de TLR3 et RIG-I sur l'induction de la réponse IFN ainsi que l'activation par phosphorylation de protéines clé comme IRF3 ou TBK1. Enfin, nous étudierons la réplication virale et l'induction de l'IFN dans des fibroblastes de souris issues d'un backcross entre CC001 et CC071 afin de mieux caractériser le contrôle génétique sous-jacent.

(4) Caractérisation de la réponse IFN induite par Zika dans des neurones primaires murins

Enfin, dans une quatrième partie, nous avons caractérisé la réponse IFN induite par le virus Zika dans des neurones primaires murins. Cette étude a montré que la capacité des neurones primaires à limiter la réplication virale est moindre que celle des fibroblastes, en raison d'un retard à l'induction de la réponse IFN. Enfin, les facteurs génétiques de l'hôte exercent un rôle critique dans ce contexte puisque les neurones primaires de CC071 ont présenté un phénotype extrême par comparaison avec des lignées plus résistantes.

Conclusions et discussion

Ce travail a permis d'apporter différents éléments de réponse à la question centrale de mon projet de thèse, qui était de déterminer si des facteurs génétiques de l'hôte pouvaient influencer la sensibilité à l'infection par le virus Zika.

Nous avons montré qu'il existe des gènes modificateurs du phénotype de sensibilité au virus Zika chez des souris *Ifnar1*^{-/-}. Nous avons établi que des combinaisons alléliques très variées permettent de décrire une gamme phénotypique de sensibilité au virus Zika extrêmement étendue et mimant le spectre d'affection clinique observé dans la population humaine. L'utilisation de modèles murins variés a également mis évidence des dé-corrélations de traits phénotypiques que l'on pensait initialement liés, comme la sévérité des lésions cérébrales et les signes cliniques de maladie. Nous avons ainsi pu décrire de nouveaux modèles de souris qui peuvent être utilisés pour étudier différents aspects de la pathogénèse du virus Zika. Ces nouveaux modèles constituent autant de nouvelles ressources à partir desquels des modèles cellulaires peuvent être utilisés pour des études mécanistiques poussées. Enfin, les résultats de notre étude ont montré qu'il existe une corrélation de sensibilité à différents Flavivirus.

Ce travail de thèse a également ouvert de nouvelles pistes de recherche qui devraient permettre d'explorer la pathogénicité du virus Zika dans de multiples directions. Parmi les pistes intéressantes à

poursuivre on peut mentionner l'identification précise des gènes contrôlant la sensibilité au virus Zika chez les souris *Ifnar1*^{-/-} et dans de nouveaux croisements ciblés entre souris génétiquement très différentes ; la caractérisation fonctionnelle des interactions entre la réponse immunitaire et la pathologie cérébrale induites par Zika ; l'utilisation des nouveaux modèles pour répondre à des questions clés sur le virus Zika telles que la transmission verticale du virus ou bien la transmission du virus de l'hôte au vecteur moustique ; la caractérisation moléculaire des défauts d'induction de la réponse IFN dans les cellules de CC071 ; et enfin l'exploration des mécanismes communs de sensibilité à divers Flavivirus.

Cette étude a, par ailleurs, mené à une réflexion sur la façon d'utiliser ces nouveaux outils que sont les populations génétiques de référence comme le CC. En effet, lors de sa création, le CC avait initialement été pensé comme « une ressource communautaire pour l'analyse génétique des caractères complexes ». Les premières estimations prévoyaient de générer environ 1 000 lignées CC différentes dont la diversité génétique permettrait d'élargir la gamme phénotypique et dont environ 200 lignées pourraient être utilisées pour des analyses QTL. Les souris CC se sont effectivement avérées comme une ressource inégalée pour l'évaluation de la diversité phénotypique des caractères complexes et notamment des maladies infectieuses. Cette variabilité peut s'exprimer au travers de nombreux traits phénotypiques, comme la perte de poids dans une étude sur le virus Influenza qui décrit une gamme phénotypique allant d'une résistance totale à une grande sensibilité, ou bien la charge bactérienne dans les organes dans une étude sur les Salmonelles. De nombreuses études ont également pu identifier des lignées aux phénotypes extrêmes, par exemple la lignée CC042 qui est particulièrement sensible à l'infection par les Salmonelles. Le CC constitue donc une plateforme expérimentale puissante pour réaliser des études phénotypiques et fonctionnelles. L'utilisation de 10 ou 15 lignées CC peut être envisagée en première intention pour un screening qui permet d'évaluer la gamme de variation phénotypique mais aussi d'identifier quelques lignées aux phénotypes intéressants. Ces lignées d'intérêt peuvent ensuite être utilisées pour des études fonctionnelles à différentes échelles, organisme, tissu, cellule etc. En fonction de la distribution et de l'héritabilité du trait phénotypique, les souris CC peuvent ensuite être utilisées pour une analyse QTL. La majorité des études ayant identifié des QTLs à partir de souris CC ont utilisé entre 60 et 130 lignées, or le CC compte aujourd'hui 80 lignées bien établies, bien loin des 1 000 lignées initialement prévues. Ce schéma d'étude génétique ne peut donc pas s'appliquer à n'importe quel caractère complexe. Une alternative consisterait alors à avoir recours à un croisement de type F2 entre deux lignées CC aux phénotypes contrastés. Cette approche permettrait d'atteindre plus facilement la puissance nécessaire pour la détection de QTLs tout en obtenant une meilleure résolution cartographique qu'avec un croisement F2 classique.

L'utilisation des souris CC dans ce projet de thèse m'a également amenée à réfléchir à la notion de modèle animal d'une maladie humaine et notamment pour l'infection par le virus Zika. En effet, les premières publications utilisant des souris pour étudier le virus Zika se sont attachées à décrire « un » modèle de la maladie. Il est vrai que le développement de modèles animaux est une étape clé pour pouvoir ensuite étudier les mécanismes d'une pathologie. En revanche, il est également légitime de se demander comment « un » modèle de souris pourra permettre de mimer une maladie aussi polymorphe que l'infection par le virus Zika. L'utilisation des souris CC peut être une approche efficace pour décrire non pas un modèle universel mais plusieurs modèles qui reflèteraient la complexité de la maladie Zika. Les souris CC ont par exemple été utilisées dans une étude sur le virus

Ebola et ont permis de décrire plusieurs catégories de présentations cliniques, avec des souris qui développent une hépatite aiguë ou des souris qui développent un syndrome hémorragique fatal. De plus, ce syndrome hémorragique, caractérisé par une augmentation des temps de coagulation, n'avait jamais été décrit précédemment chez des souris de laboratoire classiques et permettra de mieux modéliser la coagulopathie qui caractérise les formes humaines les plus sévères.

Au-delà de l'amélioration des modèles utilisés pour la recherche fondamentale, la réflexion peut être étendue aux modèles animaux utilisés en recherche préclinique pour le développement de nouveaux vaccins et médicaments. En effet, les études précliniques de pharmacocinétique, de toxicité et d'efficacité sont généralement menées sur des souris issues d'une unique lignée de laboratoire et conditionnent ensuite le lancement des études cliniques qui seront menées chez l'Homme. Or, on sait que ce schéma n'est pas idéal puisque le taux de transposition des études chez la souris vers l'Homme est relativement faible. On pourrait donc proposer d'utiliser des souris génétiquement différentes afin d'augmenter l'efficacité des études précliniques qui pourraient alors sélectionner quelques lignées de souris différentes pour mieux mimer la diversité phénotypique d'une maladie ou encore pour mieux appréhender les effets indésirables d'un médicament. Cela nécessiterait un peu plus de travail et un peu plus de moyens mais qui peuvent sembler dérisoires compte-tenu des enjeux actuels de la santé publique mondiale.

List of abbreviations

3D: Three dimensions	hiPSC: Human induced pluripotent stem cell
Ab: Antibody	HIV: Human immunodeficiency virus
ADE: Antibody-dependent enhancement	HSV: Herpes simplex virus
Areg: Amphiregulin	HTLV: Human T-cell leukemia virus
BIDMC: Beth Israel Deaconess Medical Center	i.a.: Intra-amniotic
BXD: C57BL/6J x DBA/2J	i.c.: Intra-cerebral
CC: Collaborative Cross	i.p.: Intra-peritoneal
CCR5: Chemokine (C-C motif) receptor 5	i.u.: Intra-uterine
Chr: Chromosome	i.v.: Intra-venous
CI: Confidence interval	i.vag: Intra vaginal
CLEC5A: C-type lectin domain family 5, member a	IAV: Influenza A virus
cM: CentiMorgan	Id2: Inhibitor of DNA binding 2
CNS: Central nervous system	IFIT: Interferon-induced protein with tetratricopeptide
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats	IFITM: Interferon induced transmembrane protein
Cxcl: Chemokine (C-X-C motif) ligand	IFN: Interferon
CZS: Congenital Zika syndrome	IFNAR: Interferon-alpha receptor
DEG: Differentially expressed gene	IFNGR: Interferon-gamma receptor
DENV: Dengue virus	IFNLR: Interferon-lambda receptor
DNA: Deoxyribonucleic acid	IgG: Immunoglobulin G
DO: Diversity outbred	IgM: Immunoglobulin M
ds: Double-stranded	IKKε: Inhibitor of KB kinase epsilon
EBV: Epstein-Barr virus	ILC: Innate lymphoid cells
EHT: Event horizon telescope	IRF3: Interferon regulatory factor 3
ELISA: Enzyme-linked immunosorbent assay	IRF7: Interferon regulatory factor 7
ENU: <i>N</i> -ethyl- <i>N</i> -nitrosourea	ISG: Interferon-stimulated gene
eQTL: expression QTL	IUGR: Intra-uterine growth restriction
ER: Endoplasmic reticulum	JAK: Janus kinase
FFU: Focus-forming unit	Jchain: Immunoglobulin joining chain
FPKM: Fragments per kilobase of exon per million reads mapped	JEV: Japanese encephalitis virus
GBS: Guillain-Barre syndrome	Kb: Kilobase
GO: Gene ontology	mAb: Monoclonal antibody
GRP: Genetic reference population	MA-EBOV: Mouse-adapted Ebola virus
GWAS: Genome wide-association study	MAVS: Mitochondrial antiviral signaling protein
HBV: Hepatitis B virus	Mb: Megabase
HCV: Hepatitis C virus	MEF: Mouse embryonic fibroblast
	MERS: Middle East respiratory syndrome

MGI: Mouse genome informatics
 MHV: Mouse hepatitis virus
 MPP: Multi-parental population
 MRI: Magnetic resonance imaging
 MyD88: Myeloid differentiation primary response gene 88
 nAb: Neutralizing antibody
 NHP: Non-human primate
 NIAID: National Institute of Allergy and Infectious Diseases
 NIH: National institute of health
 NK: Natural killer
 NPC: Neural progenitor cell
 NS: Non-structural
 NTR: Non-translated region
 OAS: 2'-5' oligoadenylate synthetase
 OPC: Oligodendrocyte progenitor cell
 p.i.: Post-infection
 PAMP: Pathogen-associated molecular pattern
 PANO: Pan American Health Organization
 PCR: Polymerase chain reaction
 PFU: Plaque forming unit
 Pik3cg: Phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit gamma
 PIV: Purified inactivated virus
 PKR: Protein kinase R
 PRR: Pattern recognition receptor
 Ptpn13: Protein tyrosine phosphatase, non-receptor type 13
 PVE: Percent of variance explained
 QTL: Quantitative trait locus
 R&D: Research and development
 RI: Recombinant inbred
 RIG-I: Retinoic acid-inducible gene I
 RLR: RIG-I-like receptor
 RNA: Ribonucleic acid
 RSAD2: Radical S-adenosyl methionine domain containing 2

RT-qPCR: Reverse transcription-quantitative PCR
 s.c.: Subcutaneous
 SARS: Severe acute respiratory syndrome
 SD: Standard deviation
 SEM: Standard error of the mean
 sfRNA: Subgenomic flavivirus RNA
 SNP: Single nucleotide polymorphism
 Spp.: Species
 Spp1: Secreted phosphoprotein 1
 STAT: Signal transducer and activator of transcription
 STING: Stimulator of interferon genes
 TAM: TYRO3, AXI and MER
 TBEV: Tick-borne encephalitis virus
 TBK1: TANK-binding kinase 1
 TGN: Trans-golgi network
 TIM: T-cell immunoglobulin and mucin domain
 TLR: Toll-like receptor
 TNF: Tumor necrosis factor
 TORCH: *Toxoplasma gondii*, other agents, rubella virus, cytomegalovirus, and herpes simplex virus
 TRAF: TNF receptor associated factor
 Traf1: TRAF type zinc finger domain containing 1
 Treg: T regulatory cell
 TRIF: TLR adaptor molecule 1
 TRIM56: Tripartite motif-containing 56
 USA: United States of America
 VSG: Viral-stimulated gene
 WHO: World health organization
 WNV: West-Nile virus
 YFV: Yellow fever virus
 ZIKV: Zika virus
 Zsl: ZIKV susceptibility locus

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1. INTRODUCTION

Despite many advances in the fight against infectious diseases, many pathogens continue to threaten human public health. The challenges associated with infectious diseases include the threat of “old” diseases such as malaria, plague or shigellosis but also of emerging pathogens such as Ebola virus or, more recently, Zika virus (ZIKV). The development of prophylactic and therapeutic measures is needed to contain these epidemics, but it requires an in-depth knowledge of the pathogen’s biology and pathogenesis.

While epidemiological studies in affected populations significantly contribute to our understanding of the disease, many factors can confound human studies of infectious diseases such as pathogen-related determinants (e.g. pathogen strain, dose or exposure route) and demographic factors. In addition, one shared feature of infectious diseases is that a given population exposed to a pathogen will not uniformly develop clinical signs of disease. This was well illustrated by the recent ZIKV outbreaks: while most infection cases are mild, new complications such as Guillain-Barre syndrome and congenital afflictions were associated with ZIKV infection.

Due to the limitations associated with studying infectious diseases in humans, researchers have been using animal models to investigate the mechanisms underlying the pathogenesis of infection and to test the efficacy and safety of newly developed vaccines and therapeutics. Most confounding factors can be experimentally controlled in these models, which also allow for mechanistic studies of host-pathogen interactions. Mouse models have many advantages for the study of infectious diseases including their relatively low cost, ease of handling and short generation time; but also the existence of many genetic tools. The availability of many well-characterized mouse inbred strains, as well as genetically engineered mice, has led to the generation of a variety of models of infectious diseases and has allowed to dissect the genetic mechanisms affecting the response to a specific pathogen agent. The aim of my PhD project was to study several mouse models to characterize the influence of host genetic factors in the responses to ZIKV infection, with the ultimate goal of identifying mechanisms of increased resistance or susceptibility.

2. STATE OF THE ART

2.1. Flaviviruses: a global health threat

This first part aims at introducing flaviviruses and their potential to emerge and re-emerge resulting in global scale outbreaks with a focus on the main pathogenic and most prevalent mosquito-borne flaviviruses, namely Yellow fever, Dengue, West-Nile, Japanese encephalitis and Zika viruses.

2.1.1. Biology of flaviviruses

Flaviviruses belong to the family *Flaviviridae* and share many similarities in terms of genomics, structural biology and life cycle. However, despite being genetically close (Figure 1), these viruses trigger in humans various clinical syndromes and their tissue tropisms are heterogeneous. For instance, West-Nile and Japanese encephalitis viruses are highly neurotropic and cause severe encephalitis and myelitis in human patients (David and Abraham 2016; Turtle and Solomon 2018). In contrast, both Yellow Fever and Dengue viruses can elicit a hemorrhagic fever in infected individuals though with specificities; hepatonephritis or shock syndrome respectively (Jentes *et al.* 2011; Wilder-Smith *et al.* 2019).

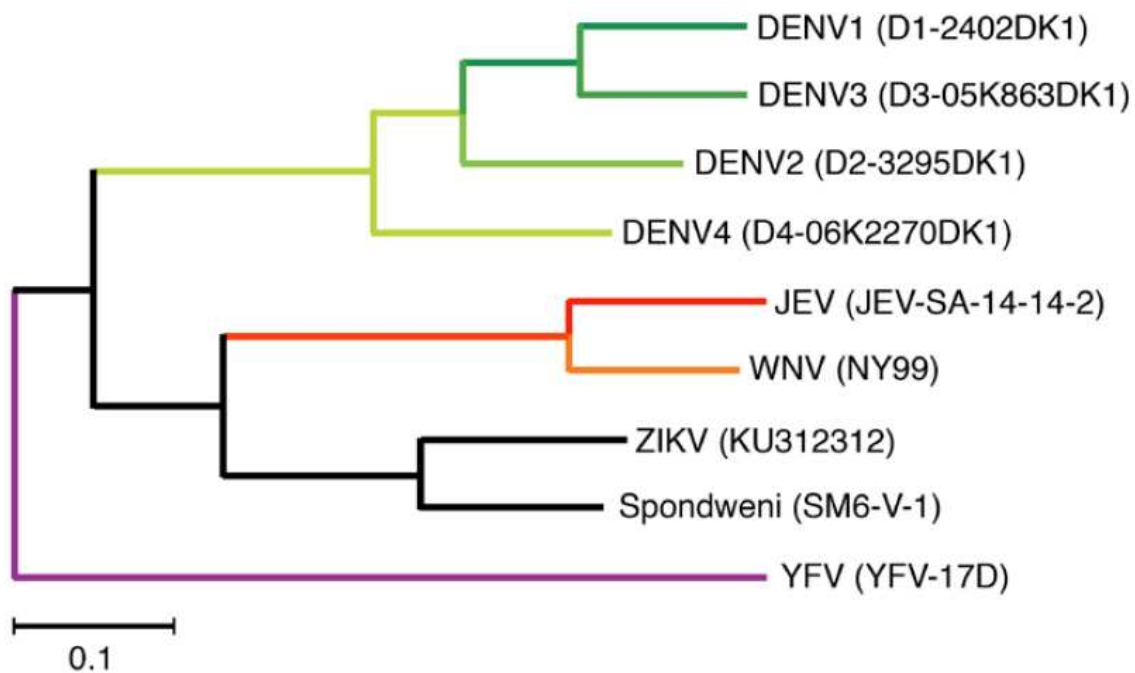


Figure 1. Phylogenetic tree of pathogenic mosquito-borne flaviviruses. Phylogenetic tree showing the genetic distances between representative strains of DENV, JEV, WNV, ZIKV, Spondweni virus and YFV. The scale bar indicates the genetic distances in substitutions per amino acid (Saron *et al.* 2018).

2.1.1.1. Flavivirus structure

Flaviviruses are small (500 Å in diameter), lipid-enveloped viruses containing a single, positive-strand, ribonucleic acid (RNA) genome with a size of approximately 10.8 kilobases (kb). The RNA has one open-reading frame and is translated into a single polyprotein encoding 3 structural proteins - capsid (C), membrane (M, which is expressed as prM, the precursor to M) and envelop (E) - and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Figure 2). The structural proteins form the skeleton of the virus particle while the non-structural proteins play a role in replication and packaging of the genome as well as in subverting the host cell machinery in favor of the virus (Hasan *et al.* 2018; Mukhopadhyay *et al.* 2005).

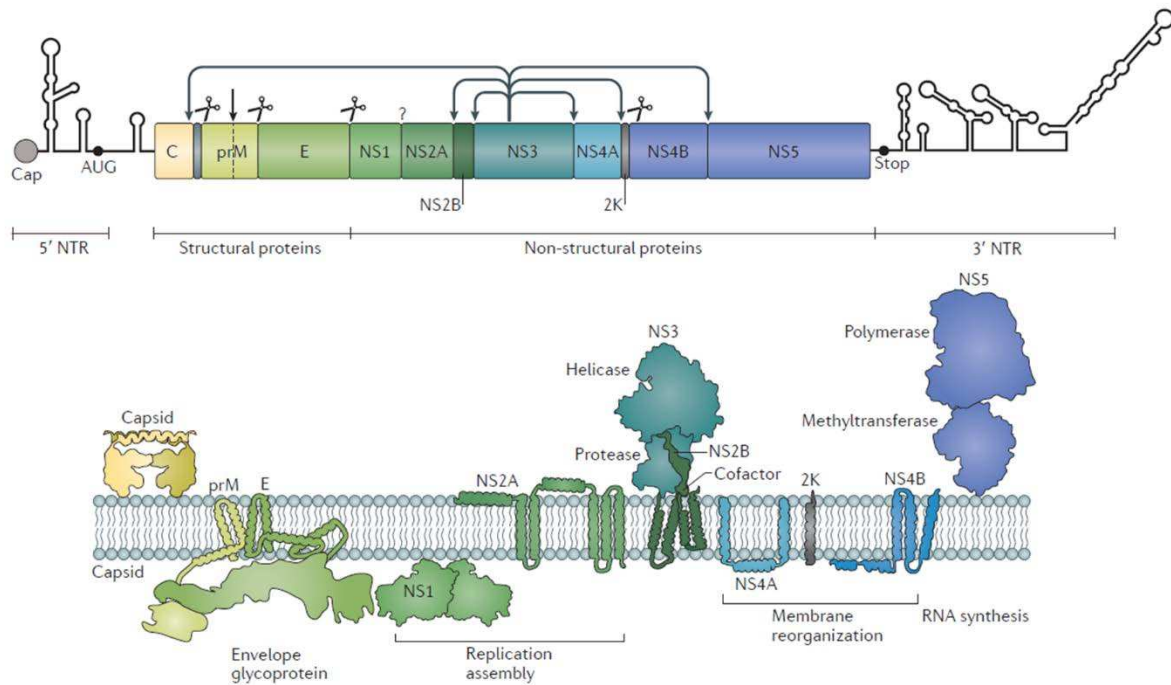


Figure 2. Flavivirus genome organization and membrane topology of mature viral proteins. (Top) The open-reading frame encoding the dengue virus (DENV) polyprotein and the predicted secondary structures of the 5' and 3' non-translating regions (NTR) of the RNA genome. Polyprotein cleavage by cellular peptidases is indicated by scissors. Arrows denote the cleavage by the viral protease, whereas the black vertical arrow indicates cleavage by the Golgi apparatus-resident protease furin. (Bottom) Polyprotein topology on the host endosomal membrane (Neufeldt *et al.* 2018).

The surface glycoproteins E and prM are subjected to significant rearrangements during the replication cycle, resulting in different states of viral particles, namely the mature and immature states (Figure 3). Immature particles are non-infectious and are composed of an icosahedral shell of 180 prM-E heterodimers associated into 60 trimeric spikes. Exposure to an acidic environment triggers trimer dissociation, followed by reorganization into 90 prM-E dimers resulting in a very different surface morphology. At the same time, maturation continues with the cleavage of the prM protein by the host protease furin. Upon exposure to neutral pH after secretion out of the cell, prM proteins dissociate from the virion resulting in an 'activated' mature particle (Rey *et al.* 2017).

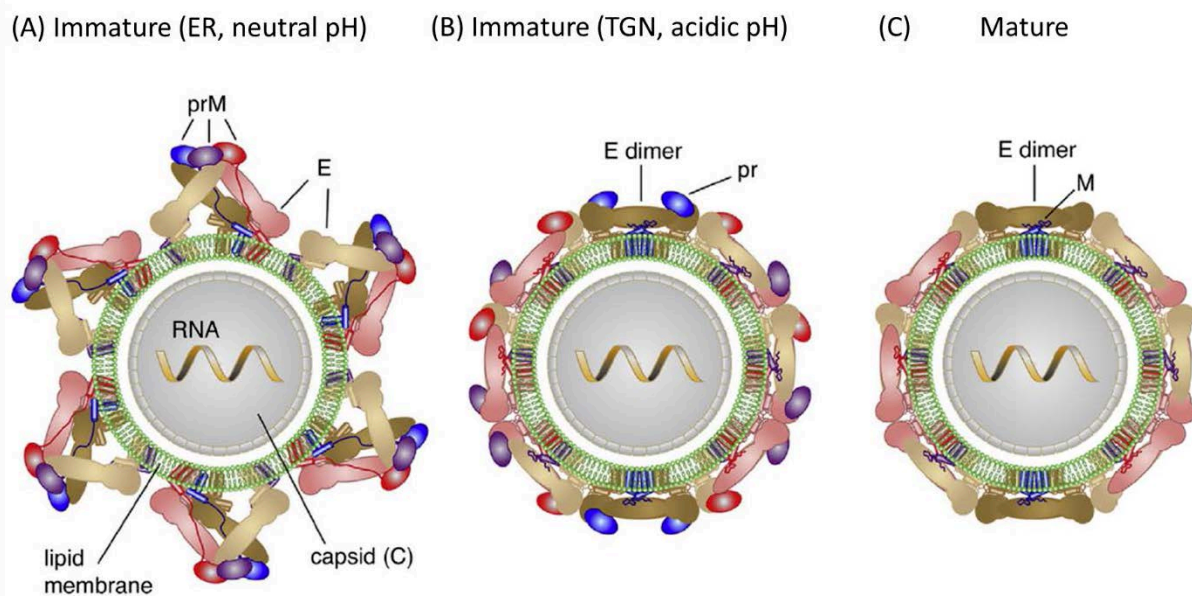


Figure 3. Structure of mature and immature flavivirus particles.

(A) Immature virion in the endoplasmic reticulum (ER). (B) Immature virion after exposure to low pH in the Trans-Golgi-Network (TGN) and rearrangement of envelop proteins. (C) Mature virion after secretion from infected cells. Adapted from (Rey *et al.* 2017).

2.1.1.2. *Flavivirus life cycle*

Flaviviruses first attach to the surface of the host cell using a variety of cellular receptors (Figure 4). As the viral E proteins harbor approximately 40% amino acid identity, different flaviviruses can have common and more specific receptors. Indeed, the number and position of glycosylated residues on the virion surface vary between different viruses, which could impact the specificity of receptor binding (Mukhopadhyay *et al.* 2005). Glycosaminoglycans such as Heparan sulfate are potential attachment factors for DENV and ZIKV. Besides, several C-type lectin receptors have been proposed as primary receptors: DC-SIGN is used by WNV, JEV and DENV, Mannose receptor and CLEC5A are also used by JEV and DENV. Additionally, $\alpha\beta3$ Integrin has been shown to be a receptor of JEV and WNV in mammalian cells. Finally, TIM (T-cell immunoglobulin and mucin domain) phosphatidylserine receptors and TAM (TYRO3, AXL and MER) receptors constitute another class of attachment molecules that are targeted mainly by DENV and ZIKV (Hasan *et al.* 2018; Laureti *et al.* 2018; Mukhopadhyay *et al.* 2005; Sirohi and Kuhn 2017).

Flaviviruses then enter the cell mainly through clathrin-dependent endocytosis (Neufeldt *et al.* 2018) (Figure 4). The virus is enclosed in the endosome where the low pH leads to the trimerization of the E protein, subsequently resulting in fusion of viral and endosomal membranes and release of the viral RNA in the cytoplasm (Hasan *et al.* 2018).

The viral single-stranded positive RNA species contains all the genetic information required for viral replication: in addition to coding for the viral polyprotein, it is used as a template for RNA replication and is integrated into newly assembled viral particles. Thus, translation, replication and packaging of the viral RNA must be coordinated in time and space as these steps cannot occur concurrently (Figure 4). For translation, the viral RNA is recognized by ribosomes, which initiate the production of the single polyprotein. Proteases of both viral and cellular origins catalyze the cleavage

of the polyprotein into structural and non-structural proteins, which are anchored to the endoplasmic reticulum (ER) membrane. Viral RNA replication is mediated by non-structural proteins and takes place in ER membrane invaginations also called replication factories. It leans on the RNA-dependent RNA polymerase activity of NS5 protein, which first synthesizes one molecule of negative-sense RNA from the positive-strand template. Subsequently, a pool of newly synthesized viral RNA molecules is produced allowing for the formation of new replication factories, for translation into viral proteins and for packaging into neo-assembled viral particles (Mazeaud *et al.* 2018; Neufeldt *et al.* 2018).

These newly assembled viral particles bud into the ER lumen and the immature virions are cleaved by the host protease furin, resulting in mature, infectious particles (Figure 3). Secretion of flaviviruses particles occur throughout the conventional secretory pathway of the cell (Hasan *et al.* 2018; Neufeldt *et al.* 2018) (Figure 4).

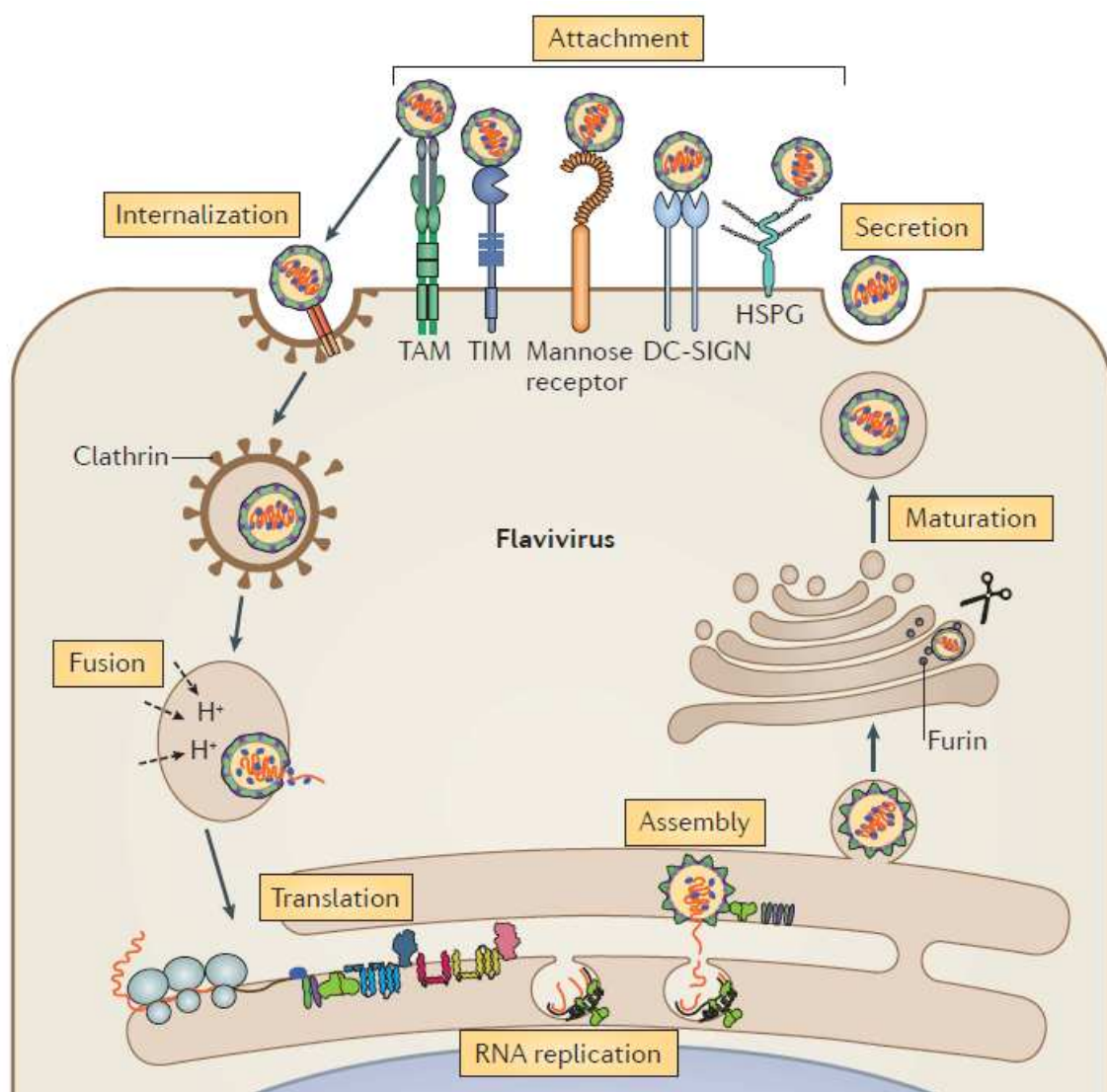


Figure 4. Flavivirus replication cycle.
Extracted from (Neufeldt *et al.* 2018).

2.1.2. Emerging flaviviruses

« Tout donc, dans la diffusion des maladies, comme dans tout phénomène biologique naturel, est affaire de circonstances. Par conséquent, sachons, en conservant ces termes commodes: endémicité, épidémicité, qu'il n'existe pas, entre les catégories qu'elles étiquettent, de barrière qu'un agent pathogène ne puisse franchir ou derrière laquelle il ne puisse s'enfermer. » Charles Nicolle, *Destin des maladies infectieuses*, 1933 (Nicolle 1933).

2.1.2.1. What is an emerging infectious disease?

The concept of emerging infectious diseases stems from the ancient observations of new diseases which were not previously recognized or of diseases which incidence has rapidly and dramatically increased. It was conceptualized by Charles Nicolle in his book “Le Destin des maladies infectieuses” in 1933 (Nicolle 1933). The term “emerging infectious disease” was then formulated as such in the 1990’s and has received variable definitions depending on authors and institutions. The Center for Disease Control (CDC) defines emerging infectious diseases as “those whose incidence in humans has increased in the past two decades or threaten to increase in the near future” (Center for Disease Control and Prevention 2018). Emergence refers to the first observation while recurrences are referred to as re-emergences.

Many examples of re-emerging infectious diseases can be found throughout history, such as the large plague pandemics (Justinian plague and middle age “Black Death”), the smallpox epidemic of the 16th century or the Spanish influenza outbreak of 1918.

Several infectious diseases, previously unknown, have emerged in the past decades in particular severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), Ebola hemorrhagic fever and Zika fever, among others. The majority of these emergent pathogens are viruses and are often zoonotic agents, infecting both animals and humans. For instance, bats have been reported to be a natural reservoir of viruses such as rabies, SARS, MERS, Nipah and Ebola viruses (Wang and Anderson 2019). Many other emerging viruses are transmitted to humans by insects such as mosquitoes and ticks and are referred to as arboviruses, shortened for arthropod-borne viruses.

Recent emergence and re-emergence of mosquito-borne viruses constitutes a major public health threat and has resulted in global scale epidemics. In 2015, the World Health Organization (WHO) gathered a coalition of experts to develop a Research and Development (R&D) Blueprint to accelerate the identification of emerging infections, promote the development of vaccines and treatments and prevent greater outbreaks, with a focus on severe diseases for which there are currently insufficient medical countermeasures. Several mosquito-borne viruses have been included in the 2018 WHO Blueprint shortlist: Chikungunya, Rift Valley Fever, West-Nile and Zika viruses (Mehand *et al.* 2018).

In this context, my PhD project was part of a coordinated “emergency action” of the LabEx “Integrative Biology of Emerging Infectious Diseases” which focused mainly on Zika virus (ZIKV) and on related mosquito-borne flaviviruses.

2.1.2.2. Emergence of mosquito-borne flaviviruses

The first pathogenic arbovirus proven to be transmitted to humans by the bite of *Aedes aegypti* mosquitoes was Yellow fever virus (YFV) (Reed 1902). Mosquito-borne flaviviruses are able to infect both humans and animals in diverse environmental conditions, sometimes switching between distinct transmission cycles. While some of these arboviruses, WNV for instance, infect people mainly from direct spillover from the enzootic cycle, viruses transmitted by *Aedes aegypti* (YFV, DENV, ZIKV) can also use humans as amplifying hosts, sustaining transmission in urban areas (Figure 5). Some of the key characteristics of the emergence of mosquito-borne flaviviruses are summarized hereafter, with a focus on the flaviviruses which are pathogenic in humans.

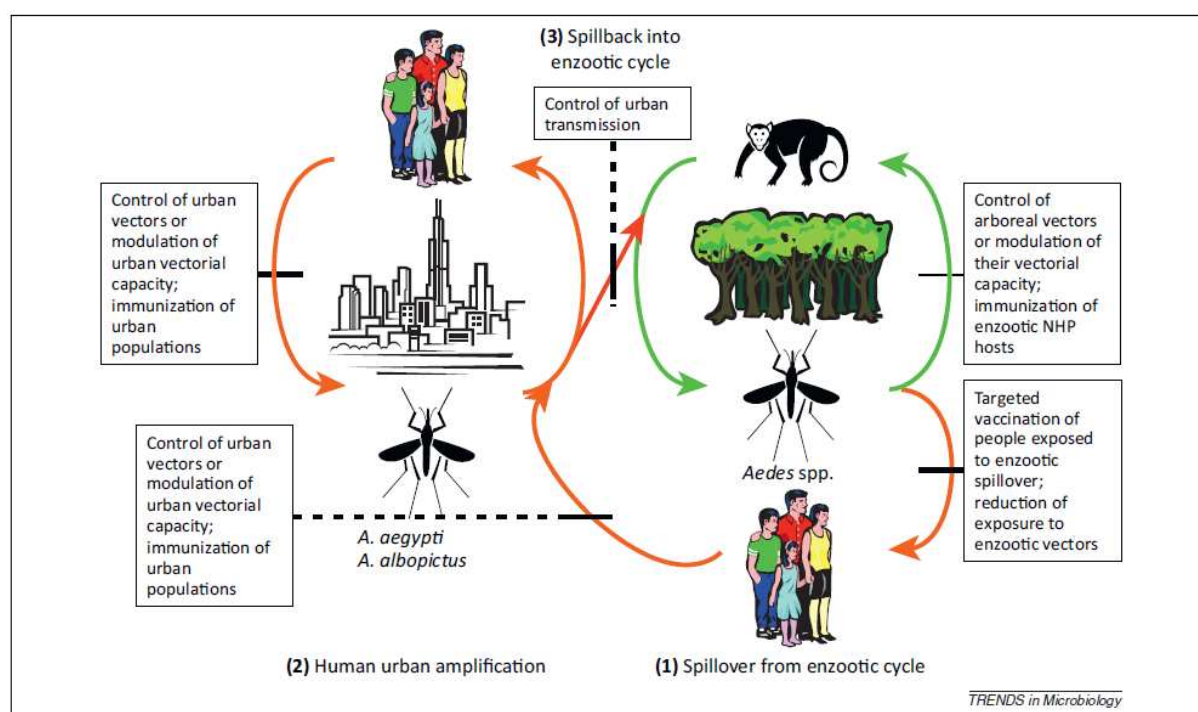


Figure 5. Emergence of urban transmission cycles for YFV, DENV and ZIKV from enzootic cycles, with potential prevention strategies.

Green arrows define the sylvatic enzootic cycle and orange arrows refer to (1) spillover from enzootic cycle, (2) human urban amplification and (3) spillback into enzootic cycle. Lines crossing the arrows indicate potential points for the implementation of prevention strategies (Weaver 2013).

2.1.2.2.1. Yellow fever virus (YFV)

YFV is a highly virulent flavivirus causing hemorrhagic fever and hepatonephritis in humans with a high fatality rate (20-50%) (Jentes *et al.* 2011). Historically, the disease was endemic to Africa, but YFV was reported as the first arbovirus to expand the geographical range of its enzootic cycle when spreading to the Americas through the transatlantic slave trade (Chippaux and Chippaux 2018). The identification of *Aedes aegypti* as the main vector species of YFV in urban areas led to the implementation of vector control measures. The development of the live-attenuated 17D vaccine was another major progress in the fight against Yellow fever. The combination of these preventive strategies led to a decrease of YFV urban transmission in South America and significantly reduced human disease burden (Chippaux and Chippaux 2018; Monath and Vasconcelos 2015).

However, YFV re-emergences continue to be reported in Africa and America (Figure 7). Notably, Brazil is experiencing a major outbreak since December 2016 with more than 2,000 confirmed cases. Several factors are likely to play a role in this re-emergence including deforestation, which brings human populations closer to wild-life hosts, vector multiplication in these new urban areas as well as insufficient vaccination coverage (Chippaux and Chippaux 2018).

2.1.2.2.2. Dengue virus (DENV)

DENV infects more than 200 million people each year and is clearly the most prominent pathogenic flavivirus (Bhatt *et al.* 2013). Dengue is now endemic to most tropical and sub-tropical regions (Figure 7) and four different serotypes of the virus are currently circulating. The infection is often asymptomatic but a secondary infection with a heterologous serotype leads in some individuals to a life-threatening hemorrhagic syndrome (Wilder-Smith *et al.* 2019).

Though endemic, the global incidence of dengue increased dramatically over the past 50 years and was multiplied by more than 30-fold (Huang *et al.* 2019). A large proportion of dengue burden originates from Asia where the fast demographic expansion and urbanization contributed to this increase of case number (Huang *et al.* 2019; Wilder-Smith *et al.* 2019). The re-colonization of forests of America by *Aedes aegypti* played a role in the re-emergence of DENV in the New-World. Finally, several dengue epidemics have been mediated in urban environments by the autochthonous transmission of the virus by *Aedes albopictus* mosquitoes, in China and Japan since 2014 but also in European countries since 2015 (Huang *et al.* 2019).

2.1.2.2.3. West-Nile virus (WNV)

West-Nile disease was initially identified in 1937 in Uganda and is now endemic to Africa, Asia, Oceania, Europe and North America (Figure 7). The virus was introduced in America in 1999 from the Middle East and triggered a large outbreak in New-York city (Huhn *et al.* 2003; Nash *et al.* 2001). WNV infection is mostly asymptomatic or triggers a self-limited flu-like syndrome. West-Nile neuroinvasive disease is observed in less than 1% of infected people and includes cases of encephalitis, meningitis and paralysis (David and Abraham 2016).

WNV is maintained in an enzootic cycle between birds and its main vector, *Culex* spp. mosquitoes (Figure 6). WNV can also infect reptilian and mammalian hosts among which horses and humans constitute “dead-end” hosts as they do not develop sufficient viremia for the virus to be transmitted to a new vector. Other viral transmission routes have been described between humans via blood transfusion and organ transplantation as well as mother-to-child transplacental transmission and lactation (David and Abraham 2016).

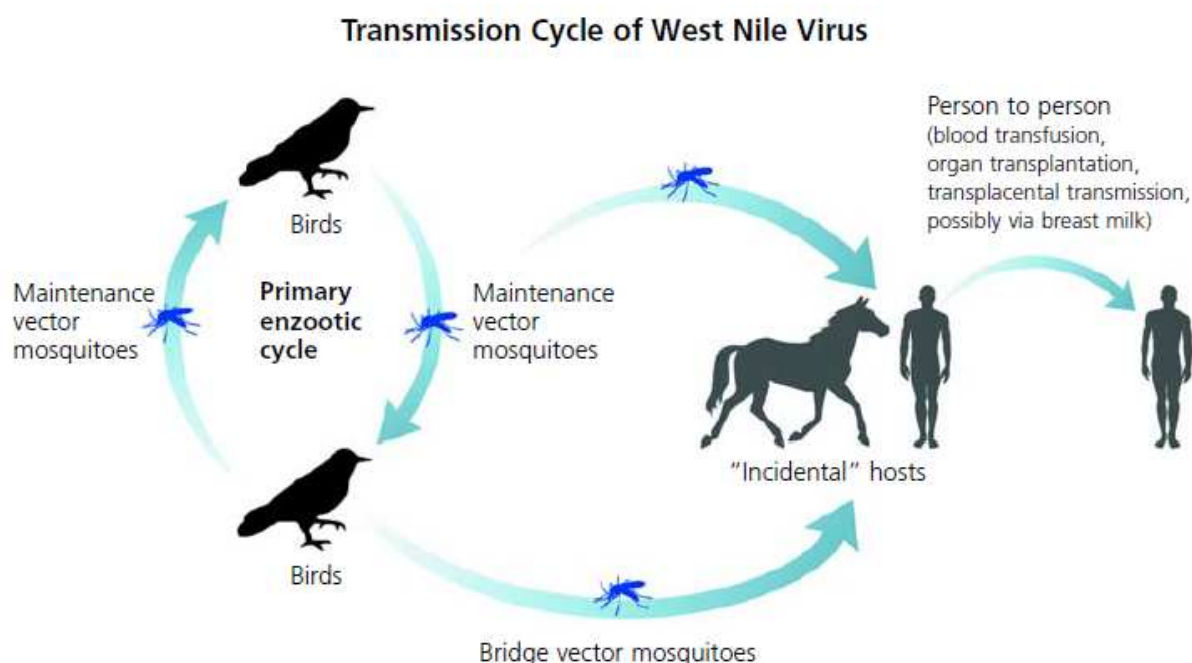


Figure 6. WNV transmission cycle.

WNV primarily cycles between *Culex* mosquitoes and birds but is able to infect non-avian incidental hosts. Human to human transmission can occur through blood transfusion, organ transplantation and mother-to-child transfer (Huhn *et al.* 2003).

The capacity of WNV to infect many different species (vectors and hosts) and to adapt to various ecological environments plays a crucial role in its geographical expansion and in emergences of new viral genotypes. WNV is now emerging in South America; circulation of the virus in horses was detected since 2005 in Colombia and spread out to neighboring regions and to Brazil where the first human case was reported in 2014 (Vieira *et al.* 2015).

2.1.2.2.4. Japanese encephalitis virus (JEV)

JEV is closely related to WNV and is endemic to Asia and Indonesia (Figure 7), where it represents the most common cause of encephalitis (Huang *et al.* 2019; Turtle and Solomon 2018). The virus cycles primarily in rural areas between *Culex* spp. mosquitoes and intermediate hosts such as birds and pigs, while horses and humans are dead-end hosts because of insufficient viremia level, as for WNV infection (Le Flohic *et al.* 2013).

The geographical distribution of JEV recently expanded to North Australian territories and to India and Nepal (Connor and Bunn 2017; Turtle and Solomon 2018). Many factors are reshaping the epidemiology of JEV, such as agricultural and geographical changes, and suggest that an increase in Japanese encephalitis incidence is likely to occur in the coming years, and that the virus has the potential to spread to new continents (Connor and Bunn 2017).

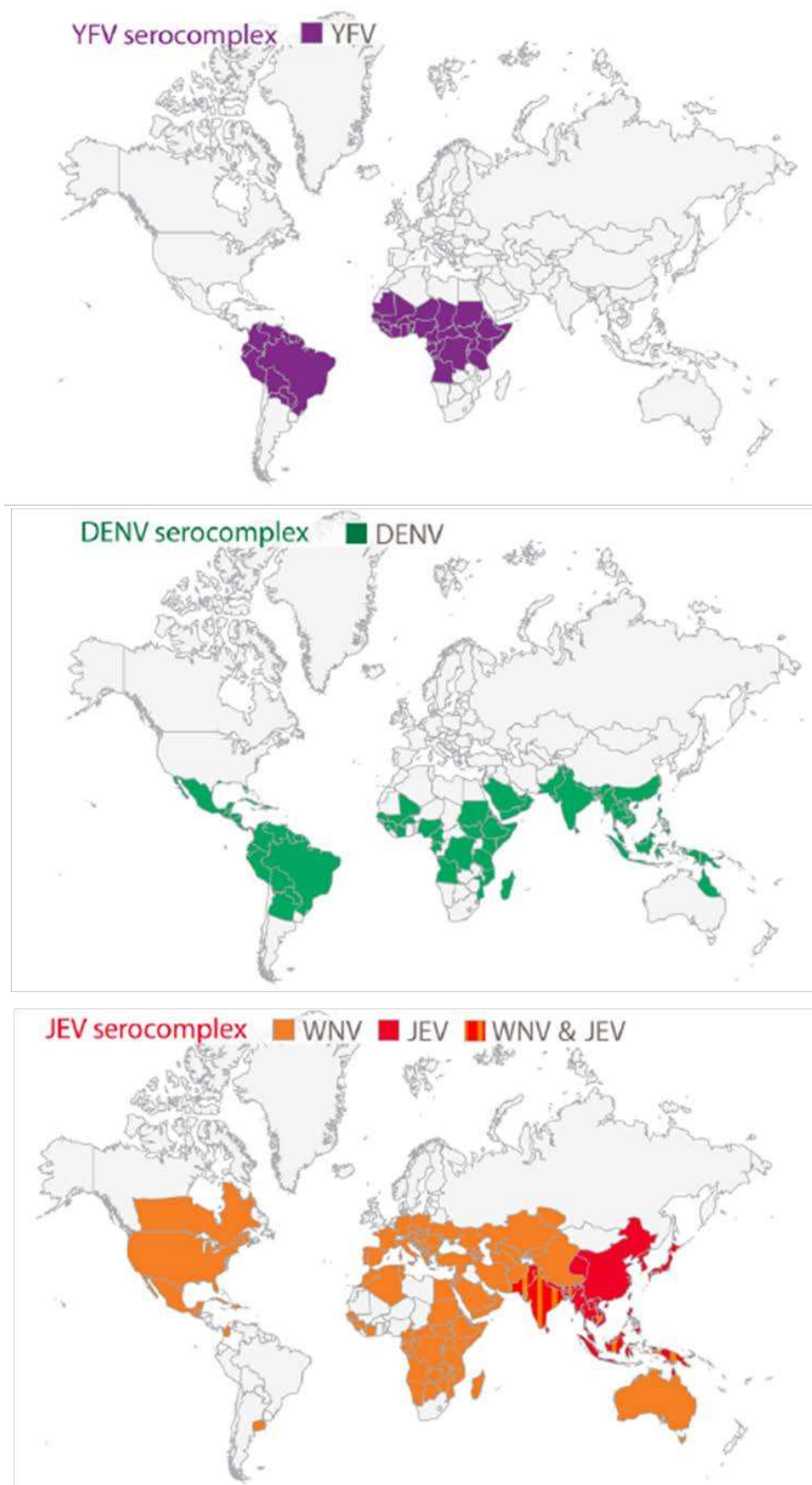


Figure 7. Global geographic distribution of YFV, DENV, WNV and JEV. Maps showing the current distributions of YFV, DENV, WNV and JEV reveal geographic regions where multiple flaviviruses co-circulate. Adapted from (Saron *et al.* 2018).

2.1.2.2.5. Zika virus (ZIKV)

The recent emergence of ZIKV was certainly unexpected and illustrates how an understudied pathogen can suddenly become a global threat for the human public health. ZIKV epidemiology and disease are described more extensively in paragraph 2.2.

2.2. Zika virus disease

This second part aims at providing an overview of what is currently known about ZIKV disease, its emergence and pathogenesis and at highlighting knowledge gaps in the field.

2.2.1. Epidemiology of ZIKV infections

2.2.1.1. *Emergence of ZIKV*

2.2.1.1.1. Discovery

ZIKV was first identified in Uganda and was named after the geographical place where it was isolated, namely the Zika forest. The virus was discovered during a surveillance study of YFV circulation and was isolated from a febrile Rhesus monkey in 1947 and then from *Aedes* mosquitoes in 1948 (Dick *et al.* 1952). The first ZIKV isolate from a human case was isolated from a 10-year-old girl in 1954 in Nigeria (Macnamara 1954). Outside Africa, ZIKV was isolated in Malaysia in 1969 from *Aedes aegypti* mosquitoes (Marchette *et al.* 1969). Before 2007, only sporadic cases of human ZIKV infection were reported and the results of several sero-surveys indicate that ZIKV is endemic to Africa and part of Asia (Musso and Gubler 2016).

2.2.1.1.2. Outbreaks

Figure 8 shows the global geographical distribution and emergence of ZIKV between 2007 and 2016.

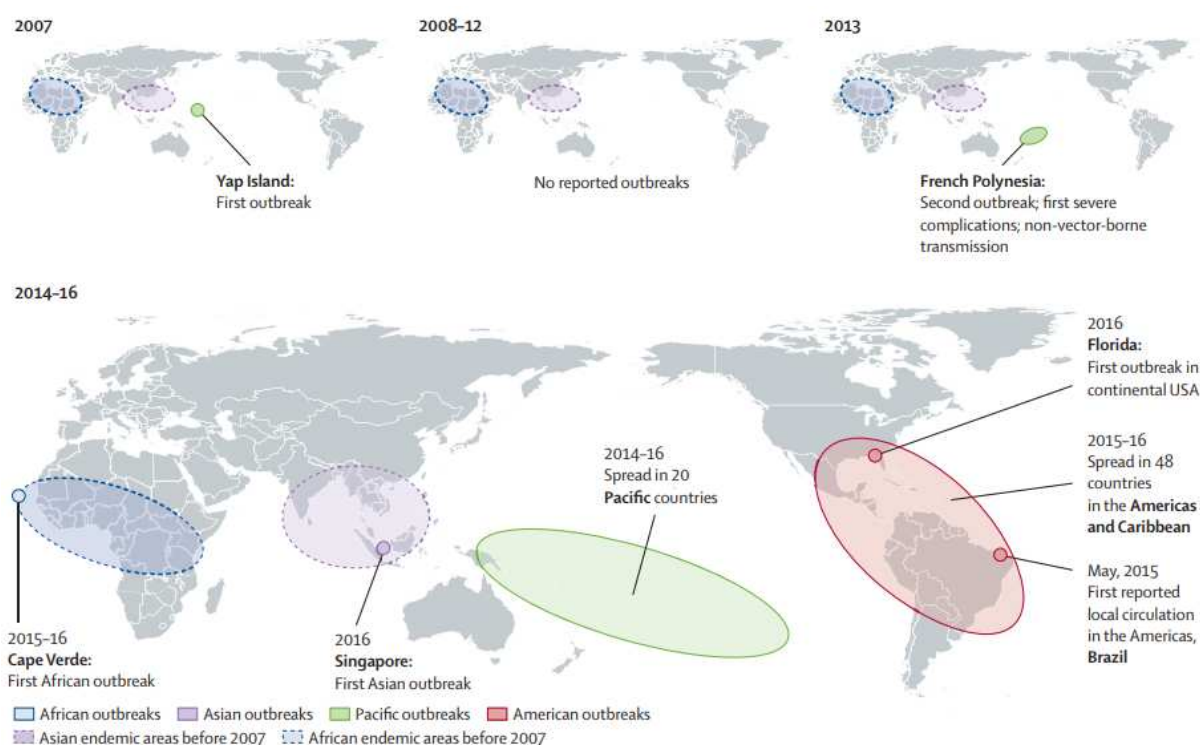


Figure 8. Zika virus outbreaks from 2007–2016.

World map of ZIKV global geographical expansion and emergence between 2007 and 2016 (Baud *et al.* 2017).

Yap, 2007

In 2007, physicians reported an outbreak of “dengue-like” disease in the Yap Islands, Micronesia, located in the Western Pacific. Although 3 patients were seropositive for DENV, physicians suspected a different disease as some patients reported symptoms which are not commonly seen in dengue, such as conjunctivitis (Duffy *et al.* 2009). Serum samples from febrile patients were sent to the CDC in Colorado and were tested positive for anti-ZIKV IgM antibodies and for ZIKV RNA. During this first outbreak, 185 cases of suspected ZIKV disease were identified among which 45 cases were later confirmed by laboratory diagnostic tests. A sero-survey was also conducted and showed that more than half of Yap residents were infected with ZIKV and that an estimate of 900 people suffered from a clinical illness which could be attributable to ZIKV infection (Duffy *et al.* 2009).

Sporadic ZIKV infections were later reported in Cambodia in 2010 (Heang *et al.* 2012), in the Philippines in 2012 (Alera *et al.* 2015) and in Thailand in 2012–2014 (Buathong *et al.* 2015) though the extent of ZIKV transmission in these countries was not assessed at that time.

French Polynesia, 2013

ZIKV transmission was not further detected in the Pacific until October 2013 when a major epidemic was reported in French Polynesia with 28,000 estimated cases of clinical ZIKV illness (Musso *et al.* 2014b). Similarly to the Yap epidemic, most patients presented with mild fever, rash, arthralgia and conjunctivitis (Cao-Lormeau *et al.* 2014).

However, in November 2013, a patient with confirmed ZIKV infection was later diagnosed with Guillain-Barré syndrome (GBS), a disease characterized by an immune-mediated ascending flaccid paralysis (Oehler *et al.* 2014). 42 cases of GBS were subsequently recorded during the French Polynesian outbreak, resulting in a 20-fold increase of GBS incidence rate correlating with ZIKV infection incidence (Figure 9) (Cao-Lormeau *et al.* 2016).

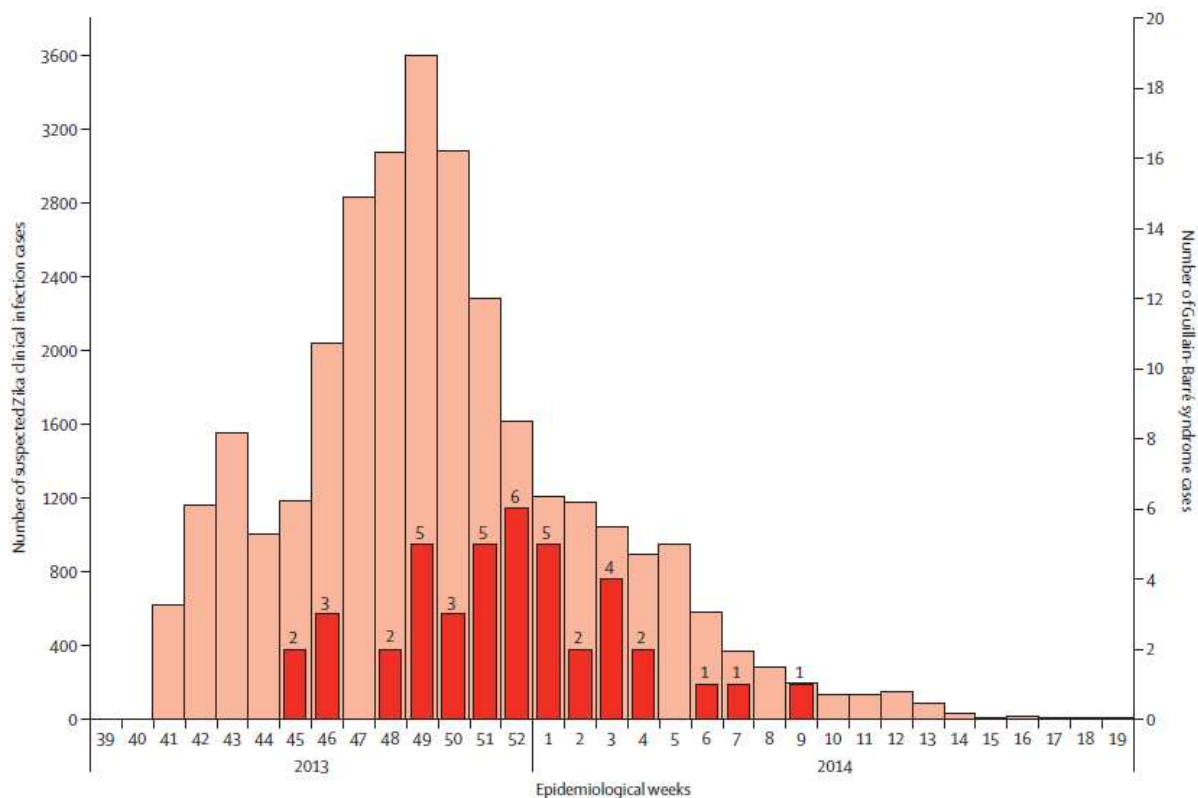


Figure 9. Temporal association between ZIKV infection and GBS cases in the French Polynesian outbreak.

Weekly cases of suspected ZIKV infections (light orange) and GBS (dark orange) in French Polynesia between October, 2013, and April, 2014 (Cao-Lormeau *et al.* 2016).

New Caledonia, 2014

Concomitantly to the French Polynesia outbreak, ZIKV spread out to other Pacific islands and triggered a new epidemic in New Caledonia in 2014. The first cases were detected in travelers returning from French Polynesia as soon as November 2013 and autochthonous transmission was observed from January 2014. Approximately 1,400 ZIKV clinical infection cases were confirmed by the end of February 2014, representing less than 1% of the population compared with 11.5% in the French Polynesian population (Dupont-Rouzeyrol *et al.* 2015). Several factors can explain this difference in epidemic magnitude: different human populations, different mosquito vectors as well as different climatic conditions (Musso and Gubler 2016).

At the same time, ZIKV infection cases were also recorded in several other islands in Oceania including the Cook Islands, Vanuatu, and the Solomon Islands (Musso and Gubler 2016).

Brazil and Cape Verde, 2015

ZIKV emerged in the Americas at the end of 2014. Outbreaks of a disease characterized by maculopapular rash, fever, arthralgia and conjunctivitis were described since February 2015 in several regions of Northeastern Brazil (Campos *et al.* 2015; Zanluca *et al.* 2015). In May 2015, ZIKV infection was confirmed in a patient from the state of Bahia, Brazil and by October 2015, this single state reported more than 50,000 suspected cases of ZIKV disease (World Health Organization 5 February 2016). Subsequent local transmission of the virus was recorded in 18 states in Brazil resulting in more than 1 million suspected cases by the end of December 2015 (Hennessey *et al.* 2016).

Between October 2015 and February 2016, Brazilian health authorities received reports of more than 4,700 cases of microcephaly in infants which represented an increase of almost 30 times that of the past five years historical average in Brazil (PANO 10 February 2016). It took several months to establish a link between these cases of microcephaly and the ZIKV outbreak. It took a few more months and mouse experiments to prove that ZIKV could actually trigger microcephaly and neuro-developmental defects (Malkki 2016). In June and July 2016, two independent studies showed that ZIKV could infect the neural progenitor cells of the developing mouse fetus and lead to microcephaly (Cugola *et al.* 2016; Li *et al.* 2016a). The epidemiological link combined with experimental evidence led health care professionals to conduct dedicated retrospective and prospective cohort studies. In August 2017, a study indeed reported 1,950 confirmed infection-related cases of microcephaly, with respect to the official diagnostic criteria at that time, which were recorded predominantly in the Northeast region of Brazil (Figure 10) (de Oliveira *et al.* 2017).

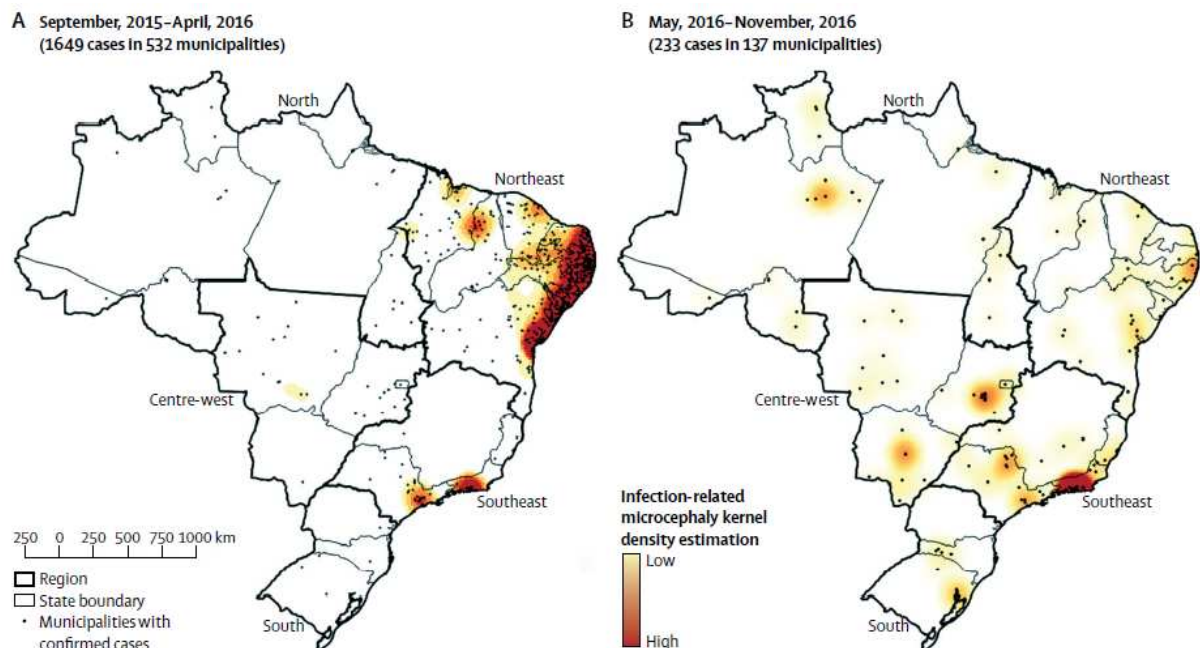


Figure 10. Distribution of confirmed infection-related microcephaly in Brazil. Cases in Brazil after the first wave of ZIKV outbreaks (A), and after the second wave, up to data closure (B) (de Oliveira *et al.* 2017).

Concomitantly, an outbreak of more than 7,000 cases of ZIKV infection was also reported in Cape Verde, Africa, in October 2015 (World Health Organization 5 February 2016), which probably originated from the Brazilian outbreak and not from African ZIKV endemic circulation.

The Americas, 2015-2016

Following the Brazilian outbreak, ZIKV rapidly spread out through South America. In February 2016, 26 countries and territories of South and Central Americas reported autochthonous transmission of the virus including Caribbean islands (World Health Organization 5 February 2016). Transmission of the virus was also observed in Florida, United States of America (USA), resulting in more than 200 confirmed infection cases (Grubaugh *et al.* 2017).

By the end of 2016, ZIKV local transmission was assessed in 48 countries and territories in the Americas with more than 175,000 laboratory-confirmed ZIKV cases and an even larger number of suspected infections (Ikejezie *et al.* 2017).

Singapore, 2016

The first outbreak of ZIKV infection in Asia occurred in August 2016 in Singapore. Despite multiple introductions of ZIKV by travelers returning from Brazil, the Singapore epidemic was not linked to the South American outbreak but probably originated from other Asian countries. Infection was confirmed in 455 cases during this 3 months outbreak (Group 2017).

Southeast Asia, 2017-2018

Improved diagnostic techniques and surveillance methods led to the investigation and reporting of autochthonous cases, and new circulation of ZIKV was described in Southeast Asia. For instance, 23 laboratory-confirmed cases of ZIKV infection have been identified in Vietnam in 2017, including one confirmed case of ZIKV-related microcephaly in a newborn girl (Moi *et al.* 2017). Even more recently, ZIKV caused an outbreak in the region of Rajasthan in India with 159 infection cases recorded by the end of 2018 (Yadav *et al.* 2019).

However, ZIKV circulation and infection incidence remain uncertain in many regions. A recent report revealed that ZIKV actually circulated in Thailand at a low level for more than 15 years, therefore suggesting that the virus could adapt to endemic persistence in favorable ecological conditions (Ruchusatsawat *et al.* 2019).

2.2.1.2. Global disease burden

According to the last WHO report (15 February 2018), evidence of ZIKV autochthonous transmission was established in 86 countries or territories worldwide (World Health Organization 15 February 2018). In January 2018, more than 220,000 local ZIKV infection cases were confirmed in the American continent (PANO 4 January 2018). Additionally, imported cases of ZIKV infection have been reported worldwide, in North America but also in Europe, in Asia and in Oceania.

However, the cumulative number of infections is most likely higher and there are many factors contributing to the underestimation of the global ZIKV disease burden. Many countries are considered at risk for ZIKV transmission but often lack suitable laboratory facilities for ZIKV infection diagnosis

as well as surveillance systems. Moreover, ZIKV generally induces a mild fever or asymptomatic infection, in which case patients do not seek medical care and ZIKV remains undetected. Finally, poor access to medical facilities also reduces the number of reported acute disease cases.

From the currently available epidemiological data, it appears that ZIKV constitutes a major threat to the global public health. The improvement of diagnostic techniques and the implementation of surveillance systems should lead to a better evaluation of ZIKV transmission and disease burden in the coming years.

2.2.1.3. Classification and evolution of ZIKV

Phylogenetic trees based on partial sequencing or on the complete coding region of the viral non-structural protein 5 (NS5) show that ZIKV is classified in a unique clade among the mosquito-borne flaviviruses and is most closely related to Spondweni virus and then to DENV (Figure 11)(Lanciotti *et al.* 2008).

The first phylogenetic analyses of ZIKV (Faye *et al.* 2014; Haddow *et al.* 2012; Lanciotti *et al.* 2008) revealed two major clusters or ZIKV lineages: the African lineage which can be divided into two sub-clades (East African and West African strains) and the Asian lineage which has been extensively studied after the recent epidemics (Figure 11).

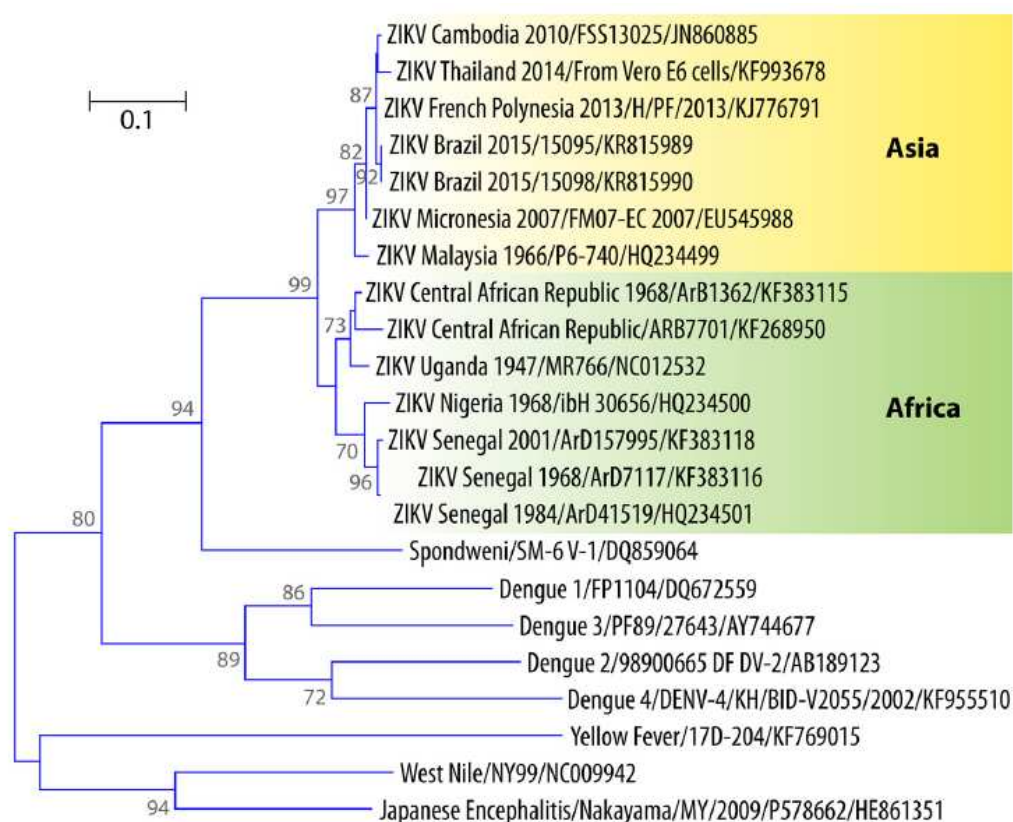


Figure 11. Phylogenetic tree of ZIKV African and Asian lineages and other pathogenic mosquito-borne flaviviruses.

Phylogenetic tree showing the genetic distances between ZIKV strains from African and Asian lineages and representative strains of Spondweni virus, DENV serotypes 1 to 4, YFV, WNV and JEV, WNV. The scale bar indicates the genetic distances in substitutions per amino acid. Support values at internal nodes illustrate the reliability of a branching split (Musso and Gubler 2016).

All the epidemic strains of the Pacific region and the Americas belong to the Asian lineage (Figure 12). Phylogenetic analyses of a high number of viral strains have increased our understanding of ZIKV evolution and geographical spread within this lineage. It is now clear that ZIKV was introduced into the Americas from French Polynesia, probably in late 2013, and then disseminated through the American continent in 2014-2015. These studies also confirmed that the 2016 Singapore outbreak strain belongs to a different sub-clade of the Asian lineage and originated directly from Asia (Liu *et al.* 2019).

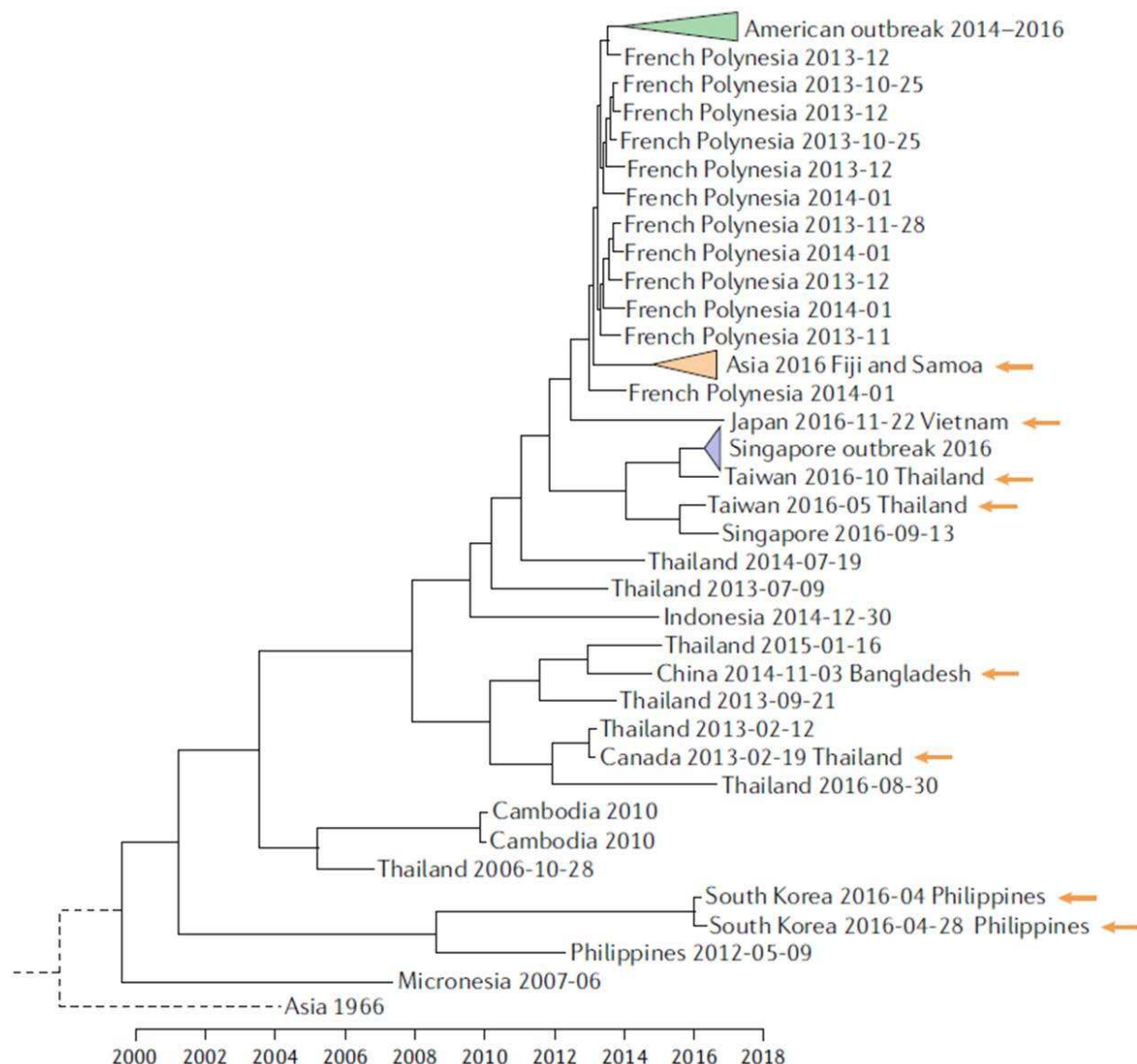


Figure 12. Phylogenetic tree of ZIKV strains of the Asian lineage.

Colored triangles illustrate the 3 sub-lineages: green, the American outbreak 2014–2016; orange, the Asian imported cases from Fiji and Samoa in 2016; and purple, the Singapore outbreak during August–September 2016. The yellow arrows indicate imported cases (Liu *et al.* 2019).

2.2.1.4. Ecology and transmission

ZIKV is mainly a vector-borne disease but additional human-to-human transmission routes have been described.

2.2.1.4.1. Vector-borne transmission

In Africa, ZIKV is enzootic and presumably cycles between non-human primates (NHPs) and mosquitoes, with periodic epizootics in monkeys, similarly to what is known for YFV and DENV. However, ecological conditions vary largely between different countries and continents, and imply diverse vector-borne transmission modes.

More than 30 different species of wild-captured mosquitoes infected with ZIKV have been reported worldwide, 22 of which belong to the *Aedes* genus which is considered as the principal mosquito taxon in ZIKV transmission. Only 6 species are implicated in the urban transmission cycle of ZIKV among which *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus*. The vector competence, i.e. the capacity to transmit the virus, of these 3 species has also been demonstrated in laboratory conditions (Gutierrez-Bugallo *et al.* 2019).

Vertebrate hosts also play an important role in the maintenance of ZIKV during the sylvatic cycle as reservoirs or amplifying hosts. NHPs are the main hosts of ZIKV in nature, although many wild species have been reported to be susceptible to ZIKV including birds, reptiles and amphibians (Gutierrez-Bugallo *et al.* 2019).

Considering the experience acquired with YFV and the explosive spread of ZIKV in the Americas, notably in Brazil, it seems that ZIKV has the potential to establish a new enzootic cycle in this region. Additionally, the global distribution of *Aedes aegypti* and *Aedes albopictus* mosquitoes will further expand, especially in unoccupied habitats such as new urban areas with suitable climatic conditions (Kraemer *et al.* 2019). This expansion is predicted to occur in all continents, including Europe and North America, and to dramatically increase the risk of ZIKV spread. Nevertheless, ecological conditions vary largely between countries and continents: for example, NHP species broadly differ between the Old and New worlds and some mosquito species have restricted geographical distributions. This results in diverse transmission cycles, which have to be investigated in each of these specific regions to get a full picture of ZIKV mosquito-borne distribution.

2.2.1.4.2. Sexual transmission

ZIKV sexual transmission represents a new mode of infection for flaviviruses. ZIKV transmission by sexual contact was first suggested in 2008, between a scientist who became infected with ZIKV in Senegal and his wife. After returning to the USA, the patient reported usual symptoms of Zika fever, and hematospermia a few days later, while at the same time, his wife developed a ZIKV-like disease. Because his wife had not traveled internationally and because the couple was sexually active, transmission via the semen was considered (Foy *et al.* 2011). This hypothesis was further supported by the isolation of the virus from the semen of a patient infected with ZIKV during the outbreak of French Polynesia in 2013 (Musso *et al.* 2015).

Later on, several studies reported excretion of ZIKV in the semen. Persistence of the virus was also highlighted as ZIKV RNA could be found in the semen of a patient 370 days after the onset of disease (Barzon *et al.* 2018). A recent study showed that ZIKV RNA could be detected in the semen of more than 60% of men with symptomatic ZIKV infection during one month after illness onset, on

average. Consistent with these results, most cases of ZIKV sexual transmission between a man and his partner have occurred within 3 weeks after the patient's illness onset (Mead *et al.* 2018).

The capacity of ZIKV to be transmitted by sexual contact raised new questions about the risk for pregnant women sexually exposed to the virus and on the impact of this pathogen on male fertility. Animal and cell culture experiments have already increased our knowledge on these subjects (briefly presented in paragraphs 2.2.4 and 2.3.2.2), but additional studies are required to better understand the effects and mechanisms of sexual transmission of ZIKV.

2.2.1.4.3. Materno-fetal and perinatal transmissions

Perinatal transmission of ZIKV was first described in two mothers and their newborns during the outbreak in French Polynesia in 2013. Both mothers and their infants were viremic a few days before and after childbirth, respectively; suggesting a transmission during delivery (Besnard *et al.* 2014).

During the Brazilian epidemic, detection of ZIKV RNA in the amniotic fluid of two women diagnosed with fetal microcephaly provided the first evidence of trans-placental transmission of ZIKV (Oliveira Melo *et al.* 2016). Materno-fetal transmission of ZIKV was confirmed in South America in prospective cohort studies of pregnant women (Hoen *et al.* 2018; Pomar *et al.* 2017) and by the detection of the virus in biological samples from infected pregnant women and their fetuses: blood, amniotic fluid, umbilical cord, placental and fetal tissue biopsies (Driggers *et al.* 2016; Martines *et al.* 2016; Melo *et al.* 2016).

Despite numerous case series reports, estimating the incidence of materno-fetal transmission remains a challenge; only symptomatic pregnant women were included as cases in most of these studies and ZIKV infection diagnostic methods can lead to false-negative and false-positive results. One study reported a vertical transmission rate of 11%, determined by the detection of ZIKV RNA or antibodies in the amniotic fluid and in samples from the neonate at birth (Pomar *et al.* 2017), which was later re-evaluated up to 18% after extensive investigation of neonatal and placental samples (Pomar *et al.* 2018). Large-scale epidemiological studies and improved diagnostic tools are needed to get a more precise estimate of materno-fetal transmission rate.

Besides, ZIKV RNA and infective particles have been detected in human breast milk (Cavalcanti *et al.* 2017b; Dupont-Rouzeyrol *et al.* 2016), which can be considered as a potentially infectious body fluid. Several systematic reviews (Mann *et al.* 2018; Sampieri and Montero 2019) reported that there is currently not enough evidence to confirm breastfeeding transmission of ZIKV. WHO guidelines regarding infant feeding in areas of ZIKV transmission remain unchanged: “the benefits of breastfeeding for the infant and mother outweigh any potential risk of Zika virus transmission through breast milk” (World Health Organization 2019a). However, a few recent case reports further suggest that ZIKV could be transmitted to the infant via maternal milk and emphasize the need for additional studies on the subject (Blohm *et al.* 2018; Giovanetti *et al.* 2018).

2.2.1.4.4. Other transmission routes

The potential of ZIKV transmission by blood transfusion was demonstrated during the outbreak in French Polynesia when ZIKV RNA could be detected in up to 3% of asymptomatic blood

donors (Musso *et al.* 2014a). Since then, two cases of ZIKV transmission by blood or platelet transfusion have been reported in Brazil (Barjas-Castro *et al.* 2016; Motta *et al.* 2016), leading WHO to emit new guidelines for blood donation in ZIKV endemic regions.

Finally, transmission of ZIKV by physical contact, via body fluids (urine, tears, saliva, sweat) has been reported, though infrequently (Swaminathan *et al.* 2016).

2.2.2. ZIKV clinical syndromes

2.2.2.1. Clinical features in adults

2.2.2.1.1. Asymptomatic infections

The majority of ZIKV infections does not trigger any sign of illness, the proportion of symptomatic ZIKV infections is generally comprised between 20 and 25% (Flamand *et al.* 2017; Fourie *et al.* 2018) and, of note, the incidence of ZIKV clinical disease is similar in pregnant women compared with the global population (Flamand *et al.* 2017). However, a higher incidence of symptomatic infections has been reported in 2014 during the outbreak in French Polynesia, with ZIKV clinical disease (Furtado *et al.* 2016) being diagnosed in approximately 50% of adults and 30% in school-children (Aubry *et al.* 2017).

2.2.2.1.2. ZIKV acute febrile illness

The incubation period of ZIKV, i.e. the delay between virus acquisition and the onset of symptoms, varies between 3 and 23 days with a median duration of 5-6 days (Fourie *et al.* 2018). Among ZIKV clinical disease cases, macopapular pruritic rash is the most frequent symptom, reported in more than 90% of patients. The other common symptoms are low-grade transient fever (65%), arthralgia (65%), myalgia (50%), non-purulent conjunctivitis (55%) and retro-orbital pain (40%). Variable clinical signs have been described in less than 30% of the cases including gastro-intestinal and respiratory symptoms as well as edema and peripheral bleeding (Brasil *et al.* 2016; Cerbino-Neto *et al.* 2016; Duffy *et al.* 2009; El Sahly *et al.* 2019). Genito-urinary symptoms have been described in a few men infected with ZIKV such as hematospermia (observed in approximately 5% of the cases) (Mead *et al.* 2018) and prostatitis (Foy *et al.* 2011).

Children who are infected by ZIKV after birth display similar symptoms to those seen in adults (Goodman *et al.* 2016; Karwowski *et al.* 2016).

2.2.2.1.3. ZIKV-associated complications

As previously mentioned, GBS in adults was the first severe complication of ZIKV infection to be reported, a temporal correlation was indeed observed between ZIKV epidemic and the increase of GBS incidence in 2013 in French Polynesia (Cao-Lormeau *et al.* 2016). Other neurologic complications were further characterized in adults infected with ZIKV and are described hereafter.

Guillain-Barre syndrome (GBS)

GBS is an immune-mediated disorder of the peripheral nervous system resulting in an acute ascending paralysis. The most frequent clinical manifestations of ZIKV-induced GBS were symmetrical limb weakness with areflexia and paresthesia (Lannuzel *et al.* 2019; Munoz *et al.* 2017). Cranial nerves involvement was also described, predominantly facial palsy or multiple cranial nerve palsy, as well as dysphagia (Dirlikov *et al.* 2018). ZIKV-induced GBS is characterized by a rapid progression to clinical nadir, the time point when the symptoms are the worst, which differs from GBS of other etiologies. The median delay was 5-10 days between ZIKV infection and GBS onset, whereas it ranges for example between 2 and 4 weeks in the case of *Campylobacter jejuni*-induced GBS (Munoz *et al.* 2017).

Based on epidemiological studies, the increased incidence of GBS was up to 20-times the baseline in French Polynesia (Cao-Lormeau *et al.* 2016) and between 2 and 10-times the baseline in the Americas (Dos Santos *et al.* 2016).

Other complications

Several observational studies have assessed the incidence and spectrum of neurologic manifestations secondary to ZIKV infection in adults (Brito Ferreira *et al.* 2017; da Silva *et al.* 2017; Lannuzel *et al.* 2019), but further studies will be required to evaluate the long-term consequences of ZIKV disease.

A cohort study in the French West Indies showed that GBS was the most frequent neurological complication of ZIKV infection followed by encephalitis or encephalomyelitis accounting for 20% of the neurological cases, cranial nerve palsies (10%) and other disorders of the peripheral nervous system (7%), and stroke (1%). A strong correlation was found between the number of suspected ZIKV infections and the incidence of ZIKV-associated neurological disease (Figure 13) and the annual rate of encephalitis in Guadeloupe was 4 times higher the baseline prevalence (Lannuzel *et al.* 2019).

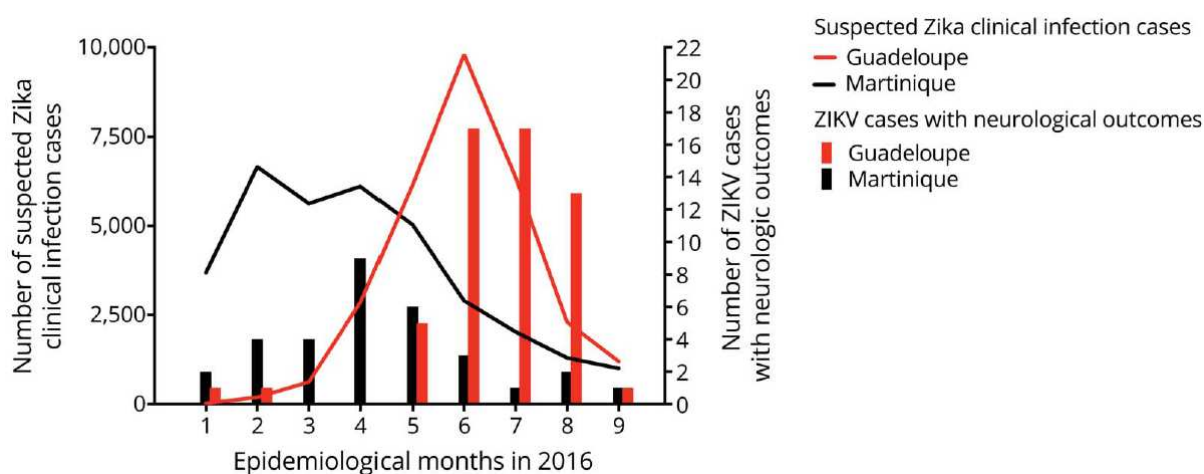


Figure 13. Correlation between suspected ZIKV infections and ZIKV cases with neurological outcomes in Guadeloupe and Martinique in 2016.

Monthly cases of suspected ZIKV infections (lines) and ZIKV cases with neurological outcomes (bars) in the French West Indies between January, 2016, and September, 2016 (Lannuzel *et al.* 2019).

A few cases of non-neurological complications have also been reported after ZIKV infection in adults including uveitis (Furtado *et al.* 2016; Kodati *et al.* 2017) and thrombocytopenia (Boyer Chammard *et al.* 2017; Karimi *et al.* 2016).

2.2.2.2. Clinical features and consequences of ZIKV infection during pregnancy

The consequences of ZIKV infection during pregnancy started to be described during the epidemic in Brazil in 2015 which was rapidly declared as a global public health emergency by WHO in 2016. Microcephaly was the first congenital malformation to be linked to ZIKV infection and the term Congenital Zika Syndrome (CZS) was subsequently coined to describe the array of birth defects associated with ZIKV infection. ZIKV is now often referred to as a member of TORCH pathogens (*Toxoplasma gondii*, other agents, rubella virus, cytomegalovirus, and herpes simplex virus), a group of pathogens causing human congenital diseases (Coyne and Lazear 2016).

2.2.2.2.1. Prevalence and risk factors of adverse outcomes

ZIKV infection during pregnancy is estimated to result in birth defects in overall 5-13% of cases (Walker *et al.* 2019). In a recent study, the rate of adverse outcomes was investigated more specifically in fetuses and newborns with confirmed congenital ZIKV infection and was estimated as follows : 20% of cases presented with mild signs, 21% had severe complications and 14% resulted in fetal loss (Pomar *et al.* 2018). However, these rates are only indicative as they depend on many factors and the true prevalence of CZS will require large cohorts and longitudinal studies to be precisely calculated. Fetal growth can also be impacted by ZIKV: several studies have shown that intra-uterine growth restriction (IUGR) was observed in approximately 9% of neonates congenitally exposed to the virus (Brasil *et al.* 2016; Walker *et al.* 2018). Placental injury and inflammation have been reported after ZIKV infection (Shapiro-Mendoza *et al.* 2017) and may lead to fetal damage or IUGR (Adibi *et al.* 2016).

As mentioned previously, pregnancy is not associated with more frequent clinical disease or complications for the mother than in other adults (Flamand *et al.* 2017). Importantly, maternal symptoms are not associated with a higher risk of fetal loss or CZS (Halai *et al.* 2017; Paixao *et al.* 2018) than in asymptomatic infected pregnant women. To date, it is still unclear whether a high or prolonged viremia represents a risk factor for adverse fetal outcomes (Driggers *et al.* 2016; Halai *et al.* 2017). As for other TORCH infections, gestational age at the time of infection has an impact on the risk for CZS: while vertical transmission can occur throughout entire pregnancy, ZIKV infection during the first and second trimesters of pregnancy is associated with a higher risk of fetal loss and CZS complications (Brady *et al.* 2019; Brasil *et al.* 2016; Hoen *et al.* 2018; Pomar *et al.* 2017).

2.2.2.2.2. Congenital Zika syndrome (CZS)

As our knowledge of ZIKV-associated adverse effects during pregnancy is still growing, the definition of CZS keeps evolving and has not yet reached a consensus. The CDC has chosen a restrictive definition which comprises five features differentiating CZS from other congenital infections: (i) severe microcephaly; (ii) thin cerebral cortices with calcifications; (iii) damage to the back of the eye; (iv) congenital contractures; and (v) marked early hypertonia (Moore *et al.* 2017). Other studies propose an inclusive definition and list all potential signs associated with CZS based on

clinical and diagnostic features (Pomar *et al.* 2018; Sanz Cortes *et al.* 2018). CZS semiology and characteristics are described in the following paragraphs.

Severe CZS

The first descriptions of the clinical findings associated with CZS were restricted to infants displaying a severe phenotype, characterized by extensive brain defects and microcephaly (Table 1). Microcephaly is most often defined by a head circumference of less than 2 standard deviations from the reference charts, and in the case of CZS, is usually associated with an atypical shape of the skull and an occipital excess of skin. Other classical features include a brain volume loss with ventriculomegaly and abnormalities of the corpus callosum (a bundle of commissural fibers connecting the cerebral hemispheres). Impaired cortical migration and disorders of cortical formation are reported in 80% of the cases and are characterized by a reduction or defects in gyration. Intracranial calcifications caused by focal necrosis are common at the gray-white matter junction but can be localized to any brain region. Cerebellar injury is also frequently reported (Del Campo *et al.* 2017; Honein *et al.* 2017; Pomar *et al.* 2017; Soriano-Arandes *et al.* 2018; Walker *et al.* 2019).

Brain abnormalities (prevalence)	Neural tube defects
Microcephaly (33-64%)	Anencephaly
Ventriculomegaly (63-92%)	Encephalocele
Intracranial calcifications (71-92%)	Spina bifida
Cerebral atrophy (92%)	
Abnormal cortical formation (79-82%)	
Corpus callosum dysgenesis (71-100%)	
Cerebellar abnormalities (21-82%)	

Table 1. CNS birth defects related to congenital ZIKV infection.

The prevalence of eye disorders in CZS is about 25% and the spectrum of abnormalities ranges from microphthalmia or anophthalmia to optic chiasma hypoplasia, coloboma (missing piece of tissue in structures that form the eye) and cataract (Table 2). Arthrogryposis and sensori-neural hearing loss can result from CNS dysfunctions described above (Del Campo *et al.* 2017; Pomar *et al.* 2017; Soriano-Arandes *et al.* 2018; Walker *et al.* 2019).

Eye abnormalities	Consequences of CNS dysfunction
Microphthalmia or anophthalmia	Contractures (arthrogryposis, congenital hip dysplasia)
Coloboma	
Congenital cataract	Sensori-neural hearing loss
Intraocular calcifications	
Chorioretinal abnormalities	
Optic nerve atrophy	

Table 2. Other birth defects related to congenital ZIKV infection.

Spectrum of congenital ZIKV-associated injury and impairments

Recent studies have started to extend the spectrum of fetal and neonatal injury linked to congenital ZIKV infection, particularly thanks to the use of more sensitive diagnostic tools such as prenatal or postnatal magnetic resonance imaging (MRI). A few studies reported several cases of progressive postnatal microcephaly developing in children that were apparently unaffected at birth (van der Linden *et al.* 2016; Vianna *et al.* 2019). Clinical neurologic impairments have also been described in both infants affected and unaffected at birth, such as dysphagia, movement disorders and epilepsy. Motor abnormalities are associated with 77-100% CZS cases, while the prevalence of epilepsy varies between 9 and 95% of congenital ZIKV infections (Alves *et al.* 2018; Del Campo *et al.* 2017; Pessoa *et al.* 2018; van der Linden *et al.* 2018).

As for other TORCH infections, congenital heart disease has been reported in CZS, though inconsistently (Cavalcanti *et al.* 2017a; Orofino *et al.* 2018). Finally, the tropism of ZIKV for the male genital organs is quite concerning as it could contribute to infertility.

Potential for long-term neurocognitive deficits?

Our knowledge of CZS spectrum keeps evolving, especially regarding the potential for long-term consequences of neuro-developmental defects. One observational study has described the outcomes of CZS in children between 19 and 24-months of age and reported concerning functional and neurological findings (Satterfield-Nash *et al.* 2017). Half of the affected children face sleeping and feeding difficulties, 60-70% display an impaired response to auditory or visual stimuli and 80% experience severe motor deficiencies. A recent study followed infants with confirmed congenital ZIKV infection between 7 and 32 months of age, and revealed that more than 30% of the children displayed below-average neurodevelopment and/or vision or hearing impairments (Nielsen-Saines *et al.* 2019).

More longitudinal studies are needed to appreciate the long-term sequelae of CZS and how they might predispose older children and adolescents to learning difficulties or psychiatric disorders. Importantly, a follow-up should be proposed to all exposed children, regardless of their status at birth, symptomatic or asymptomatic.

2.2.3. Laboratory diagnosis of ZIKV infection

Detection of Zika viral particles by titration on cultured cells is possible but is not used in daily clinical activities. Acute phase diagnosis relies mainly on the detection of ZIKV RNA or of specific antibodies targeted against the virus.

2.2.3.1. Molecular detection of ZIKV RNA

Molecular detection of ZIKV RNA is performed by reverse transcription and quantitative polymerase chain reaction (RT-qPCR) most frequently on blood and urine. The virus is present only transiently in plasma during febrile illness but the window of detection can be slightly extended by testing urine (Bingham *et al.* 2016) or whole blood (Murray *et al.* 2017). Cumulative results from different studies show that sensitivity of RT-qPCR in blood and urine during the first 2 weeks of

disease reaches 75% of serologically confirmed infections (Munoz-Jordan 2017). In addition, time of infection is always difficult to determine, therefore negative RT-qPCR results do not exclude infection.

2.2.3.2. Serology

Serological diagnosis relies on the detection of specific IgG or IgM by Enzyme-linked immunosorbent assay (ELISA). Immunodiagnosis is very important considering that the majority of ZIKV infections are asymptomatic. However, ZIKV-specific serology is challenging because of the cross-reactivity with other flaviviruses. IgM antibodies targeted against ZIKV can be detected from 4-5 days until 12 weeks after illness onset. For samples collected within the first week of disease, the combination of negative RT-qPCR and IgM antibodies results suggests the absence of recent infection (Rabe *et al.* 2016). Both molecular and serology diagnostic tests are commercially available although low cost and highly specific ZIKV testing methods still need to be developed for use in resource-limited countries.

2.2.4. Pathophysiology of ZIKV infections

Combined efforts of the scientific community and the use of complementary approaches to study ZIKV has led to great progress in understanding the biology and the pathogenesis of the virus. Current knowledge on ZIKV target cells, tissue tropism and interplay with the host immune response is briefly reviewed hereafter.

2.2.4.1. ZIKV tissue and cell tropism

ZIKV exhibits extensive cell and tissue tropism and many different cell types have been found to be permissive to ZIKV. Skin and lymphoid cells are the first to encounter the virus after a mosquito bite. Both dermal fibroblasts and epidermal keratinocytes can sustain ZIKV infection as well as most peripheral lymphoid cells: monocytes, macrophages, neutrophils and dendritic cells. The major targets of ZIKV are immune privileged organs such as the nervous system, the eyes, the genitalia and the placenta (Figure 14). Consistent with its ability to induce neurological disease, ZIKV has the capacity to infect neural cells including neural progenitor cells and mature neurons as well as astrocytes and glial cells. In the eyes, cells of the cornea, the retina and the optic nerve can be infected by ZIKV. The virus can also invade the testes, especially the spermatogonia, Sertoli and Leydig cells, and the uterus and vagina. Hofbauer cells (placental macrophages), trophoblasts and endothelial cells of the placenta are also permissive to the virus. Finally, ZIKV was also detected, though less frequently, in pneumocytes, hepatocytes and renal epithelial cells from clinical samples (Miner and Diamond 2017; Ngono and Shresta 2018; Shaily and Upadhyaya 2019).

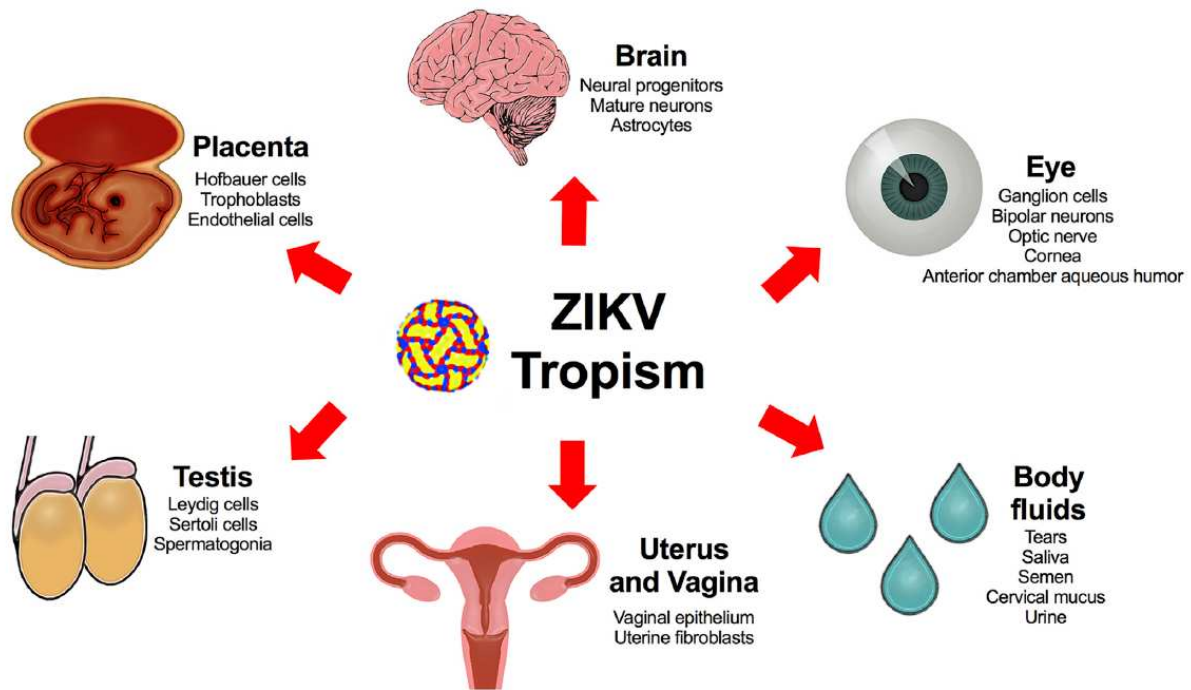


Figure 14. ZIKV tissue and cell tropism.
Extracted from (Miner and Diamond 2017).

ZIKV dissemination after a mosquito bite is comparable with other flaviviral infections, starting with initial replication of the virus at the inoculation site, which then propagates to lymphoid tissues and blood and finally spreads to the target tissues. However, several questions remain, for instance by which mechanisms the virus crosses the blood-brain barrier (BBB), the placenta or persists in specific tissues such as the testes. Insights on these issues have been brought by the use of animal models of ZIKV infection, some of them will be mentioned in part 2.3.

2.2.4.2. Immune response to ZIKV infection

Disease severity and pathogenesis of ZIKV infection are influenced by many factors including host immunity, pathogen virulence and virus-host interactions. Learning from DENV immunopathogenesis, the innate immune response appears to be the first line of defense of the host after ZIKV infection but also the target of viral counterattack. The outcome of the disease also depends on adaptive immune mechanisms among which T-cell and antibody responses.

2.2.4.2.1. Innate immune response

Host restrictions factors and the interferon (IFN) response are the primary mechanisms of innate immunity after a viral infection (Chemudupati *et al.* 2019; van den Broek *et al.* 1995). Host restriction factors are intrinsic cellular molecules demonstrating direct antiviral activity. The IFN system comprises type I interferons (IFN- α , β), type II interferon (IFN- γ) and type III interferons (IFN- λ 1-4). Sensing of the virus by cellular pattern recognition receptors (PRRs) rapidly triggers the IFN response, which induces an antiviral state within hours of viral infection.

Host restriction factors of ZIKV

Host restriction factors were first described in studies on retroviruses pathogenesis, especially on Human immunodeficiency virus (HIV). Restriction factors play a critical role in the innate immune response against viruses, and are often essential for slowing down viral replication. These proteins are usually constitutively expressed but often upregulated by the IFN system; they have very diverse functions and target almost every step of the viral life cycle. Several proteins have been shown to restrict flaviviruses: the IFITM (IFN-induced trans-membrane) proteins inhibit membrane fusion, the combination RNaseL/OAS1 degrades viral RNA, PKR (Protein kinase R) and IFIT (IFN-induced protein with tetratricopeptide repeats) proteins block viral protein translation, and Tetherin prevents virus release (Chemudupati *et al.* 2019; Kluge *et al.* 2015).

A few studies have already confirmed the role of some of these host restriction factors against ZIKV: IFITM1 and IFITM3 (Savidis *et al.* 2016), RSAD2 (Radical S-adenosyl methionine domain containing 2, also named Viperin) (Panayiotou *et al.* 2018; Van der Hoek *et al.* 2017) and TRIM56 (Tripartite motif-containing 56) (Yang *et al.* 2019).

Sensing of ZIKV infection

Multiple PRRs operate collectively to sense viral infection through the recognition of pathogen-associated molecular patterns (PAMPs) such as viral nucleic acids or proteins. Among PRRs, Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) have been shown to be implicated in ZIKV recognition. ZIKV replication takes place in the cytosol and generates both single and double-stranded RNAs that are detected by RIG-I (Figure 15). Mitochondrial antiviral-signaling protein (MAVS) is recruited upon binding and triggers the activation of the kinases IKK ϵ (Inhibitor of kappa B kinase epsilon) and TBK1 (TANK-binding kinase 1), which in turn activate IRF3/IRF7 (IFN regulatory factor) transcription factors and IFN production. ZIKV nucleic acids can also be detected in endosomal compartments by TLR3 (double-stranded RNA) and TLR7/8 (single-stranded RNA) which activate IFN production through the recruitment of TRIF (TLR adaptor molecule 1) or MyD88 (Myeloid differentiation primary response gene 88) followed by activation of IRF3/IRF7 (Ngono and Shrestha 2018; Pardy *et al.* 2019; Valdes Lopez *et al.* 2019).

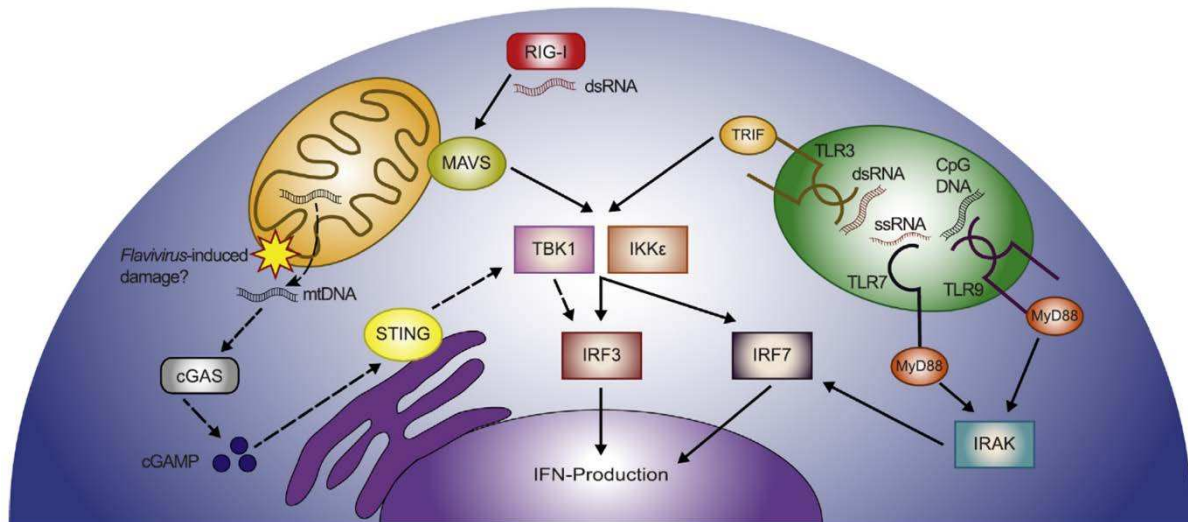


Figure 15. Sensing of ZIKV by PRRs and induction of IFN production.

ZIKV sensing by RLRs and TLRs leads to IFN production. Solid arrows indicate canonical signaling pathways for RIG-I and TLRs. Dashed arrows indicate proposed pathway by which flavivirus infection could lead to activation of cGAS-STING, another PRR. (Pardy *et al.* 2019)

IFN response and induction of antiviral state

Once secreted, type I IFNs induce the expression of hundreds of interferon-stimulated genes (ISGs) by autocrine and paracrine signaling. More explicitly, type I IFNs bind to common type I IFN receptor (IFNAR), a heterodimer between IFNAR1 and IFNAR2 chains and then signal through the JAK-STAT pathway (Janus kinase-Signal transducer and activation of transcription) (Figure 16).

In the case of DENV infection, type II IFN response not only restricts initial viral replication but also plays a role at later stages of the disease, partly by activating the transcription of inflammatory and chemo-attractant cytokines (Shrestha *et al.* 2004). IFN- γ is likely to act similarly after ZIKV infection and, indeed, has been shown to increase the recruitment of CD4⁺ and CD8⁺ T-cells (Elong Ngono *et al.* 2017). In contrast to IFNAR which is expressed in most cells, IFNLR expression is restricted on epithelial cell surfaces. In the case of ZIKV infection, IFN- λ has been shown to mediate local immunity at the maternal-fetal interface (Jagger *et al.* 2017).

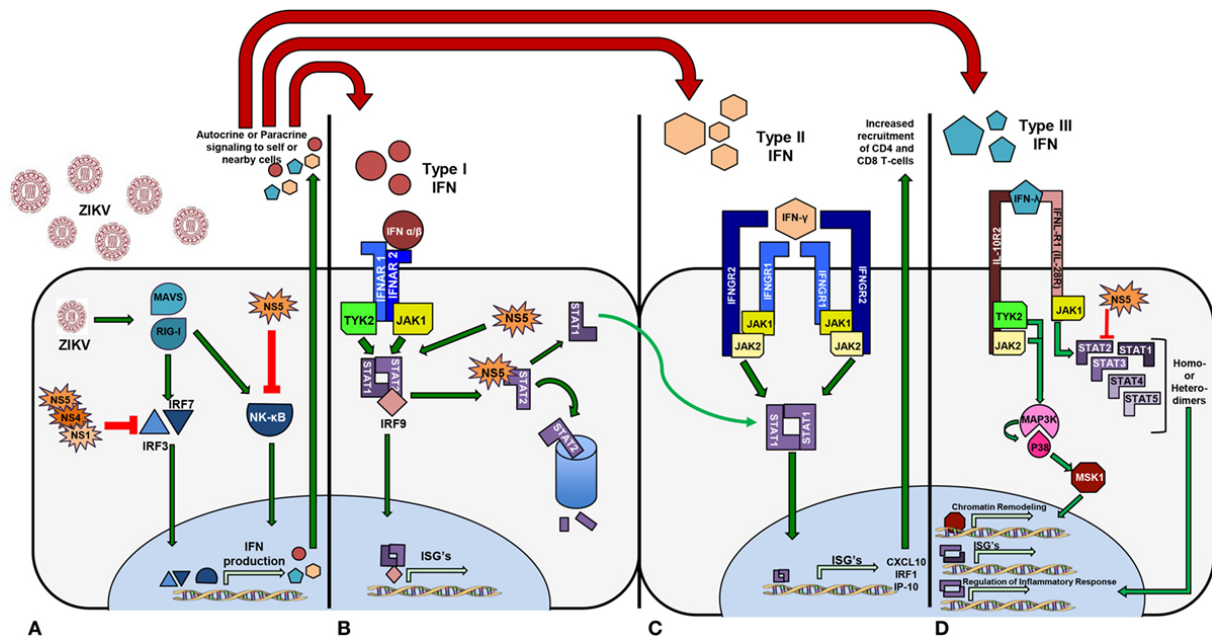


Figure 16. Induction and antagonism of the IFN system by ZIKV infection.

(A) ZIKV sensing by PRRs induces IFN production. (B) Type I IFN response signaling. (C) Type II IFN response signaling. (D) Type III IFN response signaling. (Beaver *et al.* 2018)

To date, more than 500 ISGs have been defined as genes with differential gene expression in response to IFNs (de Veer *et al.* 2001). These ISGs exert many different functions in the modulation of nucleic acid integrity (for example OAS/RNase L genes), viral entry in the cell (IFITM genes), protein translation (PKR gene) etc. Other ISGs are expressed in a second wave and act as repressors of these innate immune mechanisms, in order to shut down the IFN response upon resolution of the viral infection. While several of these ISGs are well characterized, the functions of a vast majority are still unknown (Schoggins 2014). Interestingly, part of these genes are often induced in the infected cell even in the absence of IFN signaling, through activation of IRFs and other transcription factors, and are referred to as virus stimulated genes (VSGs) (Green *et al.* 2018).

Zika virulence factors and antagonism of the innate immune response

A bunch of evidence indicate that the IFN system, and especially the type I IFN response, is the central mediator of protection against ZIKV. Almost all viruses, and ZIKV is no exception, have evolved mechanisms to evade the IFN system. In particular, the antagonism of the IFN system by DENV has been extensively investigated, revealing that multiple DENV non-structural proteins are implicated in this evasion mechanism (Table 3). For example, several steps of the RIG-I/MDA5 signaling pathway are targeted by either DENV NS2A, NS4B or NS2B-NS3 proteins. DENV NS5 protein has been reported as the most potent antagonist of the type I IFN cascade, in particular via its interaction with STAT2 leading to its proteasomal degradation. In addition, flaviviruses have the capability of producing subgenomic flavivirus RNA (sfRNA) as a result of incomplete digestion by the cellular exonucleases, which also exert antagonistic properties against the IFN response (Cumberworth *et al.* 2017; Gack and Diamond 2016; Ngono and Shrestha 2018).

Interferon antagonist	Virus	Activity
NS2A	DENV	Inhibition of the JAK/STAT signalling pathway by decreasing STAT1 phosphorylation
	KUNV	Suppression of IFN- β transcription
NS4B	DENV	Completely blocks interferon signalling (in combination with NS2A and NS4A)
	DENV, YFV, WNV	Inhibition of the JAK/STAT signalling pathway by decreasing STAT1 phosphorylation
	YFV	Interacts with STING to block RIG-I stimulation
NS2B-NS3	DENV	Cleaves MITA or STING
		Inhibits IFN production by interacting directly with I κ B kinase ϵ , disrupting RIG-I signalling, blocking serine 386 phosphorylation, and inhibiting IRF3 nuclear translocation
NS5	DENV	Targets STAT2 for ubiquitin mediated proteasomal degradation involving interactions with UBR4
	ZIKV	Induces ubiquitin mediated proteasomal degradation of STAT2
	YFV	Binds and inhibits STAT2 following IFN-I induced phosphorylation of STAT1, requires K6 ubiquitination
	WNV	Inhibits STAT1 phosphorylation
	JEV	Blocks Tyk2 phosphorylation
sfRNA	DENV-2	Sequesters G3BP1, G3BP2, and CAPRIN1, Binds and inhibits TRIM25
	ZIKV	Inhibits IFN-I response downstream of RIG-I & MDA5
	WNV	Inhibits IFN-I response through unknown mechanism
	JEV	Inhibits IRF-3 phosphorylation and nuclear localisation

Note. DENV = dengue virus; IFN = interferon; IFN-I = type I interferon; IRF = IFN-regulatory factor; JAK = Janus kinase; JEV = Japanese encephalitis virus; KUNV = Kunjin virus; NS = nonstructural; sfRNA = subgenomic flavivirus RNA; STAT = signal transducer and activation of transcription; STING = stimulator of the IFN genes; TRIM = tripartite motif-containing protein; Tyk2 = tyrosine kinase 2; UBR4 = ; Ubiquitin protein ligase E3 component N-Recognin 4; WNV = West Nile virus; YFV = yellow fever virus; ZIKV = Zika virus.

Table 3. Antagonism of type I IFN system by flaviviruses. Extracted from (Cumberworth *et al.* 2017).

An increasing number of studies reveals that ZIKV has evolved similar mechanisms to escape the host immune defense. ZIKV NS4A protein specifically binds to MAVS thus preventing any interaction with RLRs (Ma *et al.* 2018); NS1 and NS4B target TBK1 and thus inhibit type I IFN production, whereas NS2B-NS3 impairs JAK-STAT signaling pathway downstream of IFN receptors (Wu *et al.* 2017); and NS5 protein interacts with STAT2 and targets it to degradation (Bowen *et al.* 2017; Kumar *et al.* 2016) (Figure 16). Finally, ZIKV sfRNA has recently been shown to antagonize both RIG-I and MDA5 (Donald *et al.* 2016).

Overall, the interplay between ZIKV and the host immune response is complex but understanding these mechanisms and interconnections is fundamental for the development of new antiviral drugs (see 2.2.5.3).

2.2.4.2.2. Adaptive immune response

For decades, paradoxical effects have been attributed to the adaptive immune response against flaviviruses, especially DENV, with potential dual roles in protection and pathogenesis (Ngono and

Shrestha 2018). Epidemiological studies show that severe dengue disease most often occurs in individuals experiencing a second DENV infection by a different serotype. Several non-mutually exclusive hypotheses were proposed to explain this phenomenon including antibody-dependent enhancement (ADE). Considering the high homology between DENV and ZIKV, cross-reactive adaptive immune responses are also a concern in the context of ZIKV infection. The roles of T-cell and antibody responses in ZIKV protection versus pathogenesis are summarized below.

T-cell response

T-cell immune response against DENV has been extensively studied and recent lines of evidence tend to favor the hypothesis of a protective rather than pathogenic response, which correlates with recent findings on ZIKV. In patients infected with ZIKV, T-cell epitopes have been mapped to the complete viral proteome (Grifoni *et al.* 2017; Ricciardi *et al.* 2017). Further exploration of the role of T-cell in ZIKV immunity was performed in animal models of ZIKV infection.

For instance, the peak of activation of T-cells correlates with the clearance rate of Zika viral load in the blood of NHPs (Dudley *et al.* 2016). Mouse studies characterized ZIKV-specific CD8⁺ T-cells as poly-functional and cytotoxic, and showed that depletion of these cells resulted in an increase of viral load in tissues (Elong Ngono *et al.* 2017). Several studies have resorted to multiple approaches, such as adoptive transfer of specific T-cells between mice, and have demonstrated the critical role played by T-cells, both ZIKV-specific (Huang *et al.* 2017; Manangeeswaran *et al.* 2016; Winkler *et al.* 2017a) or DENV cross-reactive T-cells (Wen *et al.* 2017), in the protection against ZIKV infection. However, an exacerbated CD8⁺ T-cell infiltration in response to ZIKV infection has been shown to induce major cytotoxic effects and tissue damage, especially in the CNS, thus contributing to neuro-inflammation in mice (Jurado *et al.* 2018).

Together, these findings suggest a protective role of the T-cell response against ZIKV though a balance in T-cell mediated cytotoxicity seems required to avoid tissue injury and severe disease.

Antibody response

Antibody response, especially the development of neutralizing Abs (nAbs), is crucial in the immune response to viral infection. Indeed, ZIKV-specific antibodies are sufficient for protection as passive transfer of ZIKV-IgG from vaccinated NHPs into naïve animals prevented viremia development upon ZIKV infection (Abbink *et al.* 2016).

The E, prM and NS1 proteins are the main targets of nAbs in flavivirus infection (Rey *et al.* 2018). The ectodomain of the E protein comprises three distinct domains: DI contains the N-terminus, DII is a finger-like structure and contains a fusion loop mediating viral fusion, and DIII is an immunoglobulin-like structure involved in attachment to target cells (Robbiani *et al.* 2017). Considering the important role of the E protein in ZIKV replication cycle, this protein is an ideal target for nAbs. The characterization of B-cells from patients infected with ZIKV allowed identifying epitopes on the E protein of highly neutralizing and protective antibodies. Antibodies directed against the epitope in DIII, the quaternary sites covering DI-II-III across the E protein dimer (EDE), and epitopes within DI or DII were strongly neutralizing (Robbiani *et al.* 2017; Rogers *et al.* 2017; Sapparapu *et al.* 2016;

Stettler *et al.* 2016). On the contrary, cross-reactive antibodies elicited against the conserved fusion loop in DII were poorly neutralizing and could lead to ADE (Stettler *et al.* 2016; Zhao *et al.* 2016).

As mentioned previously, ADE was first considered in the case of heterotypic DENV infections to explain for the higher risk of developing a severe form of the disease. In this context of DENV infections, evidence in favor of this hypothesis has been brought by a considerable number of *in vitro* (Ayala-Nunez *et al.* 2016; Goncalvez *et al.* 2007) and *in vivo* (Goncalvez *et al.* 2007; Pierson 2010; Shresta *et al.* 2006; Zompi *et al.* 2012) studies. Epidemiological observations also support these findings. A recent report on more than 6,000 Nicaraguan children established that pre-existing DENV-specific antibodies were directly correlated with DENV disease severity (Katzelnick *et al.* 2017). ADE is now a concern in the context of infections by distinct but closely-related flaviviruses such as DENV and ZIKV; research was rapidly initiated to investigate whether ADE resulting from previous DENV infection could enhance ZIKV infection and increase viral pathogenicity (Figure 17).

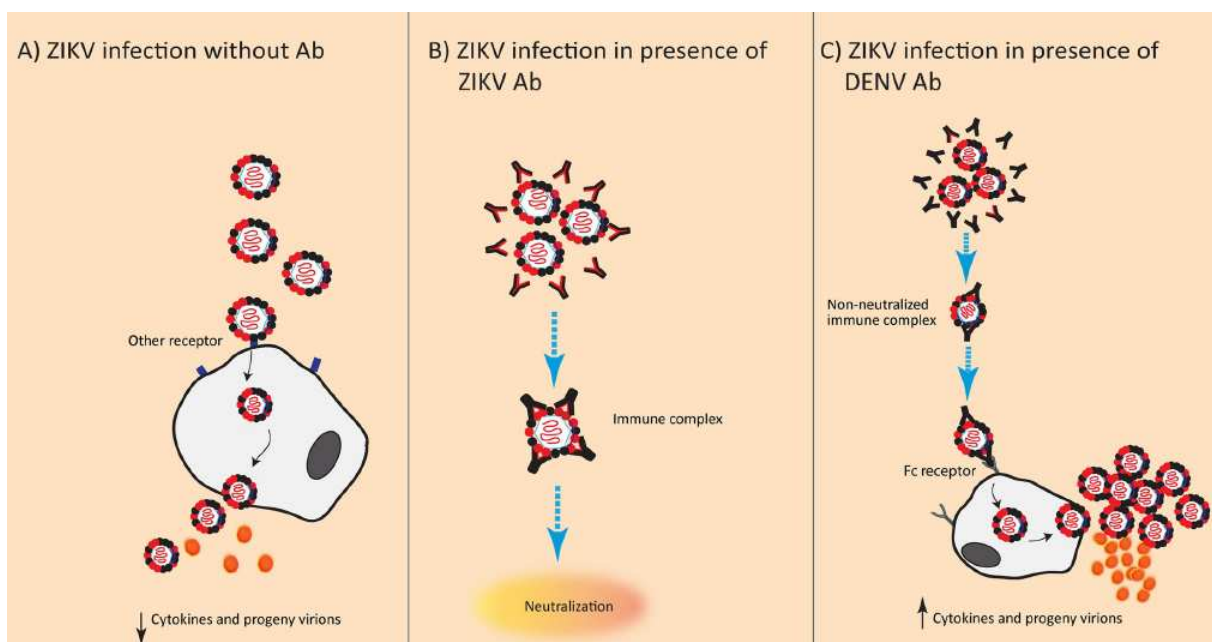


Figure 17. Proposed mechanism of ADE of ZIKV infection mediated by cross-reactive anti DENV antibodies.

(A) Primary ZIKV infection in naïve individuals. Entry occurs via receptors other than Fc receptor and leads to virus and cytokine production. (B) Secondary ZIKV infection in a ZIKV pre-immune individual. Neutralization occurs effectively and blocks infection. (C) ZIKV ADE (black antibodies: pre-existing antibodies against primary infecting DENV); low-affinity Abs can cross-react with ZIKV without neutralizing it but allowing entry of the virus–antibody complexes into cells via the Fc receptor, leading to higher viral load along with higher levels of cytokines than cells infected in absence of antibodies (Langerak *et al.* 2019).

Early studies actually revealed an enhancement of ZIKV infection *in vitro* by various anti-flavivirus antibodies (Langerak *et al.* 2019). On the other hand, these findings are supported by only one (Bardina *et al.* 2017) among many (Kam *et al.* 2017; McCracken *et al.* 2017; Pantoja *et al.* 2017; Stettler *et al.* 2016; Swanstrom *et al.* 2016) *in vivo* studies, while ADE of DENV infection in the presence of cross-reactive ZIKV antibodies has been confirmed by *in vivo* studies (Stettler *et al.* 2016; Swanstrom *et al.* 2016). So far, epidemiological data regarding ADE of ZIKV infection is sparse, but a study in ZIKV-infected pregnant women showed that DENV antibodies at the time of infection were

not associated with an increased risk of CZS (Halai *et al.* 2017). Based on these diverging results, ADE of ZIKV cannot be either confirmed not disproved and still warrants further investigation.

Despite many studies, we are still far from getting a full picture of ZIKV-induced immunity; but understanding the mechanisms regulating the balance between protection and pathogenesis of these immune responses is crucial for the development of safe and effective ZIKV therapeutics and vaccines (see 2.2.5).

2.2.5. Prevention and control measures

With the recent outbreaks of ZIKV infection and the description of CZS, the development of new antivirals and vaccines has been strongly encouraged by international funding agencies. Nonetheless, vector control strategies are also required to restrict ZIKV and other arboviruses transmission.

2.2.5.1. Vector control strategies

Aedes aegypti and *Aedes albopictus* mosquito species are particularly difficult to control in the long term because of their capacity to breed in both natural and artificial containers nearby households. Consequently, WHO recommends an integrated vector management (World Health Organization 2012), i.e. the use of a combination of various methods in order to limit transmission of pathogens, reduce the vector populations etc.

Classical approaches include informing and advising people on personal protection measures and on how to reduce mosquito sources locally. Large-scale pesticides fogging has been implemented by mosquito control agencies, though with a limited efficacy because of indoor resting habits of *Aedes* mosquitoes and insecticide resistance. Biopesticides are also used, such as larvicides, to avoid the problem of resistance to chemical compounds; well-known examples include microbial control agents and insect growth regulators such as methoprene and pyriproxyfen. The field of vector control is evolving rapidly with novel approaches, which target the adult stage of the mosquito and use the modified male mosquito as a delivery vehicle. Several of these methods are under development, including the sterile insect technique, the release of insects carrying dominant lethal genes and the release of *Wolbachia*-infected mosquitoes (Kauffman and Kramer 2017; Singh *et al.* 2018).

2.2.5.2. Vaccines

Following the sudden outbreaks of ZIKV infection, international public health agencies alerted the medical and scientific communities on the urgent need to develop a ZIKV vaccine. One important challenge of ZIKV vaccine development is to produce a safe, efficient and low cost vaccine to be administered to pregnant women. Indeed, WHO is advising to prioritize the vaccination of women of reproductive age, including pregnant women, to prevent CZS (World Health Organization and United Nations International Children's Emergency Fund 2017).

Many vaccine subtypes are currently under development. About a dozen vaccine candidates are in clinical trial phase I or II (Table 4) and belong to three categories of vaccine technologies: (i)

inactivated vaccines, (ii) subunit vaccines and (iii) live-attenuated vaccines. Several of these vaccines have been shown to confer good protection in preclinical animal models (Alves Dos Santos and Fink 2018) and results from 3 phase I clinical trials were published recently (Gaudinski *et al.* 2018; Modjarrad *et al.* 2018; Tebas *et al.* 2017).

Vaccine	Platform	Clinical trial	Sponsor Name	Phase	Published results
GLS-5700	DNA	NCT02809443 NCT02887482	GeneOne Life Science Inovio Pharma.	I	(Tebas <i>et al.</i> 2017)
AGS-v	Peptide	NCT03055000	NIH	I	
MV-Zika	Recombinant vector	NCT02996890	Themis Bioscience	I	
mRNA-1325	mRNA	NCT03014089	Moderna Therapeutics	II	
VRC-ZKADNA 085-00-VP	DNA	NCT02840487	NIAID	I	(Gaudinski <i>et al.</i> 2018)
VRC-ZKADNA 090-00-VP	DNA	NCT02996461 NCT03110770	NIAID NIAID	I II	(Gaudinski <i>et al.</i> 2018)
ZIKV PIV	Inactivated virus	NCT02963909 NCT02952833 NCT02937233 NCT03008122	NIAID NIAID BIDMC NIAID	I I I I	(Modjarrad <i>et al.</i> 2018) (Modjarrad <i>et al.</i> 2018) (Modjarrad <i>et al.</i> 2018)
PIZV or TAK-426	Inactivated virus	NCT03343626	Takeda	I	
VLA1601	Inactivated virus	NCT03425149	Valneva Austria GmbH	I	
rZIKV/ D4Δ30-713	Recombinant vector	NCT03611946	NIAID	I	
BBV121	Inactivated virus	CTRI/2017/05/008539	Bharat Biotech	I	

Table 4. ZIKV vaccines in clinical trials.

Data extracted from (World Health Organization 2019b), last update on January 2019. NIH, National Institute of Health; NIAID, National Institute of Allergy and Infectious Diseases; BIDMC, Beth Israel Deaconess Medical Center.

Inactivated and subunit vaccines are non-infectious and therefore require a vaccination protocol with prime and boost injections whereas live-attenuated vaccines usually provide quick and long-lasting immunity after a single injection. A vaccine with single-dose efficacy would be particularly suited in case of new ZIKV outbreaks in low-income countries, but non-infectious vaccines would be more appropriate for pregnant women and immunocompromised individuals. The diversity of epidemiological situations and populations at risk for ZIKV infection therefore justifies the development of various and complementary ZIKV vaccine platforms (Richner and Diamond 2018; Shan *et al.* 2018).

In summary, tremendous efforts have been undertaken to develop an effective vaccine against ZIKV infection and several candidates have already been identified. Nonetheless, to succeed in this enterprise, many hurdles and knowledge gaps will have to be addressed, in particular the declining incidence of ZIKV infection, interferences with pre-existing DENV Abs and the risk of inducing ADE of DENV infections (Figure 18).

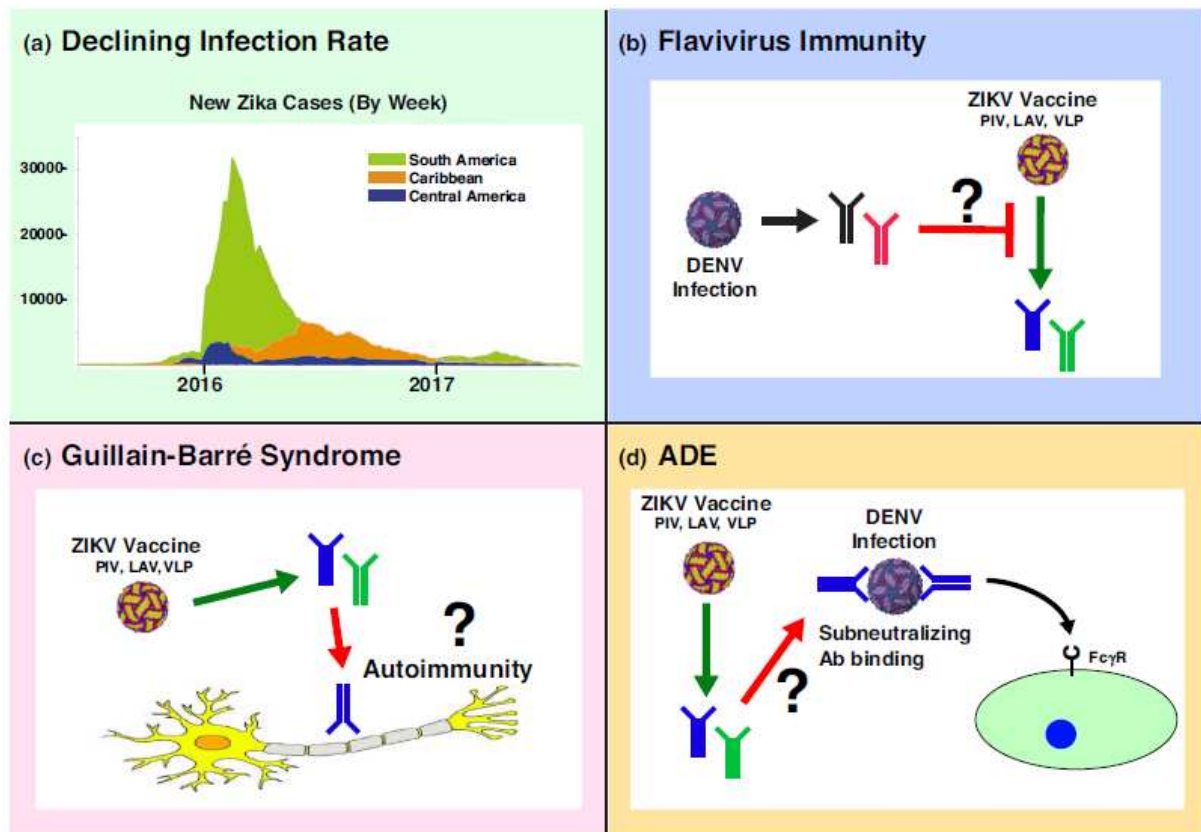


Figure 18. Challenges and knowledge gaps to address in the development of ZIKV vaccines. (a) The infection rate has declined since the peak in 2016, which complicates vaccine efficacy study design. (b) Pre-existing immunity against DENV or other flaviviruses could inhibit the immune response against ZIKV vaccines. (c) Vaccination might induce antiviral antibodies that would cross-react with host proteins on glial cells and lead to GBS. (d) Vaccine-induced anti-ZIKV antibodies that cross-react with DENV could lead to ADE and worsen DENV disease (Richner and Diamond 2018).

2.2.5.3. Antiviral therapies

Antiviral drugs typically target specific and vulnerable steps of the virus life cycle; some interact with the viral components while others interact with host proteins. Using large screening strategies as well as re-purposing of currently available antiviral drugs, many compounds have been found to have *in vitro* activity against ZIKV, but only a few of them have shown antiviral effects *in vivo*. Several inhibitors of ZIKV NS5 RNA polymerase have demonstrated antiviral effects in preclinical models. Sofosbuvir, an approved therapeutic agent of Hepatitis C, blocks ZIKV replication *in vitro* and *in vivo* and also prevents ZIKV vertical transmission in pregnant mice (Ferreira *et al.* 2017; Mesci *et al.* 2018). Similarly, Ribavirin, which was historically used against Hepatitis C virus, interferes with ZIKV RNA processing and reduces viral load *in vivo* (Kamiyama *et al.* 2017). The 2 compounds NSC157058 and Temoporfin are able to block the interaction between ZIKV NS2B and NS3 proteins, therefore inhibiting the protease activity of this complex and reducing viral load in mice (Sacramento *et al.* 2017; Shiryayev *et al.* 2017b). On the other side, Chloroquine is a classical antimalarial agent which inhibits autophagy and 25-hydroxycholesterol limits viral entry in host cells; these 2 molecules prevent virus-host cell interactions and have been proven efficacious in infected mice (Cao *et al.* 2017; Li *et al.* 2017; Shiryayev *et al.* 2017a).

Great endeavors have led to the description of a broad range of therapeutic agents against ZIKV but the fraction that will go through all the stages of the development pipeline, from *in vitro* experiments to *in vivo* and then clinical studies, is very limited. More basic research on virus-host interaction mechanisms will be necessary to the discovery of original antiviral compounds.

A few years after the onset of ZIKV outbreak in South America, intense scientific research on ZIKV disease has been conducted internationally and will continue as billions of people live in at-risk areas for transmission. Multitudes of investigations have increased our understanding of the biology of the virus among which many studies have relied on animals models. The use of suitable animal models is indeed a critical concern in the field of infectious diseases, for the study of pathogenic mechanisms but also for the establishment of reliable pre-clinical models.

2.3. Animal models of ZIKV infection

This third part summarizes the advances in the development and the use of animal models of ZIKV infection and pathogenesis.

2.3.1. Using mice to study flaviviruses

2.3.1.1. Propagation of flaviviruses in the brain of mice

The use of experimental animal models to study flaviviruses was first reported when the Rockefeller Foundation instigated research programs on Yellow fever in the beginning of the 20th century. Rhesus macaques had been described as natural hosts of YFV (Balfour 1914) and were initially used in experimental infections (Hudson 1928). In 1930, M. Theiler managed to propagate a strain of YFV in mice for the first time (Theiler 1930) (Figure 19). This was an important finding as it offered researchers an alternative experimental model, much more convenient and affordable than the use of NHPs. After M. Theiler's publication, mice became widely used to study human pathogenic viruses. They were instrumental to assess the pathogenicity of unknown viruses, to propagate and isolate strains of viruses, to investigate the immune response to these viruses and to evaluate vaccine candidates.

SPECIAL ARTICLES

SUSCEPTIBILITY OF WHITE MICE TO THE VIRUS OF YELLOW FEVER¹

It has been found that if yellow fever virus is injected into the brains of white mice, a certain number become ill and die. The virus can be propagated in mice indefinitely by the intracerebral injection into normal mice of the brain of an infected mouse.

The virus is highly neurotropic in mice. Infection can be produced by the injection of the virus into the brain, spinal cord or eye. Other routes of injection, such as intraperitoneal, subcutaneous, etc., very seldom cause illness and death, though a considerable proportion of such mice are rendered immune to a subsequent intracerebral injection of the passage virus.

MAX THEILER

DEPARTMENT OF TROPICAL MEDICINE,
HARVARD MEDICAL SCHOOL

Figure 19. Extract from M. Theiler's first publication on the propagation of YFV in mice. Extracted from (Theiler 1930).

Interestingly, M. Theiler described that adult mice, in contrast to adult NHPs, could be infected with YFV by intracerebral injection but not by classical subcutaneous (s.c.) or intraperitoneal (i.p.) injection (Figure 19). G. Dick reported similar observations when he isolated the first strain of ZIKV. Mice which were infected intraperitoneally with the serum from a febrile Rhesus monkey did not show any abnormality while mice inoculated intracerebrally started showing signs of illness 10 days after infection (Dick *et al.* 1952). Only several decades later, scientists began to understand why peripheral inoculation of flaviviruses does not trigger disease in mice.

2.3.1.2. Interplay between flaviviruses and the innate immune system in mice

In the 1990's, M. Aguet and his team generated mice carrying a constitutive deletion of the *Ifnar1* gene, encoding the subunit 1 of the type I IFN receptor (IFNAR1). These *Ifnar1*-deficient mice (*Ifnar1*^{-/-} mice) proved to be much more susceptible to diverse viruses such as vesicular stomatitis virus or Semliki Forest virus, displaying higher tissue viral loads and mortality (Muller *et al.* 1994). Those *Ifnar1*^{-/-} mice were later shown to be susceptible to various flaviviruses after peripheral inoculation, including DENV (Shresta *et al.* 2004), WNV (Samuel and Diamond 2005) and YFV (Meier *et al.* 2009). These studies elucidated the critical role of type I IFN response in mouse resistance to many viruses, including flaviviruses.

In 2010, Ashour *et al.* demonstrated that some interactions between DENV and host proteins are species-specific. Precisely, they showed that DENV NS5 protein is able to associate and degrade human STAT2 but not mouse STAT2, and therefore that the natural resistance of mice to DENV probably results from an inability of DENV to inhibit the type I IFN response as it does in humans (Ashour *et al.* 2010). Similarly, it was later reported that DENV NS2B3 protein is not able to degrade the mouse STING (Stimulator of interferon genes protein), which acts as a facilitator of innate immune signaling and promotes the production of type I IFN through the recruitment of IRF3 (Aguirre *et al.* 2012). Surprisingly, Stabell *et al.* discovered that in most primates implicated as reservoir species, DENV could not inactivate STING; this was explained by a small region in the STING protein that differs between humans and primates (Stabell *et al.* 2018). Recently, similar mechanisms

were described in the case of ZIKV infection in mouse cells, for both STAT2 (Grant *et al.* 2016) and STING (Ding *et al.* 2018).

WNV infection in mice stands out as an exception in flaviviruses. Indeed, most classical laboratory strains of mice are susceptible to WNV infection by peripheral inoculation. The susceptibility of these mice to WNV has been assigned to a loss-of-function mutation in the 2'-5' oligoadenylate synthase 1b gene (*Oas1b*) (Mashimo *et al.* 2002; Perelygin *et al.* 2002), an ISG involved in the degradation of viral RNA, and is detailed in part 2.5.3.

Overall, these findings illustrate that the inability of several flaviviruses, with the exception of WNV, to cause disease in mice is linked to their failure to antagonize the mouse IFN system through their non-structural proteins. This species-specific restriction mechanism certainly represents a limitation of mouse models of flaviviral infection and has to be taken into account when developing and using such experimental models.

2.3.2. Mouse models of ZIKV infection

A variety of mouse models have been used to study the pathogenesis of ZIKV disease, the mechanisms of sexual transmission and congenital infection; they are described hereafter.

2.3.2.1. Mouse models of ZIKV systemic infection

2.3.2.1.1. Immunocompromised adult mice

As ZIKV replicates poorly in wild-type mice due to its inability to antagonize the type I IFN response, the most commonly used models of ZIKV pathogenesis include mice with targeted deficiencies in this type I IFN pathway (Table 5).

Ifnar1^{-/-} mice were one of the first models used to characterize ZIKV infection. 2 mouse strains carry this *Ifnar1* knock-out mutation. The mutation was engineered on the 129S2/SvPas genetic background and later backcrossed to the C57BL/6J background to yield a congenic strain. Similarly to what was observed after infection with DENV or YFV, *Ifnar1*^{-/-} mice display enhanced susceptibility to ZIKV infection. After peripheral inoculation of ZIKV (i.p., s.c. or i.v.), they develop moderate to severe clinical signs, which manifest as general features of lethargy, hind limb weakness or paralysis, often leading to death. The severity of the disease in *Ifnar1*^{-/-} mice depends on several factors including the viral strain and dose, the inoculation route, the age of the mice and the mouse genetic background (Table 5) (Dowall *et al.* 2016; Dowall *et al.* 2017; Lazear *et al.* 2016; Rossi *et al.* 2016; Smith *et al.* 2018; Tripathi *et al.* 2017).

Ifnar1^{-/-} *Ifngr1*^{-/-} double knock-out mice (often referred to as "AG129") lack both the type I and type II IFN receptors and showed the greatest vulnerability to ZIKV infection which was uniformly fatal whatever the viral strain or dose used in the experiment (Aliota *et al.* 2016; Rossi *et al.* 2016). Other genetically immunocompromised mice sustaining ZIKV infection include *Irf3*^{-/-}, *Irf7*^{-/-} double knock-out mice, *Irf3*^{-/-}, *Irf5*^{-/-}, *Irf7*^{-/-} triple knock-out mice, *Stat2*^{-/-} and *Stat1*^{-/-} knock-out mice (Kamiyama *et al.* 2017; Kawiecki *et al.* 2017; Lazear *et al.* 2016; Li *et al.* 2016b; Tripathi *et al.* 2017).

Finally, mice with a transient, pharmacologically induced, immune deficiency have also been used successfully as models of ZIKV infection. C57BL/6J mice treated with a monoclonal antibody (mAb) blocking the type I IFN receptor (MAR1-5A3, in particular) before infection sustain viral replication independently of the viral strain but display signs of disease only after inoculation with a high dose of ZIKV from the African lineage (Lazear *et al.* 2016; Smith *et al.* 2017; Zhao *et al.* 2016). One study also described ZIKV infection and disease in BALB/c mice treated with an immunosuppressive dose of dexamethasone (Chan *et al.* 2016).

Strain (Background)	Target / Treatment	Age in weeks	ZIKV strain	Dose, route	Viremia	Disease	Mortality	References
AG129 (129 Sv/Ev)	<i>Ifnar1</i> ^{-/-} , <i>Ifngr1</i> ^{-/-}	3	FSS13025 (Cambodia, 2010)	10 ⁵ , i.p.	+	++	100%	(Rossi <i>et al.</i> 2016)
		3-8	H/PF/2013 (French Polynesia, 2013)	10 ⁵ , s.c.	+	++	100%	(Aliota <i>et al.</i> 2016)
<i>Ifnar1</i> ^{-/-} (129 Sv/Ev) (also referred to as A129)	<i>Ifnar1</i> ^{-/-}	5-6	MP1751 (Uganda, 1962)	10 ⁶ , s.c.	+	++	100%	(Dowall <i>et al.</i> 2016)
		6-8	MP1751 (Uganda, 1962)	10 ⁶ , s.c.	+	++	100%	(Dowall <i>et al.</i> 2017)
		6-8	PRVABC59 (Puerto Rico, 2015)		+	-	0%	
		3	FSS13025 (Cambodia, 2010)	10 ⁵ , i.p.	+	++	100%	(Rossi <i>et al.</i> 2016)
		5			NR	+	50%	
		11				+	0%	
<i>Ifnar1</i> ^{-/-} (C57BL/6J)	<i>Ifnar1</i> ^{-/-}	5-6	MR 766 (Uganda, 1947)	10 ² /10 ³ , s.c.	NR	++	100%	(Lazear <i>et al.</i> 2016)
			H/PF/2013 (FP, 2013)		+	++	80%	
		5-6	MR 766 (Uganda, 1947)	10 ³ , s.c.	NR	++	100%	(Tripathi <i>et al.</i> 2017)
			DAKAR 41519 (Senegal, 1984)			++	100%	
			P6-740 (Malaysia, 1966)			+	0%	
			FSS13025 (Cambodia, 2010)			+	30%	
			PRVABC59 (Puerto Rico, 2015)			+	0%	
		10	DAKAR 41519 (Senegal, 1984)	10 ⁵ , i.p.	NR	++	100%	(Smith <i>et al.</i> 2018)
			CPC-0740 (Phillipines, 2012)			++	90%	
			SV0127-14 (Thailand, 2014)			-	10%	
<i>Irf3</i> ^{-/-} , <i>Irf5</i> ^{-/-} , <i>Irf7</i> ^{-/-} TKO (C57BL/6J)	<i>Irf3</i> ^{-/-} , <i>Irf5</i> ^{-/-} , <i>Irf7</i> ^{-/-}	5-6	MR 766 (Uganda, 1947), H/PF/2013 (French Polynesia, 2013)	10 ² , s.c.	NR	++	100%	(Lazear <i>et al.</i> 2016)
						++	100%	
		5-6	FSS13025 (Cambodia, 2010)	10 ³ , i.v.	NR	+	NR	(Li <i>et al.</i> 2016)
<i>Irf3</i> ^{-/-} , <i>Irf7</i> ^{-/-} DKO (C57BL/6J)	<i>Irf3</i> ^{-/-} , <i>Irf7</i> ^{-/-}	6-10	MR766 (Uganda, 1947)	10 ⁶ , s.c.	+	+	30%	(Kawiecki <i>et al.</i> 2017)
<i>Stat2</i> ^{-/-} (C57BL/6J)	<i>Stat2</i> ^{-/-}	5-6	MR 766 (Uganda, 1947)	10 ³ , s.c.	+	++	100%	(Tripathi <i>et al.</i> 2017)
			DAKAR 41519 (Senegal, 1984)		+	++	100%	
			P6-740 (Malaysia, 1966)		+	+	0%	
			FSS13025 (Cambodia, 2010)		+	+	20%	
			PRVABC59 (Puerto Rico, 2015)		+	+	0%	
<i>Stat1</i> ^{-/-} (unspecified)	<i>Stat1</i> ^{-/-}	7-9	MR 766 (Uganda, 1947)	10 ⁴ , s.c.	+	++	100%	(Kamiyama <i>et al.</i> 2017)
C57BL/6J	IFNAR1-blocking mAb (MAR1-5A3)	4-5	H/PF/2013 (French Polynesia, 2013)	10 ³ , s.c.	+	-	0%	(Lazear <i>et al.</i> 2016)
		5	DAKAR 41525 (Senegal, 1984)	10 ⁶ , i.p.	+	++	100%	(Smith <i>et al.</i> 2017)
				10 ⁶ , s.c.	+	++	40%	
		4-5	DAKAR 41519 (Senegal, 1984)	10 ⁵ , s.c.	+	++	90%	(Zhao <i>et al.</i> 2016)
BALB/c	Dexamethasone	6-8	PRVABC59 (Puerto Rico, 2015)	10 ⁶ , i.p.	+	++	100%	(Chan <i>et al.</i> 2016)

Table 5. Immunocompromised mouse models of ZIKV infection.

TKO, triple knock-out, DKO, double knock-out; i.p., intraperitoneal, s.c., subcutaneous, i.v., intravenous; NR, not reported

2.3.2.1.2. Immunocompetent neonatal mice

ZIKV infection has been studied in immunocompetent newborn mice (Fernandes *et al.* 2017; Li *et al.* 2018b; Manangeeswaran *et al.* 2016; Miner *et al.* 2016b; Snyder-Keller *et al.* 2019; Yuan *et al.* 2017). As the neuro-developmental processes continue after birth in rodents, neonatal mice can be used to study how the virus impairs neurogenesis in a period corresponding to the third trimester of pregnancy in humans (Snyder-Keller *et al.* 2019). Peripheral inoculation of 1-day-old C57BL/6J and Swiss mice with ZIKV strains from Cambodia and Brazil, respectively, resulted a few days later in neurological symptoms such as tremors, ataxia and paralysis (Fernandes *et al.* 2017; Manangeeswaran *et al.* 2016). In both studies, ZIKV infection of the brain was confirmed. Additionally, as ZIKV infection of neonatal mice is often non-fatal, it allows for the investigation of long-term neurological sequelae and their behavioral consequences. Indeed, a recent study demonstrated that ZIKV infection of newborn mice one day after birth led to behavioral abnormalities in adulthood such as hyperactivity and incoordination, which correlated with the presence of calcifications in the brain (Snyder-Keller *et al.* 2019).

2.3.2.1.3. Immunocompetent adult mice

Results from recent ZIKV studies confirmed that fully immunocompetent mice sustain very low levels of viral replication after peripheral inoculation. Low and transient levels of viremia could be detected in young (5 week-old) C57BL/6J and 129Sv/Ev mice after s.c. inoculation with ZIKV (Dowall *et al.* 2016; Lazear *et al.* 2016). BALB/c and SJL mice were shown to sustain higher plasma viral loads without developing disease (Larocca *et al.* 2016) though SJL mice are known to have several atypical immunological features (Glineur *et al.* 2011; Hutchings *et al.* 1986; Spindler *et al.* 2001). Lately, Gorman *et al.* generated an immunocompetent mouse model by replacing the mouse *Stat2* gene with its human counterpart. These mice displayed a slightly enhanced ZIKV infection and disease compared to wild-type C57BL/6J mice when infected with a mouse-adapted viral strain of ZIKV, specifically developed to have higher intrinsic infectivity (Gorman *et al.* 2018).

In summary, no fully immunocompetent adult mouse model of ZIKV infection has been identified so far, that would mirror the susceptibility of human adults. Depending on the scientific issue to be solved, obtaining a reliable mouse model of ZIKV infection will require to adjust the natural infection system, whether by changing the route of administration as described almost 100 years ago, by using a mouse-adapted viral strain, or by using mice with targeted immune deficiencies.

2.3.2.2. Mouse models of ZIKV infection in the genital tract and sexual transmission

Sexual transmission has been reported in humans and was confirmed in mouse studies, which also investigated the pathogenesis of ZIKV infection in the genital tract as well as the persistence of the virus in the male genital organs (Figure 20).

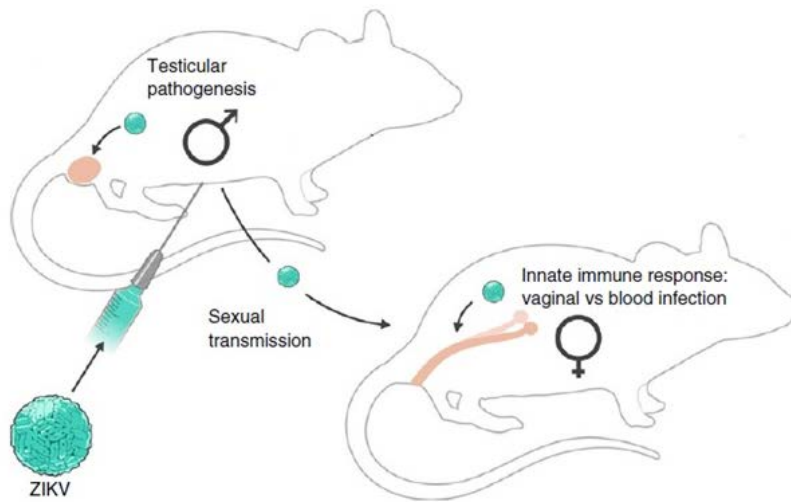


Figure 20. ZIKV infection of the genital tract and sexual transmission in mouse models.

Mouse models have demonstrated that ZIKV infection leads to replication and pathology in the testes, that the female reproductive tract is permissive to ZIKV infection and that the virus can be sexually transmitted from a ZIKV-infected male to a naïve female. Adapted from (Winkler and Peterson 2017).

Several studies have shown that ZIKV targets the male genital organs and is able to induce severe inflammation and tissue injury in the testes and epididymis of *Ifnar1*^{-/-} and anti-IFNAR mAb-treated mice (Clancy *et al.* 2018; Govero *et al.* 2016; Ma *et al.* 2016; Uraki *et al.* 2017; Winkler *et al.* 2017a). ZIKV was shown to induce an innate immune response and the production of inflammatory cytokines in Leydig, Sertoli and epididymal cells (Ma *et al.* 2016). Besides, Govero *et al.* reported that ZIKV-induced inflammation in the testis can lead to a reduction in the levels of sex hormones and to a destruction of cells resulting in subfertility in these male mice (Govero *et al.* 2016).

In addition, the permissiveness of the female genital tract to ZIKV infection was assessed in several studies using intra-vaginal (i.vag.) inoculation in wild-type mice with or without anti-IFNAR mAb treatment, in *Ifnar1*^{-/-} and in AG129 mice (Clancy *et al.* 2019; Khan *et al.* 2016; Scott *et al.* 2018; Tang *et al.* 2016b; Yockey *et al.* 2016). After i.vag. infection, ZIKV replication was detected in vaginal tissues of wild-type C57BL/6J mice while it resulted in disseminated infection with high viral loads in multiple tissues of *Ifnar1*^{-/-} mice (Yockey *et al.* 2016).

Finally, direct animal-to-animal sexual transmission was evaluated in a few reports. Infection of immunodeficient females occurred after sexual contact with a ZIKV-infected male and resulted in viral replication in the female reproductive organs (Figure 20) (Duggal *et al.* 2018; Winkler *et al.* 2017b).

2.3.2.3. Mouse models of ZIKV congenital infection

The dramatic consequences of ZIKV infection in infants born from infected pregnant women elicited significant efforts to develop animal models of ZIKV pathogenesis in developing fetuses. Different strategies have been used to model CZS in the mouse. ZIKV has been inoculated to pregnant dams, most often with targeted deficiencies of the innate immune response, or directly to the fetuses of wild-type pregnant mice.

Systemic infection (s.c. or i.vag. injection) of pregnant *Ifnar1*^{-/-} mice between embryonic day 6.5 (E6.5) and E9.5 resulted in placental infection, vertical transmission to the fetuses and fetal demise (Jagger *et al.* 2017; Miner *et al.* 2016a; Yockey *et al.* 2016). Interestingly, fetal resorptions were less frequently observed in ZIKV-infected pregnant wild-type mice pre-treated with an anti-IFNAR mAb. In this model, ZIKV was shown to induce IUGR, placental injury and viral replication in placental and fetal tissues (Miner *et al.* 2016a; Sapparapu *et al.* 2016; Shan *et al.* 2017; Valentine *et al.* 2018; Winkler *et al.* 2017b). Similar findings, though to a lesser degree, were described in wild-type C57BL/6J mice infected with ZIKV by i.p., i.v. or i.vag. injection (Paul *et al.* 2018; Szaba *et al.* 2018; Yockey *et al.* 2016).

In contrast to these experimental systems, which imply trans-placental transmission of the virus, other models have been described in wild-type mice, in order to assess the pathogenic effects of ZIKV infection in the developing fetus after intra-uterine (i.u.) (Shi *et al.* 2018; Vermillion *et al.* 2017), intra-amniotic (i.a.) (Cui *et al.* 2017) or intra-cerebral (i.c.) (Li *et al.* 2016a; Wang *et al.* 2017; Wu *et al.* 2016; Zhu *et al.* 2018) inoculation. A broad range of brain malformations were described in the pups infected *in utero*, including microcephaly, brain volume loss and ocular defects, overall recapitulating CZS features observed in human newborns.

2.3.3. Non-human primate models of ZIKV infection

2.3.3.1. Adult NHP models of ZIKV infection

NHPs have also been employed to evaluate aspects of ZIKV biology and disease. ZIKV infection has been characterized in several species of NHPs, including cynomolgus macaques, Rhesus macaques, pigtail macaques and marmosets. ZIKV is usually administered to NHPs by s.c. injection; infection in the adult recapitulates many features of human disease, such as minimal bodyweight loss, slight fever and mild rash around the inoculation site. ZIKV can be detected in the blood of NHPs, plasma viral load often peaks within 2 to 6 days after challenge and the virus is cleared from the bloodstream after around 10 days (Dudley *et al.* 2019; Li *et al.* 2016c; Nguyen *et al.* 2017; Osuna *et al.* 2016). Dissemination of ZIKV was reported in body fluids: semen, vaginal secretions, urine and saliva; and in peripheral tissues: lymph nodes, reproductive tract, gastro-intestinal tract and CNS (Dudley *et al.* 2019; Koide *et al.* 2016; Osuna *et al.* 2016). Finally, ZIKV-infected macaques also mount specific antibody and cellular immune responses which protect against subsequent challenge with homologous or heterologous viruses, suggesting that NHPs can be useful as pre-clinical models to study vaccines efficacy (Aid *et al.* 2017; Dudley *et al.* 2016; Osuna *et al.* 2016).

Additionally, a recent study described a model of infant Rhesus macaques infected with ZIKV post-natally (Mavigner *et al.* 2018). ZIKV replication kinetics and dissemination was similar to those observed in adult ZIKV-infected macaques, showing tropism for cerebellar neurons and microglial cells. Interestingly, functional follow-up of these young infected macaques with MRI showed persistent brain damage such as enlargement of lateral ventricles and altered functional connectivity, correlating with behavioral abnormalities. This model could be of interest to gain insights into the potential outcomes of human infants infected with ZIKV during pregnancy or in early childhood.

2.3.3.2. NHP models of ZIKV congenital infection

NHPs represent appropriate models to study CZS, as both the placental barrier and the gestational development closely resemble those of humans. NHPs studies have been conducted in very diverse conditions, using different viral strains, as well as timing, route, and dose of inoculation (Adams Waldorf *et al.* 2018; Adams Waldorf *et al.* 2016; Mohr *et al.* 2018; Nguyen *et al.* 2017). Some studies have used doses in the range that is thought to be delivered by mosquitoes (10^4 – 10^5 plaque-forming units (PFU)) (Hirsch *et al.* 2018; Martinot *et al.* 2018; Mohr *et al.* 2018; Nguyen *et al.* 2017; Seferovic *et al.* 2018) while others have used higher doses to maximize the possibility of vertical transmission and fetal damage (Adams Waldorf *et al.* 2018; Adams Waldorf *et al.* 2016). Additionally, one group has used intra-amniotic inoculation of the macaque fetus to ensure direct fetal exposure to ZIKV (Coffey *et al.* 2018).

All these infection modalities have resulted in productive infection of the pregnant female macaque, vertical transmission and detection of ZIKV in placental and/or fetal tissues. Interestingly, ZIKV-infected pregnant Rhesus macaques had a prolonged viremia, lasting up to 55 days, similarly to what has been reported in infected pregnant women (Driggers *et al.* 2016; Dudley *et al.* 2016; Martinot *et al.* 2018). However, if ZIKV-induced injury to fetal brain was substantial after high-dose or i.a. challenge, only one study using conventional s.c. inoculation of ZIKV described CNS pathology in the fetus, characterized by a reduction of brain volume, calcifications and hemorrhagic lesions (Martinot *et al.* 2018). The uncommon detection of fetal brain damage in ZIKV-infected NHPs is somehow consistent with the low incidence of apparent brain malformations in human CZS. This is a limitation in the use of NHPs to model the natural history of congenital ZIKV infection; indeed uncovering CZS anomalies would require large number of animals, which is ethically and financially inconceivable, or more artificial ZIKV-inoculation methods.

2.3.4. Other animal models of ZIKV infection

A few alternative animal models of ZIKV infection have been tested, including guinea pigs, rats, hamsters and piglets. After ZIKV infection, guinea pigs developed clinical signs of fever and lethargy and ZIKV replication was described in the blood as well as in the spleen and brain (Kumar *et al.* 2017). However, after infection of pregnant female guinea pigs, ZIKV could not be detected in the placenta and in the fetuses, and no pregnancy adverse outcome was reported. A similar model was established in pregnant female rats resulting in this case in a reduction of the cerebral cortex of the offspring associated with increased cell death in the hippocampus (Sherer *et al.* 2019). One study evaluated the susceptibility to ZIKV of Syrian golden hamsters, which sustained weak and short viral replication in the blood after inoculation with a viral strain from the African lineage (Miller *et al.* 2018). Finally, other models have been developed in piglets infected with ZIKV *in utero*, which recapitulate some features of CZS neurodevelopmental pathology (Darbellay *et al.* 2017; Wichgers Schreur *et al.* 2018).

Though each one of the aforementioned animal models has imperfections, their use in different conditions, and in a myriad of studies, has increased our understanding of ZIKV biology and have provided decent pre-clinical models to test antiviral drugs and vaccines against ZIKV (Figure 21).

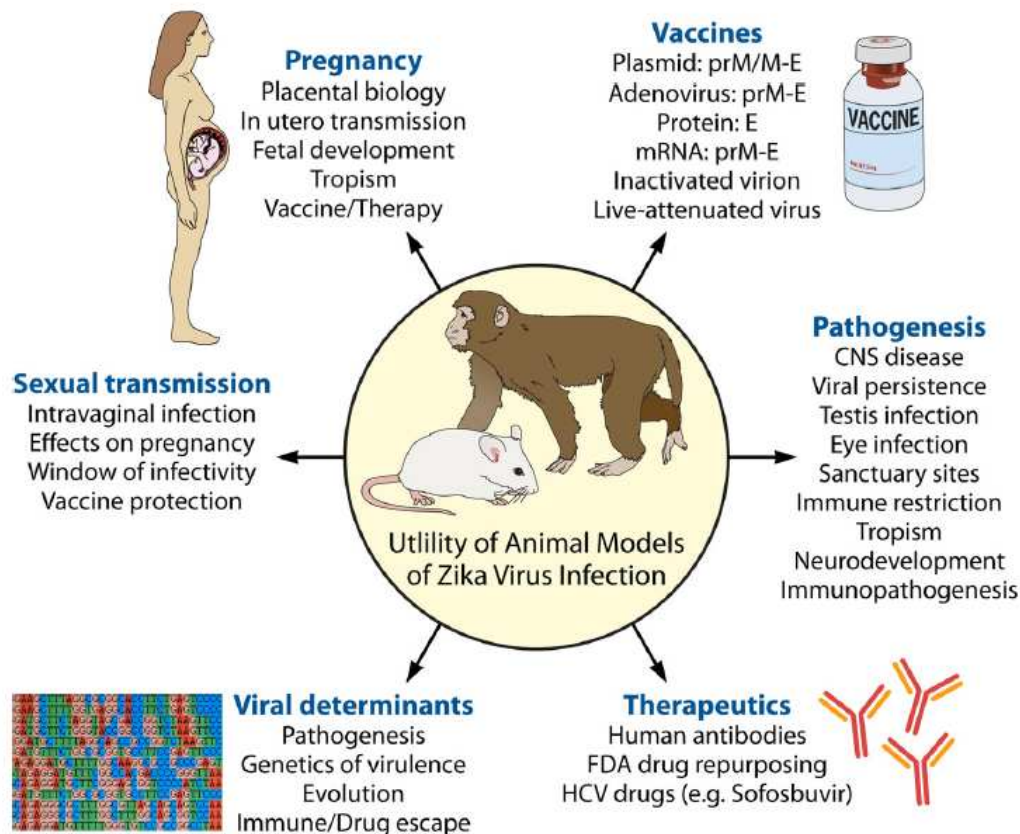


Figure 21. Use of animal models to study ZIKV infection.
 Extracted from (Morrison and Diamond 2017).

2.3.5. Insights from animal models on ZIKV neuro-pathogenesis

Considering the distinctive feature of ZIKV to trigger neuro-developmental defects during pregnancy, potential underlying mechanisms uncovered thanks to animal models are summarized hereafter.

Following the detection of ZIKV in brain clinical samples of human fetuses from infected pregnant women, an *in vitro* model based on human induced pluripotent stem cells (hiPSCs) provided the first evidence that cortical neural progenitor cells (NPCs) can be targeted by the virus (Tang *et al.* 2016a). Using three-dimensional (3D) cultures of brain organoids, several groups also showed that ZIKV is able to infect astrocytes, microglial cells and oligodendrocyte progenitor cells (OPCs) (Cugola *et al.* 2016; Dang *et al.* 2016; Garcez *et al.* 2016). The use of animal models further confirmed ZIKV neuro-tropism and target cells in the developing brain (Cugola *et al.* 2016; Li *et al.* 2016a; Shao *et al.* 2016; Wu *et al.* 2016), but were especially instrumental to study the mechanisms leading to neuro-pathology after ZIKV infection (Figure 22).

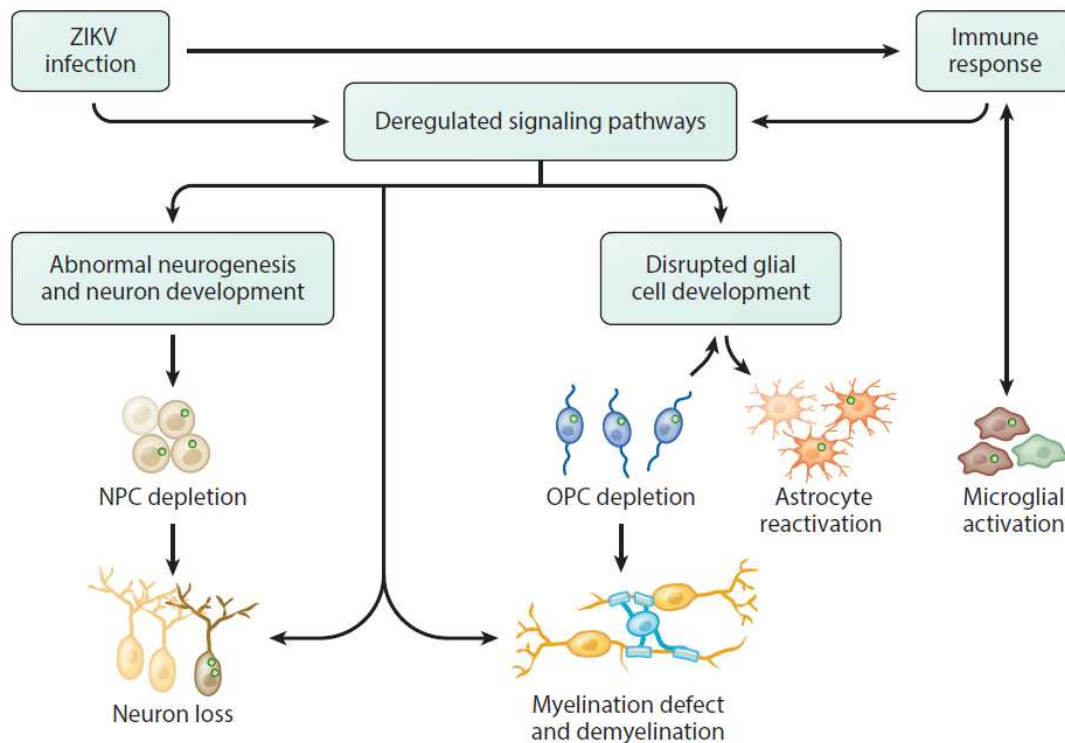


Figure 22. Mechanisms underlying ZIKV-induced neuro-pathology. Extracted from (Xu *et al.* 2019).

Neurogenesis can be strongly impaired by a deregulation in cell cycle progression of NPCs, and indeed, NPC proliferation was reduced in ZIKV-infected developing mouse brains. The ventricular zone of the fetal cortex was shown to contain a decreased number of mitotic cells (Li *et al.* 2016a; Nguyen *et al.* 2016; Wu *et al.* 2016), several studies demonstrated that most ZIKV-infected cerebral cells were blocked in the S-phase of the cell cycle (Li *et al.* 2016a; Shao *et al.* 2016). ZIKV infection also leads to a decrease in the proliferation of NPCs in the adult mouse, especially in the hippocampus region of the brain (Li *et al.* 2016b).

Cell-death is another process contributing to ZIKV-induced neuropathology, excessive apoptosis was actually reported in the neocortex of mouse and NHPs fetuses (Martinot *et al.* 2018; Shao *et al.* 2016). Caspase-3 activation, indicative of apoptosis, was reported *in vivo* and correlated with a reduced pool of NPCs and with smaller brain size (Li *et al.* 2016a; Miner *et al.* 2016a; Shao *et al.* 2016; Wu *et al.* 2016). Interestingly, ZIKV has been shown to trigger the unfolded protein response, a cellular stress reaction related to the ER, in NPCs of mouse fetuses. This reaction leads to a reduction in the number of intermediate neurons generated by the NPCs and to an increase of susceptibility of their neuronal progeny to the unfolded protein response-dependent apoptosis (Gladwyn-Ng *et al.* 2018). Moreover, activation of the innate immune response may also mediate ZIKV-induced apoptosis and impaired neurogenesis. Notably, many genes of the innate immune response (*Rsad2*, *Isg15*, *Ifnb1* etc.), and genes involved in inflammatory cytokines production (*Il1b*, *Cxcl10*, *Ccl5* etc.) are strongly up-regulated in ZIKV-infected mouse brain cells (Gorman *et al.* 2018; Li *et al.* 2016a; Shao *et al.* 2016).

Glial cell development and microglial activation following infection are also implicated in ZIKV neuro-pathogenesis (Wu *et al.* 2016). Glial cells represent at least half of the total brain cell

population; their number was dramatically decreased in neonatal mouse brains exposed to ZIKV *in utero* and their progenitors (OPCs) were less proliferative (Li *et al.* 2018a). In several animal-models, a major increase of Iba1 positive cells in ZIKV-infected brain samples indicates a strong microglial activation and neuro-inflammation (Gurung *et al.* 2019; Huang *et al.* 2016; Shao *et al.* 2016; Smith *et al.* 2017). Actually, glial cells have been shown to trigger the inflammasome pathway leading to the production of inflammatory cytokines such as IL-1 β . (Meertens *et al.* 2017). Finally, microglial reaction can also drive neuronal and synaptic loss, more specifically characterized by the elimination of post-synaptic termini in the hippocampal region of the brain from ZIKV-infected mice (Garber *et al.* 2019).

Microglial activation and inflammation could also engender vascular anomalies such as alterations in permeability or breakdown of the BBB. ZIKV infection has been shown to alter blood vessel density and structure in the brain of mouse fetuses (Shao *et al.* 2016). In addition, BBB disruption has been detected in adult mice only at late time points after ZIKV infection, consistent with the hypothesis that vascular leakage is triggered by the inflammation rather than by the virus itself (Papa *et al.* 2017).

Currently, elucidating the mechanisms implicated in ZIKV pathogenesis remains a field of intense research. The establishment of animal models of ZIKV infection has greatly facilitated the study of ZIKV biology and has enabled to start defining the underlying mechanisms. In addition to studying the linear course of ZIKV disease, animal models also provide a solid platform to investigate the genetic determinants of ZIKV pathogenicity and vectorial transmission, of both viral and host origins, which are the subject of the next two parts (2.4 and 2.5).

2.4. Viral genetic determinants of ZIKV pathogenicity

The large-scale ZIKV epidemics in the Americas and the associated disease severity, with neurological damage in adults and in fetuses, were neither previously seen nor expected. One straightforward hypothesis is that the virus has acquired mutations that have enhanced its virulence and its pathogenicity. Determining if and how ZIKV has evolved to cause these massive outbreaks, to be sexually transmitted and to trigger neurological syndromes has already been the subject of several publications whose results are mentioned in this part.

One way to determine if viral mutations are implicated in the recent outbreaks in the Americas is to compare currently circulating strains with archived strains of ZIKV. As for other RNA viruses, mutations occur as a result of error-prone viral replication. Some mutations have negative effects and will be removed quickly, others are selectively neutral and can be maintained over time, and finally some mutations can confer a selective advantage and increase the virus fitness and thus change the course of the epidemiological situation. Among the numerous mutations arising in the RNA genome of ZIKV, those occurring at the nodes of phylogenetic branches are good candidates as all viral strains

in that branch have inherited them. Once identified, the phenotype resulting from these mutations of interest can be analyzed through a reverse genetics approach: new viruses can be engineered with these mutations and then compared to the initial wild-type virus (Liu *et al.* 2019; Rossi *et al.* 2018).

2.4.1. Phenotypic differences of ZIKV lineages

Despite the fact that the Asian lineage has been responsible for most ZIKV epidemics and associated with severe disease, evidence from *in vitro* and *in vivo* experiments rapidly indicated that African strains are actually more pathogenic than strains of the Asian lineage (Smith *et al.* 2018). ZIKV African strains yield higher titers and have stronger cytopathic effects in cell culture (Anfasa *et al.* 2017; Bowen *et al.* 2017; Sheridan *et al.* 2018; Simonin *et al.* 2019); they are also more pathogenic in animal models (Dowall *et al.* 2017; Rossi *et al.* 2016; Shao *et al.* 2016), usually resulting in severe disease and mortality in *Ifnar1*^{-/-} mice by comparison with mice infected with Asian-lineage viruses (Table 5). One important limitation of many of these studies is that they used old ZIKV African strains, such as the MR 766 strain that was serially passaged on mouse brains, and are unlikely to reflect currently circulating African ZIKV.

These findings therefore suggested that Asian lineage viruses (Asian and American ZIKV strains) may be less pathogenic than strains of the African lineage even though they lead to neurological disease, and especially to CZS. Considering these contradictory results, researchers then decided to specifically focus on the Asian lineage through a genomic comparison between pre- and post-epidemic strains.

2.4.2. Contribution of mutations in ZIKV Asian-lineage to the recent outbreaks

Several studies have investigated the effects of several mutations in ZIKV genome, most of which affect structural or non-structural proteins of the virus and therefore impact viral transmission or infection.

2.4.2.1. PrM S139N mutation contributes to increased neuro-pathogenesis

Yuan *et al.* compared three ZIKV isolates from the South-American outbreak with a pre-epidemic clinical isolate from Cambodia 2010 (Yuan *et al.* 2017). All 3 epidemic strains showed increased infectivity in mouse NPCs and caused a more severe microcephaly phenotype in mouse fetuses after i.c. inoculation of ZIKV *in utero*. They identified several mutations of interest thanks to phylogenetic analyses, which were then engineered back into the Cambodian infectious clone. In the Cambodian clone, only one serine-to-asparagine (S139N) mutation exhibited significantly enhanced neurovirulence, with more severe microcephaly and higher mortality rates in mouse neonates.

Advanced evolutionary analyses revealed that this S139N substitution probably originated during the French Polynesia outbreak and has been maintained since 2013 (Pettersson *et al.* 2016). However, Yuan *et al.* did not include any African ZIKV strain in their comparison and did not assess neurovirulence after peripheral inoculation *in vivo*, thus restricting the relevance of their results.

Indeed, a recent study demonstrated, in a mouse model of vertical transmission, that African ZIKV can infect mouse fetuses, resulting in neurological damage, and that the S139N substitution does not seem essential for ZIKV to trigger fetal injury (Jaeger *et al.* 2019). The phenotype of S139N mutation of ZIKV prM needs to be further characterized in order to fully appreciate its contribution to ZIKV neuro-pathogenesis.

2.4.2.2. NS1 A188V mutation increases mosquito transmission of ZIKV

Liu *et al.* compared a ZIKV clinical isolate from Venezuela to a pre-epidemic strain from Cambodia in terms of transmission of the virus from the host (an infected mouse) to the mosquito vector *Aedes aegypti* (Liu *et al.* 2017). Their study revealed that, in spite of similar viremia levels, the plasmatic levels of NS1 were increased in mice infected with the Venezuelan strain and correlated with a higher prevalence of infected mosquitoes which fed on these mice. Subsequently, they identified two mutations in the NS1 segment among which the alanine-to-valine A188V substitution was able to both increase NS1 secretion in the host and enhance mosquito infectivity. Evolutionary analysis showed that this mutation likely appeared in South Asia between 2003 and 2007 (Delatorre *et al.* 2017).

NS1 is a multifunctional glycoprotein that plays a critical role in viral replication as well as in evasion from the host immune response (cf 2.2.4.2.1), accordingly the NS1 A188V mutation might have multiple functional consequences. Xia *et al.* reported that this A188V mutation provides NS1 with the capacity to antagonize the induction of the type I IFN response (Xia *et al.* 2018).

In summary, ZIKV mutations that may have contributed to the major outbreaks have just started to be identified and characterized. More mutations are likely to be reported in the coming years and accumulation of field and experimental data will be necessary to evaluate precisely their roles in the rapid spread of ZIKV and the severity of the recent outbreaks.

However, answering this outstanding question will require more complex hypotheses and much broader investigations. Indeed, a vast majority of studies on ZIKV have made the assumption that the severity of the epidemics are primarily virus-mediated, and have neglected the contribution of genetic variations of the host and of the vector. Assessing the current knowledge on the influence of host genetic factors in the susceptibility to ZIKV is the subject of the following part (2.5).

2.5. Host genetic determinants of susceptibility to ZIKV

One shared feature of infectious diseases is that a given population exposed to a pathogen will not uniformly develop clinical signs of disease. Individual variations in disease severity are the rule. In other words, the infection by a pathogenic agent is necessary but not sufficient for an individual to be clinically affected. Natural genetic variants are implicated in these individual phenotypic differences

and can affect disease severity and outcome. For instance, host genetics shapes the kinetics and amplitude of the innate and adaptive immune responses, resulting in differences in susceptibility to infectious diseases within and between populations. Unlike host restriction factors, which are conserved molecules demonstrating a direct antimicrobial activity, host genetic determinants of susceptibility or resistance to a viral infection are defined as polymorphisms in the genome that can be associated with differential clinical severity or pathogenesis, and that affect the expression, the function or the interactions of very diverse host factors.

2.5.1. Evidence for a role of host genetics in the susceptibility to human ZIKV infection

Epidemiological studies often bring the first clues that host genetic factors are implicated in the differential susceptibility to a pathogen. Actually, ethnicity was hypothesized to be a risk factor for the severity of ZIKV disease in few epidemiological reports (El Sahly *et al.* 2019; Flamand *et al.* 2017). Flamand *et al.* described that the proportion of symptomatic infections in pregnant women varied substantially between the different regions of French Guiana during the South American outbreak (Figure 23). The population of the interior regions is mainly composed of Maroons, while the population in the coastal area is more diverse, including Creoles, people of European ancestry and migrants from Asia and South America. One of the authors' hypotheses to explain these differences is thus that ethnicity may affect the risk of developing symptoms after ZIKV infection (Flamand *et al.* 2017).

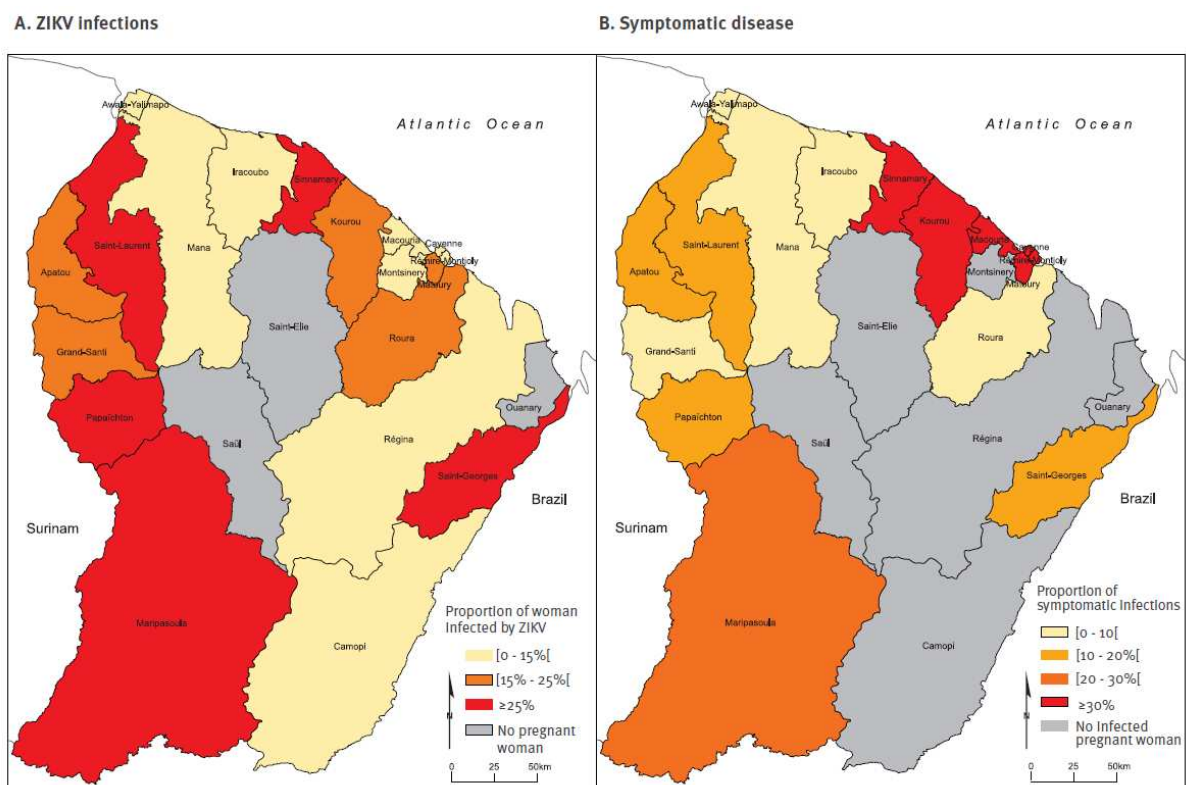


Figure 23. Proportion of pregnant women infected by ZIKV and proportion of symptomatic infections in regions of French Guiana between February and June 2016 (Flamand *et al.* 2017).

Another observation suggesting the role of host genes came from the study of a small cohort of pairs of twins exposed to ZIKV infection during pregnancy. Most of the dizygotic twins (genetically different twins) were clinically discordant for CZS, which suggested that the genetic makeup of the individual could impact its susceptibility to CZS (Caires-Junior *et al.* 2018). Moreover, the authors also showed that ZIKV infection of NPCs derived from affected neonates led to higher viral titers compared to cells from non-affected neonates; bringing a first experimental indication that host genetic background influences the response to ZIKV infection.

The identification of host genetic factors controlling these differences in susceptibility to ZIKV infection could improve our understanding of pathophysiological mechanisms and lead to the discovery of new biomarkers and therapies. Several strategies to identify genetic determinants of other viral infections in humans are illustrated below.

2.5.1. Strategies and challenges to identify host genetic factors affecting susceptibility to viral infections in humans

The identification of human genetic factors affecting susceptibility to a viral infection is based either on candidate gene or whole-genome approaches. These strategies have been successful to discover host genetic determinants with diverse biological functions, although they face limitations inherent to their methodologies and to the complexity of viral diseases in human populations.

2.5.1.1. Single-gene versus whole-genome approaches

2.5.1.1.1. Candidate gene study

Candidate gene studies have been successfully used for the identification of disease susceptibility loci in individuals displaying increased illness severity or rare complications (Kenney *et al.* 2017). This approach is “hypothesis-driven”, the tested genes are chosen based on their role in pathogenesis established by previous *in vitro* or animal experiments. These studies then consist in genotyping or sequencing the polymorphisms in these candidate genes and test them for association with phenotypes of case and control individuals (Chapman and Hill 2012).

One well-documented example of this approach has been the identification of *IFITM3* as a genetic determinant of influenza A virus (IAV) infection (Kenney *et al.* 2017). *IFITM3* was shown to both mediate cellular resistance to IAV (Brass *et al.* 2009) and to restrict the mortality in mice infected with IAV (Everitt *et al.* 2012). The *IFITM3* gene was thus sequenced in patients with severe influenza disease during the 2009 pandemic of H1N1 IAV. Several studies found a significant association between the *IFITM3* variant rs12252-C and severe influenza disease (Everitt *et al.* 2012; Mills *et al.* 2014; Zhang *et al.* 2013). In another example, *TNF* (Tumor necrosis factor) variant rs1800629-A has been linked to an increased risk of cervical cancer in several cohorts of women infected with Human Papilloma virus (Kenney *et al.* 2017).

Despite some successes, the candidate gene approach also suffers from several limitations. The first one is inherent to the “hypothesis-based” strategy, which focuses on already known pathways and mechanisms. The second reason is that many of these studies have failed to be replicated. Several

factors could explain this low reproducibility: small population sample sizes, unknown or ignored population stratification or inadequate statistical analyses (Chapman and Hill 2012).

2.5.1.1.2. Genome-wide linkage analysis

Genome-wide linkage analysis relies on familial co-segregation of polymorphic markers and loci controlling the disease phenotype, and leads to the identification of a chromosomal region containing the genetic determinant of the disease (Clementi and Di Gianantonio 2006).

Examples of successful genome-wide linkage studies in human infectious diseases include the identification of a susceptibility locus to Human T-cell leukemia virus type 1 (HTLV-1) mapped in African children to chromosome 6 (Plancoulaine *et al.* 2006) and a region associated with increased risk of cold sore in individuals infected with Herpes simplex virus (HSV) mapped to chromosome 21 (Hobbs *et al.* 2008). While the first study did not identify the causal locus, the second study pointed at a poorly characterized gene but did not yet lead to further functional dissection (Kenney *et al.* 2017).

Linkage analyses of infectious diseases are limited by the difficulty in recruiting a sufficient number of families comprising more than one infected individual. They also lack statistical power to detect small contributions of several genomic regions acting concomitantly to control complex diseases such as infections (Burgner *et al.* 2006; Chapman and Hill 2012).

2.5.1.1.3. Genome-wide association study (GWAS)

GWAS is currently the favored strategy to identify variants involved in infectious diseases. In these studies, millions of genetic polymorphisms spanning the whole genome are tested for genotype-phenotype associations in large patient cohorts. This hypothesis-neutral approach has the potential to identify unsuspected genetic associations and actually led to the discovery of susceptibility genes to various viral infections (Kenney *et al.* 2017).

Several GWASs have provided the evidence that Human leukocyte antigen *HLA-B*57* is protective in the context of HIV infection, being associated with decreased viral load and lessened decline of CD4⁺ T-cells in infected individuals (Altfeld *et al.* 2003; Fellay *et al.* 2007). GWASs have also enable the discovery of candidate genes controlling susceptibility to Hepatitis C virus (HCV) and Hepatitis B virus (HBV) infections. Two single nucleotide polymorphisms (SNPs) close to *IFNL3* have been associated with spontaneous clearance of HCV and one intronic variant in *STAT4* gene has been associated with increased risk of HBV-induced hepatocellular carcinoma (Kenney *et al.* 2017).

Despite major advances in genotyping and sequencing technologies, GWASs still face several limitations. The probability to detect a true association, i.e the study power, depends on the sample size, the allelic frequencies, the effect size and the significance threshold (Chapman and Hill 2012). GWASs therefore require large cohorts of thousands of individuals and to take into account potential population stratification. In addition, GWASs are usually designed to detect common variants, for which the minor allele frequency reaches at least 5%, and thus explain only part of the heritability of a trait and are not well-powered to detect rare variants influencing susceptibility to a disease (Chapman and Hill 2012).

2.5.1.1.4. Whole exome sequencing study (WES)

WES studies have been developed recently and allow for the discovery of such rare variants. Because the coding regions represent only 1% of the human genome, WES can be an efficient method for the identification of rare, strong-effect genetic determinants underlying specific or uncommon disease phenotypes (Casanova *et al.* 2013; Chapman and Hill 2012; Kenney *et al.* 2017).

For instance, loss-of-function mutations were sequenced in the *IRF7* gene of a 2.5-year-old girl infected with H1N1 and who suffered from a life-threatening acute respiratory distress syndrome (Ciancanelli *et al.* 2016; Ciancanelli *et al.* 2015). These mutations impaired the IRF7 protein function and led to very high levels of viral replication in cells derived from the patient. Elsewhere, a recent study identified a gain-of-function mutation in *NLRP1* leading to inflammasome activation in two brothers suffering from juvenile-onset recurrent respiratory papillomatosis (Drutman *et al.* 2019). These studies prove that WES can be a powerful method to discover rare variants and that single-gene inborn errors of immunity can cause severe viral disease.

If WES can be a cost-effective strategy for discovery of primary immunodeficiencies and rare genetic determinants of infectious diseases, this kind of study is not always straightforward and needs to be carefully designed and interpreted. According to Meyts *et al.*, the major challenge lies in the identification of a few candidate variants among a myriad of sequenced polymorphisms (Meyts *et al.* 2016). Combining a solid genetic hypothesis (based on inheritance mode, clinical penetrance, etc.) with bioinformatics approaches should help identifying plausible candidate variants, which would eventually need to be functionally validated (Meyts *et al.* 2016).

2.5.1.2. Biological mechanisms impacted by known host genetic determinants of viral infection

In addition to this phenotypic classification (variant size effect and viral disease specificity), these host genetic determinants can be grouped according to their biological functions and mechanisms (Kenney *et al.* 2017). In the context of a viral infection, host genetic factors can be implicated at many different stages of the infection (Figure 24), some of which are illustrated hereafter.

Genetic variants in viral receptors can prevent the virus from entering in the target cells: for instance, CCR5 (Chemokine (C-C motif) receptor 5) acts as a co-receptor of HIV and individuals carrying defective copies of this chemokine receptor are highly resistant to the infection (Dean *et al.* 1996). Viral sensing is another key step that can be impacted by genetic mutations with deleterious effects. A dominant negative form of TLR3, has been linked to an increased risk of encephalitis in children infected with HSV (Zhang *et al.* 2007). Genetic variability in key antiviral restriction factors can also have drastic effects on the susceptibility to viral infections. For example, one SNP in the *APOBEC3G* gene has been associated with decline in CD4⁺ T-cells and rapid progression towards acquired immune deficiency syndrome in HIV-positive individuals (An *et al.* 2004). Genetic variants in cytokine genes are frequently associated with either increased vulnerability to viruses, including Respiratory syncytial virus, HTLV-1 and Human Papilloma virus, or resistance to infection, such as HCV and Epstein-Barr virus (EBV) (Kenney *et al.* 2017). Finally, mutations can affect the development of adaptive immune cell and therefore the outcome of the disease. For example,

deficiency in ITK (IL-2-inducible T-cell kinase), which is required for the development of natural killer T-cells, is associated with fatal lymphomas induced by EBV (Kenney *et al.* 2017).

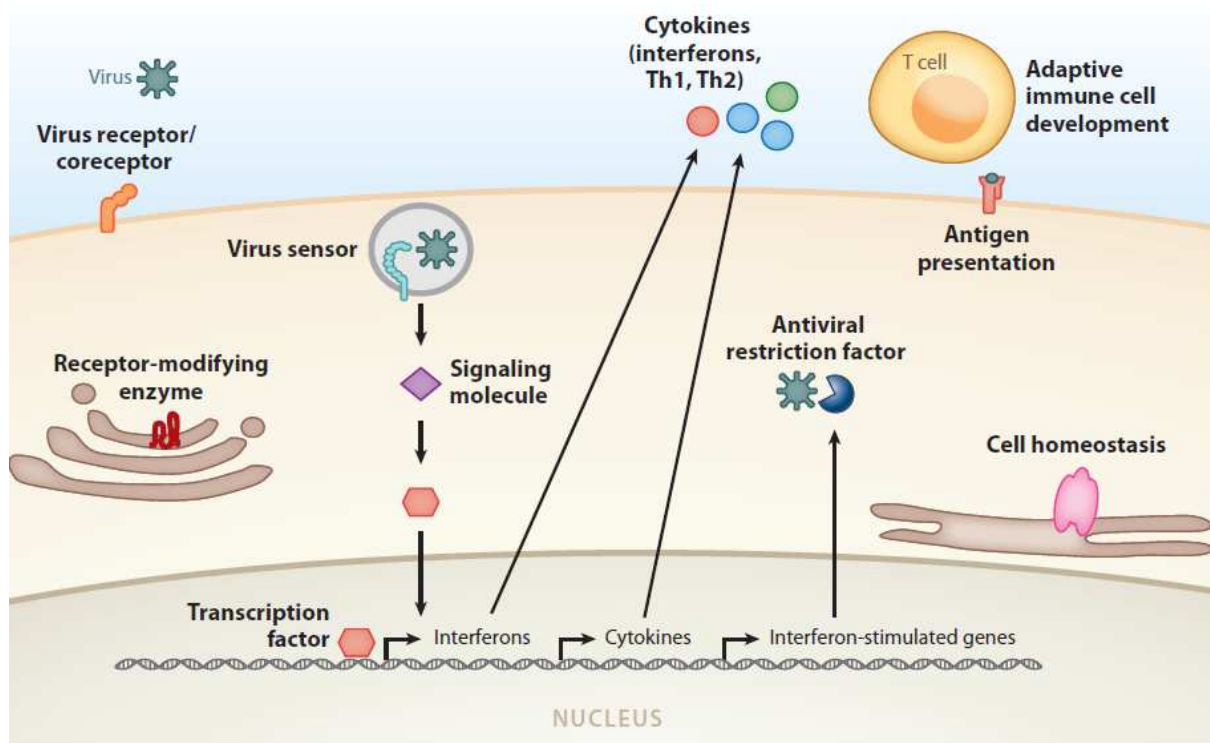


Figure 24. Functional categories of genetic variants of susceptibility/resistance to viral infections. Human genetic determinants of viral diseases can be categorized based on the distinct cellular functions of their associated proteins (bold text). Th, T helper (Kenney *et al.* 2017).

2.5.1.3. Challenges of human genetic studies of susceptibility to viral infections

In addition to methodological issues, human genetic studies of viral diseases are hindered by the complexity of infection conditions in human populations.

As mentioned previously, clinical presentations of a given viral infection can be very heterogeneous among patients and thus complicate the phenotype definition of both cases and controls. For example, clinical syndromes have to be carefully diagnosed using precisely defined criteria, which should be common between multiple medical centers. Similarly, standardized methods should be used for the evaluation of exposure to the virus, which is critical for the recruitment of valid controls (Chapman and Hill 2012; Newport and Finan 2011).

In addition, many parameters of infection vary between individuals or between populations and most of them cannot be assessed in human studies. These include the viral strain or serotype of infection, the viral dose and the infection route, the time of infection, the physiological state of the individual and potential co-morbidities. Other environmental factors also interfere with the course of infection, such as intercurrent treatments that patients could receive either for the infection of interest or for unrelated pathologic conditions. Finally, recruiting patients in the acute phase of infection is challenging and partly explains why many genetic studies have investigated chronic viral diseases (Kenney *et al.* 2017).

In comparison with other viral infections, human genetic studies on the susceptibility to ZIKV are hampered by at least three additional challenges due to the epidemiology of the infection. First, the unexpected emergence and rapid dissemination of the virus in naïve populations excluded this large number of cases from carefully designed cohort studies. Second, passed the peak of epidemics, most of the population exposed to the virus had been infected and had seroconverted, which resulted in a massive reduction in the number of new cases. Lastly, the populations exposed to ZIKV are also at risk of dengue; previous infection with DENV is likely to modify (reduce by Ab cross-protection or increase by ADE) the innate susceptibility to ZIKV infection.

Congenital afflictions represent a singular clinical syndrome of ZIKV infection, which could also be subjected to genetic studies. Such studies will probably turn out to be very challenging due to additional limitations. As the whole spectrum of ZIKV congenital complications is not yet known, defining the phenotype will certainly be a major hurdle to overcome. Multiple but robust inclusion criteria will be required for the recruitment of both cases and controls. Finally, the genomes of both the mother and the fetus can play a role in the development of congenital anomalies; genetic association studies should therefore be cautiously designed and interpreted. One study recently reported an association between polymorphisms in maternal adenylate cyclase genes with CZS (Rossi *et al.* 2019). If this study is a honest attempt to identify host genetic factors controlling the risk for human CZS, its results should be read carefully with respect to several major limitations. Indeed, the study was conducted on a very small cohort of patients, 28 cases and 24 controls, in a restricted population coming from Northeast Brazil and should therefore be replicated in larger and unstratified cohorts. Moreover, only maternal genome was investigated for association with severe CZS, and it is not stated whether precautions were taken for the isolation of maternal DNA from blood, as up to 20% of blood cell-free DNA is of fetal origin in pregnant women (Wong and Lo 2016).

Altogether, these difficulties have motivated searching for host susceptibility genes in mouse models of viral infections.

2.5.2. The identification of susceptibility genes in mouse models of viral infections

Mouse genetic studies have many advantages compared to human studies for the dissection of the genetic architecture underpinning susceptibility to viral infections (Flint and Eskin 2012; Leist and Baric 2018). First of all, a number of infectious diseases of humans can be modelled in the mouse, either because the mouse is naturally susceptible to the infectious agent of humans (e.g. rabies, influenza, etc) or can be rendered susceptible by adapting the viral strain (e.g. Ebola), by introducing the human receptor into the mouse by transgenesis (e.g. poliovirus) or by attenuating the mouse immune response (e.g. flaviviruses). Environment can be strictly controlled, including the microbial environment, the physiological state of the animals, the infection parameters etc. These standardized experimental conditions allow to lower the background noise that can reduce study power and also to investigate gene-by-environment interactions. Another major asset of mouse studies is that many phenotypic traits can be evaluated and measured in any tissue, whereas invasive procedures are obviously restricted in humans thus limiting access to organs of interest (Leist and Baric 2018).

Moreover, mouse models also provide a platform for functional validation of candidate genes for example by modifying the genome through homologous recombination or genome editing. Finally, mouse genetic studies are much easier and less expensive than human studies (Flint and Eskin 2012). Mouse genetic studies thus appear as a valuable strategy to complement human genetic studies. Similarly to human genetic studies, various approaches have been used to identify host genetic determinants of viral infections in mice and are discussed below.

2.5.2.1. Forward genetic approaches

Forward genetics are unbiased approaches which aim at identifying genes underlying phenotypic variations between individuals. It takes advantage from natural variation in the mouse species and does not make any assumption on the mechanisms (Leist and Baric 2018).

More specifically, it begins with the description of contrasted phenotypes in response to infection between two genetically different mouse strains. For example, in 1961 J. Lindenmann found that a particular mouse inbred strain, called A2G, was more resistant to IAV compared to other common mouse laboratory strains (Lindenmann *et al.* 1963). This was the starting point of decades of research that led to the discovery of murine protein MX1, for Myxovirus resistance protein 1. This protein was molecularly characterized (Staeheli *et al.* 1986a), which further allowed locating the *Mx1* gene to mouse chromosome 16 (Staeheli *et al.* 1986b). Additional work showed that wild-derived mice carry a functional *Mx1* allele, whereas most laboratory strains carry loss-of-function mutations in this gene (Staeheli *et al.* 1988).

Once a divergence in susceptibility to viral infection has been characterized between mouse strains, the standard method to identify genetic loci controlling these phenotypic differences is to perform genetic mapping (for monogenic, binary traits) or quantitative trait locus (QTL) analysis (for multigenic, quantitative traits). As for human GWAS, the objective of QTL studies is to search for statistically significant genotype-phenotype associations. A classical approach is to investigate genetic segregation in a mouse population generated by crossing the 2 phenotypically divergent mouse strains. The most common types of crosses used for QTL mapping are the F2 intercross and the backcross (Figure 25). The resulting F2 or N2 progeny are phenotyped and genotyped using markers distributed across the whole genome. Statistical association analysis leads to the identification of genetic loci underlying the resistance or susceptibility phenotype (Figure 25).

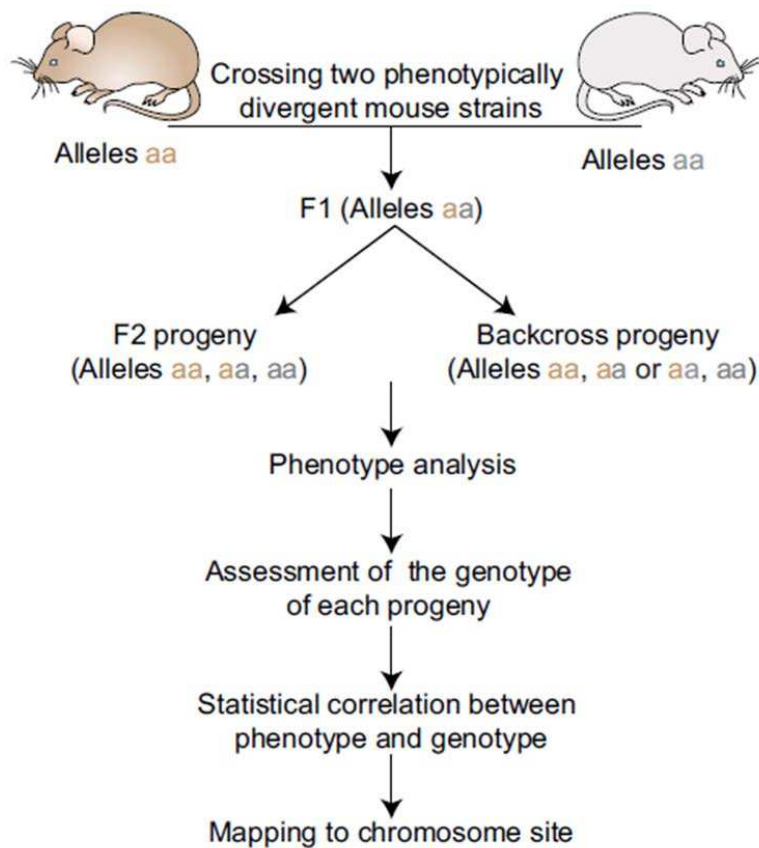


Figure 25. QTL mapping in backcrossed and intercrossed mice.

F1 progeny are generated by crossing two phenotypically divergent strains. F1 mice can be intercrossed to produce F2 progeny or backcrossed to either parental strains to produce N2 progeny. All progenies are phenotyped and genotyped using informative markers across the genome. Statistical phenotype/genotype correlations are carried out to identify genetic loci underlying the given phenotype (Moussa *et al.* 2012).

This strategy has been used extensively to identify host genetic factors controlling various phenotypic traits, including response to viral infection. For example, the WNV resistance locus was mapped to chromosome 5, using several backcrosses between susceptible and resistant mice (Mashimo *et al.* 2002; Urošević *et al.* 1995); and further led to the identification of a loss-of-function mutation in the *Oas1b* gene (see (Manet *et al.* 2018) for more details).

Interestingly, a recent study demonstrated strain differences in susceptibility to ZIKV in a neonatal mouse model. Specifically, the authors investigated the effects of ZIKV infection on the neuro-development of four genetically different inbred mouse strains (C57BL/6J, 129S1/SvImJ, FVB/NJ, and DBA/2J); they highlighted inter-strain variations in neuro-pathology of the brain as well as in long-term behavioral abnormalities (Snyder-Keller *et al.* 2019). These results indicate that host genetic background influences the vulnerability to ZIKV infection and suggest that phenotypically divergent mouse strains could be used to dissect the genetic mechanisms of susceptibility to ZIKV.

2.5.2.2. ENU mutagenesis

Natural variation in the susceptibility to a given viral infection is not always observed among mouse populations. In this case, *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis can be used to produce

new phenotypic variants, which subsequently undergo forward genetics analysis. Large-scale ENU mutagenesis programs have been implemented with the aim of creating new phenotypic variants of traits for which no or little natural variation is observed (Auwerx *et al.* 2004).

ENU is a strong mutagen that induces point mutations in the DNA at a high rate, up to one point mutation per million base pairs (Concepcion *et al.* 2004). This alkylating agent preferentially affects spermatogonial stem cells, allowing for the transmission of the mutation from a male to its progeny (Caignard *et al.* 2014). When transmitted to the progeny, the effects of the mutations are phenotypically evaluated thanks to infectious screens, with the objective to identify “pheno-deviant” individuals. The mutations are then molecularly characterized first by mapping to the mouse genome and then by selecting candidate causative mutations thanks to genome sequencing. Indeed, genomic data allow for variant discovery which can be subsequently filtered for quality control and functional annotation (Caignard *et al.* 2014).

Both B. Beutler and S. Vidal’s groups have been successful in using ENU mutagenesis to dissect the genetic architecture of viral infectious diseases.

Notably, B. Beutler *et al.* established an ENU screen for murine cytomegalovirus (MCMV) (Beutler *et al.* 2005). Over the years, more than 22,000 C57BL/6J mice of the third generation, carrying ENU-induced mutations, were infected with MCMV (Moresco and Beutler 2011). A low-dose viral inoculum was used in this screen to identify extremely susceptible animals. In addition, pheno-deviant mice identified in other screens and displaying singular phenotypes (immunological, developmental etc.) were also tested for MCMV susceptibility (Moresco and Beutler 2011). Altogether, these studies have led to the identification of many mutations underlying resistance to MCMV infection, including genes of the TLR signaling pathway, the type I IFN response, cellular immunity and cell homeostasis (Caignard *et al.* 2014; Moresco and Beutler 2011); and supporting a mechanistic model of cooperation between NK cells, monocytes, and dendritic cells in the establishment of host defense against MCMV (Moresco and Beutler 2011).

S. Vidal’s group, which has focused mainly on bacterial and parasitic infectious diseases, has also discovered in an ENU screen a new susceptibility gene for HSV-induced encephalitis (Caignard *et al.* 2014). This study has led to the identification of a loss-of-function mutation in the Receptor-type tyrosine-protein phosphatase C gene (*Ptpnc*), encoding the tyrosine phosphatase CD45. Mice carrying this mutation displayed abnormal thymic and B-cell development and were affected by intense inflammation in their brain stems (Caignard *et al.* 2013).

In summary, ENU mutagenesis is an unbiased and powerful approach to explore the host genetic determinism of viral infections. This strategy provides a way to expand mouse phenotypic diversity by inducing neo-mutations that mimic naturally occurring variants. Notably, ENU creates various types of variants, including hypomorphic, gain-of-function or loss-of-function mutations. Thus, ENU mutagenesis nicely complements the genetic mapping and QTL analysis of natural variants as well as the reverse genetics approach, presented in the following paragraph.

2.5.2.3. Reverse genetics

Reverse genetics is an approach that starts with a *de novo* genetic modification and analyzes the resulting phenotype. Prior knowledge about the gene of interest increases the chance of phenotypic impact in this hypothesis-driven approach (Leist and Baric 2018). Reverse genetics initially relied on transgenic strategies, then on homologous recombination and recently on CRISPR–Cas9 gene-editing technologies to create knock-out or knock-in mice (Nadeau and Auwerx 2019). Reverse genetics also constitutes a powerful strategy for the functional validation of candidate genes. Beyond this role of identification of susceptibility or resistance gene, transgenic mice studies are instrumental in elucidating the functions of the genes associated with viral infections (Ermann and Glimcher 2012).

After the discovery of IAV-resistance gene *Mx1*, numerous knock-out mice have been used to identify other genes underlying resistance and susceptibility to IAV infection (Ciancanelli *et al.* 2016). Examples include knockout mice lacking RIG-I signaling (Kato *et al.* 2006) and *Ifitm3*-deficient mice (Everitt *et al.* 2012), which are both highly susceptible to IAV infection. On the opposite, IAV infection of *Il15*, *Il17* or *Ccr2* knock-out mice results in a reduced level of leukocyte, neutrophil or macrophage infiltration and thus in increased survival compared to wild-type mice (Medina and Garcia-Sastre 2011). These studies illustrate how reverse genetics can provide mechanistic knowledge of viral pathogenesis.

Another advantage of the reverse genetics approach is that it allows for the identification of susceptibility genes that are common to different viruses, belonging to different genera and families. This is typically the case of *Ifnar1*^{-/-} mice, which, as mentioned previously, proved to be susceptible to very diverse viruses, such as Lymphocytic Choriomeningitis virus, Semliki Forest virus, Theiler's virus and Vesicular Stomatitis virus (Muller *et al.* 1994; van den Broek *et al.* 1995).

2.5.2.4. Genetic reference populations

Early genetic mapping studies often reported large confidence intervals containing hundreds of genes and among which only a minority of causal variants could be identified. The development of mouse genetic reference populations (GRPs) was a compelling progress for genetic studies.

A GRP is a collection of inbred strains displaying broad, balanced and predictable genetic variation, and constitutes a shared and renewable resource (Noll *et al.* 2019; Saul *et al.* 2019). GRPs thus provide new experimental platforms for systems genetics approaches, which couple genetics with genome-scale molecular phenotyping to characterize the biological networks underlying complex traits. In other words, it integrates genomic variation with gene expression, protein activation and abundance, molecular and environmental interactions to dissect the biological mechanisms regulating complex diseases (Noll *et al.* 2019; Saul *et al.* 2019).

GRPs usually gather multiple recombinant inbred (RI) strains, which are generated by intercrossing two or more parental inbred strains to produce recombinant mice, with genomic contributions from each founder, and are later bred to homozygosity (Noll *et al.* 2019; Saul *et al.* 2019). GRPs are permanent resources: they can be used in different laboratories, in different conditions, and big datasets can be gathered and shared for comparisons. Examples of mouse GRPs and their use to study viral infections are illustrated below.

2.5.2.4.1. Bi-parental recombinant inbred strains

Bi-parental RI strains are generated by sibling mating between F2 intercross animals until the resulting progeny is fully inbred (Figure 26). The first RI strain panel was developed by D. Bailey in the 1970's by intercrossing BALB/cJ with C57BL/6J mice, hence called "CXB", with the objective to map the major histocompatibility locus (Noll *et al.* 2019). Other bi-parental RI panels include the AXB/BXA (A/J x C57BL/6J, C57BL/6J x A/J), the BXH (C57BL/6J x C3H/HeJ) and the BXD (C57BL/6J x DBA/2J), which is the largest and most widely used collection. Production of the BXD family started in 1971 by B.A. Taylor and now comprises approximately 150 RI strains (Schughart and Williams 2017). These panels were originally intended for studying monogenic traits and build genetic maps of markers but were rapidly used for the genetic dissection of complex traits including infectious diseases (Ashbrook *et al.* 2019; Noll *et al.* 2019).

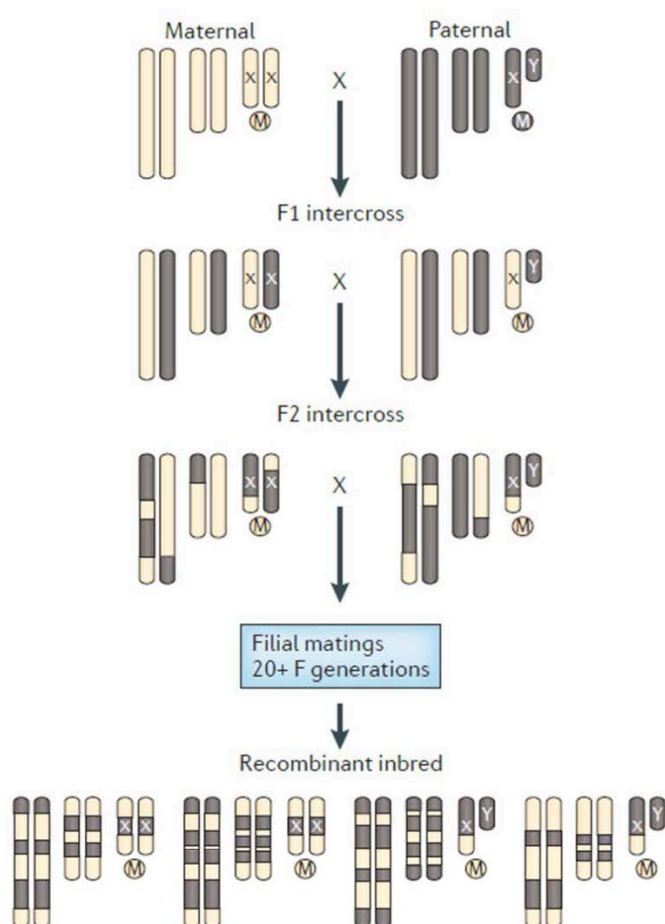


Figure 26. Breeding schemes for mouse QTL analysis in recombinant inbred strains.

RI strains are generated by sibling mating F2 intercross animals until the resulting progeny is fully inbred, at least 20 generations later (Flint and Eskin 2012).

For example, the BXD family was used to study host genetic control of IAV infection. Using 53 BXD strains, Nedelko *et al.* identified 2 significant and 3 suggestive QTLs controlling susceptibility to H1N1 IAV infection (Nedelko *et al.* 2012). QTLs for body weight and survival showed a time-dependent profile and suggested that host genetic determinants in these QTLs control the host response in a temporal fashion. Data mining based on gene functions, gene expression and

sequence variants between C57BL/6J and DBA/2J parental strains led to a selection of 31 candidate causal genes (Nedelko *et al.* 2012).

Classic bi-parental RI strains harbor several advantages for QTL mapping compared to F2 or backcrossed mice. First, with more recombination events per genome they allow for higher mapping resolution (Schughart and Williams 2017). Second, unlike F2 or backcross individuals which are unique, RI strains are inbred populations in which phenotypes can be measured on multiple identical individuals and replicated. However, if these panels are great tools for the study of complex traits, causal gene identification can be challenging partly because of the lack of genetic diversity between the two parental strains, resulting in “blind-spots” for genetic mapping where the two parental strains share the same haplotypes. It thus appeared to mouse geneticists that the ability to investigate and dissect the mechanisms of complex traits could be improved through the use of larger and more genetically diverse GRPs, and that is what motivated the creation of the Collaborative Cross (Threadgill *et al.* 2002).

2.5.2.4.2. The Collaborative Cross

To increase the genetic diversity in GRPs, an ambitious project of multi-parental populations (MPP) of mice was designed in the early 21st century and carried out the next 10 years (Churchill *et al.* 2004; Threadgill *et al.* 2002), with the objective to promote complex traits genetic analyses and systems genetics studies. The resource that came out of this proposition is now known as the Collaborative Cross, and was followed by the creation of the Diversity Outbred.

The CC is a large collection of RI strains derived from eight genetically diverse founder strains. The CC founders include five classical laboratory strains among which 3 are extensively studied strains (A/J, C57BL/6J, and 129S1/SvImJ) and 2 are used as models for common human diseases (NOD/ShiLtJ for type 1 diabetes and NZO/HILtJ for metabolic syndrome and obesity). To increase the pool of segregating genetic variants, 3 wild-derived mouse strains were added to the breeding scheme and represent different phylogenetic origins of the murine species: *Mus musculus castaneus* (CAST/EiJ); *Mus musculus musculus* (PWK/PhJ); *Mus musculus domesticus* (WSB/EiJ) (Collaborative Cross Consortium 2012).

These 8 founder strains were intercrossed in a breeding scheme to generate recombinant mice with genomic contributions from each parental strain, which were then brought to near homozygous state by siblings mating to finally produce CC RI strains (Figure 27). Breeding was performed by 3 institutions: Oak Ridge National Laboratory in Oak Ridge, USA, which moved to the University of North Carolina at Chapel Hill, USA (Chesler *et al.* 2008); the International Livestock Research Institute in Nairobi, Kenya, which moved to the Tel Aviv University in Tel Aviv, Israel (Iraqi *et al.* 2008); and the Western Australian Institute for Medical Research/Geniad Ltd in Perth, Australia (Morahan *et al.* 2008). Although the initial goal was to reach 1,000 CC strains, hundreds of strains became extinct during the inbreeding process, mainly because of male infertility and genomic incompatibility between wild-derived alleles (Shorter *et al.* 2017).

The Diversity Outbred (DO) is a resource that was developed to complement the CC. DO mice are derived from the same set of founder strains, and was produced by intercrossing over mice from 170 developing CC strains. The DO is maintained as an outbred population through random mating. It

is a valuable tool for high-resolution genetic mapping thanks to the large number of recombination events (Svenson *et al.* 2012). Indeed, early studies successfully led to identification of genes controlling atherosclerosis (Smallwood *et al.* 2014) or benzene-induced toxicity (French *et al.* 2015). DO mice are available from the Jackson laboratory.

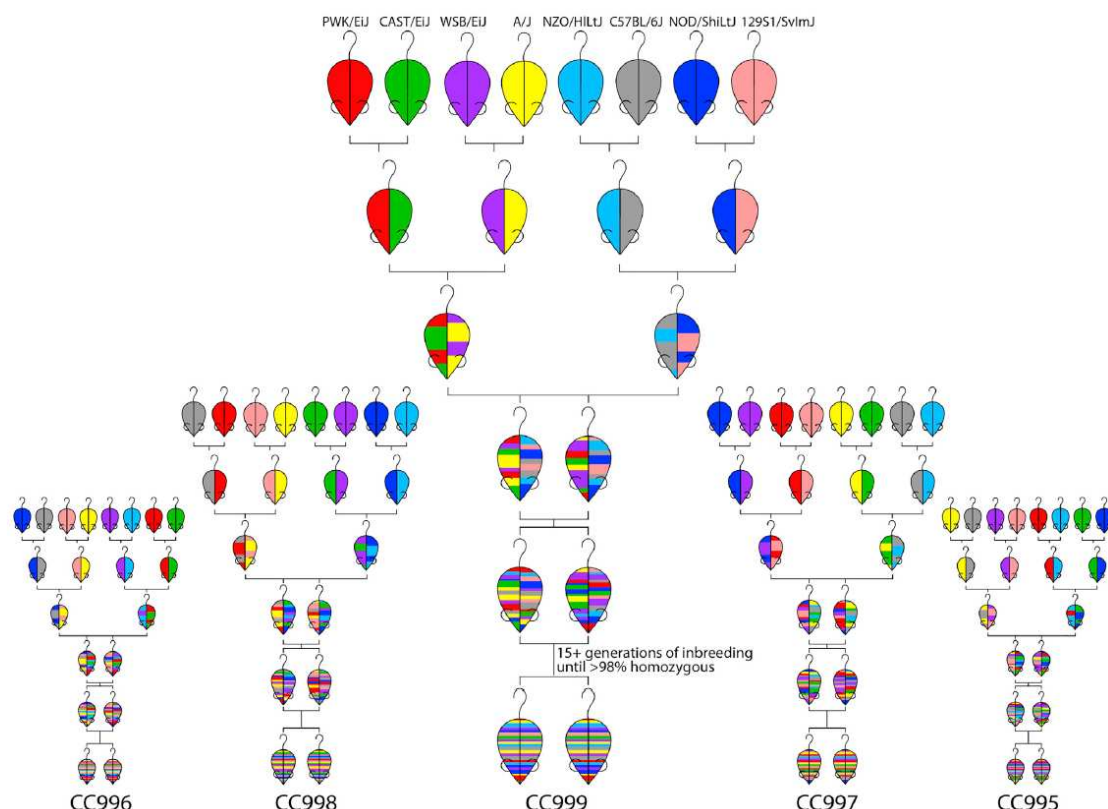


Figure 27. Breeding scheme and representative CC funnels.

CC founder strains were crossed in funnel breeding schemes to produce progeny with genetic contributions from each of the eight founders, at which point they were inbred for generations until reaching near homozygosity. Many different funnels were set up, each to produce a unique CC RI strain (Noll *et al.* 2019).

The level of genetic diversity in the CC population is comparable to the one found across all human populations and the CC was shown to capture about 90% of the genetic variants segregating in laboratory mice (Roberts *et al.* 2007). In addition to this unprecedented genetic variation, novel allelic combinations lead to an extended range of phenotypes, beyond the scope observed in classical laboratory strains and in the CC founder strains. The CC is a new resource but has already been used in various disciplines such as immunity (Collin *et al.* 2019; Graham *et al.* 2017; Kristic *et al.* 2018), toxicology (Venkatratnam *et al.* 2018; Zeiss *et al.* 2019), metabolic diseases (Abu-Toamih Atamni *et al.* 2017; Atamni *et al.* 2016) and susceptibility to infectious diseases (discussed hereafter). There are currently around 80 CC strains available for distribution, from the UNC Systems Genetics Core Facility (UNC Systems Genetics) and/or from the Jackson laboratory (The Jackson Laboratory).

The CC and DO MPPs were specifically designed for complex traits analysis hence they form a powerful platform for systems genetics studies and for data integration. Notably, the CC is particularly suited to study the influence of host genetic factors in host-pathogen interactions.

The CC, a powerful tool to study host genetic control of susceptibility to viruses

Over the past decade, the CC has turned out to be a convenient and powerful resource to study infectious diseases. Initial studies using pre-CC lines (i.e. which were not yet fully inbred) provided proof-of-concept by identifying QTLs controlling the susceptibility to *Aspergillus fumigatus* (Durrant *et al.* 2011) and to *Klebsiella pneumoniae* (Vered *et al.* 2014). These early studies confirmed the considerable phenotypic diversity in response to infection and sometimes identified completely novel disease phenotypes. A few examples of utilization of the CC to probe the role of host genetics in response to viral infections are illustrated below.

Ebola virus (EBOV)

Recently, EBOV has been responsible for several major outbreaks in West Africa, causing severe hemorrhagic fever in humans with a high mortality rate. Classical mouse laboratory strains sustain viral infection with a mouse-adapted EBOV (MA-EBOV), though this model does not recapitulate the typical clinical features of Ebola clinical syndrome such as rash and coagulopathy. Rasmussen *et al.* investigated the response to MA-EBOV in the CC founder strains and in a panel of F1 crosses between CC strains (CC-F1s) (Rasmussen *et al.* 2014). The authors described an extensive variation in disease phenotype, from complete resistance to lethality, and a spectrum of clinical and anatomo-pathological findings (from normal to hepatic injury and hemorrhagic syndrome) comparable to the range found in humans. Representative susceptible and resistant strains were subsequently used to study the transcriptional response to infection. A few differentially expressed genes were unique to susceptible mice, among which the endothelial tyrosine kinase *Tek*, for which haplotypes across the CC-F1s correlated with MA-EBOV-induced mortality. The causal role of *Tek* variants has yet to be established.

Influenza A virus (IAV)

Maurizio *et al.* determined the heritability of IAV-induced disease in CC-F1s and in F1 crosses between CC parental strains (Maurizio *et al.* 2018). The heritability of body weight loss 4 days after infection was 57% and was mostly attributable to the haplotype of *Mx1*, a major restriction factor of IAV. Transcriptomic analyses are also valuable to study the influence of CC genetic diversity on the susceptibility to a viral infection. Indeed, mouse genetic background of 11 CC strains had a strong influence on the transcriptional signature and played a major role in the severity of Influenza disease (Kollmus *et al.* 2018). Besides, human studies have identified changes in the blood transcriptomic signature between infected and non-infected people, which could be useful to identify specific biomarkers and for patient prognosis. Schughart *et al.* found a good correlation between the transcriptomic profiles of IAV-infected people and of the CC founder strains (Elbahesh and Schughart 2016). A similar study revealed that the top-regulated genes in humans were also differentially expressed in 11 CC strains infected with IAV (Kollmus *et al.* 2018).

CC mice were also used to search for QTLs and expression QTLs (eQTLs) controlling Influenza disease severity. Bottomly *et al.* selected a sample of pre-CC lines (i.e. which were not yet fully inbred), classified as low or high responders to IAV (as determined by the extent of viral replication and weight loss), performed transcriptional analysis and genetic mapping, and identified 21

significant eQTLs controlling transcriptional response to infection (Bottomly *et al.* 2012). In another study, QTL analysis of Influenza disease phenotypic traits (clinical disease, viral replication, virus-induced inflammation, pathology and transcription) was completed on 155 pre-CC lines and led to the identification of 4 significant QTLs, including one strong QTL spanning over the well-known IAV resistance gene *Mx1* (Ferris *et al.* 2013). Interestingly, a new allelic variant of *Mx1* deriving from CAST/EiJ was discovered, which appeared to less efficiently restrict viral replication. Consistent with this finding, Leist *et al.* found that, among CC founders, CAST/EiJ displayed a singular response to the H3N2 strain of IAV, characterized by high viral loads in the lungs, low granulocytic infiltration and elevated recruitment of macrophages. This unique phenotype correlated with a transcriptomic signature showing an abnormal response in leukocytes recruitment (Leist *et al.* 2016).

West-Nile virus

Graham *et al.* assessed the susceptibility to WNV infection in a panel of CC-F1s and described a broad range of disease phenotypes, from resistance to high susceptibility. They also noticed a new outcome category in which mice have high viral loads in the brain without displaying any clinical symptoms. The authors performed a detailed phenotyping analysis on one specific CC-F1 ((CC032/GeniUnc x CC013/GeniUnc)-F1), in which surviving mice presented with a “chronic form” of WNV disease. These mice were characterized by a rapid innate immune response capable of restricting but not clearing viral replication; and by a specific immunoregulatory profile with an elevated number of T regulatory cells (Graham *et al.* 2016).

In this study, a global correlation was found between WNV susceptibility and the haplotype of *Oas1b*, a well-known flavivirus resistance gene. However, some extent of phenotype variability was observed within groups of mice sharing the same *Oas1b* haplotype. Green *et al.* further investigated the impact of *Oas1b* haplotype on the immune transcriptional response to WNV infection. The authors found that the *Oas1b* haplotype influenced WNV susceptibility and disease parameters, but revealed that the presence of a functional *Oas1b* allele in heterozygous mice did not always predicted protection against disease (Green *et al.* 2017).

In summary, the CC combines the advantages of mouse GRPs and extensive genetic diversity, thus representing a unique resource to study complex traits, including infectious diseases. As illustrated previously, the CC allows for both phenotypic characterization and genetic mapping analyses, potentially leading to the development of new mouse models and to the discovery of disease-related mechanisms.

2.5.3. Mouse and human genes controlling the susceptibility to flaviviruses

As a focused introduction to my experimental work, we published in the journal *Mammalian Genome* the current knowledge on host genetic control of mosquito-borne flavivirus infection, including ZIKV, in mice and humans (Manet *et al.* 2018).

Title

Host genetic control of mosquito-borne flavivirus infections.

Abstract

Flaviviruses are arthropod-borne viruses, several of which represent emerging or re-emerging pathogens responsible for widespread infections with consequences ranging from asymptomatic seroconversion to severe clinical diseases and congenital developmental deficits. This variability is due to multiple factors including host genetic determinants, the role of which has been investigated in mouse models and human genetic studies. In this review, we provide an overview of the host genes and variants which modify susceptibility or resistance to major mosquito-borne flaviviruses infections in mice and humans.

Aim of the review

In this review, we summarize the evidence demonstrating the role of host genes in the susceptibility or resistance to flaviviruses in mice and humans, with emphasis on innate immunity. We focus on infections caused by WNV, DENV, ZIKV, JEV, and YFV. While many host genes have been shown to interfere with virus biology in cultured cells, we considered only genes for which variants have been associated with differential clinical severity. Many mouse studies have focused on WNV, while human studies investigated mostly susceptibility to highly prevalent DENV, thus limiting comparisons between the two species.



Host genetic control of mosquito-borne Flavivirus infections

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Abstract

Flaviviruses are arthropod-borne viruses, several of which represent emerging or re-emerging pathogens responsible for widespread infections with consequences ranging from asymptomatic seroconversion to severe clinical diseases and congenital developmental deficits. This variability is due to multiple factors including host genetic determinants, the role of which has been investigated in mouse models and human genetic studies. In this review, we provide an overview of the host genes and variants which modify susceptibility or resistance to major mosquito-borne flaviviruses infections in mice and humans.

Introduction

Flaviviruses constitute a large genus of arthropod-borne viruses, several of which represent emerging or re-emerging pathogens. Important members of this genus include West Nile (WNV), dengue (DENV), Zika (ZIKV), Japanese encephalitis (JEV), and yellow fever (YFV) viruses, all of which are transmitted by mosquitoes. In humans, flavivirus infections can remain asymptomatic, trigger flu-like symptoms, or progress towards severe complications such as encephalitis, hemorrhagic fever or, in the case of ZIKV, Guillain–Barré syndrome and congenital brain developmental deficits. Because of the rapid progression of these infections, the innate immune response plays a key role in the quick control of viral multiplication and dissemination

(Suthar et al. 2013). Flavivirus cell entry and genome replication trigger multiple sensing events, activation of antiviral effectors through the type I interferon (IFN) pathway, cellular stress reaction, and inflammation (Valadao et al. 2016). Like other viruses, they have evolved a variety of mechanisms to block the IFN pathway at different steps through interactions between their non-structural proteins (Cedillo-Barrón et al. 2018), in particular NS5 (Best 2017; Laurent-Rolle et al. 2010), and molecular components of this pathway (Cumberworth et al. 2017; Wu et al. 2017).

Mouse models have been developed to study the pathophysiology of mosquito-borne flavivirus infections, to model the complications observed in humans (in particular encephalitis and neuroinvasive disease, but also intrauterine growth restriction and fetal demise caused by ZIKV infection during pregnancy) and to test novel preventive and therapeutic countermeasures (Julander and Siddharthan 2017). However, while most laboratory strains of mice are naturally susceptible to WNV (Mashimo et al. 2002) and JEV (Wang and Deubel 2011), their infection with DENV fails to elicit overt signs of disease (Zellweger and Shresta 2014) and they are somewhat refractory to ZIKV with the exception of very young mice or genetically manipulated strains with immune deficit (Julander and Siddharthan 2017). In humans, DENV and ZIKV inhibit type I IFN response by STING cleavage (Aguirre et al. 2012; Ding et al. 2018) and by NS5-induced STAT2 degradation (Best 2017; Grant et al. 2016), but these restriction mechanisms are inefficient in mice (Best 2017; Ding et al. 2018; Miorin et al. 2017). Efficient infection is obtained in mice genetically deficient in the receptors for type I (encoded by the *Ifnar1* and *Ifnar2* genes) and/or type

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II (*Ifngr1* and *Ifngr2* genes) IFNs. Alternatively, the type I IFN receptor can be blocked pharmacologically by anti-IFNAR antibody injection prior to infection (Sheehan et al. 2006, 2015). Recently, an immunocompetent mouse model for ZIKV infection has been produced by infecting with a mouse-adapted ZIKV strain mice in which the *Stat2* gene had been replaced with its human version (Gorman et al. 2018).

The mechanisms underlying the variable severity of symptoms in human patients and between mouse inbred strains remain poorly understood, although the viral strain and inoculum are obvious contributing factors. Host microbiota can also influence susceptibility to flaviviruses, as shown in mice by oral antibiotic treatment (Thackray et al. 2018). Host genetic determinants affect the susceptibility of humans or animal species to infections. In this review, we will summarize the evidence demonstrating the role of host genes in the susceptibility or resistance to flaviviruses in mice and humans, with emphasis on innate immunity. We will focus on infections caused by WNV, DENV, ZIKV, JEV, and YFV. While many host genes have been shown to interfere with virus biology in cultured cells, we will consider only genes for which variants have been associated with differential clinical severity. Many mouse studies have focused on WNV, while human studies investigated mostly susceptibility to highly prevalent DENV, thus limiting comparisons between the two species.

Mouse models

In mice, the effect of specific genetic variants has been identified either through a genome-wide, forward genetic approach (from phenotypic variation to underlying gene variants) or by testing the consequences of genetic ablation of specific genes chosen from their function (reverse genetic approach). Table 1 presents a cross-compilation of all mouse genes which have been shown to influence clinical severity and/or lethality after infection with WNV, DENV, ZIKV, YFV, or JEV. The corresponding experimental details, results, and references are provided in Table 2.

Genome-wide search for genetic association

The forward genetic approach to identify flavivirus-resistance genes has been successfully applied so far only to WNV infection, taking advantage of the contrast between the high susceptibility of several laboratory inbred strains and the strong resistance of wild-derived inbred strains, one of the first examples of inherited resistance to a pathogen to be described in mice (Webster 1937; Webster and Clow 1936). Flavivirus resistance was found to be inherited in a monogenic, autosomal dominant manner (Darnell et al.

Table 1 List of mouse genes which have been shown to affect clinical outcome and/or mortality following infection with WNV, DENV, ZIKV, YFV, and JEV

Gene (synonym)	WNV	DENV	ZIKV	YFV	JEV
<i>Atg16l1</i>			X		
<i>Ccr2</i>	X	X			
<i>Ccr4</i>		X			
<i>Ccr7</i>	X				
<i>Clec5a</i>		X			
<i>Cxcl10</i>	X	X			
<i>Cxcr3</i>	X	X			
<i>Ddx58 (RIG-1)</i>	X				
<i>Dhx58 (LGP2)</i>	X				
<i>Ifih1 (MDA5)</i>	X				
<i>Ifi2712a</i>	X				
<i>Ifit2</i>	X				
<i>Ifitm3</i>	X				
<i>Ifna</i>	X				
<i>Ifnb</i>	X				
<i>Ifng</i>	X				
<i>Ifnar1</i>	X	X	X	X	
<i>Ifngr1</i>	X	X	X	X	
<i>Ifnlr1</i>	X		X	X	
<i>Il10</i>	X				
<i>Il12a</i>	X				
<i>Il12b</i>	X				
<i>Il23a</i>	X				
<i>Irf1</i>	X	X			
<i>Irf3</i>	X	X	X		
<i>Irf5</i>	X	X	X		
<i>Irf7</i>	X	X	X		
<i>Isg15</i>			X		
<i>Mavs (IPS-1)</i>	X				
<i>Mb21d1 (cGas)</i>		X			
<i>Myd88</i>	X				
<i>Oas1b</i>	X				
<i>Rag1</i>		X	X		
<i>Rag2</i>		X			
<i>Rnasel</i>	X				
<i>Rsad2</i>	X				
<i>Sema7a</i>	X				
<i>Stat1</i>	X	X	X	X	
<i>Stat2</i>		X	X		
<i>Tlr3</i>	X				
<i>Tlr7</i>	X				
<i>Tnfrsf9</i>					X

Details of supporting studies are given in Table 2

1974; Sangster et al. 1993) and was mapped to chromosome 5 (Urošević et al. 1995). Two groups simultaneously identified a loss-of-function mutation in the 2'-5' oligoadenylate

Table 2 Detailed information on mouse genetic studies supporting the role of specific genes on susceptibility or resistance to flaviviruses

Gene symbol (synonym)	Function/pathway	Virus	Mouse strains/cell type	Experimental conditions	Phenotype, observations	References
Genes identified by forward genetics approaches						
<i>Oas1b</i>	Activation of RNA decay pathway by dsRNA recognition	WNV	Susceptible BALB/cByJ, C57BL/6J vs resistant MAI/Pas and MBT/Pas, backcrosses and congenic strains	WNV strain IS-98-ST1, 1000 ffu ip, 6-week-old mice	Most laboratory inbred mouse strains carry a non-sense, loss-of-function mutation making them highly susceptible	Mashimo et al. (2002)
		WNV	MEFs from susceptible C3H/He and resistant C3H.PRI- <i>Flv</i> ^r congenic strain	WNV strain Eg101 In vitro infection of MEFs transfected with cDNAs of candidate genes	C3H.PRI- <i>Flv</i> ^r congenic strain carries a non-sense mutation resulting in higher viral replication in MEFs	Pereygin et al. (2002)
		WNV	Collaborative Cross (CC) and F1 crosses of CC mice	WNV strain TX-2002-HC, 10 ² pfu footpad, 8–10-week-old mice	<i>Oas1b</i> plays a role in susceptibility to WNV but a wide range of WNV-associated phenotypes are observed for a given <i>Oas1b</i> allele	Graham et al. (2015)
<i>Stat1</i>	Promotes transcription of ISG and IFN subtypes	WNV	Backcross between C57BL/6- <i>MHCII</i> - <i>Aa</i> /β-/- and C3H	WNV strain Kunjin, 10 ³ pfu footpad, 8-week-old mice	Highly susceptible <i>MHCII</i> - <i>Aa</i> /β-/- mice have acquired a spontaneous amino acid substitution mutation	Larena and Lobigs (2017)
Genes identified by their function in host immune responses						
<i>Ccr2</i>	Chemokine receptor expressed on Ly6c ^{hi} inflammatory monocytes and other leukocyte subtypes	WNV	C57BL/6J WT and <i>Ccr2</i> KO	WNV strain NY99-35262, 100 pfu sc, 8–12-week-old mice	Increased mortality, large and selective reduction of Ly6c ^{hi} monocyte accumulation in the brain, sustained monocytopenia	Lim et al. (2011)
<i>Ccr7</i>	Chemokine receptor expressed by numerous cell types, which regulates the homing of T cells into lymphoid organs	WNV	C57BL/6J WT and <i>Ccr7</i> KO	WNV strain NY99, 10 ⁴ pfu footpad, 8–12-week-old mice	Increased mortality and CNS viral loads, leukocyte accumulation in the CNS with neuroinflammation and reduced viral clearance	Bardina et al. (2017)
<i>Cxcl10</i>	Chemokine of the CXCR3 subfamily which binds to CXCR3 receptor	WNV	C57BL/6J WT and <i>Cxcl10</i> KO	WNV strain NY99, 10 ⁴ pfu footpad, 5–9-week-old mice	Enhanced morbidity and mortality, increased viral burden in the brain, decreased CXCR3 ⁺ CD8 ⁺ T cell trafficking	Klein et al. (2005)
<i>Cxcr3</i>	Receptor for chemokines CXCL9, CXCL10 and CXCL11 which stimulates leukocyte trafficking	WNV	C57BL/6J WT and <i>Cxcr3</i> KO	WNV strain 3000.0259, 10 ² pfu footpad, 5–8-week-old mice	Enhanced mortality with increased viral burden and reduced CD8 ⁺ T cell trafficking specifically in the cerebellum	Zhang et al. (2008)

Table 2 (continued)

Gene symbol (synonym)	Function/pathway	Virus	Mouse strains/cell type	Experimental conditions	Phenotype, observations	References
<i>Ddx58 (RIG-I)</i>	Cytoplasmic sensor of viral ssRNA and dsRNA	WNV	WT and <i>Ddx58</i> KO (mixed genetic background)	WNV isolate TX 2002-HC, 100 pfu footpad, 6–12-week-old mice	Increased mortality. Decreased innate immune signaling and virus control in MEFs. <i>Ddx58-Iflh1</i> double KO are extremely susceptible	Errett et al. (2013)
<i>Ddx58 (LGP2)</i>	Cytoplasmic sensor of viral RNA and inhibitor of antiviral RNAi by association with Dicer	WNV	C57BL/6J WT and <i>Ddx58</i> KO	WNV isolate TX 2002-HC, 100 pfu footpad, 6–12-week-old mice	Increased mortality despite activation of innate immune response. LGP2 regulates CD8 ⁺ T cell survival and effector functions	Suthar et al. (2012)
<i>Iflh1 (MDA5)</i>	Cytoplasmic sensor of viral dsRNA	WNV	C57BL/6J WT and <i>Iflh1</i> KO	WNV isolate TX 2002-HC, 100 pfu footpad, 6–12-week-old mice	Increased mortality. Decreased innate immune signaling and virus control in MEFs. <i>Ddx58-Iflh1</i> double KO are extremely susceptible	Errett et al. (2013)
<i>Ifl27l2a</i>	ISG expressed at higher levels in granule cell neurons than in cortical neurons	WNV	C57BL/6J WT and <i>Iflh1</i> KO	WNV strain 3000.0259, 10 ² –10 ⁴ pfu footpad, 9–12-week-old mice	Reduced survival. Increased viral burden primarily in the CNS	Lazear et al. (2013)
<i>Ifl2</i>	Aka ISG54, member of the IFN-induced proteins with tetratricopeptide repeats	WNV	C57BL/6J WT and <i>Ifl2</i> KO	WNV strain 3000.0259, 100 pfu footpad, 8–12-week-old mice	Increased susceptibility. Viral burden higher only in the CNS, associated with reduced cell death	Lucas et al. (2015)
<i>Iflm3</i>	ISG inhibiting virus entry and preventing viral fusion and release of viral contents into the cytosol	WNV	C57BL/6J WT and <i>Iflm3</i> KO	WNV strain 3000.0259, 100 pfu footpad or 10 pfu ic, 8–10-week-old mice	Increased susceptibility with higher viral burden in peripheral organs and CNS. Decreased B cells, CD4 ⁺ T cells and Ag-specific CD8 ⁺ T cells	Cho et al. (2013)
<i>Iflna</i>	Interferon- α (type I) with multiple members	WNV	C57BL/6J treated with anti-IFN- α mAb	WNV NY1999, 100 pfu sc, 8–9-week-old mice	Increased susceptibility with higher viral burden in peripheral organs and CNS. Decreased B cells, CD4 ⁺ T cells and Ag-specific CD8 ⁺ T cells	Gorman et al. (2016)
<i>Iflnb</i>	Interferon- β (type I)	WNV	C57BL/6J WT and <i>Iflnb</i> KO	WNV strain 3000.0259, 100 pfu footpad or 10 pfu ic, 8–12-week-old mice	Increased lethality when mAb injected one day prior and two days following WNV infection	Sheehan et al. (2015)
		WNV	C57BL/6J WT and <i>Iflnb</i> KO	WNV strain 3000.0259, 100 pfu footpad or 10 pfu ic, 8–12-week-old mice	Increased mortality with enhanced viral replication in peripheral tissues and the CNS	Lazear et al. (2011)

Table 2 (continued)

Gene symbol (synonym)	Function/pathway	Virus	Mouse strains/cell type	Experimental conditions	Phenotype, observations	References
<i>Ifng</i>	Interferon- γ (type II)	WNV	C57BL/6J WT and <i>Ifng</i> KO	WNV strain 3000.0259, 100 pfu footpad or 10 pfu ic, 8–12-week-old mice	Increased mortality with enhanced viremia and viral replication in lymphoid tissues, earlier detection of WNV the CNS	Shrestha et al. (2006)
<i>Ifnar1</i>	Receptor of type I (α and β) interferons	WNV	C57BL/6J WT and <i>Ifnar1</i> KO 129SvEv WT and <i>Ifnar1</i> KO	WNV strain 3000.0259, 100 pfu footpad or 10 pfu ic, 8–10-week-old mice	High susceptibility with death in 4 days and increased infection in macrophages, B cells, and T cells in the spleen	Samuel and Diamond (2005)
<i>Ifngr1</i>	Receptor of type II (γ) interferon	WNV	C57BL/6J WT and <i>Ifng</i> KO	WNV strain 3000.0259, 100 pfu footpad or 10 pfu ic, 8–12-week-old mice	Increased mortality with enhanced viremia and viral replication in lymphoid tissues, earlier detection of WNV the CNS	Shrestha et al. (2006)
<i>Ifnlr1</i>	Receptor of type III (λ) interferon	WNV	C57BL/6J WT and <i>Ifnlr1</i> KO	WNV strain 3000.0259, 100 pfu footpad, 5–12-week-old mice	Increased viral load in the brain due to enhanced blood–brain barrier permeability	Lazear et al. (2015)
<i>Il10</i>	Pleiotropic cytokine with immunosuppressive properties	WNV	C57BL/6J WT and <i>Il10</i> KO	WNV strain 2741, 2000 pfu ip, 7–8-week-old mice	Markedly reduced infection with increased production of antiviral cytokines	Bai et al. (2009)
<i>Il12a</i>	p35 subunit of IL12 which activates NK cells and induces differentiation of CD4+ T cells into IFN- γ producing Th1 cells	WNV	C57BL/6J WT and <i>Il12a</i> KO	WNV strain 2741, 2000 pfu ip, 8–12-week-old mice	No difference in susceptibility with wildtype controls	Town et al. (2009)
<i>Il12b</i>	p40 subunit of IL12	WNV	C57BL/6J WT and <i>Il12b</i> KO	WNV strain 2741, 2000 pfu ip, 8–12-week-old mice	Increased mortality	Town et al. (2009)
<i>Il23a</i>	Associates with IL12B to form IL23, which can activate STAT4 and stimulate the production of IFN- γ	WNV	C57BL/6J WT and <i>Il23a</i> KO	WNV strain 2741, 2000 pfu ip, 8–12-week-old mice	Increased mortality	Town et al. (2009)
<i>Irf1</i>	Transcriptional regulator of interferon-stimulated genes	WNV	C57BL/6J WT and <i>Irf1</i> KO	WNV strain 3000.0259, 100 pfu footpad, 8–12-week-old mice	Increased mortality, elevated viral burdens in peripheral tissues and the CNS	Brien et al. (2011)
<i>Irf3</i>	Transcriptional regulator of interferon-stimulated genes	WNV	C57BL/6J WT and <i>Irf3</i> KO	WNV strain 3000.0259, 100 pfu footpad, 8–12-week-old mice	Increased mortality, elevated viral burdens in peripheral tissues and the CNS	Daffis et al. (2007)
<i>Irf5</i>	Transcriptional regulator of interferon-stimulated genes	WNV	C57BL/6J WT and <i>Irf5</i> KO	WNV strain 3000.0259, 100 pfu footpad, 9–10-week-old mice	Increased lethality with elevated infection in peripheral organs and the CNS	Thackray et al. (2014)

Table 2 (continued)

Gene symbol (synonym)	Function/pathway	Virus	Mouse strains/cell type	Experimental conditions	Phenotype, observations	References
<i>Irf7</i>	Transcriptional regulator of interferon-stimulated genes	WNV	C57BL/6J WT and <i>Irf7</i> KO	WNV strain 3000.0259, 100 pfu footpad, 8–12-week-old mice	Increased lethality with blunted systemic type I IFN response. Increased viral titers in primary macrophages, fibroblasts, dendritic cells, and cortical neurons	Daffis et al. (2008b)
<i>Mavs (IPS-1)</i>	Adaptor molecule of RIG-I and MDA5	WNV	C57BL/6J WT and <i>Mavs</i> KO	WNV isolate TX 2002-HC, 100 pfu footpad, 6–12-week-old mice	High susceptibility in 8 days with enhanced viral replication and dissemination (similar to <i>Ddx58-Iflh1</i> double KO)	Suthar et al. (2010); Errett et al. (2013)
<i>Mb21d1 (cGas)</i>	Cytosolic DNA sensor, cGMP-AMP synthase, activator of <i>Tmem173</i> (STING)	WNV	C57BL/6J WT and <i>Mb21d1</i> KO	WNV NY1999, 100 pfu sc, 8–9-week-old mice	Increased mortality but no increase in brain viral load	Schoggins et al. (2014)
<i>Myd88</i>	Adaptor molecule of TLR7	WNV	C57BL/6J WT and <i>Myd88</i> KO	WNV strain 2741, 2000 pfu ip, 8–12-week-old mice	Increased mortality. Increased viral burden systemically and in the CNS	Town et al. (2009)
		WNV	C57BL/6J WT and <i>Myd88</i> KO	WNV strain 3000.0259, 100 pfu footpad, 8–10-week-old mice	Increased mortality. Increased viral burden primarily in the CNS	Szretter et al. (2010)
<i>Rnasel</i>	Ribonuclease L (2',5'-OAS-dependent)	WNV	C57BL/6J WT and <i>Rnasel</i> KO	WNV strain 3000.0259, 100 pfu footpad or 10 pfu ic, 8–10-week-old mice	Increased mortality (even higher if combined with <i>Eif2ak2</i> (PKR) knockout). Increased viral burden in tissues and CNS	Samuel et al. (2006)
<i>Rsad2</i>	Aka viperin: ISG expressed in response to type I and II interferons. Modulates cholesterol and isoprenoid biosynthesis, and lipid raft formation	WNV	C57BL/6J WT and <i>Rsad2</i> KO	WNV strain 3000.0259, 100 pfu footpad or 10 pfu ic, 8–10-week-old mice	Increased lethality. Modest increase of viral replication in macrophages. No difference in cortical neurons	Szretter et al. (2011)
<i>Sema7a</i>	A membrane-associated/secreted protein involved in connecting the neuronal and immune systems	WNV	C57BL/6J WT and <i>Sema7a</i> KO	WNV strain 2741, 1000 pfu ip, 6–8-week-old mice	Increased survival, reduced viral burden and blood–brain barrier permeability	Sultana et al. (2012)
<i>Tlr3</i>	Recognition of dsRNA	WNV	C57BL/6J WT and <i>Tlr3</i> KO	WNV strain 2741, 1000 pfu ip, 6–10-week-old mice WNV strain 3000.0259, 100 pfu footpad, 8–12-week-old mice	Reduced mortality, reduced viral load, inflammatory responses and neuropathology in the brain Increased mortality with higher viral burden in the CNS	Wang et al. (2004); Daffis et al. (2008a)

Table 2 (continued)

Gene symbol (synonym)	Function/pathway	Virus	Mouse strains/cell type	Experimental conditions	Phenotype, observations	References
<i>Tlr7</i>	Recognition of ssRNA	WNV	C57BL/6J WT and <i>Tlr7</i> KO	WNV strain 2741, 2000 pfu ip, 8–12-week-old mice	Increased mortality. Increased viral burden systemically and in the CNS	Town et al. (2009)
<i>Ccr2</i>	Chemokine receptor expressed on Ly6C ^{hi} inflammatory monocytes and other leukocyte subtypes	DENV	C57BL/6J WT and <i>Ccr2</i> KO	Mouse-adapted DENV-2 strain P23085, 20 pfu ip, 8–10-week-old male mice	Enhanced survival associated with decreased liver damage, decreased cell activation, and decreased cytokine storm	Guabiraba et al. (2010)
<i>Ccr4</i>	Chemokine receptor which regulates leukocytes trafficking	DENV	C57BL/6J WT and <i>Ccr4</i> KO	Mouse-adapted DENV-2 strain P23085, 20 pfu ip, 8–10-week-old male mice	Enhanced survival associated with decreased hemoconcentration, thrombocytopenia, liver damage, systemic inflammation, and leukocyte activation	Guabiraba et al. (2010)
<i>Clec5a</i>	C-type lectin which regulates cell adhesion, cell–cell signaling during immune response	DENV	<i>Stat1</i> KO (unspecified genetic background) treated with anti-CLEC5A mAb	Mouse-adapted DENV-2 strain New Guinea C-N, 10 ⁵ pfu ip and ic simultaneously, unspecified mouse age	Decreased plasma leakage and TNF- α serum level without suppression of viral replication and overall reduced lethality	Chen et al. (2008)
<i>Cxcl10</i>	Chemokine of the CXCR3 family which binds to CXCR3 receptor	DENV	C57BL/6J WT and <i>Cxcl10</i> KO (unspecified genetic background)	Mouse-adapted DENV-2 strain New Guinea C-N, 2 \times 10 ⁵ pfu ic, 6–8-week-old mice	Increased mortality rate but unchanged number of infiltrating T cells in the brain. <i>Cxcl10</i> KO mice tend to be more susceptible than <i>Cxcr3</i> KO mice	Hsieh et al. (2006); Ip and Liao (2010)
<i>Cxcr3</i>	Receptor for chemokines CXCL9, CXCL10 and CXCL11 which stimulates leukocyte trafficking	DENV	C57BL/6J WT and <i>Cxcr3</i> KO (unspecified genetic background)	Mouse-adapted DENV-2 strain New Guinea C-N, 2.10 ⁵ pfu ic, 6–8-week-old mice	Increased mortality rate with high viral load associated with a decrease in CD8 ⁺ T cells in the brain	Hsieh et al. (2006)

Table 2 (continued)

Gene symbol (synonym)	Function/pathway	Virus	Mouse strains/cell type	Experimental conditions	Phenotype, observations	References
<i>Ifnar1</i>	Receptor of type I (α and β) IFNs	DENV	C57BL/6J WT and <i>Ifnar1</i> KO	Mouse-adapted DENV-2 strain D220 or DENV-2 strain D2S10, 10^{5-6-7} pfu iv, 6–8-week-old mice	Susceptible to both viral strains with 100% morbidity. Mortality rate varies between 15% and 100% according to the viral strain and dose. Viral replication is observed in blood, liver and blood marrow. Increased serum levels of TNF- α and IL-10	Shrestha et al. (2004); Orozco et al. (2012)
		DENV	129 Sv/Ev WT and <i>Ifnar1</i> KO (also called A129 mice)	DENV-2 S221 strain, 10^{11-12} genomic equivalents (GE) iv, 5–6-week-old mice	100% morbidity with observation of systemic disease but no sign of limb paralysis. Mortality rate varies between 0% and 100% according to the inoculum dose. Viral replication is observed in blood, spleen, and blood marrow	Prestwood et al. (2012)
<i>Ifnar1/Ifngr1</i>	Receptors of type I (α and β) and type II (γ) IFNs	DENV	129 Sv/Ev WT and <i>Ifnar1-Ifngr1</i> double KO (also called AG129 mice)	DENV-2 strain PL046, $10^{5-6-7-8}$ pfu iv or mouse-adapted DENV-1 strain Mochizuki, 4×10^4 pfu iv, 5–6-week-old mice	Highly susceptible to both viral strains. Mortality rate varies between 0 and 100% depending on DENV-2 infectious dose. Clinical signs include limb paralysis. Viral replication is observed in blood, spleen, lymph nodes, and CNS	Shrestha et al. (2004); Prestwood et al. (2012)
<i>Irf1/Irf3/Irf5/Irf7</i>	Transcriptional regulators of IFN-stimulated genes	DENV	C57BL/6J WT and <i>Irf3-Irf5-Irf7</i> triple KO (TKO) C57BL/6J WT and <i>Irf1-Irf3-Irf5-Irf7</i> quadruple KO (QKO)	DENV-2 S221 strain, 5×10^6 ffu iv, under ADE conditions, 5–6-week-old male and female mice	Both TKO and QKO mice sustain viral replication but only QKO mice succumb to infection. DENV infection of TKO mice results in minimal type I IFN production but a robust type II IFN response	Carlin et al. (2017)
<i>Rag1</i>	Protein involved in activation of immunoglobulin V(D)J recombination during B and T cell development	DENV	C57BL/6J WT and <i>Rag1</i> KO (deficient in B and T lymphocytes)	DENV-2 strain PL046, $10^{5-6-7-8}$ pfu iv or mouse-adapted DENV-1 strain Mochizuki, 4×10^4 pfu iv, 5–6-week-old mice	Increased mortality rate with mouse-adapted DENV-1 strain but no detection of viral replication in peripheral organs	Shrestha et al. (2004)

Table 2 (continued)

Gene symbol (synonym)	Function/pathway	Virus	Mouse strains/cell type	Experimental conditions	Phenotype, observations	References
<i>Rag2</i>	Protein involved in the initiation of V(D)J recombination during B and T cell development	DENV	129 Sv/Ev WT and <i>Rag2</i> KO (deficient in B and T lymphocytes)	DENV-2 strain PL046, $10^{5.6-7.8}$ pfu iv or mouse-adapted DENV-1 strain Mochizuki, 4×10^4 pfu iv, 5–6-week-old mice	Increased mortality rate with mouse-adapted DENV-1 strain but no detection of viral replication in peripheral organs	Shrestha et al. (2004)
<i>Stat1</i>	Promotes transcription of ISG and IFN subtypes	DENV	129 Sv/Ev WT and <i>Stat1</i> KO	DENV-2 strain PL046, 10^{7-8} pfu iv or mouse-adapted DENV-1 strain Mochizuki, 4×10^4 pfu iv, 5–6-week-old mice	Increased mortality rate with both DENV strains but viral replication is only transient (DENV is undetected at day 3 p.i. in various tissues)	Shrestha et al. (2005); Perry et al. (2011)
<i>Stat2</i>	Promotes transcription of ISG and IFN subtypes	DENV	C57BL/6J WT and <i>Stat2</i> KO	Mouse-adapted DENV-2 D2S10 strain, 106 ffu iv, under ADE conditions, unspecified mouse age	Extended viral detection in spleen and lymph nodes but no mortality	Perry et al. (2011)
<i>Stat1/Stat2</i>	Promotes transcription of ISG and IFN subtypes	DENV	129 Sv/Ev WT and <i>Stat1-Stat2</i> double KO	DENV-2 S221 strain, 2×10^5 pfu iv, 5–6-week-old mice	Highly susceptible, 100% morbidity and lethality contrary to single-deficient mice	Perry et al. (2011)
<i>Atg16l1</i>	Part of a protein complex necessary for autophagy	ZIKV	C57BL/6J WT and <i>Atg16l1</i> tm (hypomorphic) treated with anti-IFNAR mAb	ZIKV strain Paraiba 2015 (Asian), 10^3 ffu sc, 8–10-week-old pregnant female mice	Restricted ZIKV vertical transmission and placental/fetal damage. Overall improved placental and fetal outcomes	Cao et al. (2017)
<i>Ifnar1</i>	Receptor of type I (α and β) IFNs	ZIKV	C57BL/6J WT and <i>Ifnar1</i> KO	Several ZIKV strains (African and Asian), 10^3 ffu sc, 5–6-week-old male mice ZIKV strain MR766 (African) or PF13 (Asian), 10^2 ffu sc, 5–6-week-old male mice	Highly susceptible to the African strains, 100% morbidity and lethality. Depending on the Asian strains, mortality rate varies between 0 and 100%. Viral replication is observed in all cases in blood, brain, spleen, liver, and testis	Lazear et al. (2016); Tripathi et al. (2017); Other results reviewed in Winkler and Peterson (2018)
		ZIKV	129 Sv/Ev WT and <i>Ifnar1</i> KO (also called A129 mice)	ZIKV strain MP1751 (African) or PRV/ABC59 (Asian), 10 or 10^6 ffu sc, 6–8-week-old male mice ZIKV strain FSS13025 (Asian), 10^5 ffu ip, 3–5–11-week-old mice	Highly susceptible to the African strain, 100% morbidity and lethality. Viral dissemination to the brain, spleen, liver, and testis. Susceptibility to Asian strain is age-dependent, only mice under 6 weeks of age display morbidity and lethality. Viral replication is observed in all cases in blood, brain, spleen, liver, and testis	Dowall et al. (2017); Rossi et al. (2016)

Table 2 (continued)

Gene symbol (synonym)	Function/pathway	Virus	Mouse strains/cell type	Experimental conditions	Phenotype, observations	References
<i>Ifnar1/Ifngr1</i>	Receptors of type I (α and β) and type II (γ) IFNs	ZIKV	129 Sv/Ev WT and <i>Ifnar1-Ifngr1</i> double KO (also called AG129 mice)	ZIKV strain PF13 (Asian), 10^5 pfu sc, 3–4–8-week-old mice	Highly susceptible regardless of mouse age or infectious dose. 100% morbidity and mortality. Viral replication is observed in blood and organs including spleen, liver, and CNS but is associated with severe pathological findings only in the brain and muscle	Aliota et al. (2016); Other results reviewed in Winkler and Peterson (2018)
<i>Ifnlr1</i>	Receptor of type III (λ) interferon	ZIKV	C57BL/6J WT and <i>Ifnlr1</i> KO	ZIKV strain Paraiba 2015 (Asian), 10^3 pfu footpad	Increased ZIKV replication in the placenta and fetus	Jagger et al. (2017)
<i>Irf3/Irf7/Irf7</i>	Transcriptional regulators of IFN-stimulated genes	ZIKV	C57BL/6J WT and <i>Irf3-Irf5-Irf7</i> triple KO (TKO)	ZIKV strain MR766 (African) or PF13 (Asian), 10^2 ffu sc, 5–6-week-old mice	Highly susceptible to both ZIKV strains, 100% morbidity and lethality	Lazear et al. (2016)
<i>Isg15</i>	Ubiquitin-like protein activated by type I IFNs	ZIKV	C57BL/6J WT and <i>Isg15</i> KO	ZIKV strain PRVABC59 (Asian), 10^4 pfu intravitreally in the right eye, 4–6-week-old male and female mice	Severe chorioretinitis associated with increased retinal cell death and higher ZIKV RNA and protein levels	Singh et al. (2017)
<i>Rag1</i>	Protein involved in activation of immunoglobulin V(D)J recombination during B and T cell development	ZIKV	C57BL/6J WT and <i>Rag1</i> KO (deficient in B and T lymphocytes) treated with anti-IFNAR mAb	Mouse-adapted ZIKV strain Dakar 41,519 (African), 10^6 ffu sc, 7-week-old male mice ZIKV strain Paraiba (Asian), 10^4 pfu ip, 8–10-week-old male mice	ZIKV replication is observed in male reproductive organs. Enhanced morbidity with signs of severe disease. Viral replication is observed at high level in the brain and testes	Governo et al. (2016); Winkler et al. (2017)
<i>Stat1</i>	Promotes transcription of ISG and IFN subtypes	ZIKV	129 Sv/Ev WT and <i>Stat1</i> KO (unspecified genetic background)	ZIKV strain MR766 (African), 10^4 pfu sc, 7–8-week-old male mice	Highly susceptible, 100% morbidity and lethality. High viral load in the blood and in the brain	Kamiyama et al. (2017)
<i>Stat2</i>	Promotes transcription of ISG and IFN subtypes	ZIKV	C57BL/6J WT and <i>Stat2</i> KO	ZIKV strain MR766 (African), 10^3 ffu sc, 5–6-week-old female mice	Highly susceptible, 100% morbidity with neurological symptoms and 100% lethality. High viral loads in the CNS, gonads, spleen and liver	Tripathi et al. (2017)

Table 2 (continued)

Gene symbol (synonym)	Function/pathway	Virus	Mouse strains/cell type	Experimental conditions	Phenotype, observations	References
<i>Ifnar1</i>	Receptor of type I (α and β) interferons	YFV	129 and <i>Ifnar1</i> KO	YFV Asibi and Angola wildtype strains, 10^4 pfu footpad and YFV 17D-204 vaccine strain, 10^6 pfu footpad, 3–4 week-old mice	Highly susceptible to viscerotropic YFV strain but subclinical infection with 17D vaccine strain	Meier et al. (2009)
		YFV	C57BL/6J and <i>Ifnar1</i> KO	YFV 17D vaccine strain, 10^4 pfu ip, 3–4 week-old mice	Viscerotropic disease with mortality by ip but not by im, footpad, or sc routes	Erickson and Pfeiffer (2015)
<i>Ifngr1</i>	Receptor of type III (γ) interferon	YFV	129 and <i>Ifngr1</i> KO	YFV Asibi and Angola wildtype strains, 10^4 pfu footpad and YFV 17D-204 vaccine strain, 10^6 pfu footpad, 3–4 week-old mice	Resistant to YFV wildtype and 17D vaccine strains	Meier et al. (2009)
<i>Ifnar1/Ifngr1</i>	Receptors of type I (α and β) and type II (γ) interferons	YFV	129 and <i>Ifnar1-Ifngr1</i> double KO	17D-204 vaccine strain, 10^6 pfu footpad, 3–4 week-old mice	Highly susceptible to 17D-204 vaccine strain	Meier et al. (2009); Thibodeaux et al. (2012)
		YFV	129 and <i>Ifnar1-Ifngr1</i> double KO	17D-204 vaccine strain, 2.105 pfu ip, 7–8 week-old mice	Highly susceptible to 17D-204 vaccine strain, with neurotropic and viscerotropic infection (high viral titers in brain and liver)	Meier et al. (2009); Thibodeaux et al. (2012)
<i>Ifnlr1</i>	Receptor of type III (λ) interferon	YFV	C57BL/6J WT and <i>Ifnlr1</i> KO	17D vaccine strain, 10^{6-7} pfu iv, 2–6 months old	KO resistant to vaccinal strain 17D like <i>Ifnar1/2</i> mice, but mice with double <i>Ifnar1/2</i> and <i>Ifnlr1</i> KO highly susceptible	Douam et al. (2017)
<i>Stat1</i>	Promotes transcription of ISG and IFN- α subtypes	YFV	129 and <i>Stat1</i> KO	YFV Asibi and Angola wildtype strains, 10^4 pfu footpad and YFV 17D-204 vaccine strain, 10^6 pfu footpad, 3–4 week-old mice	Highly susceptible to viscerotropic YFV strain but subclinical infection with 17D vaccine strain	Meier et al. (2009)
<i>Tnfrsf9</i>	Aka CD137, T cell co-stimulatory molecule	JEV	C57BL/6 WT and <i>Tnfrsf9</i> KO	JEV Beijing-1 strain, $1.5-3 \times 10^5$ pfu ip, 4–5 week-old mice	Reduced mortality rate and reduced viral burden in extra-neural tissues and the CNS	Kim et al. (2015)

synthetase 1b (*Oas1b*) mouse gene (Mashimo et al. 2002; Perelygin et al. 2002) and experimentally confirmed its causative role in knocked-in (Scherbik et al. 2007) and transgenic mice (Simon-Chazottes et al. 2011). As pointed out by Mashimo et al., the susceptibility to WNV of almost all laboratory strains most likely results from the shared inheritance of a haplotype carrying the *Oas1b* mutation from one of the very few progenitors at the origin of laboratory mice (Mashimo et al. 2002).

Oas1b is one of the hundreds of IFN-stimulated genes (ISG) which have antiviral and immune modulatory activity to limit viral replication and spread. The mouse *Oas* gene cluster includes *Oas1*, *Oas2*, *Oas3*, and *OasL* genes. The *Oas1* gene has eight copies (*Oas1a* to *Oas1h*), compared to one copy (OAS1) in humans (Mashimo et al. 2003). Most *Oas* genes encode 2'-5' oligoadenylate synthetases (OAS) which bind to double-stranded RNA (dsRNA) and polymerize ATP into 2'-5'-linked oligoadenylates (2-5A). 2-5A bind and activate ribonuclease L (RNase L), a latent endoribonuclease. Upon activation during viral infection, RNase L cleaves viral and cellular single-stranded RNAs (ssRNAs). RNase L contributes to host resistance to WNV since RNase L-deficient mice showed increased mortality (Samuel et al. 2006). RNase L products of RNA degradation can also bind and activate RIG-I-like receptors (RLR), resulting in enhanced innate immune signaling (Choi et al. 2015). However, *Oas1b* lacks 2-5A activity (Elbahesh et al. 2011). Moreover, RNase L has an antiviral effect against WNV infection in mouse embryonic fibroblasts carrying either functional or deficient *Oas1b* alleles, indicating that *Oas1b* controls WNV infection through another, RNase L-independent, mechanism (Elbahesh et al. 2011; Scherbik et al. 2006). Further insight into this mechanism may come from the identification of molecular partners of *Oas1b* (Courtney et al. 2012) and from the analysis of innate immune gene signatures that correlate with variations in *Oas1b* gene dosage, in genetically diverse mouse populations such as the Collaborative Cross (Green et al. 2017). This collection of recombinant inbred strains with large genetic diversity (Churchill et al. 2004) is an ideal platform for modeling a large range of phenotypes and has led to the development of several new models for human WNV disease where *Oas1b* is not the sole determinant (Graham et al. 2015, 2016).

Despite differences in the genomic organization of the members of this gene family across mammals, the conservation of the OAS pathway and of its role in host response to WNV allowed to successfully identify a variant of the equine OAS1 gene associated with symptomatic forms of WNV disease in horses (Rios et al. 2010).

An amino acid substitution in the *Stat1* gene, resulting in partial inactivation of the IFN pathway, was recently identified in a backcross involving an MHC-II knockout mouse strain, through genome-wide SNP genotyping followed by

sequence capture and sequencing of the candidate interval (Larena and Lobigs 2017).

Similar genetic association studies have not yet been reported with other flaviviruses. In the case of Dengue and Zika viruses, they are more difficult to perform due to the necessity to analyze mice with an abrogated type I IFN response.

Functional analysis of candidate genes

Host genetic factors involved in mouse susceptibility to flavivirus infections have also been identified through reverse genetic approaches, by evaluating the phenotype resulting from a specific genetic modification. Most candidate genes have been tested based on their role in immune responses and validated through functional analysis using mice carrying loss-of-function mutations. In particular, the IFN signaling pathways are crucial in the innate immune response against flaviviruses (Miorin et al. 2017), although adaptive immunity also plays a significant role, as illustrated by the enhanced susceptibility to DENV or ZIKV of mice lacking *Rag1* or *Rag2* (Shresta et al. 2004; Winkler et al. 2017), two genes critical for B and T cell development (Table 2). The importance of several mechanisms in the host susceptibility to these infections has been confirmed in vivo by reverse genetics (Fig. 1). These host genetic determinants are reviewed hereafter according to their function in the immune responses to viral infection.

Interferon responses

Type I IFNs are secreted by infected cells, induce an antiviral state, and promote immune responses against viral pathogens in an autocrine and paracrine manner. These signaling pathways are finely regulated by host factors at multiple levels starting from viral sensing and recognition to transduction and regulation of transcription.

A variety of pattern recognition receptors (PRRs) are involved in the recognition of the virus, including Toll-like receptors (TLRs) and RLRs. Among the RLRs, *Ddx58* and *Ifih1* (which encode viral nucleic acid sensors known as RIG-I and MDA5, respectively) are essential PRR genes: mice deficient for either of them showed increased lethality after WNV infection, and mice lacking both genes were extremely susceptible (Errett et al. 2013; Lazear et al. 2013). Likewise, mice deficient for *Mavs*, the downstream adaptor molecule of these PRRs which coordinates pathways leading to the activation of NF κ B, IFN regulatory factors (IRFs) 3 and 7, showed enhanced WNV replication and dissemination, with high mortality (Suthar et al. 2010). LGP2, another member of the RLR family, is not essential for induction of innate immune response but promotes antigen-specific CD8⁺ T cell survival, proliferation, and antiviral effector

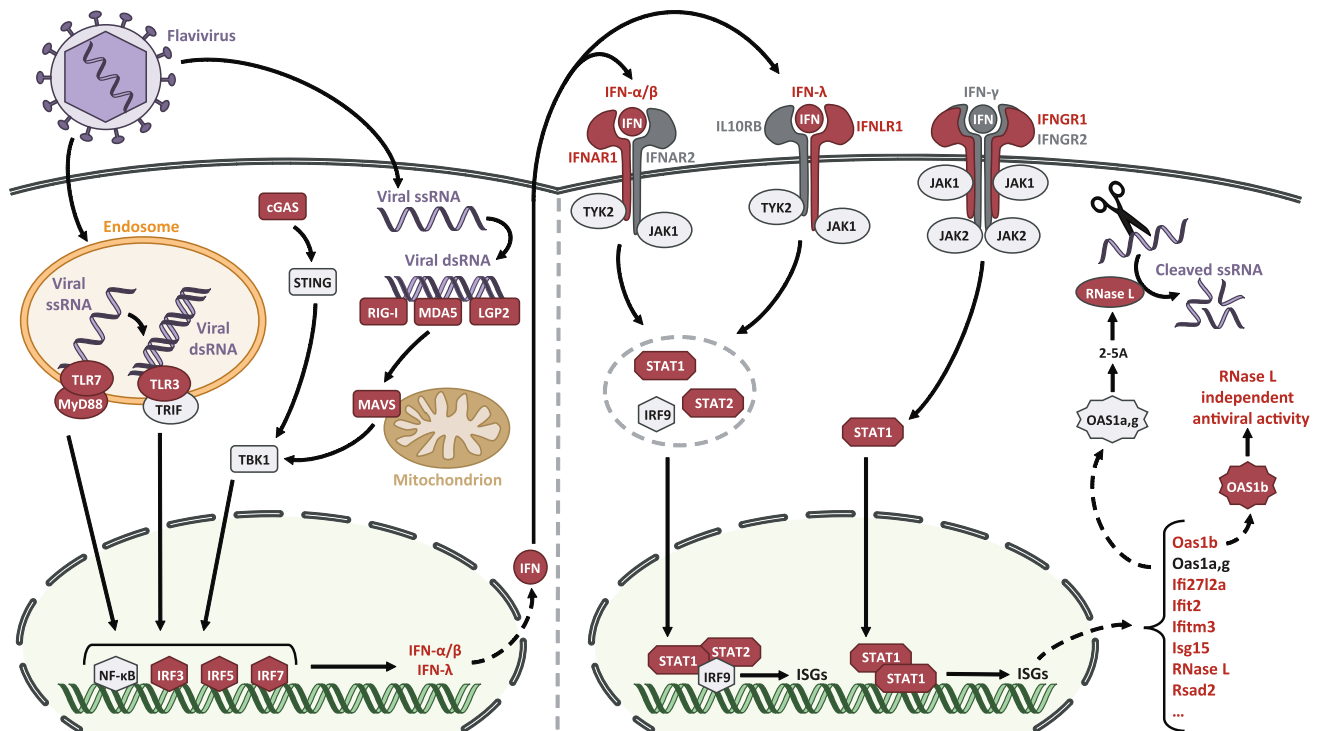


Fig. 1 Innate immune response triggered by flavivirus infection, highlighting mouse genes involved in susceptibility or resistance. Following infection by a flavivirus, viral RNA is detected by TLRs and RLRs which induce activation of several transcription factors such as NF- κ B and IRFs which, in turn, promote the transcription of type I, type II, and type III IFNs. Once secreted, each IFN subtype (α/β , γ , λ) binds to its specific receptor (IFNAR, IFNGR, IFNLR) which leads to the activation of the JAK/STAT transduction pathways. IFNAR and IFNLR both signal through STAT1, STAT2, and IRF9, whereas only

STAT1 is activated by IFNGR. STAT proteins activate the transcription of hundreds of ISGs, including *Oas* genes, some of which sense viral dsRNA and further promote viral ssRNA cleavage by activating RNase L. Genes of the IFN signaling pathway identified in the mouse as host genetic factors of susceptibility to flaviviral infections are depicted in red. *TLR* toll-like receptor, *RLR* RIG-I-like receptor, *IFN* interferon, *IRF* IFN regulatory factor, *ISG* IFN-stimulated gene, *ssRNA* single-stranded RNA, *dsRNA* double-stranded RNA. (Color figure online)

functions (Suthar et al. 2012). It was recently demonstrated that LGP2 associates with DICER and blocks the cleavage of viral dsRNAs, therefore inhibiting antiviral RNA interference (van der Veen et al. 2018). TLR7, a sensor for ssRNA, is another critical host sensor of WNV. *Tlr7* inactivation in mice led to an ineffective viral clearance and resulted in increased susceptibility to lethal WNV encephalitis (Town et al. 2009). MYD88 is considered the exclusive adaptor molecule for TLR7 and is required for signal transduction after viral RNA sensing and for an effective IFN response against WNV (Wang et al. 2004). Accordingly, *Myd88* KO mice displayed the same highly susceptible phenotype as *Tlr7* KO mice following WNV infection (Szretter et al. 2010; Town et al. 2009). By contrast, the analysis of *Tlr3*-deficient mice has led to contradictory results. A first study found that *Tlr3* KO mice were more resistant to WNV infection than controls, with decreased viral load in the brain and reduced blood–brain barrier permeability (Wang et al. 2004), while another study reported higher mortality and increased viral burden in the central nervous system (CNS) (Daffis et al. 2008a). Discrepancies were attributed to differences in

passage history of the virus, and to dose or route of inoculation. Finally, MB21D1, also known as cGAS, is a cytosolic sensor of dsDNA and a cGMP-AMP synthase which plays a key role in restriction of DNA viruses (Ma and Damania 2016). Interestingly, it is also important for innate immunity against RNA viruses with no DNA intermediates in their life cycle, as demonstrated by the increased susceptibility of *Mb21d1* KO mice to WNV (Schoggins et al. 2014). cGMP activates TMEM173, better known as STING (also MITA or MYPS). DENV protein NS2B3 is able to cleave human, but not mouse STING (Aguirre et al. 2012; Ma and Damania 2016; Stabell et al. 2018; Yu et al. 2012), a phenomenon which contributes to the natural resistance of mice to DENV. NS2B3 protein from WNV, ZIKV, and JEV, but not YFV, showed the same property (Ding et al. 2018). However, *Tmem173*-deficient mice did not exhibit increased susceptibility to ZIKV (Ding et al. 2018).

Viral recognition by TLRs or RLRs activates multiple transcription factors including NF- κ B and IRFs, directly or through TBK1. *Irf3*, *Irf5*, and *Irf7* seem to have redundant functions since these three genes need to be inactivated

to induce susceptibility to DENV or ZIKV (Carlin et al. 2017; Lazear et al. 2016). Although signaling through the same pathway, IRFs can influence host susceptibility through cell and tissue-specific processes. Mice deficient for either *Irf1*, *Irf3*, *Irf5*, or *Irf7* showed increased mortality upon WNV infection with intact IFN- β production (Brien et al. 2011; Daffis et al. 2007, 2008b; Thackray et al. 2014). IRFs stimulate in turn the transcription of type I IFNs. Mice deficient for the *Ifnb* gene, or injected with an antibody directed against IFN- α and/or IFN- β before and during WNV infection, displayed increased lethality, highlighting the critical contribution of type I IFNs in antiviral responses against WNV (Lazear et al. 2011; Sheehan et al. 2015). Likewise, mice deficient for the type II IFN *Ifng* gene showed enhanced mortality with higher viremia and replication in lymphoid tissue (Shrestha et al. 2006).

IFN receptors are the central players of the IFN system and are activated upon binding of their subtype-specific IFN. Noteworthy, mice deficient in *Ifnar1* and in both *Ifnar1* and *Ifngr1* genes (often referred to as A129 and AG129, respectively, when bred on the 129S2/SvPas background) are currently the most widely used models in YFV, DENV, and ZIKV studies. *Ifnar1* KO mice were more susceptible than WT controls to WNV (Samuel and Diamond 2005), ZIKV (Dowall et al. 2017; Lazear et al. 2016; Rossi et al. 2016; Tripathi et al. 2017), DENV (Orozco et al. 2012; Prestwood et al. 2012; Shresta et al. 2004), and YFV (Meier et al. 2009) infections, with high levels of viral replication and disease manifestations allowing for pathogenesis and mechanistic studies in vivo. Morbidity and lethality were very high with WNV, ZIKV, and YFV, and were dose-dependent with DENV. By contrast, *Ifngr1* KO mice were resistant to YFV (Meier et al. 2009), moderately susceptible to DENV (Shresta et al. 2004), but highly susceptible to WNV (Shrestha et al. 2006). Mice lacking the receptor for type III IFN (*Ifnlr1* gene) were resistant to YFV but exhibited enhanced WNV neuroinvasion with increased blood–brain barrier permeability (Lazear et al. 2015) and enhanced ZIKV transplacental transmission (Jagger et al. 2017). For all the above viruses, *Ifnar1*/*Ifngr1* double KO mice displayed very high susceptibility with 100% mortality (Aliota et al. 2016; Meier et al. 2009; Prestwood et al. 2012; Shresta et al. 2004; Thibodeaux et al. 2012). Likewise, *Ifnar1*/*Ifnlr1* double KO mice were highly susceptible to YFV (Douam et al. 2017). These mouse models have allowed to decipher the roles of the different IFNs and IFN-receptor subtypes in response to flaviviruses. For example, in DENV infection, IFNAR signaling limits initial viral replication and controls its subsequent dissemination. By contrast, IFNGR-mediated responses appear to act at later stages of dengue disease by restricting viral replication in the periphery and eliminating virus from the CNS (Shresta et al. 2004).

IFN receptors signal through different tyrosine kinases which recruit signal transducers and activators of transcription (STAT). STAT proteins are key mediators of the IFN response. IFNAR and IFNLR signal through both STAT1 and STAT2, whereas only STAT1 is activated after IFNGR stimulation. STAT2 is one of the targets of the NS5 protein of flaviviruses, a potent and specific antagonist of IFN signaling which acts through virus-specific mechanisms (Grant et al. 2016). ZIKV NS5 binds to and targets human (Kumar et al. 2016), but not mouse, STAT2 for proteasomal degradation, which provides the mechanism underlying the natural resistance of mice to ZIKV and DENV (Best 2017; Grant et al. 2016; Miorin et al. 2017). The difference between human and mouse STAT2 for the binding of DENV NS5 was mapped to the coiled-coil domain (Ashour et al. 2010). To bind STAT2, DENV and YFV require E3 ubiquitin ligases UBR4 (Best 2017; Grant et al. 2016) and TRIM23 (Laurent-Rolle et al. 2014), respectively. WNV NS5 binds to prolidase, a cellular peptidase, to suppress IFNAR maturation and cell surface expression (Laurent-Rolle et al. 2010; Lubick et al. 2015). By analyzing multiple crosses between mice inactivated for either *Ifnar1*, *Ifngr1*, *Stat1*, or *Stat2* genes, Perry et al. have demonstrated the importance of STAT proteins in the host immune response against DENV. They have shown that the combined loss of STAT1 and STAT2 resulted in severe disease and death in mice challenged with DENV. They also showed, using high virus doses, that *Stat1* KO mice succumbed to dengue disease unlike *Stat2* KO mice, and concluded that STAT1 plays a more prominent role than STAT2 in anti-DENV responses (Perry et al. 2011). *Stat1* KO mice were also highly susceptible to ZIKV and YFV infections although morbidity and mortality rates varied according to viral strains (Kamiyama et al. 2017; Meier et al. 2009). After ZIKV infection, *Stat2* KO mice displayed neurological symptoms and viral dissemination to the brain and gonads. Interestingly, clinical signs of Zika fever were delayed and milder in *Ifnar1* KO mice compared with *Stat2* KO mice, pointing to a possible protective role of INF- λ response (Tripathi et al. 2017).

Once activated, STAT proteins stimulate the transcription of hundreds of ISGs. In addition to *Oas1b*, several ISGs have been shown to influence mouse susceptibility to WNV infection. Mice lacking *Rsad2* (also known as viperin), *Ifi2712a*, *Ifitm3*, or *Ifit2* were all more vulnerable to lethal WNV challenge and allowed higher viral replication mainly in the CNS (Cho et al. 2013; Gorman et al. 2016; Lucas et al. 2015; Szretter et al. 2011). *Rsad2* inhibits ZIKV replication by inducing proteasome-dependent degradation of ZIKV NS3 (Panayiotou et al. 2018) and the synthesis of a replication-chain terminator (Gizzi et al. 2018), and has cell-type-specific activity in the CNS (Lindqvist et al. 2018). *Ifitm* proteins alter the properties of cell and viral membranes and can inhibit the replication of a wide range of pathogenic

viruses (Perreira et al. 2013). They induce similar restriction of primary and antibody-dependent enhancement secondary DENV infections in human leukemia cells (Chan et al. 2012). *Ifitm3* inhibits the early stages of Zika virus replication and can prevent Zika virus-induced cell death (Savidis et al. 2016). In a model of direct inoculation of ZIKV in the eye, *Isg15* KO mice showed increased ocular tissue pathology, characterized by a severe chorioretinitis with enhanced retinal cell death (Singh et al. 2017).

Overall, many studies have emphasized the crucial role of IFN responses, and especially the type I IFN pathway, which constitute an essential line of defense for the host after infection by flaviviruses.

Cytokines and chemokines regulating the immune response

The role of cytokines and chemokines as important regulators of immune responses has also been investigated in flaviviral infections using mice deficient for cytokines, chemokines, or chemokine receptors.

After WNV infection, *Il10* KO mice had a decreased mortality rate, suggesting that IL-10, which has immunosuppressive properties, promotes WNV pathogenesis (Bai et al. 2009). Mice lacking IL-12b or IL-23a were more susceptible to WNV induced encephalitis, but not mice deficient for IL-12a, indicating that survival required intact IL-23 as opposed to IL-12 responses (Town et al. 2009).

Chemokines and chemokine receptors, which modulate leukocytes trafficking, play an important role in the regulation of immune responses. Several of them have been implicated in host susceptibility to flaviviruses, sometimes with opposing effects depending on the virus and its cell tropism. Deficiency in *Cxcr3*, the receptor for chemokines CXCL9, CXCL10, and CXCL11, resulted in increased lethality in mice infected by DENV or WNV, with a decrease in T lymphocytes in the brain, CD8⁺ T cells in particular (Hsieh et al. 2006; Zhang et al. 2008). Mice lacking CXCL10 also showed enhanced susceptibility to DENV infection with higher viral loads in the brain but unchanged number of infiltrating T cells, suggesting that CXCL10 protective effect in dengue might be due to its direct antiviral activity rather than its role in lymphocyte recruitment (Hsieh et al. 2006, Ip and Liao 2010). *Cxcl10* KO mice showed enhanced susceptibility to WNV with increased viral burden in the brain and a decrease in CXCR3⁺ CD8⁺ T cell trafficking, supporting a neuroprotective role for *Cxcl10* in the brain (Klein et al. 2005). *Ccr2* KO mice displayed increased survival time associated with decreased liver damage following DENV infection, whereas they showed a higher mortality rate combined with a reduction of monocyte accumulation in the brain after WNV infection (Guabiraba et al. 2010; Lim et al. 2011). Upon WNV infection, *Ccr7*-deficient mice

exhibited enhanced mortality and CNS viral load, associated with marked leukocyte accumulation in the brain. These results indicate that CCR7 contributes to viral clearance and effectively modulates neuroinflammation in a model of WNV encephalitis (Bardina et al. 2017).

Other stimulatory molecules of immune cells have crucial functions in promoting or restricting flaviviral infections. CLEC5a is a C-type lectin which regulates cell adhesion and cell–cell signaling during the immune response. CLEC5a has been shown to act as a susceptibility factor in DENV infection. *Stat1* KO mice treated with an anti-CLEC5a antibody showed increased survival associated with a reduction of plasma leakage and TNF- α serum level (Chen et al. 2008). Mice deficient in *Tnfrsf9*, a T cell co-stimulatory factor, displayed reduced mortality rate in a model of Japanese encephalitis, highlighting a detrimental role of this molecule in the immune response against JEV (Kim et al. 2015).

Other mechanisms

Autophagy is an essential mechanism which targets cellular components for lysosomal degradation. In the immune system, autophagy has many functions in both innate and adaptive responses, such as intracellular pathogen detection, modulation of the inflammatory processes, as well as regulation of lymphocytes homeostasis. Mice carrying a hypomorphic variant of *Atg16l1*, a key autophagy gene, and treated with an anti-IFNAR antibody showed reduced ZIKV vertical transmission and placental damage. This phenotype was shown to result from a placental cell-autonomous effect of autophagy activity indicating that autophagy promotes ZIKV pathogenesis during gestation (Cao et al. 2017).

Semaphorins constitute a group of proteins that are involved in connecting the neuronal and immune systems. Following WNV infection, *Sema7A* KO mice exhibited increased survival, correlated with a reduction of blood–brain barrier permeability. SEMA7A thus appears to play a deleterious role during WNV infection in vivo (Sultana et al. 2012).

Human versus mouse genetics

While mouse genetics studies improve our understanding of the function of genes and identify new susceptibility genes in genome-wide screens, the main obstacle in studying flaviviruses using mouse models is the inherent resistance of mice to most mosquito-borne flaviviruses (such as DENV and ZIKV). Several mouse models frequently used in flaviviral research are constitutively deficient for the IFN type I and/or type II responses, and therefore do not reflect an intact functioning human immune system. Moreover, these models are generally more susceptible to mouse-adapted

viral strains, which are genetically different from human pathogens. Therefore, human cohorts are important to understand the role of the genes in protection or pathogenesis of human diseases, although human genetic studies are highly dependent on patient numbers.

Human genetic studies

Multiple approaches have been taken to identify genetic variants in human populations associated with susceptibility or resistance to flaviviruses, in particular case–control studies to test candidate genes, genome-wide association studies, association with specific HLA alleles, and allelic selection in exposed populations or ethnicities. Many studies have focused on favorable or disadvantageous immunological mechanisms involved in the pathogeny of infection but few have identified underlying genetic variants.

West Nile virus

WNV is a neurotropic virus and is transmitted to humans, who are a dead end host, by *Culex* mosquitoes. WNV causes a self-limiting febrile illness in most individuals that occasionally progresses to severe neurological disease including meningitis and encephalitis (Colpitts et al. 2012). Currently, there is no vaccine licensed in humans. Although there were a few human genetic studies on WNV, the most significant data resulted from the confirmation of the findings of the mouse model. Two polymorphisms of human OAS1, a splicing variant (rs10774671) (Lim et al. 2009) and an intron 2 variant (rs34137742) (Bigam et al. 2011), were associated with symptomatic WNV seroconversion. Another study found a polymorphism of human OASL (rs3213545) associated with hospitalized WNV fever, meningitis, and/or WNV encephalitis (Yakub et al. 2005). Two studies have identified associations between the clinical severity of WNV infection and HLA Class I and II alleles (Lanteri et al. 2011; Sarri et al. 2016).

Dengue virus

Classification of dengue cases

DENV, which is the most common mosquito-borne viral infection, is spreading worldwide. There is one dengue vaccine licensed. The strategic Advisory Group of Experts on Immunisation (SAGE) recommended limited usage of the vaccine in only seropositive individuals since April 2018 (http://www.who.int/immunization/diseases/dengue/revised_SAGE_recommendations_dengue_vaccines_apr2018/en/). There are four serotypes of DENV co-circulating (DENV-1 to DENV-4). Infection by one of the four can

result in a spectrum of clinical outcomes ranging from asymptomatic to inapparent infection (patients developed mild disease but not enough to seek medical advice) to undifferentiated fever, classical dengue fever (DF) with or without hemorrhage, dengue hemorrhagic fever (DHF) with plasma leakage leading to shock (dengue shock syndrome (DSS)), and other organ involvement (such as hepatitis, encephalitis).

There are several difficulties in performing genetic studies of dengue even though there are a high number of cases. First, clinical case definition can be based on either WHO (1997) or WHO (2009) criteria, which define severity of dengue in different ways. The WHO (1997) utilizes well-defined criteria focused on plasma leakage to differentiate DF, DHF, and DSS. The WHO (2009) criteria are designed for patient management and rely on more subjective criteria including several signs and symptoms of multiple organ involvement, hence complicating our understanding of disease pathogenesis. Therefore, most human genetic studies used WHO (1997) criteria, which is specific for plasma leakage.

Secondly, previous infection history and infecting dengue serotypes are the two most important confounding factors for genetic studies but are expensive, time consuming, and not always possible to determine. Most studies, which are designed to distinguish primary from secondary infection and can identify the infecting dengue serotype, indeed showed interaction of these variables and genetic factors (Simon-Loriere et al. 2015; Stephens et al. 2002).

Genetic study of dengue

There were more human genetics studies of DENV compared to mouse genetic studies, which were more focused on candidate genes related to IFN pathways. There are several excellent recent reviews on human genetic susceptibility to dengue, including a meta-analysis (Xavier-Carvalho et al. 2017a). Associations could be replicated in at least two populations for only one locus and six genes (Xavier-Carvalho et al. 2017a). The region which was replicated in many populations is the HLA locus on chromosome 6 including HLA-A*24 (Loke et al. 2001; Malavige et al. 2011; Nguyen et al. 2008), MHC class I polypeptide-related sequence B MICB (rs3132468) (Dang et al. 2014; Khor et al. 2011; Whitehorn et al. 2013), and tumor necrosis factor TNF (-308, rs1800629) (Fernandez-Mestre et al. 2004; Fernando et al. 2015; Sam et al. 2015; Santos et al. 2017). The other six genes are C-type lectin, CD209 (rs4804803) (Sakuntabhai et al. 2005; Wang et al. 2011; Xavier-Carvalho et al. 2013), C-type lectin domain containing 5A CLEC5A (rs1285933) (Xavier-Carvalho et al. 2013, 2017b), immunoglobulin heavy chain receptor FcγR IIA (Arg131His-rs1801274) (Garcia et al. 2010; Mohsin et al. 2015; Noecker

et al. 2014), cytokine IL-10 (-1082/-819/-592) (Fernando et al. 2015; Perez et al. 2010), alpha tryptase 1 TPSAB1 (Velasquez et al. 2015), and phospholipase C epsilon 1 PLCE1 (rs3765524, and rs3740360) (Dang et al. 2014; Khor et al. 2011; Whitehorn et al. 2013). Among these genes, how CLEC5A contributes to pathology has been investigated in a mouse model for DENV (Chen et al. 2008).

Although there was a bias of patient selection among the studies (more studies on DHF/DSS than DF), there was evidence that different sets of genes are associated with DF (mild clinical dengue) and/or DHF/DSS (severe dengue). While the more severe form of dengue is associated with HLA class I, and genes associated with inflammation and immune response (CLEC5A) (Xavier-Carvalho et al. 2013, 2017b), cytokine response (IL-10), NK cell (MICB), mast cell activity (TPSAB1) (Velasquez et al. 2015), and lipid metabolism (PLCE1, PLCB4, OSBPL10, RXRA) (Sierra et al. 2017), DF is associated with genes in xenobiotic pathway (CHST10, AHRR, PPP2R5E, and GRIP1) (Oliveira et al. 2018) and HLA class II (LaFleur et al. 2002; Sierra et al. 2007; Stephens et al. 2002; Weiskopf et al. 2015). Genes involved in viral entry (CD209, FcγRII) and TNFα pathway were associated with both forms of diseases. In addition, a non-synonymous variant of OAS3 was associated with severe dengue caused by DENV serotype 2 (Simon-Loriere et al. 2015). Adaptive immunity could play a more important role in eliminating the virus and in the development of clinical and severe dengue disease. More human genetic studies with well-characterized patients are needed in order to understand protection and pathogenesis of mild clinical and severe dengue in humans.

Protective and enhancing HLA class I and class II alleles in dengue virus infections

An association between HLA class I alleles and DHF susceptibility was shown in a large cohort of Vietnamese patients. More specifically, it was found that children with HLA-A*33 were less likely to develop DHF, whereas children with HLA-A*24 were at increased risk of developing DHF (Loke et al. 2001). Analyses of NS3- and NS5-specific CD8 T cell responses in different donors suggest opposing roles for T cells in both protection and development of DHF. Likewise, a more recent study in Vietnam confirmed the HLA association between HLA-A*24 and DHF or DSS and showed that HLA-A*24 with histidine at codon 70 is a susceptible allele, whereas the HLA-DRB1*0901 class II allele is protective against development of DSS, in patients with DENV-2 infection (Nguyen et al. 2008).

A second and larger case-control study in ethnic Thai patients revealed a variety of HLA class I association with the severity of clinical disease during secondary DENV infections (Stephens et al. 2002). The HLA-A*0203 allele

was in particular associated with less severe DF, regardless of the secondary infecting virus serotype. By contrast, HLA-A*0207 was associated with susceptibility to the more severe DHF in patients with secondary DENV-1 and DENV-2 infections. Conversely, HLA-B*51 was associated with the development of DHF in patients with secondary infections, and HLA-B*52 was associated with DF in patients with secondary DENV-1 and DENV-2 infections. Moreover, HLA-B44, B62, B76, and B77 also appeared to be protective against developing clinical disease after secondary dengue virus infection. Interestingly, at least for the HLA-A*02 class I alleles, the strong binding potential to viral peptides was suggested to enable T cell activation and protection observed in these patients with secondary DENV infections.

Another study of HLA polymorphism in the Cuban population revealed an increased frequency of HLA-A*31 and -B*15 class I alleles in symptomatic dengue virus infection compared with controls, and conversely, an elevated frequency of HLA-DRB1*07 and DRB1*04 class II alleles in control subjects compared with dengue case patients (Sierra et al. 2007). In a Mexican population, HLA-B*35 was negatively associated with symptomatic disease, whereas HLA-DQB1*0302 was positively associated with DHF, and HLA-DQB1*0202 was positively associated with DF only (Falcon-Lezama et al. 2009). Finally, in a Sri Lankan population, it was found that HLA-A*31 and HLA-DRB1*08 were associated with susceptibility to DSS, during secondary infection, and HLA-A*24 and HLA-DRB1*12 were strongly associated with DHF during primary dengue infection (Malavige et al. 2011).

One of the reasons for the association observed between certain HLA alleles and dengue disease severity is linked to the ability of these class I or class II alleles to induce a strong CD8+ or CD4+ T cell response against dengue epitopes. In that respect, it was found that T cell responses that were weak in magnitude, for example those observed against dengue epitopes restricted by HLA-A*0101 and HLA-A*2401, correlated with disease susceptibility, whereas strong and polyfunctional CD8+ T cell responses were observed in HLA-B*3501 individuals and were negatively associated with symptomatic disease (Weiskopf et al. 2013). Likewise, a higher resistance and susceptibility to severe dengue clinical outcome were shown to be associated with a more vigorous CD4+ T cell response in the context of DRB1*0401 and DRB1*0802, respectively (Weiskopf et al. 2015).

Protective or enhancing killer immunoglobulin-like receptors (KIRs) in DENV infection

An association between KIR-ligand pairs and susceptibility to dengue in Southern Brazil was also detected, in the context of DENV3 infection (Beltrame et al. 2013). Although

the exact role of NK cells expressing either activating or inhibitory KIRs has yet to be determined during DENV infection in humans, several studies strongly support the ability of DENV- or Flavivirus-derived conserved peptides, to stimulate or to inhibit KIR2DS2 or KIR3DL1 NK cells in vitro against NS1 or NS3 peptides, and in the context of HLA-C*0102 or HLA-B*57, respectively (Naiyer et al. 2017; Townsley et al. 2016).

The HLA transgenic mice as models to study the immune protection against DENV infection

To allow the identification of HLA-restricted peptides derived from the viral proteins, several HLA class I and class II transgenic mice have been developed to study the T cell response against the whole virus or the peptides derived from the viral proteins (Boucherma et al. 2013; Pascolo et al. 1997). Strikingly, most DENV-derived T cell epitopes inducing a T cell response in HLA class I mice correspond to the peptides identified from human individuals after DENV infection (Duan et al. 2015; Elong Ngono et al. 2017; Rivino et al. 2013a, b; Weiskopf et al. 2011, 2013). Importantly, as the magnitude of the T cell response reflects the binding affinity of the different peptides to an HLA allele, analysis of the magnitude of T cell response in these transgenic mice against the different peptides covering the whole sequence should allow the identification of the most immunogenic peptides inducing a long-lasting immunity.

An improvement of this mouse model involves a transient blockade of type I interferon signaling, after treatment with anti-IFNAR antibody. Commonly used in wildtype mice to study the cellular effectors of the immune response to different viruses, such as the WNV or ZIKV (Lazear et al. 2016; Ng et al. 2015; Pinto et al. 2011; Sheehan et al. 2015; Zhao et al. 2016), this procedure is currently adapted to the HLA transgenic mice, with the objective to study the role of peptide-specific T cells in the induction of long-lasting immunity against DENV and ZIKV infection.

Perspectives

A number of studies on flaviviruses have focused on the influence of viral virulence factors on viral multiplication, on the inhibition of immune responses of the infected host, and on disease severity. By contrast, the role of host genetic determinants on clinical severity of flavivirus infections remains elusive, with the notable exception of type I IFN responses. The few examples of genes identified which are not directly related to the type I IFN responses demonstrate that genome-wide, unbiased approaches will be essential in identifying novel actors. Despite differences in host–virus interactions, the mouse can successfully serve as

an experimental model to assess the role of specific genes and to query the genome for host genetic determinants in genetic reference populations with vast genetic polymorphism, such as the Collaborative Cross (Aylor et al. 2011) and the Diversity Outbred mice (Recla et al. 2014). Their IFN response could be abrogated by antibodies directed against the type I IFN receptor (Lazear et al. 2016). These resources will be highly valuable to address the complex host–virus interactions through systems biology approaches.

Along with these innovative mouse model, well-characterized human cohorts of flaviviral infections should be collected. More DENV cohorts with well-characterized virological, immunological, and clinical parameters from different countries are needed to understand human genetic basis of susceptibility to severe dengue, host–viral interaction, and genetic susceptibility to ADE phenomenon. In addition, cohorts of asymptomatic DENV-infected patients will help us understand protective immunity and will serve as the best control for symptomatic and severe dengue. Beyond DENV and WNV, ZIKV infection represents an ongoing public health challenge because of its complications. Collecting a sufficient number of samples from ZIKV-infected donors living in different endemic regions is a necessary step forward to be able to identify host genetic factors influencing persistent infection, susceptibility to neurological complications, mother-to-child transmission, and susceptibility to brain pathology.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding authors state that there is no conflict of interest.

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2.5.4. Possible strategies for identifying susceptibility genes to ZIKV in the mouse

While reverse genetics has established the role of several innate immune response genes in the control of ZIKV infection, much remains to be done to identify novel genes controlling susceptibility or resistance to ZIKV, whether acting in isolation or in interaction with others. This can be achieved only by forward genetics and quantitative approaches. Compared with other viruses to which mice are naturally susceptible, the identification of mouse genes affecting the outcome of ZIKV infection is complicated by the inability of the virus to replicate in immunocompetent adult mice.

Considering the different strategies to identify host genetic factors controlling complex traits that have been discussed previously, several options could be considered and adapted to study ZIKV infection.

1- Induce mutation (by ENU-mutagenesis) in *Ifnar1*^{-/-} mice, which sustain ZIKV replication and disease because of their type I IFN response deficiency.

2- Engineer an *Ifnar1* knock-out mutation in genetically diverse strains thanks to CRISPR-Cas9 gene-editing technology.

3- Investigate ZIKV infection in genetically diverse mouse strains previously treated with an Ab blocking the IFNAR receptor, as this method has already been described as a potential model to study ZIKV infection in adult immunocompetent mice.

We adopted this last strategy for my PhD project and used the CC panel of strains that currently gathers the broadest genetic diversity. The objectives and approaches that we used in this project are described in part 3.

3. OBJECTIVES AND OUTLINE

Very little was known about ZIKV until 2015 but the recent epidemics have led to a major increase in bio-medical research on this newly emerging pathogen. In the past four years, a plethora of studies have increased our understanding of ZIKV biology, pathogenesis and disease and have led to the pre-clinical development of prophylactic and therapeutic measures. While several groups have investigated how viral genetic variability can affect ZIKV transmission and disease, the influence of host genetic diversity on the susceptibility to ZIKV has barely been taken into account.

Considering the expertise of the *Mouse Genetics* laboratory in genetics of infectious diseases and the context of international emergency actions engaged to study ZIKV, my PhD project was initiated in 2016 in collaboration with the unit of human “*Functional genetics of infectious diseases*” at the Institut Pasteur, which has long interest in host genetic susceptibility to dengue virus.

The objectives of my project were to characterize, for the first time, the influence of host genetic factors on ZIKV pathogenesis and disease using mouse strains from the Collaborative Cross and to identify genes and mechanisms driving the differences in susceptibility to ZIKV. To this end, I used a combination of complementary approaches, from forward quantitative genetics to functional and transcriptomic analyses. The interplay between the different studies of my PhD project are presented in Figure 28, and the results are presented in part 5.

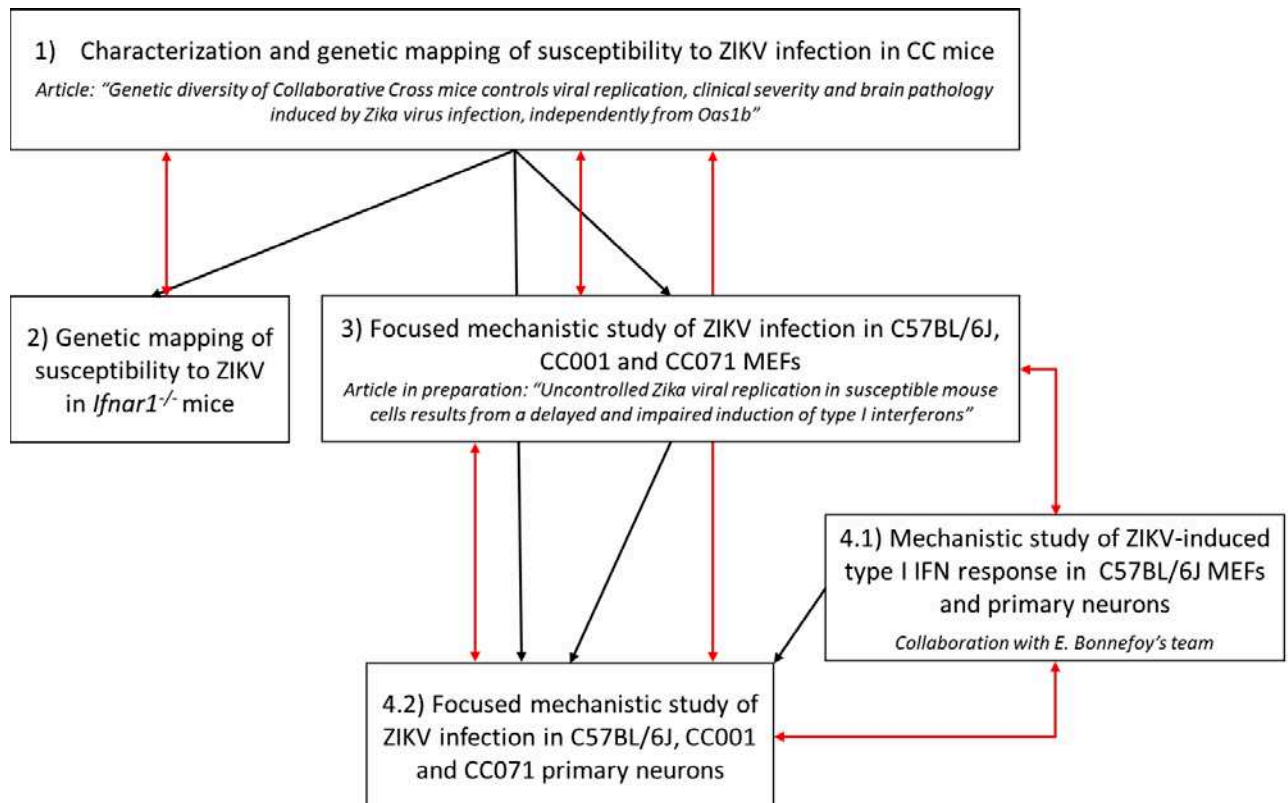


Figure 28. Interconnexions between the different studies of my PhD project. Black arrows indicate the logical and temporal progression between studies. Red arrows indicate possible comparisons and crossed-interpretations between results obtained in the different studies.

4. MATERIAL AND METHODS

Material and methods of part 5.1 are detailed in the article “Genetic diversity of Collaborative Cross mice controls viral replication, clinical severity and brain pathology induced by Zika virus infection, independently from *Oas1b*”. The methods are listed below for convenience.

Mice

Cell lines

Viruses

Mouse experiments

Mouse embryonic fibroblasts (MEFs) isolation and infection

Focus-forming assay

Viral genome quantification by RT-qPCR

Western Blot analysis

Histopathology

Genetic analysis

Statistical analysis

Material and methods of parts 5.2, 5.2 and 5.4 (complementary to those of part 5.1) are specified hereafter.

F2 cross

(B6-*Ifnar1* x 129-*Ifnar1*) F1 mice were generated at the Institut Pasteur by crossing B6-*Ifnar1* females with 129-*Ifnar1* males; (B6-*Ifnar1* x 129-*Ifnar1*) F2 mice (referred to as B6129(*Ifnar1*)F2 mice) were produced by intercrossing F1 mice. A total of 192 F2 mice, males and females, of 6-8 weeks of age were infected with ZIKV FG15 strain and phenotyped as described in 5.1 (ZIKV IP infection).

Mouse genotyping

A subset of 94 F2 mice displaying contrasted susceptibility to ZIKV infection was selected for further genetic analysis. Tail biopsies were sent to Neogen (Neogen/Geneseek, Inc, Lincoln, NE, USA) for whole-genome genotyping using miniMUGA array containing 9,914 SNP markers.

QTL analysis

QTL analysis was performed using J/qtl software version 1.3.5. The survival rate was analyzed as a binary trait and the time to death was analyzed using a non-parametric model. Significance thresholds of genome-wide LOD scores were estimated by 1,000 permutations of experimental data. Loci that exceeded the 95th percentile of the permutation distribution were defined as significant ($p < 0.05$) and those exceeding the 37th percentile were suggestive ($p < 0.63$). 95% confidence intervals were estimated in J/qtl using the Bayesian Credible Interval function.

Flow cytometry analysis

The following antibodies were used: anti-ZIKV E protein (4G2, purified from the ATCC hybridoma), AlexaFluor-488-573 conjugated anti-mouse IgG (Invitrogen). For stainings, cells were fixed with 4% PFA and permeabilized with 0.1% Triton (100X) in PBS, followed by 45-minutes incubations with primary and then secondary antibodies, diluted in a solution of 1% BSA in PBS. Samples were acquired with an Attune NxT flow cytometer (ThermoFischer Scientific), and data were analyzed in FlowJo.

ZIKV-binding and entry assays

MEFs were incubated for 1 hour at 4°C with ZIKV FG15 at a MOI of 5. For ZIKV-binding assay, cells were washed 5 times with cold PBS and lysed for total RNA extraction. For ZIKV-entry assay, the inoculum was replaced with fresh medium and cells were further incubated for 2 hours at 37°C and then with Trypsin-EDTA 0.05% (Gibco) for 30 minutes at 37°C. After Trypsin inactivation with complete medium and centrifugation, cell pellet was rinsed with PBS and lysed for total RNA extraction.

MEFs stimulation assays

For stimulation with IFN- α , MEFs were treated with 300 IU/mL IFN- α (Miltenyi Biotec), incubated for 8 to 24 hours at 37°C and then lysed for total RNA extraction. For intracellular

stimulation with Poly(I:C) or with 3p-hpRNA, MEFs were transfected with 1 µg/mL Poly(I:C) (HMW VacciGrade, InvivoGen) or 0.5 µg/mL 3p-hpRNA (InvivoGen) using 5 µL Lipofectamine LTX and 1 or 0.5 µL Plus Reagent (ThermoFischer Scientific), according to the manufacturer's instructions. Cells were incubated for 8 to 48 hours at 37°C and then lysed for total RNA extraction. Transfection efficiency was assessed by Immunofluorescence using an anti-dsRNA primary antibody (Kerafast, ES2001).

Mouse primary neurons isolation and infection

Primary neurons were prepared from mouse fetuses at day 16.5 of gestation. Isolated cortices were rinsed in HBSS medium (Gibco) and digested with 1 mg/mL Trypsin-EDTA (Gibco) and 0.5 mg/mL DNase I (Merck) in HBSS medium for 15 minutes at 37°C. B-27 supplement (Life Technologies) was added to inactivate Trypsin and mechanical dissociation of the cortices was performed by passages through a narrowed glass pipet. The cell suspension was centrifuged for 10 minutes at 200g and cell pellet was re-suspended in Neurobasal medium (Gibco) supplemented with 2% B-27, 0.2% L-glutamine (Gibco) and 1% penicillin-streptomycin-fungizone (Life Technologies). Cells were plated at identical densities in culture plates pre-coated with polyD-lysine (Merck) and Laminin (Merck).

Primary cultured neurons were infected with ZIKV FG15 strain at a MOI of 5 at 12 days of *in vitro* culture, for network maturation. After 2 hours of incubation at 37°C, the inoculum was replaced with fresh medium.

Total RNA extraction from cells

For MEFs and primary neurons total RNA extraction, cells were lysed in 350 µL RLT buffer (Qiagen) supplemented with 1% β-mercaptoethanol. Total RNA extraction was performed with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. On-column digestion with DNase I (Qiagen) was performed to avoid genomic DNA contamination.

RNA sequencing

RNA integrity and quantification were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). High quality RNA samples (RIN > 9.2) representing biological triplicates of infected or mock-infected MEFs were submitted to Novogene for commercial RNA-sequencing (Novogene Beijing, China).

A total amount of 1 µg RNA per sample was used as input material for the RNA sample preparations. Poly-A selected RNA was used for paired-end library preparation and transcriptome sequencing. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. The library preparations were sequenced on an Illumina platform and paired-end reads were generated.

Approximately 28.6 million raw reads and 28.3 million clean reads were generated on average per sample, with phred Q30 values averaging 92.7%. Reads were filtered for quality and aligned to the GRCm38 - mm10 mouse genome using HISAT2 software. ZIKV RNAs do not contain poly-A signals, therefore it was not necessary to map reads to the viral genome.

Differential expression analysis between two conditions (3 biological replicates per condition) was performed using DESeq2 R package. The resulting p values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an adjusted p value < 0.05 found by DESeq2 were assigned as differentially expressed.

Heatmaps and clusters of DEGs were generated using pheatmap R package. Genes in each cluster were grouped according to their Gene Ontology (GO) annotation (<https://go.princeton.edu/cgi-bin/GOTermFinder>).

Reverse-transcription and qPCR

Samples (500 ng RNA) were used for reverse-transcription with SuperScript II Reverse Transcriptase (ThermoFischer Scientific). qPCR was performed with *Power* SYBR Green PCR Master Mix (ThermoFischer Scientific) on a QuantStudio 12K Flex (ThermoFischer Scientific) Real-Time PCR System and specific primers (Table 6).

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>Aldolase</i>	AGCAGAATGGCATTGTACCC	ACAGGAAAGTGACCCCAAGTG
<i>Ddx58</i> (RIG-I)	GGGCAACAGGAATGACGCTCCC	TGCCTTCATCAGCGACCGAGG
<i>Ifitm3</i>	ACTGTGATCAACATGCCAGAG	CTTCCGATCCCTAGACTTCACG
<i>Ifna4</i>	TGATGAGCTACTACTGGTCAGC	GATCTCTTAGCACAAGGATGGC
<i>Ifnb1</i>	CAGCTCCAAGAAAGGACGAAC	GGCAGTGTAACCTTTCTGCAT
<i>Il3</i>	GAACCTCTGCCTTAGCTGAGGT	ATTCCCGTTTTCTCCGACACG
<i>Irf7</i>	CAGCGAGTGCTGTTTGGAGAC	AAGTTCGTACACCTTATGCGG
<i>Isg15</i>	GGTGTCTGTGACTAACTCCAT	TGGAAAGGGTAAGACTGTCCT
<i>Oas1b</i>	GAGGTGCCGACGGAGGT	TCCAGATGAAGTCTTCCCAAAG
<i>Eif2ak2</i> (PKR)	TTCACACGTGCTTCACGGAGT	ATGTCTCAGGTCGGTCCTTGG
<i>Rplp0</i>	CACTGGTCTAGGACCCGAGAAG	GGTGCCTCTGGAGATTTTCG
<i>Stat1</i>	CCGAGAACATACCAGAGAATC	AGTAGCAGGAAGGAATCACAG
<i>Stat2</i>	GCATAACTTGCGAAAATTCAGCC	TCAGAATCCTTTGCTCTTCCAGA
<i>Tbp</i>	AGAACAATCCAGACTAGCAGC	GGGAACTTCACATCACAGCTC
<i>Tnf</i>	AGCCGATGGGTTGTACCTTG	ATAGCAAATCGGCTGACGGT
<i>Zika-Env</i>	CCGCTGCCCAACACAAG	CCACTAACGTTCTTTTGCAGACAT

Table 6. Sequence of primers used for qPCR experiments.

5. RESULTS

5.1. Host genetics control of susceptibility to Zika virus in Collaborative Cross mice

Article published in the Journal of Virology:

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(Manet *et al.* 2019)



Genetic Diversity of Collaborative Cross Mice Controls Viral Replication, Clinical Severity, and Brain Pathology Induced by Zika Virus Infection, Independently of *Oas1b*

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ABSTRACT The explosive spread of Zika virus (ZIKV) has been associated with major variations in severe disease and congenital afflictions among infected populations, suggesting an influence of host genes. We investigated how genome-wide variants could impact susceptibility to ZIKV infection in mice. We first describe that the susceptibility of *Ifnar1*-knockout mice is largely influenced by their genetic background. We then show that Collaborative Cross (CC) mice, which exhibit a broad genetic diversity, in which the type I interferon receptor (IFNAR) was blocked by an anti-IFNAR antibody expressed phenotypes ranging from complete resistance to severe symptoms and death, with large variations in the peak and the rate of decrease in the plasma viral load, in the brain viral load, in brain histopathology, and in the viral replication rate in infected cells. The differences in susceptibility to ZIKV between CC strains correlated with the differences in susceptibility to dengue and West Nile viruses between the strains. We identified highly susceptible and resistant mouse strains as new models to investigate the mechanisms of human ZIKV disease and other flavivirus infections. Genetic analyses revealed that phenotypic variations are driven by multiple genes with small effects, reflecting the complexity of ZIKV disease susceptibility in the human population. Notably, our results rule out the possibility of a role of the *Oas1b* gene in the susceptibility to ZIKV. Altogether, the findings of this study emphasize the role of host genes in the pathogeny of ZIKV infection and lay the foundation for further genetic and mechanistic studies.

IMPORTANCE In recent outbreaks, ZIKV has infected millions of people and induced rare but potentially severe complications, including Guillain-Barré syndrome and encephalitis in adults. While several viral sequence variants were proposed to enhance the pathogenicity of ZIKV, the influence of host genetic variants in mediating the clinical heterogeneity remains mostly unexplored. We addressed this question using a mouse panel which models the genetic diversity of the human population and a ZIKV strain from a recent clinical isolate. Through a combination of *in vitro* and *in vivo* approaches, we demonstrate that multiple host genetic variants determine viral replication in infected cells and the clinical severity, the kinetics of blood viral load, and brain pathology in mice. We describe new mouse models expressing high degrees of susceptibility or resistance to ZIKV and to other flaviviruses. These models will facilitate the identification and mechanistic characterization of host genes that influence ZIKV pathogenesis.

KEYWORDS Zika virus, flavivirus, mouse model, host genetics, genetic diversity, Collaborative Cross, Zika

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Zika virus (ZIKV) is a mosquito-borne flavivirus isolated in 1947 from a febrile rhesus monkey in Uganda (1). Until 2007, ZIKV had circulated in Africa and Asia, causing mild flu-like syndromes, with rare reported clinical cases (2). However, during recent epidemics, ZIKV infection triggered severe complications, including Guillain-Barré syndrome and encephalitis in adults (3, 4) and congenital malformations in the fetuses of infected pregnant women (5, 6). Viral mutations may have contributed to the enhanced pathogenicity of ZIKV (7, 8) but only partly explain the variable proportion of symptomatic infections between populations (9) and the increased incidence of congenital Zika syndrome (CZS) in Polynesia (10) and Brazil (11), suggesting a role for host genetic variants. Recent evidence indicates that the regulation of innate immunity genes is driven by the host genetic background in human fetal brain-derived neural stem cells (hNSCs) infected *in vitro* with ZIKV (12). Additionally, the analysis of pairs of dizygotic twins exposed to ZIKV during pregnancy and discordant for CZS suggests multigenic host susceptibility to ZIKV-induced brain malformations (13).

Multiple mouse models have been proposed to decipher the mechanisms of ZIKV disease pathogenesis (14, 15). These models allow the investigation of several key features of human infection, such as neuronal damage (16, 17), sexual and vertical transmission (18–21), and fetal demise and CZS (22–25). However, while nonstructural ZIKV proteins efficiently inhibit the innate antiviral responses in humans (26, 27), allowing viral replication, ZIKV replicates poorly in wild-type mice due to the inability of its NS5 protein to antagonize the STAT2 protein and the type I interferon (IFN) response as it does in humans (28). Effective systemic infection in mice occurs when this response is abrogated by genetically inactivating the *Ifnar1* gene (29) or by blocking the type I IFN receptor (IFNAR) with the MAR1-5A3 monoclonal antibody (MAb) (30, 31). So far, the host genetic factors involved in mouse susceptibility to ZIKV infection have been investigated mainly through reverse genetic approaches, by studying the consequences of genetic ablation of specific genes, such as innate or adaptive immunity genes (29, 32–35). While these models have contributed to our understanding of the mechanisms of ZIKV disease, they do not model the simultaneous contribution of variants in multiple pathways like those that would most likely be observed in the natural population. A recent study has reported strain-specific differences in susceptibility to neonatal ZIKV infection across four mouse laboratory strains, affecting neuropathology and behavior in adulthood (36). More extensive studies investigating the role of genome-wide genetic variations on susceptibility to ZIKV infection, using mouse models that reflect the phenotypic and genetic diversity of the human population, are needed (37).

In this study, we addressed this question using two types of susceptible mouse models. First, since the phenotype resulting from a single gene modification often varies under the influence of modifier genes (38, 39), we assessed the effect of host genetic background on the susceptibility of *Ifnar1*-deficient mice. We then investigated the impact of host genetic diversity on the susceptibility to ZIKV infection in the Collaborative Cross (CC), a panel of recombinant inbred mice produced via a funnel breeding scheme that combined eight founder inbred strains, including five classic laboratory strains and three wild-derived strains (40). As a result, every CC strain has inherited a unique and balanced contribution from each of the eight founder strains. These founder strains capture approximately 90% of the genetic variants present in the *Mus musculus* species (41), and the resulting CC strains, which segregate an estimated 45 million polymorphisms, have more genetic diversity than the human population (42). Extensive variations in pathogenic phenotypes have been previously reported in the CC panel after viral (43–50), bacterial (51, 52), and fungal (53) infections, demonstrating that this resource is ideally suited for investigating the role of host genetic variants in the pathophysiology of infectious diseases (54).

Susceptibility to ZIKV in *Ifnar1*-deficient mice was strongly influenced by the genetic background, which has practical implications for future virology studies to identify modifier genes. The challenge of 35 immunocompetent CC strains with ZIKV after MAR1-5A3 MAb treatment allowed efficient viral replication. We show that the genetic

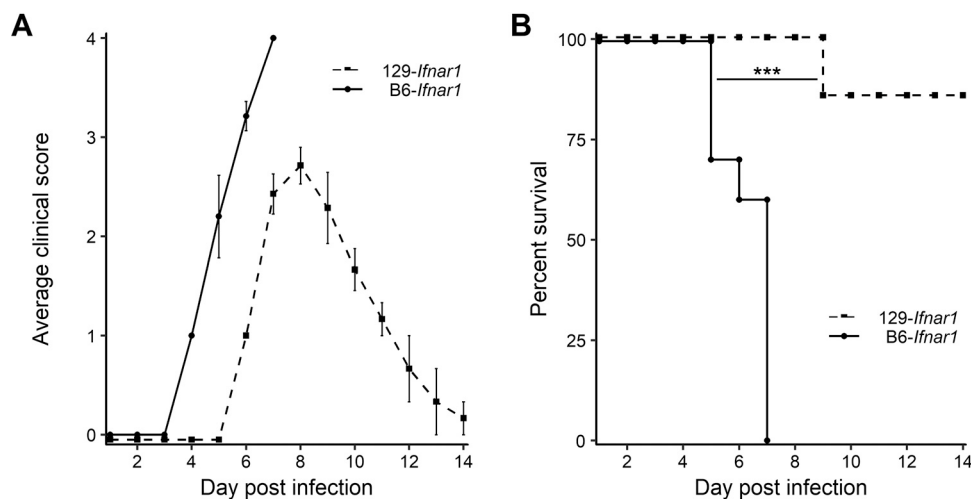


FIG 1 ZIKV disease severity in *Ifnar1*-deficient mice is driven by the genetic background. Six- to 7-week-old 129-*Ifnar1* ($n = 7$) and B6-*Ifnar1* ($n = 10$) mice were infected i.p. with 10^7 FFUs of ZIKV FG15 and monitored for 14 days. (A) Average clinical score, with numerical values given as follows: 0, no symptoms; 1, ruffled fur; 2, emaciation, hunched posture, and/or hypoactivity; 3, hind limb weakness, prostration, and/or closed eyes; and 4, moribund or dead. (B) Kaplan-Meier survival curves showing 100% lethality in B6-*Ifnar1* mice at day 7 p.i. and the survival of 6/7 129-*Ifnar1* mice, ***, $P = 0.0002$ (log-rank test). B6-*Ifnar1* mice developed early symptoms, which rapidly evolved to death, while 129-*Ifnar1* mice developed symptoms 2 days later, which eventually resolved in most mice.

diversity in the CC panel enabled large variations in the clinical severity of ZIKV disease, in the peak and the kinetics of the plasma viral load, and in the severity of ZIKV-induced brain pathology. The genetic diversity also resulted in differences in the permissiveness of CC mouse cells to viral replication, and these differences likely contribute to the *in vivo* phenotypic range. We found that the differences in the susceptibility of a subset of CC strains to ZIKV correlated with the differences in the susceptibility of the strains to dengue virus (DENV) and West Nile virus (WNV), suggesting shared underlying mechanisms. We identified highly susceptible and resistant mouse strains as new models to investigate the mechanisms of human ZIKV disease and other flavivirus infections. Finally, genetic analysis revealed that the susceptibility to ZIKV in the CC is driven by multiple loci with small individual effects and that *Oas1b*, a major determinant of mouse susceptibility to WNV, is not involved.

(This article was submitted to an online preprint archive [55].)

RESULTS

Genetic background controls the susceptibility of *Ifnar1*-deficient mice to ZIKV.

Many studies have used *Ifnar1*-knockout mice on the 129S2/SvPas mouse (here abbreviated 129 mouse) (56, 57) or C57BL/6J mouse (here abbreviated B6 mouse) (23, 29, 34) inbred background, but the differences in ZIKV susceptibility between these two strains have not been reported and remain unclear due to heterogeneous experimental conditions between studies. We compared the susceptibility of age-matched 129S2/SvPas *Ifnar1*^{−/−} (129-*Ifnar1*) and C57BL/6J *Ifnar1*^{−/−} (B6-*Ifnar1*) mice infected intraperitoneally (i.p.) with 10^7 focus-forming units (FFUs) of ZIKV FG15. B6-*Ifnar1* mice showed increasingly severe symptoms, with body weight loss, ruffled fur, ataxia, and hind limb paralysis from day 4 postinfection (p.i.), and all (10/10) B6-*Ifnar1* mice were moribund or dead by day 7 p.i. In contrast, 129-*Ifnar1* mice developed mild symptoms (ruffled fur, hunched back) starting on day 6 p.i. and declining over the second week of infection, with one mouse dying on day 9 p.i. (Fig. 1), demonstrating that the susceptibility to ZIKV infection conferred by *Ifnar1* genetic inactivation is critically influenced by the host genetic background.

MAb blockade of IFNAR is a robust model to study ZIKV infection in CC mice.

Ifnar1 genetic deficiency permanently abrogates alpha/beta IFN (IFN- α/β)-mediated

TABLE 1 Origin of *Oas1b* and *Ifnar1* alleles in the 35 CC strains tested^a

Strain	<i>Oas1b</i>	<i>Ifnar1</i>
CC001	B	F
CC002	A	B
CC003	H	B
CC004	G	B
CC005	B	E
CC006	E	D
CC007	D	B
CC009	E	D
CC011	B	G
CC012	H	A
CC013	E	E
CC017	H	D
CC018	D	C
CC019	H	C
CC021	C	D
CC024	E	A
CC025	H	A
CC026	A	H
CC027	B	G
CC032	B C *	B
CC037	B	C
CC039	H	B
CC040	B D *	D
CC041	F	D
CC042	H	F
CC043	C	C
CC045	C	H
CC049	B D *	D
CC051	E	B
CC059	E	B
CC060	D	F
CC061	E	A
CC068	B	E G *
CC071	C	F
CC072	C	D

functional

not functional

A/J allele

B6 allele or identical

CAST allele

PWK allele

^aData are from <http://csbio.unc.edu/CCstatus/CCGenomes/#genotypes>.
*, heterozygous strain; A, A/J mice; B, C57BL/6J mice; C, 129S1/SvImJ mice; D, NOD/ShiLtJ mice; E, NZO/HiLtJ mice; F, CAST/EiJ mice; G, PWK/PhJ mice; H, WSB/EiJ mice.

immune responses but is not currently available on diverse genetic backgrounds. We therefore tested the suitability of transient IFNAR blockade mediated by MAb treatment as a model to study ZIKV infection in genetically diverse mice, like the CC mice. Since the MAR1-5A3 MAb was generated in a laboratory strain (the 129-*Ifnar1* mouse) (30) and since the CC strains differ in their *Ifnar1* allele (Table 1), we first compared the coding sequence of the *Ifnar1* gene across the eight founder strains. While 129S1/SvImJ, NOD/ShiLtJ, NZO/HiLtJ, and WSB/EiJ mice carry the same *Ifnar1* allele as C57BL/6J mice, the *Ifnar1* alleles of A/J, PWK/PhJ, and CAST/EiJ mice differ by 1, 3, and 7 amino acids from the C57BL/6J mouse reference sequence, respectively (Table 2). Notably, all three PWK/PhJ variants were found in CAST/EiJ mice. Therefore, we assessed the efficacy of the MAR1-5A3 MAb by Western blot analysis on mouse embryonic fibroblasts (MEFs) isolated from two CC strains (strains CC001 and CC071),

TABLE 2 Variants in the coding sequence of the *Ifnar1* gene

Position ^a	dbSNP ^b annotation	Nucleotide in the <i>Ifnar1</i> gene of the CC founder strains:								Amino acid position	Amino acid change ^c
		Reference (B6)	129S1/SvImJ	A/J	CAST/EiJ	NOD/ShiLtJ	NZO/HILtJ	PWK/PhJ	WSB/EiJ		
91,485,366	rs235809125	C	— ^d	—	T	—	—	—	—	8	A → V
91,485,368	rs255866699	G	—	—	A	—	—	—	—	9	A → T
91,499,433	rs31418313	G	—	A	—	—	—	—	—	274	R → H
91,499,468	rs223171563	A	—	—	G	—	—	—	—	286	N → D
91,499,532	rs240163845	A	—	—	G	—	—	G	—	307	H → R
91,501,623	rs263194450	T	—	—	G	—	—	G	—	376	I → R
91,501,631	rs246470801	G	—	—	A	—	—	A	—	379	E → K
91,505,284	rs219805193	C	—	—	T	—	—	—	—	549	A → V

^aThe sequences at positions 91,485,344 to 91,505,411 on chromosome 16 were retrieved from the Sanger Institute Mouse Genomes Project (https://www.sanger.ac.uk/sanger/Mouse_SnpViewer/rel-1505).

^bdbSNP, Single Nucleotide Polymorphism Database.

^cAmino acid changes compared with the reference (B6 mouse) sequence (CCDS database; <https://www.ncbi.nlm.nih.gov/CCDS/>).

^d—, no nucleotide change.

both of which inherited the *Ifnar1* allele from the most divergent CAST/EiJ strain (58), by comparison with that on B6 MEFs. IFNAR stimulation by IFN- α/β activates the JAK1/TYK2 pathway and results in the phosphorylation of STAT1. We found that in B6, CC001, and CC071 MEFs, STAT1 phosphorylation was equally induced by murine IFN- α and fully inhibited by the MAR1-5A3 MAb (Fig. 2A).

To assess MAR1-5A3 MAb efficacy *in vivo*, we infected the CC001 and CC071 strains with 10⁷ FFUs of ZIKV FG15 i.p. and we measured the kinetics of the plasma viral load in mice with and without 2 mg MAb treatment 24 h prior to infection. Consistent with previous studies in B6 (31, 59) and BALB/c (60) mice, the viral load was consistently 4 to 5 log₁₀ units higher in MAb-treated mice of both the CC001 and CC071 strains than in untreated mice, demonstrating that MAR1-5A3 MAb treatment successfully increases CC mouse permissiveness to ZIKV replication (Fig. 2B).

We then measured the kinetics of the plasma viral load in the 129-*Ifnar1* strain as well as in four MAb-treated CC strains infected with 10⁷ FFUs of ZIKV FG15 i.p. We established that the peak plasma viral load occurred in most individuals at day 2 p.i., independently of the mouse genetic background (Fig. 2C).

In previous studies, viral loads have been measured either by FFU titration or by reverse transcription (RT)-quantitative PCR (qPCR) quantification of viral genome copies. We compared these two methods in the B6-*Ifnar1* and 129-*Ifnar1* strains and in 10 MAb-treated CC strains (Fig. 2D). We performed focus-forming assays (FFA) to measure the viral particles in the plasma at day 2 p.i. and confirmed the production of infectious ZIKV in the blood of all strains. Next, we compared the plasma viral load measured by RT-qPCR and by FFA. We found that these two parameters were strongly correlated over a 2-log₁₀ range (Pearson's coefficient [r^2] = 0.89, $P = 9.9 \times 10^{-17}$), with the number of genome copies being, on average, 3 log₁₀ units higher than the number of FFUs (Fig. 2D). We therefore validated that the plasma viral load measured by RT-qPCR, a more labor-efficient method, could be used as a proxy for viremia throughout the study.

Finally, we compared the plasma viral loads at day 2 p.i. between male and female 129-*Ifnar1* mice and both sexes of 4 MAb-treated CC strains that had been tested. We found no significant difference between sexes across diverse genetic backgrounds (two-way analysis of variance [ANOVA], $P = 0.24$) (Fig. 2E), validating the use of merged data from males and females in mouse ZIKV infection experiments.

CC genetic diversity drives ZIKV disease severity and plasma viral load. To explore broad genetic variation, we assessed the susceptibility of MAb-treated mice of 35 CC strains. B6-*Ifnar1*, 129-*Ifnar1* and MAb-treated B6 mice were included as reference strains. Among non-*Ifnar1*-deficient mice, only mice of three CC strains developed symptoms, as shown in Fig. 3A, which summarizes the clinical observations made at day 7 p.i. CC021 and CC026 mice recovered and survived, while symptoms worsened in 7/9 (78%) CC071 mice, which were moribund or died between days 7 and 9 p.i.

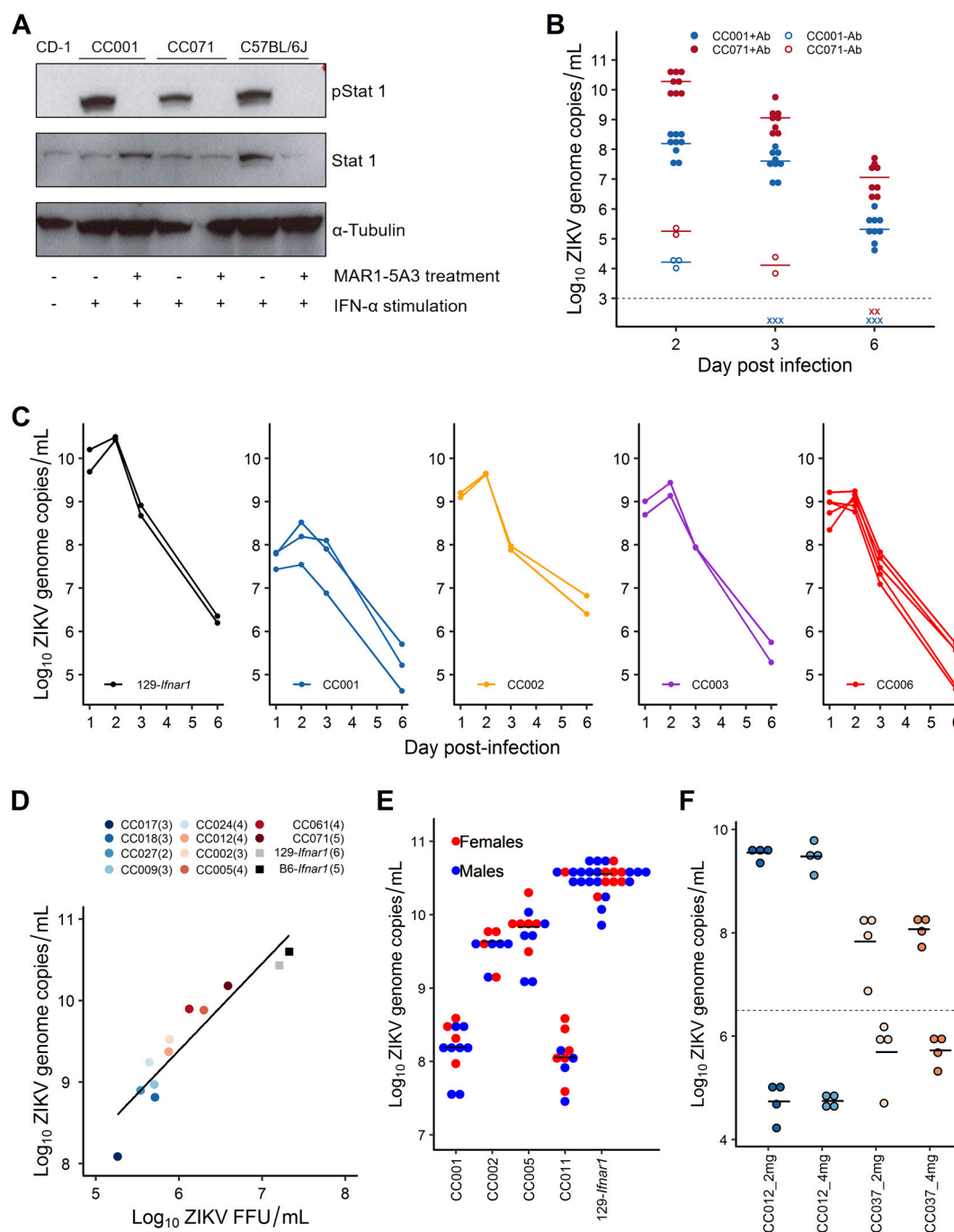


FIG 2 Establishment and validation of the experimental conditions for assessing susceptibility to ZIKV in CC strains. (A) The efficacy of the MAR1-5A3 MAb (100 μ g for 5×10^6 cells) at blocking the IFNAR receptor in diverse mouse genetic backgrounds was determined by assessing STAT1 phosphorylation (pStat 1) by Western blotting on mouse embryonic fibroblasts (MEFs) derived from the C57BL/6J, CC001, CC071, and CD-1 strains. (B) Plasma viral load, measured on days 2, 3, and 6 p.i. by RT-qPCR, in 6- to 8-week-old mice of the CC001 and CC071 strains that had been treated with MAb MAR1-5A3 24 h prior to ZIKV infection (filled circles; $n = 9$ and 8, respectively) or untreated (open circles; $n = 3$ and 2, respectively). x, a sample with a level below the detection level. (C) Kinetics of the plasma viral load in 129-Ilfar1 mice and 4 CC strains measured by RT-qPCR. Each circle represents the result for a 6- to 8-week-old mouse analyzed on days 1, 2, 3, and 6. (D) Correlation between the plasma viral load determined by FFA (x axis) and RT-qPCR (y axis) in 46 blood samples from 129-Ilfar1 and B6-Ilfar1 mice and 10 CC strains (circles show the mean for each strain; the number of 6- to 8-week-old mice per strain is shown in parentheses). (E) Plasma viral load, measured by RT-qPCR at day 2 p.i., in 6- to 8-week-old males and females of the 129-Ilfar1 strain and of 4 CC strains ($n \geq 4$ mice per group). (F) Effect of the dose of MAR1-5A3 antibody treatment on the rate of decrease of the plasma viral load. Seven- to 9-week-old mice of the CC012 and CC037 strains (4 mice per group) received 2 mg of the MAR1-5A3 MAb 1 day prior to being inoculated i.p. with 10^7 FFUs of ZIKV FG15. The groups receiving 4 mg of the MAb received additional i.p. injections of 1 mg of MAb on days 2 and 4 p.i. The plasma viral load was measured on days 2 and 6 p.i. (and the results are presented above and below the dashed line, respectively).

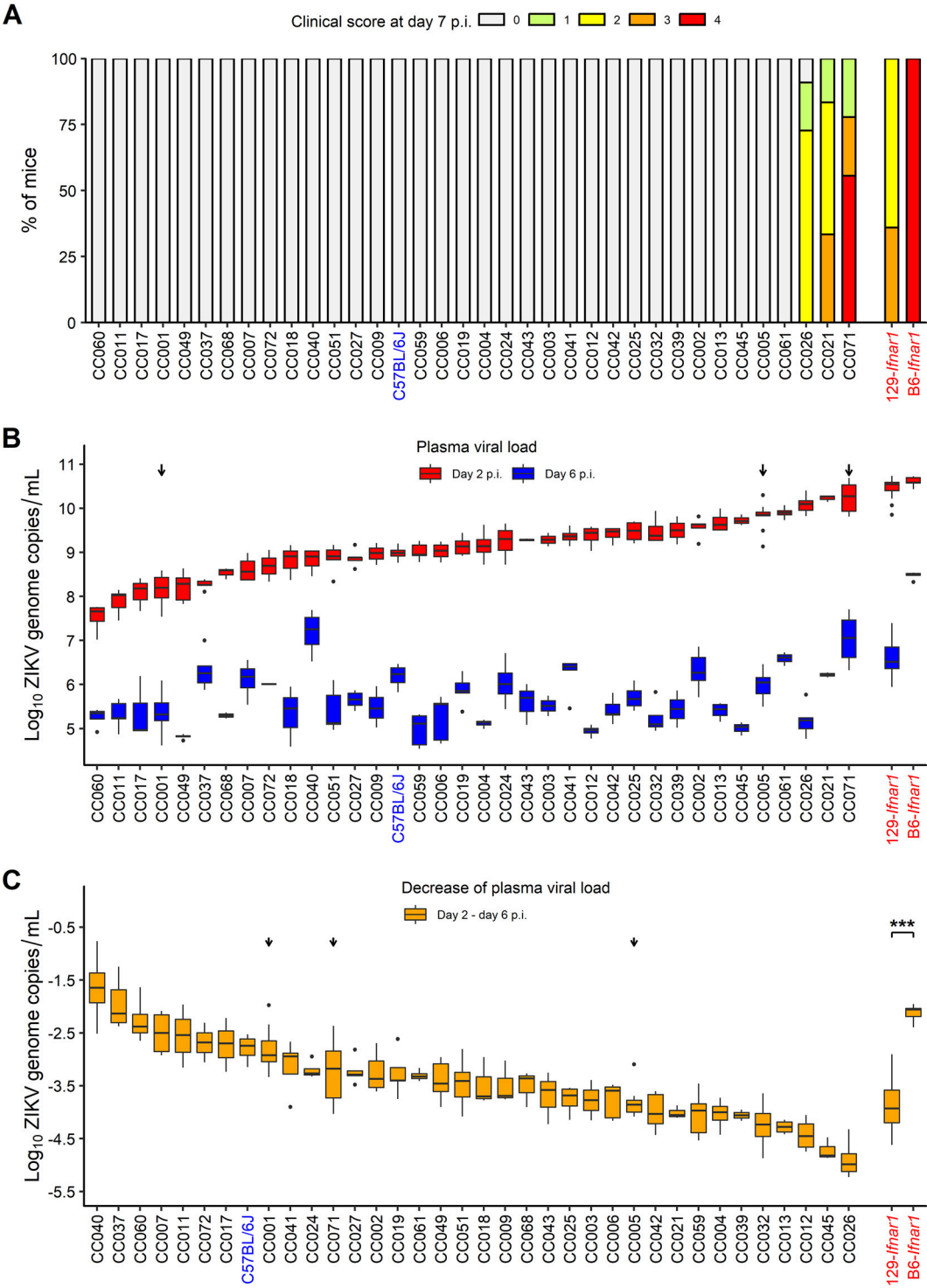


FIG 3 CC genetic diversity strongly impacts the clinical severity of infection and the plasma viral load. Thirty-five CC strains ($n = 2$ to 9 6- to 8-week-old mice per strain) were infected i.p. with 10^7 FFUs of ZIKV FG15 at 24 h after i.p. injection of 2 mg of the MAR1-5A3 MAb. 129-*Ifnar1* ($n = 24$) and B6-*Ifnar1* ($n = 5$) mice were similarly infected without MAb treatment. (A) Clinical scores at day 7 p.i. as the percentage of mice in the five levels of severity (as described in the legend to Fig. 1). (B) Plasma viral load at days 2 p.i. (upper values) and 6 p.i. (lower values) quantified by RT-qPCR, shown as box-whisker plots, with outliers shown as dots (the strains are shown in the same order in which they are shown in panel A). (C) Difference between plasma viral loads at days 2 and 6 p.i. Strains are sorted by increasing absolute difference and are therefore in an order different from that in panels A and B. (B and C) Arrows indicate the subset of CC mouse strains selected for detailed study.

The plasma viral load was measured on days 2 and 6 p.i. At day 2 p.i., which corresponded to the time of peak viral load, the viral load was generally characterized by small within-strain heterogeneity and large interstrain variations spread over a 2.8-log_{10} range (Fig. 3B), demonstrating a strong effect of host genes (Kruskal-Wallis test, $P = 4.8 \times 10^{-15}$) with a broad-sense heritability of 86% (61). The three symptomatic CC strains showed the highest peak viral load, close to that of B6-*Ifnar1* and 129-*Ifnar1* mice. However, other strains (such as CC005 and CC061) had similarly high viral loads but never showed any clinical signs of disease, indicating that the peak viral load is unlikely the sole factor controlling clinical severity. At day 6 p.i., within-strain variations were larger and more heterogeneous, but we still observed highly significant interstrain differences (Kruskal-Wallis test, $P = 1.1 \times 10^{-10}$). Interestingly, the viral loads on days 2 and 6 p.i. were only moderately correlated (Pearson's coefficient [r^2] = 0.46; $P = 0.004$), indicating that the viral load at day 2 p.i. was not predictive of the viral load at day 6 p.i. (see, for example, the results for CC018 and CC040 mice or CC026 and CC071 mice in Fig. 3B).

We used the difference in the \log_{10} plasma viral loads between days 2 and 6 p.i. to estimate the clearance rate of the virus from the bloodstream (Fig. 3C, in which the order of the strains sorted by increasing clearance rate was therefore different from that in Fig. 3A and B). This rate varied over a 3.3-log_{10} range between strains, demonstrating a strong effect of host genes (Kruskal-Wallis test, $P = 2.2 \times 10^{-12}$) with a broad-sense heritability of 76%. B6-*Ifnar1* mice showed a slower decrease in the viral load than 129-*Ifnar1* mice (Wilcoxon rank-sum test, $P = 1.7 \times 10^{-5}$), despite similar peak viral loads at day 2 p.i. To confirm that the differences between CC strains were not due to differential rates of MAR1-5A3 antibody turnover, we treated CC012 mice (which showed a 4.4-log decrease) and CC037 mice (which showed a 1.9-log decrease) with 2 mg of MAb 24 h before ZIKV infection and then treated or did not treat them with two additional injections of 1 mg of MAb on days 2 and 4 postinfection. No differences in plasma viral load on days 2 and 6 p.i. were observed between the two conditions (Fig. 2F).

Overall, the genetic diversity in the CC panel controlled the clinical severity of the ZIKV infection, mouse survival, the peak plasma viral load, and the clearance rate of the plasma viral load. Of note, there was no association, across the 35 CC strains tested, between the peak plasma viral load and the *Ifnar1* allele inherited from the founder strain (ANOVA, $P > 0.09$). The findings from this analysis confirm our *in vitro* data (Fig. 2A) and indicate that the variations in peak plasma viral load do not result from differences in MAb treatment efficacy due to MAb turnover or due to the *Ifnar1* alleles.

From this screening, we identified several strains with extreme phenotypes, in particular, strain CC071, which was the most susceptible to ZIKV infection; strains CC001, CC011, CC017, and CC060, which had low peak plasma viral loads; strain CC040, which had a slowly decreasing plasma viral load; and strains CC045 or CC026, which had high peak plasma viral loads but fast-decreasing plasma viral loads.

CC mice show correlated susceptibility to ZIKV, DENV, and WNV. We further characterized three CC strains (indicated by arrows in Fig. 3B and C) among those showing the lowest peak viral loads (strain CC001) and the highest peak viral loads with (strain CC071) or without (strain CC005) clinical symptoms. To establish whether the above-described differences were specific to the FG15 ZIKV strain of the Asian lineage, we first assessed the susceptibilities of the three selected strains to the HD78788 ZIKV strain of the African lineage. 129-*Ifnar1* mice and MAb-treated CC mice were infected i.p. with 10^3 FFUs of ZIKV HD78788, which proved to be highly pathogenic in *Ifnar1*-deficient mice, with rapid and severe symptoms and 100% mortality (Fig. 4A). CC001 mice were fully resistant, with no or mild clinical signs (Fig. 4A, left and center). In contrast, all CC071 mice were moribund or dead by day 10 p.i., with early and quickly aggravating symptoms, almost like those in 129-*Ifnar1* mice. Only one of the five CC005 mice developed symptoms and died. The peak viral load (day 2 p.i.) varied over a 2.4-log_{10} range, and the differences between strains were similar to those observed with the FG15 ZIKV strain (Fig. 3B). Here again, the plasma viral load at day 2 p.i. was

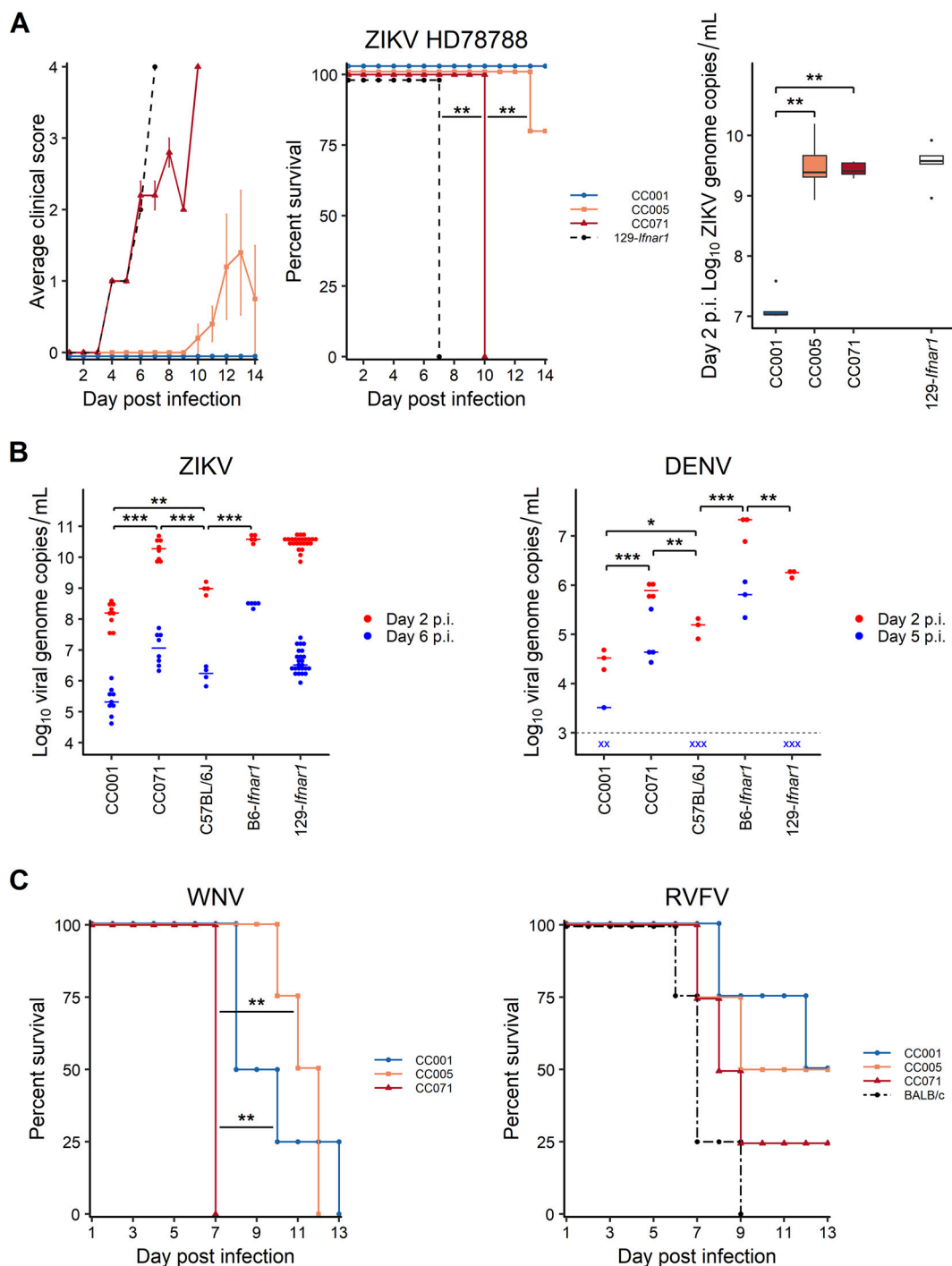


FIG 4 The differences in susceptibility to ZIKV between CC strains correlate with the differences in susceptibility to other flaviviruses. (A) Six- to 8-week-old mice from three selected CC strains treated with the MAR1-5A3 MAb and 129-*Ifnar1* mice were infected intraperitoneally with 10^3 FFUs of ZIKV HD78788. (Left) Average clinical score, with numerical values given as described in the legend to Fig. 1; (center) Kaplan-Meier survival curves (log-rank test); (right) plasma viral load at day 2 p.i., measured by RT-qPCR (P values were determined by the Wilcoxon rank-sum test). (B) The viral loads after ZIKV infection (left; data extracted from Fig. 3) and DENV infection (right; data for i.v. infection with 2×10^6 FFUs of DENV KDH0026A) in MAb-treated 6- to 8-week-old CC001, CC071, and B6 mice and in 129-*Ifnar1* and B6-*Ifnar1* were compared (P values were determined by the t test). (C) (Left) Kaplan-Meier survival curves for four 8- to 12-week-old male mice of each of the three selected CC strains infected i.p. with 1,000 FFUs of WNV strain IS-98-ST1 and monitored for 14 days (P values were determined by the log-rank test); (right) Kaplan-Meier survival curves for four to five 8- to 12-week-old male mice of the BALB/cByJ strain and each of the three selected CC strains infected i.p. with 100 PFUs of RVFV strain ZH548 and monitored for 14 days (log-rank test, $P > 0.05$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the highest in the very susceptible CC071 and 129-*Ifnar1* strains and low in the resistant CC001 strain, but it was also very high in CC005 mice, which were moderately susceptible, confirming that clinical severity does not depend solely on the peak plasma viral load.

To evaluate whether these differences in susceptibility were specific to ZIKV or extended to other flaviviruses, we assessed the phenotypes of a few strains after infection with DENV and WNV, two other members of the *Flaviviridae* family.

We measured the plasma viral load after intravenous (i.v.) infection with 2×10^6 FFUs of DENV DKH0026A (a DENV serotype 1 [DENV-1] strain) in MAb-treated CC001, CC071, and B6 mice and in 129-*Ifnar1* and B6-*Ifnar1* mice (Fig. 4B, right). Most interstrain differences observed with the ZIKV FG15 strain (Fig. 4B, left, with data from Fig. 3B) were also observed with DENV, with the CC071 mice displaying the highest plasma viral load after MAb treatment. DENV infection was, overall, much less clinically severe, since only B6-*Ifnar1* mice developed nonlethal symptoms, including ruffled fur, hunched back, and ataxia.

We also investigated the susceptibility of the selected CC strains to WNV. *Oas1b* was previously shown to be a major host genetic determinant of susceptibility to WNV in mice (62). Of note, the three selected CC strains carry the same truncated, nonfunctional allele of *Oas1b* inherited from the laboratory strain founders (Table 1), conferring to them susceptibility to WNV infection. CC mice were infected i.p. with 10^4 FFUs of WNV IS-98-ST1 and monitored for 14 days p.i. (efficient WNV infection does not require anti-IFNAR MAb treatment in mice carrying the truncated *Oas1b* allele). All CC071 mice died by 7 days p.i., which was significantly faster than the time to death for the CC001 and CC005 mice (log-rank test, $P < 0.01$) (Fig. 4C, left), indicating that the genetic diversity between CC strains also influences their susceptibility to WNV even in the context of *Oas1b* deficiency.

To assess whether the differences in susceptibility between these CC strains also applied to other viruses, we infected them with 10^2 PFU of Rift Valley fever virus (RVFV) ZH548 i.p. No significant difference was found between the three CC strains (log-rank test, $P > 0.3$ for all pairwise comparisons) (Fig. 4C, right), which succumbed late in the infection, like the commonly used BALB/cByJ mice. Only CC001 mice died significantly later than BALB/cByJ mice (log-rank test, $P = 0.04$).

Genetic analysis suggests polygenic control of susceptibility to ZIKV in CC mice. To identify host genetic factors controlling the susceptibility to ZIKV in the CC strains, we performed a genome-wide association study between the plasma viral loads at days 2 and 6 p.i. or the rate of decrease in the plasma viral load and the genotypes of the 35 CC strains. Genetic associations were plotted as logarithm of the odds (LOD) scores. We did not find any genome locations at which the LOD scores reached the minimum 0.1 significance threshold for any of the three traits (Fig. 5), which would be expected if phenotypic variations were controlled by one or two loci with strong effects. Therefore, these results suggest that the plasma viral load is controlled by multiple small-effect genetic variants.

The genetic diversity of CC strains controls brain viral load and pathology. To assess the influence of host genetics on the brain pathology caused by ZIKV infection, we further characterized the three previously selected CC strains. We measured the viral load in the brain 6 days after i.p. infection with ZIKV FG15 in MAb-treated CC mice and 129-*Ifnar1* mice (Fig. 6A). CC005 and CC071 mice, which had higher peak plasma viral loads than CC001 mice, also had higher brain viral loads (mean = $6.5 \log_{10}$ copies/ μ g RNA for CC005 and CC071 mice, compared with $5 \log_{10}$ copies/ μ g RNA for CC001 mice). As expected, 129-*Ifnar1* mice showed the highest viral load in the brain. These results indicate an overall correlation between plasma and brain viral loads.

Histopathological analysis, carried out with brain tissue from the same mice (Fig. 6B to N), revealed different lesion profiles between the four mouse strains (Fig. 6B, F, I, and L for low-magnification images). 129-*Ifnar1* mice clearly displayed the most severe inflammatory lesions with subacute leptomeningoencephalitis (i.e., infiltration of

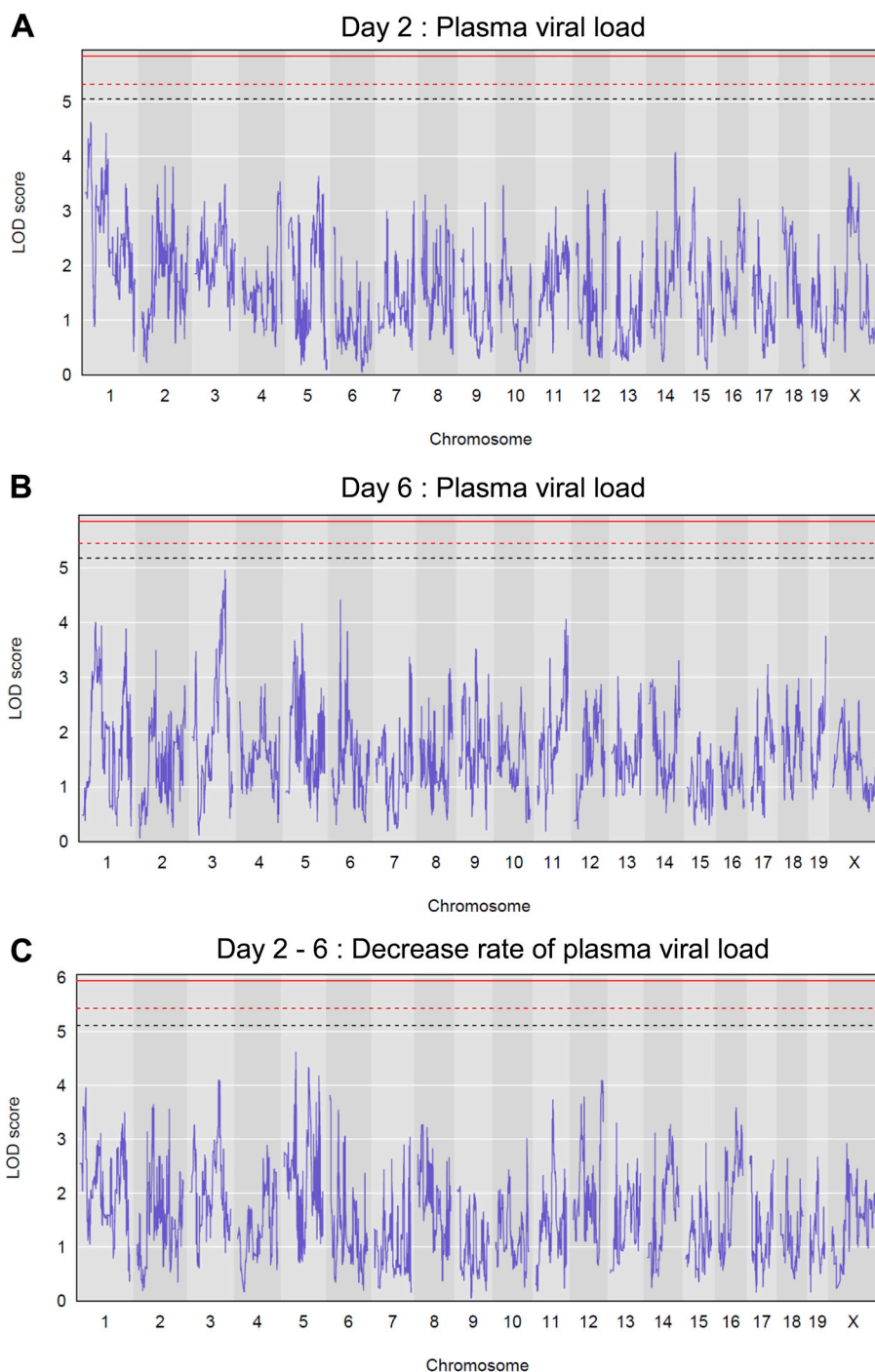


FIG 5 Genetic analysis of susceptibility to ZIKV fails to identify simple genetic control. Genome-wide linkage analysis for the plasma viral load at day 2 p.i. (A), the plasma viral load at day 6 p.i. (B), and the rate of decrease in the plasma viral load (C) for the 35 CC strains for which the results are shown in Fig. 3. The x axis represents the genomic location; the y axis is the LOD score, representing the statistical association between the phenotype and the genomic location. Genome-wide thresholds of P equal to 0.1, 0.05, and 0.01, computed from 1,000 permutations, are represented by dashed black, dashed red, and plain red lines, respectively. No genome location reached the threshold of P equal to 0.05.

perivascular spaces and leptomeninges by lymphocytes, plasma cells, and macrophages; Fig. 6B to E) and activation of microglial cells with microglial nodules (Fig. 7B to D). In contrast, almost no histological lesions were detected in the brains of CC001 mice (Fig. 6F to H), which showed normal, nonactivated, microglial cells (Fig. 7E to G).

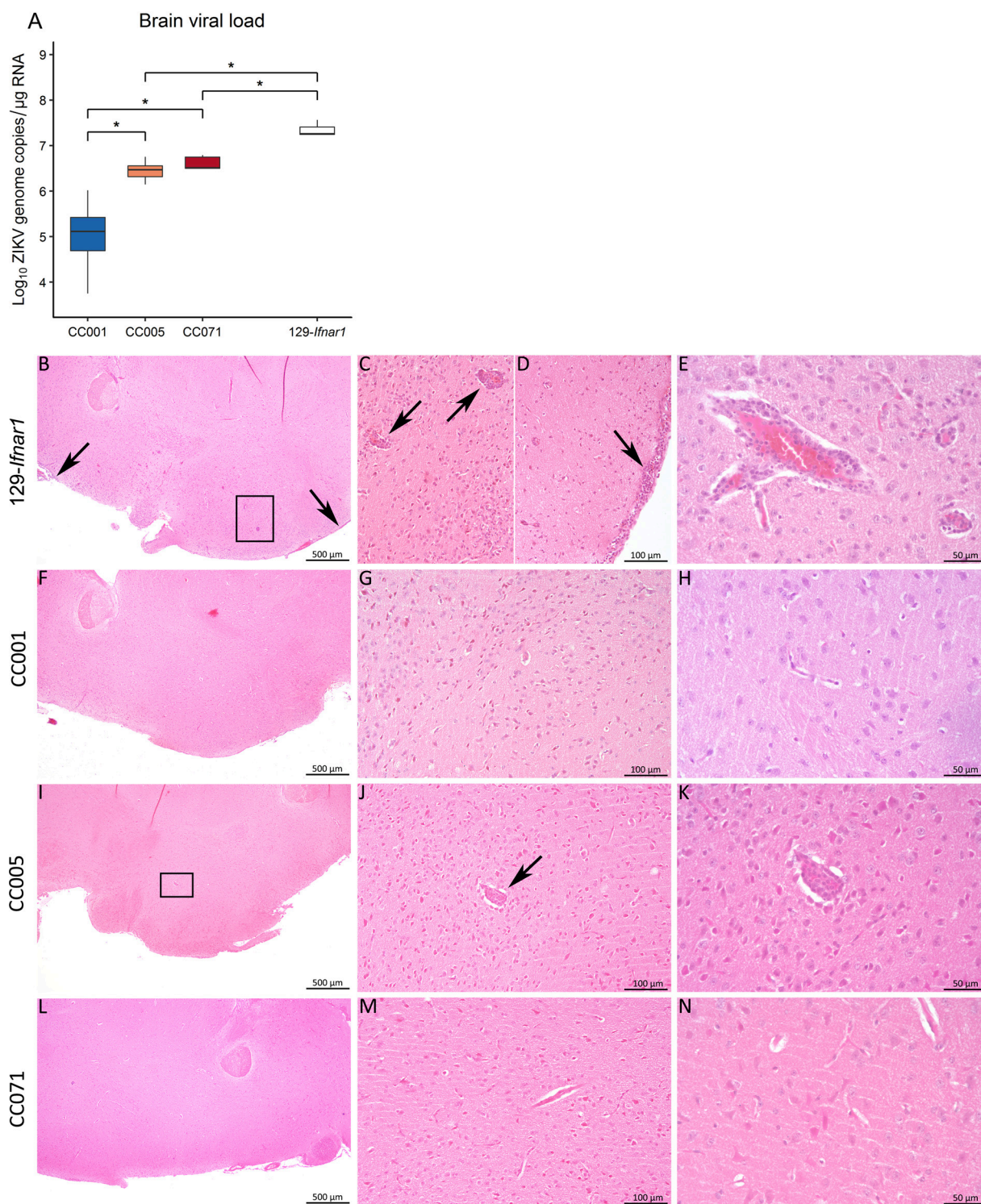


FIG 6 Genetic variations between CC strains control the brain viral load and histological profile in infected mice. Four to five 6- to 8-week-old mice of the 129-*Ifnar1* strain and three selected CC strains were infected i.p. with 10^7 FFUs of ZIKV FG15 24 h after i.p. injection of 2 mg of the MAR1-5A3 MAb. (A) Brain viral load measured by RT-qPCR at day 6 p.i. *, $P < 0.05$, Wilcoxon rank-sum test. (B to N) Representative HE-stained brain sections at three different magnifications. (B to E) 129-*Ifnar1* mice ($n = 3$). Black rectangle, encephalitis with perivascular lymphocyte cuffs. (B and D) Arrows, lesions of subacute leptomeningoencephalitis. (C) Arrows, perivascular lymphocyte cuffs. (F to H) CC001 mice ($n = 5$). (I to K) CC005 mice ($n = 5$). Black rectangle, encephalitis with perivascular lymphocyte cuffs; arrow, perivascular cuffing. (L to N) CC071 mice ($n = 4$).

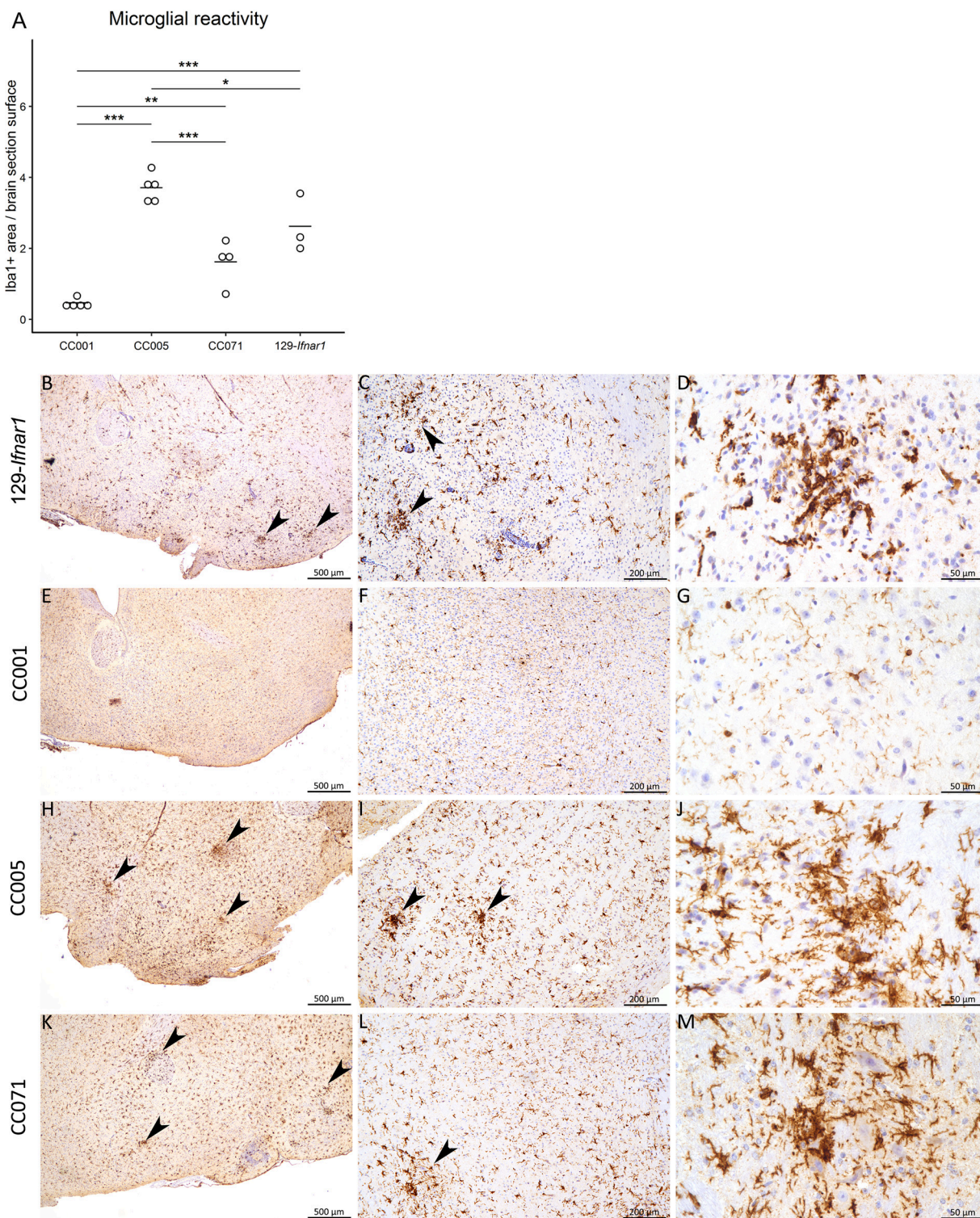


FIG 7 Genetic variations between CC strains control brain neuroinflammation in infected mice. Microglial reactivity was assessed on brain sections from the same mice described in the legend to Fig. 6 by anti-Iba1 immunohistochemistry. (A) Quantification of the Iba1 labeling signal on the brain sections. P values were determined by t tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B to M) Representative anti-Iba1 immunohistochemistry of brain sections at three different magnifications. (B to D) 129-Ilfnar1 mice ($n = 3$); (E to G) CC001 mice ($n = 5$); (H to J) CC005 mice ($n = 5$); (K to M) CC071 mice ($n = 4$). Arrowheads, nodules of activated microglial cells.

Only very rare small clusters of activated microglial cells were detected. CC005 mice displayed moderate inflammatory lesions characterized by perivascular cuffing (Fig. 6I to K) and activation of microglial cells (hyperplasia and thickening of cell processes) and microglial nodules (Fig. 7H to J). CC071 mice displayed almost no lesions upon hematoxylin-eosin (HE) staining (Fig. 6L to N) but inflammatory lesions of intermediate severity with activation of microglial cells and microglial nodules (Fig. 7K to M) similar to those in 129-*Ifnar1* mice (Fig. 7A), as revealed by Iba1 immunolabeling.

The nature and intensity of brain histological lesions may depend on the circulating viral load, on the capacity of the virus and of the MAb to cross the blood-brain barrier, and on the permissiveness of brain cells (in particular, neurons and microglia). To assess the differences in the susceptibility of brain cells between CC strains, we performed intracerebral (i.c.) infections to deliver the virus directly into the brain tissue. 129-*Ifnar1* and MAb-untreated CC mice received 10^5 FFUs of ZIKV FG15 in the left ventricular region of the brain and were followed for 3 weeks. Mild and transient symptoms (ruffled fur, hunched back) were observed in a few mice of the three strains, and one CC005 mouse died on day 19 p.i. A second group of CC mice was infected similarly and euthanized at day 6 p.i. for histological analysis. The differences in the brain viral loads between CC strains were similar to those observed after i.p. infection, with CC005 and CC071 mice showing significantly higher brain viral loads than CC001 mice (Fig. 8A). The lesions seen in 129-*Ifnar1* mice after infection by the i.c. route were mostly similar to the lesions seen after infection by the i.p. route, with marked subacute leptomeningoencephalitis (Fig. 8B to E) and activation of microglial cells (Fig. 9B to D). In contrast, the lesion profiles were clearly different in the three CC strains. Two of the five CC001 mice displayed no significant histological lesions, with normal resting microglial cells, while the other three displayed minimal lesions, with gliosis (Fig. 8F to H) and rare small clusters of activated microglial cells (Fig. 9E to G). By the i.c. route of infection, CC005 mice displayed heterogeneous lesion profiles, with either suspected meningitis and gliosis (Fig. 8I to K; 4/5 mice) or moderate leptomeningoencephalitis (1/5 mice). Activation of microglial cells with variable severity was detected in all animals (Fig. 9H to J). Strikingly, all CC071 mice displayed marked leptomeningoencephalitis (Fig. 8L to O), with the strongest activation being of microglial cells (Fig. 9A and K to M).

These results indicate that CC strains differ in their permissiveness to viral replication in the brain and in their susceptibility to ZIKV-induced histological brain damage. The comparison between the i.p. and i.c. infection routes shows that these variations cannot be explained only by the possibility of the differential dissemination of ZIKV from the circulation to the brain between CC strains.

Viral replication in CC071 mouse cells is increased *in vitro*. The differences in the peak plasma viral loads and the results from i.c. infections suggested that different efficiencies of viral replication could contribute to the variations in susceptibility between CC strains. To address this point, we measured the production of infectious viral particles in MEFs derived from the CC001 and CC071 strains. Cells were infected with ZIKV FG15 at a multiplicity of infection (MOI) of 5. While the viral titers remained stable in CC001 mice between 24 and 72 h, the production of viral infectious particles by CC071 MEFs increased very significantly over the same period (Fig. 10). These results suggest that the increased replication efficiency in CC071 mice could contribute to their susceptible phenotype.

DISCUSSION

ZIKV is a serious public health concern, considering the occurrence of severe neurological complications in adults and the congenital malformations that can result from the infection of pregnant women. The variable outcomes of ZIKV infection in humans have led investigators to hypothesize a role for host genetic factors (9, 13), although this has never been demonstrated thus far. Like for other infectious diseases, human genetic studies on susceptibility to ZIKV would require large cohorts of patients and would be confounded by pathogen genetics, pathogen dose, mosquito-dependent

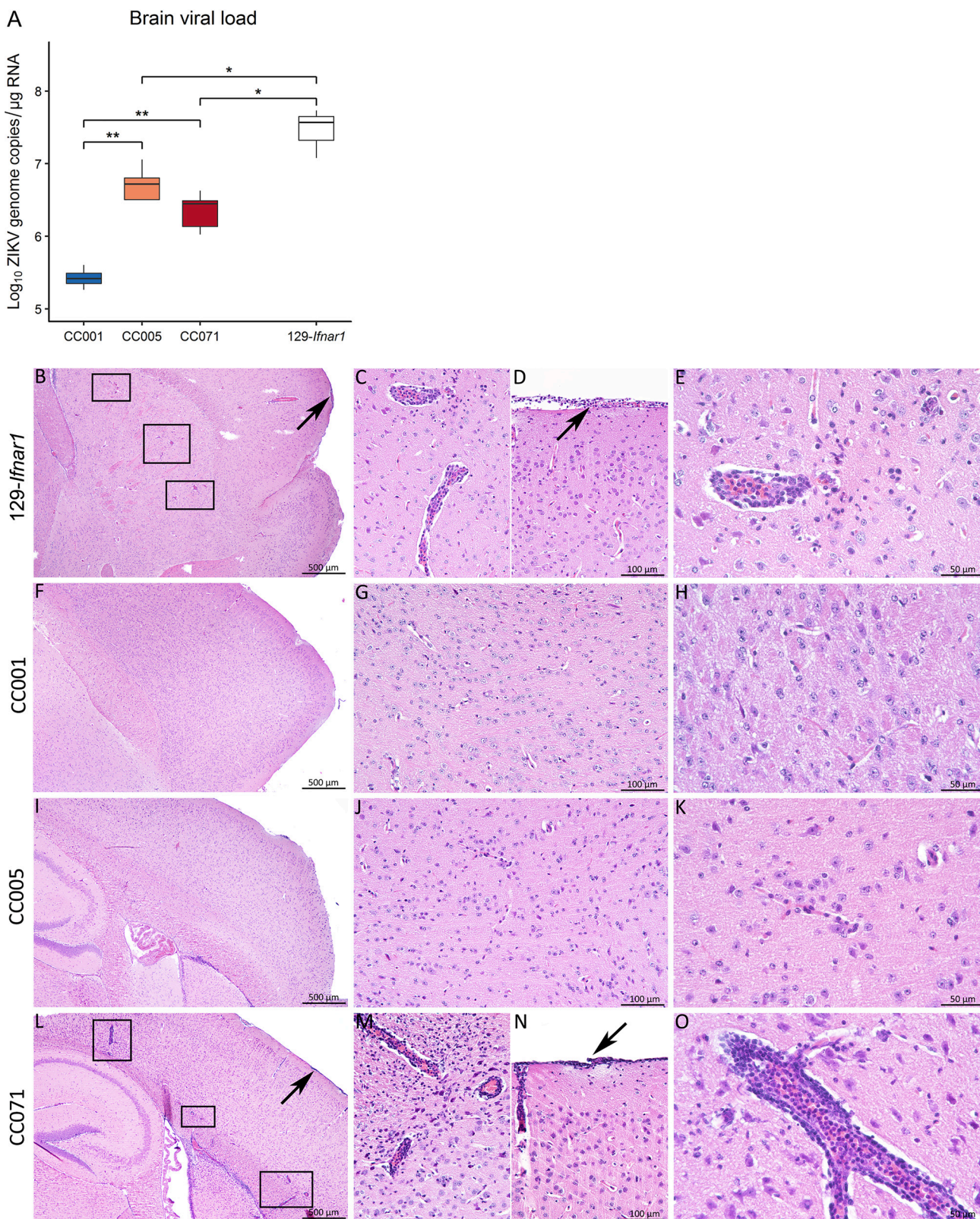


FIG 8 Intracranial ZIKV FG15 infection results in a strain-dependent viral load and brain histological lesions. Groups of 5- to 6-week-old mice of the 129-*Ifnar1* strain and three selected CC strains (3 to 5 mice per strain) were infected i.c. with 10^5 FFUs of ZIKV FG15 in the absence of prior anti-IFNAR treatment. (A) Brain viral load measured by RT-qPCR at day 6 p.i. *P* values were determined by the Wilcoxon rank-sum test. *, $P < 0.05$; **, $P < 0.01$. (B to O) Representative HE-stained brain sections at three different magnifications. (B to E) 129-*Ifnar1* mice ($n = 4$). Black rectangles, encephalitis with perivascular lymphocyte cuffs; arrows, leptomeningitis. (F to H) CC001 mice ($n = 5$). (I to K) CC005 mice ($n = 4$). (L to O) CC071 mice ($n = 5$). Black rectangles, encephalitis with perivascular lymphocyte cuffs; arrows, leptomeningitis.

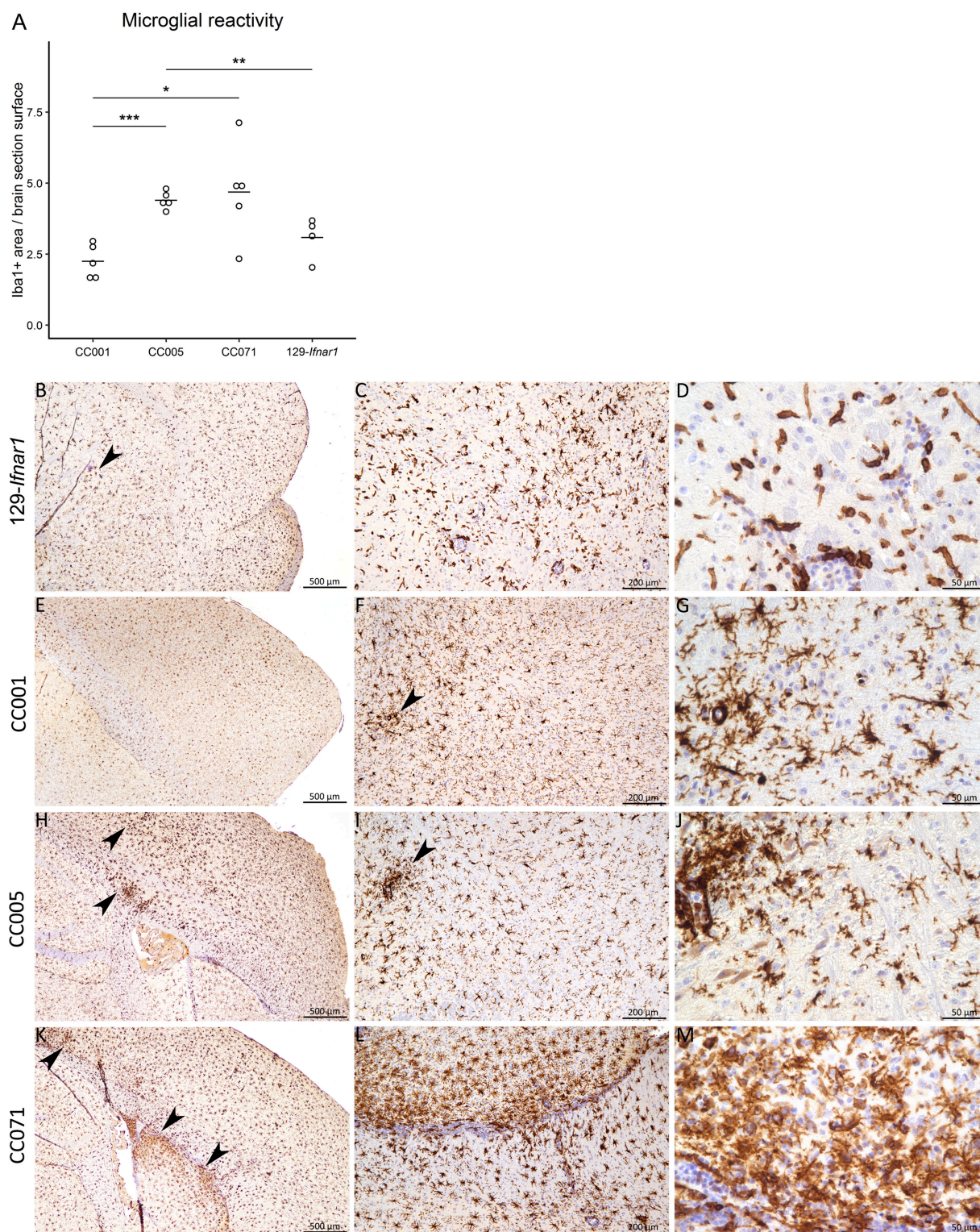


FIG 9 Intracranial ZIKV FG15 infection results in strain-dependent neuroinflammation of the brain. Microglial reactivity was assessed on brain sections from the same mice described in the legend to Fig. 8 by anti-Iba1 immunohistochemistry. (A) Quantification of the Iba1 labeling signal on the brain sections. *P* values were determined by *t* tests. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. (B to M) Representative anti-Iba1 immunohistochemistry of brain sections at three different magnifications. (B to D) 129-Irfar1 mice (*n* = 4); (E to G) CC001 mice (*n* = 5); (H to J) CC005 mice (*n* = 4); (K to M) CC071 mice (*n* = 5). Arrowheads, nodules of activated microglial cells.

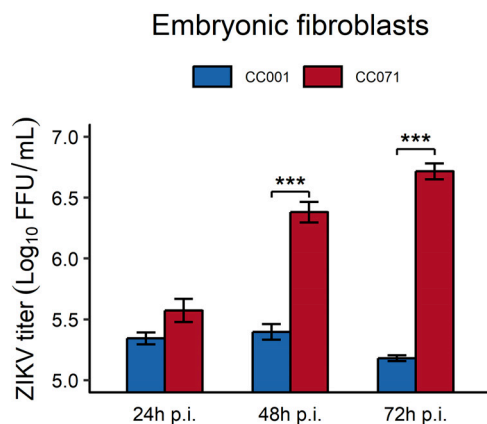


FIG 10 Enhanced ZIKV replication in CC071 MEFs compared with CC001 MEFs. MEFs derived from CC001 and CC071 mouse embryos were infected with ZIKV FG15 at an MOI of 5. The ZIKV titer in the supernatant was quantified by a focus-forming assay at 24, 48, and 72 h p.i. The data represent the mean \pm SEM from 3 biological replicates. The results were replicated in 3 independent experiments (t tests, ***, $P < 0.001$).

factors, and multiple environmental parameters. Also, genetic analyses are complicated by the randomness of virus infections and heterogeneity in clinical assessment.

Several mouse models of human ZIKV infection have already been described and have substantially improved our understanding of viral tropism, dissemination, pathogenesis, persistence, transmission, and vaccine protection. To overcome the inability of ZIKV to inhibit IFN induction and signaling pathways in mice, as was observed in humans (27), most studies have been performed using *Ifnar1*-deficient mice, which have become a reference model. However, high levels of viral replication can also be achieved by temporary inhibition of IFN signaling by anti-IFNAR MAb treatment (30, 31, 63) or even in immunocompetent mice by infecting neonates (36, 64–66) or using a combination of mouse-adapted ZIKV strains and human STAT2-knock-in mice (67).

The choice of ZIKV strain used in an animal model is important to maximize the relevance of mouse studies to human infection. Previous mouse studies have used different ZIKV strains from the African or Asian lineage. Mouse-adapted strains of the African lineage derived from a large number of serial passages are more pathogenic in mice at lower doses than strains of the Asian lineage (34) but carry mutations that may bias the translatability of the results to humans. To avoid this limitation, mouse studies have often used different ZIKV strains derived from clinical isolates of the Asian lineage. Genetic differences between these two lineages are suspected to be responsible for the emergence of symptomatic cases in humans starting with the Yap Island epidemics in 2007 (7, 8). Therefore, while the ZIKV strain needs to be standardized in experimental studies, for generalization of the results obtained with one viral strain, those results must be confirmed using another viral strain. Because of the incidence of neurological complications associated with infections caused by strains of the Asian lineage of ZIKV, we used for our genetic screening a low-passage strain derived from a 2015 case in French Guiana, at an early stage of the South American epidemics. Since this strain had not been adapted to the mouse, high doses were required to achieve high circulating viral loads.

Most mouse studies have used either B6-*Ifnar1* or 129-*Ifnar1* strains without a specific rationale, and their results cannot be directly compared due to many experimental differences, such as the ZIKV strain, dose, and route of inoculation (35). Under strictly identical conditions, we found that B6-*Ifnar1* mice developed more rapid and severe clinical symptoms and higher mortality than 129-*Ifnar1* mice, despite similar levels of plasma viral RNA at day 2 p.i. We also found that the viral load persisted longer in B6-*Ifnar1* mice. These results show that, under our experimental conditions, these two *Ifnar1*-deficient strains have clearly distinct susceptibilities to ZIKV. To our knowledge,

these two strains have been compared in only one study, which found no difference in survival after WNV infection (68). However, their extreme susceptibility might have prevented the identification of any difference. Our results have practical implications for many studies based on *Ifnar1*-deficient mice and motivate further genetic studies to identify the determinants and mechanisms controlling differences in susceptibility between B6 and 129 mouse inbred backgrounds.

To further investigate the role of host natural genetic variants on ZIKV susceptibility, we leveraged the genetic diversity across CC strains. The CC has been developed as a collection of inbred strains that more accurately reproduce the genetic diversity and phenotypic range seen in the human population (69). To enable systemic ZIKV replication after parenteral inoculation in mice with diverse genetic backgrounds, we blocked the type I IFN response using the MAR1-5A3 MAb (23, 31).

Several lines of evidence demonstrate that the MAb treatment was effective in all CC strains. First, we showed a similar abrogation of IFN- α -induced STAT1 phosphorylation in MEFs from B6 mice and two CC strains carrying the most divergent *Ifnar1* haplotypes (Fig. 2A). Moreover, the differences in the peak plasma viral load across 35 CC strains were not associated with the *Ifnar1* allele that each CC mouse strain received from the founder strains. Second, the peak viral load in all MAb-treated CC mice was at least 2.5 logs higher than that in untreated CC071 mice (Fig. 2B and 3B). Finally, we showed, in two CC strains, that increasing the dose of MAb to 4 mg did not modify the plasma viral loads at days 2 and 6 p.i. (Fig. 2F), indicating that the 2-mg dose was not limiting. This is consistent with the 5.2-day half-life of the MAR1-5A3 antibody previously reported (30). These results validate that the MAR1-5A3 MAb is effective across a broad range of mouse genetic backgrounds, which will be useful for the development of new models of viral infections.

A single injection of the MAR1-5A3 MAb at 24 h before ZIKV infection resulted in moderate to very high levels of viral RNA in the blood and brain. ZIKV infection was symptomatic in a minority of CC strains (3/35), as has been observed in infected humans (9, 70), and mortality was observed only in CC071 mice. These results confirm that ZIKV can replicate and establish viremia without inducing symptoms (29). Moreover, while all symptomatic strains had high peak viral loads, other strains with similarly high viral loads (like the CC005 or CC061 strains) never developed any signs of acute illness, indicating that other pathogenic mechanisms are required to result in symptomatic infection and that the viral load alone does not reliably predict the clinical outcome of ZIKV infection in a genetically diverse mouse population.

Since all experimental parameters were carefully standardized between strains (in particular, the microbiological environment in which they were bred), which resulted in small intrastrain variations, and since the MAR1-5A3 MAb treatment was effective across all CC strains, differences in peak viral loads between strains can be confidently attributed to host genetic variants. The 86% broad-sense heritability further indicates that the genetic background is the principal factor driving the peak viral load across CC strains.

Viremia decreased between days 2 and 6 p.i., as previously reported in several studies (56, 71, 72) but not in others (29, 57), for reasons that have not been discussed and that remain unclear. In our study, the rate of decrease, which was estimated as the difference of the \log_{10} plasma viral load between day 2 and day 6 p.i., showed remarkable homogeneity between individuals of the same CC strain and very large variations across CC strains. Injecting additional doses of MAb during the course of infection did not modify the kinetics of the viral load (Fig. 2F), ruling out the potential role of differential rates of antibody turnover. These results demonstrate a strong influence of host genes on the rate of ZIKV clearance from the bloodstream. The decrease in the circulating viral load is the net result of ZIKV production in infected tissues, dissemination to the bloodstream, and elimination from the circulation. Therefore, host genes could control the kinetics of the viral load through multiple mechanisms.

After exploring the range of susceptibility to ZIKV across a broad range of genetic

diversity, we focused our study on a few CC strains exhibiting contrasting phenotypes with the aim of characterizing new models (54). The CC001 strain was one of the strains least permissive to ZIKV, with a low peak viral load. At the other extreme of the distribution, the CC005 and CC071 strains had similarly high plasma viral loads, while only the CC071 strain showed symptoms and high mortality. These differences between CC strains were strikingly conserved with the African, mouse-adapted, HD78788 strain (Fig. 4A). The use of lower infectious doses with the HD78788 virus was supported by its higher pathogenicity resulting from mouse adaptation. The consistency between these two experiments suggests that the large phenotypic diversity that we have reported should apply to most ZIKV strains.

Overall, the brain viral load and brain pathology after i.p. infection were consistent with the peak plasma viral load. In CC mice, the most notable microscopic lesions included signs of neuroinflammation, evidenced by Iba1 immunohistochemistry. Neuroinflammation was similar in 129-*Ifnar1*, CC005, and CC071 mice. These changes were less pronounced than those in a previous study, which reported more severe central nervous system lesions in MAR1-5A3-treated B6 mice (31) infected with a more virulent African lineage ZIKV strain. The variable severity of the lesions observed in CC mice, ranging from very mild abnormalities in CC001 mice to inflammatory lesions with perivascular cuffing, activation of microglial cells, and microglial nodules in CC005 mice, indicates that the genetic background also controls ZIKV neuropathogenesis.

The less severe histological lesions observed in CC mice than in 129-*Ifnar1* mice could be due to the limited access to the brain of the virus or of the MAb, which does not appreciably cross the blood-brain barrier (29). Therefore, intracerebral infection aimed at comparing brain lesions between strains while controlling for the amount of virus effectively delivered. Surprisingly, CC001 and CC005 mice showed similar types and severities of lesions (although not all CC001 mice showed lesions), while CC071 mice developed much more severe signs of leptomeningoencephalitis with massive neuroinflammation, similar to those seen in 129-*Ifnar1* mice. This last result suggests that the milder lesions observed in CC071 mice than in 129-*Ifnar1* mice after i.p. infection were likely due to reduced viral dissemination to the brain. Importantly, mice did not receive prior MAb treatment, allowing for the development of local and systemic antiviral responses. These results emphasize the complex interplay between infected cells and effectors of the immune response, which likely differs between CC strains under the control of host genes.

The viral replication rate was investigated in resistant CC001 mice and highly susceptible CC071 mice, as it is a plausible mechanism for the differences in susceptibility between these two strains. MEFs are a semipermanent source of cells which have been extensively used to assess viral replication (73, 74), including with ZIKV (75, 76). In CC001 MEFs, the production of infectious viral particles was stable between 24 and 48 h and decreased between 48 and 72 h, while it steadily increased over time in CC071 MEFs, leading to significantly higher viral titers starting at 48 h. Our data are consistent with the observation by Caires-Junior et al., who reported an increased ZIKV replication rate in induced pluripotent stem cell-derived neuroprogenitor cells from CZS-affected babies compared with their unaffected dizygotic twins (13). Therefore, our results strongly suggest that the increased replication rate in CC071 mice compared with CC001 mice likely contributes to their higher plasma and brain viral loads and to their higher overall susceptibility to ZIKV.

Investigating the genetic diversity of a large number of CC strains has significantly extended the range of phenotypes induced by ZIKV infection in mice, and these mice better model the heterogeneity of the human population. It has allowed the testing of important factors, such as mouse gender and the method of viral load measurement across multiple host genetic backgrounds, providing robust conclusions (37). Importantly, we found no differences between male and female mice in susceptibility to ZIKV disease or in the peak viral load (Fig. 2E). We also found a high correlation between the viral loads measured by titration and by qRT-PCR over a 2- \log_{10} range (Fig. 2D). This is in contrast to the findings of a study of Ebola virus, which showed that, in spleen and

TABLE 3 Summary of main features of ZIKV infection in MAb-treated CC strains and 129-*Ifnar1* mice^a

Mouse strain	Symptoms	Mortality (%)	Peak plasma viral load	Rate of decrease of plasma viral load	Systemic infection		Intracerebral infection		Viral replication <i>in vitro</i> (24–72 h)
					Brain viral load	Brain pathology	Brain viral load	Brain pathology	
CC001	—	0	+	Moderate	+	—	+	++	Decreasing
CC005	—	0	+++	Fast	+++	+++	+++	+++	ND
CC071	+++	78	+++	Moderate	+++	++	+++	++++	Increasing
129- <i>Ifnar1</i>	++	12.50	+++	Fast	+++	+++	+++	++++	ND

^a—, absent; +, ++, +++, and +++++, present at increasing levels; ND, not done.

liver, the susceptible mice produced similar amounts of viral genomes but 1 to 2 log₁₀ more infectious virions than the resistant mice (43).

Genetic diversity also allowed us to assess the correlations between traits, which cannot be achieved with a single strain. We showed that the brain viral load at day 6 after i.p. infection was consistent with the plasma viral load at day 2 p.i. but that the plasma viral loads at days 2 and 6 p.i. were only moderately correlated. Likewise, we found that clinical severity did not correlate with the intensity of brain histological lesions and neuroinflammation, as summarized in Table 3. These dissociations between phenotypes provide evidence for partly distinct mechanisms and genetic control (37) and lead to distinct mouse models.

A recent study has reported strain-dependent variations in the long-term neuropathological and behavioral consequences of ZIKV infection after neonatal infection between four mouse inbred strains known to differ in their susceptibility to pathogens (36). Since they are all laboratory strains, they do not cover the same genetic variation used in our study, and it is likely that even more diverse phenotypes would be observed in this model with the CC panel.

Genetic analysis of our results strongly suggests that, in contrast to other viruses for which major host genetic determinants have been identified (e.g., *Oas1b* for WNV [62] or *Mx1* for influenza virus [77]), susceptibility to ZIKV in CC strains is under polygenic control. This is supported by the continuous distributions, across the CC strains, of the values of the peak plasma viral load (Fig. 3B) and of the rate of viral decrease (Fig. 3C) and by the absence of any regions of the genome significantly associated with variations in viral loads (Fig. 5). Calculations based on the CC genotypes show that, with 35 strains and an average of 5 mice per strain, we had an 80% power to detect a biallelic quantitative trait locus (QTL) explaining 30% or more of the phenotypic variance (78). This clearly rules out the possibility that the phenotypic variations measured across CC strains were controlled by one or a few genes with major effects, as observed with *Oas1b* for WNV (47). Dissecting the genetic architecture of resistance and susceptibility to ZIKV in these strains will require combining complementary strategies. Intercrosses between contrasted strains, such as strains CC001 and CC071, will reduce the genetic complexity and may result in an increased power for QTL mapping due to the larger sample size (54). However, the mapping resolution achieved in an F2 hybrid or a backcross leads to large genetic intervals. Other approaches, such as gene expression studies on various cell types (such as immune cells, neurons, or glial cells) will be used to identify differentially activated host response pathways and reduce the number of candidate genes in these intervals.

Oas1b is an interferon-stimulated gene and a major determinant of mouse susceptibility to WNV (45). A variant in *OAS3*, a member of the gene family in humans homologous to *Oas1b*, has been associated with an increased severity of dengue (79). Most laboratory strains, including five of the eight CC founders, carry the same nonfunctional allele of *Oas1b*, which renders them susceptible to WNV infection (62), while the three wild-derived CC founders carry polymorphic but functional alleles and are resistant (45). CC strains therefore carry either functional or nonfunctional *Oas1b* alleles. Our results provide multiple lines of evidence to rule out the possibility of a significant role of *Oas1b* in the variations in susceptibility to ZIKV across CC strains. First,

since MAb-mediated blockade of the type I IFN response likely temporarily inhibits *Oas1b* induction, the *Oas1b* allele is unlikely to explain the differences in the peak viral load at day 2 p.i. Moreover, our QTL mapping analysis showed that the mouse genotype at *Oas1b* (located on distal chromosome 5) did not significantly contribute to variations in the viral load at day 2 or day 6 p.i. or in the rate of viral load decrease (Fig. 5). Finally, since the CC001, CC005, and CC071 strains carry the *Oas1b* truncated allele (Table 1), the differences in clinical severity, brain pathology, and replication rate in infected cells among these strains must be controlled by other genetic variants. Interestingly, because of the large difference in the survival times after WNV infection between CC071 mice and CC001 or CC005 mice, these strains provide ideal strain combinations to identify novel genes controlling susceptibility to this virus.

Out of this large series of CC strains, we identified several new mouse models of ZIKV disease. CC071 mice were the most susceptible to ZIKV infection and were more susceptible than MAb-treated B6 mice (Fig. 3A and B). MAb treatment was required to achieve a high circulating viral load (Fig. 2B), showing that CC071 mice have a functional type I IFN response. CC071 mice were also very susceptible to DENV and WNV, two flaviviruses related to ZIKV. However, they are not uniformly susceptible to infectious agents, since they showed susceptibility to RVFV similar to that of BALB/c, CC001, and CC005 mice and intermediate susceptibility to *Salmonella enterica* serovar Typhimurium (52). Together with other susceptible strains, like CC021 and CC026, which developed symptoms, or CC005, which developed severe brain lesions, CC071 mice will help identify the mechanisms of severe ZIKV infection and their genetic control. In contrast, CC001 mice were highly resistant, even to a strongly pathogenic African ZIKV strain, despite a blockade of type I IFN signaling. Extensive analysis of these CC strains with extreme phenotypes may elucidate how genetic variants affect susceptibility as well as innate and adaptive immune responses to flaviviral infection (54) and provide a deeper understanding of the pathophysiology of severe complications of human ZIKV disease.

MATERIALS AND METHODS

Mice. All Collaborative Cross (CC) mice (purchased from the Systems Genetics Core Facility, University of North Carolina, and bred at the Institut Pasteur) (80), C57BL/6J mice (purchased from Charles River Laboratories France), and BALB/cByJ and *Ifnar1*-knockout mice (mice with the *Ifnar1*^{tm1Agt} allele on the 129S2/SvPas or C57BL/6J background, designated 129-*Ifnar1* and B6-*Ifnar1*, respectively, and bred at the Institut Pasteur) were maintained under specific-pathogen-free conditions with a 14-h light and 10-h dark cycle and *ad libitum* food and water in the Institut Pasteur animal facility. All CC strains bred at the Institut Pasteur were included in this study. In all experiments, mice were killed by cervical dislocation. All experimental protocols were approved by the Institut Pasteur Ethics Committee (projects 2013-0071, 2014-0070, 2016-0013, 2016-0018, and dap190107) and authorized by the French Ministry of Research (decisions 00762.02, 7822, 6463, 6466, and 19469, respectively), in compliance with French and European regulations.

Cell lines. Vero cells (ATCC CRL-1586) were cultured at 37°C in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Eurobio). C6/36 cells (ATCC CRL-1660) were cultured at 28°C in Leibovitz medium (L-15 medium; Gibco) supplemented with 10% FBS, 1% nonessential amino acids (Life Technologies), and 1% tryptose phosphate broth (Life Technologies).

Viruses. The FG15 Asian Zika virus (ZIKV) strain, isolated from a patient during a ZIKV outbreak in French Guiana in December 2015, was obtained from the Virology Laboratory of the Institut Pasteur of French Guiana. The HD78788 African ZIKV strain, isolated from a human case in Senegal in 1991, was obtained from the Institut Pasteur collection. The KDH0026A DENV serotype 1 (DENV-1) strain, isolated from a patient in Thailand in 2010, was previously described (81). Viral stocks were prepared from the supernatant of infected C6/36 cells, clarified by centrifugation at 800 × *g*, and titrated on Vero cells by a focus-forming assay (FFA). Stocks were kept at −80°C. West Nile virus (WNV) strain IS-98-ST1 (or Stork/98) was obtained, cultured, and used as described by Mashimo et al. (62). Rift Valley fever virus (RVFV) strain ZH548 was obtained, cultured, and used as described by Tokuda et al. (82).

Mouse experiments. All infection experiments were performed in a biosafety level 3 animal facility. The mice were maintained in isolators.

(i) ZIKV and DENV systemic infection. CC mice received 2 mg of an IFNAR-blocking mouse MAb (MAb MAR1-5A3; BioXCell) by intraperitoneal (i.p.) injection 1 day before ZIKV or DENV infection (83). Groups of 6- to 8-week-old mice were inoculated i.p. with 10⁷ focus-forming units (FFUs) of ZIKV FG15 or 10³ FFUs of ZIKV HD78788 in 200 μl phosphate-buffered saline (PBS). For DENV infection, mice were anesthetized by i.p. injection with a solution of xylazine (5 mg/kg of body weight) and ketamine (80 mg/kg) and afterwards were inoculated by intravenous (i.v.) injection in the retro-orbital sinus with 2 × 10⁶ FFUs of DENV-1 KDH0026A in 100 μl PBS. Survival and clinical signs were monitored daily for

up to 14 days. Clinical signs were scored as follows: 0, no symptoms; 1, ruffled fur; 2, emaciation, hunched posture, and/or hypoactivity; 3, hind limb weakness, prostration, and/or closed eyes; and 4, moribund or dead. Blood samples were collected at several time points from the retromandibular vein for plasma viral load assessment.

(ii) ZIKV intracerebral infection. Mice were anesthetized by i.p. injection with a solution of xylazine (5 mg/kg), ketamine (75 mg/kg), and buprenorphine (0.03 mg/kg). Groups of 5- to 6-week-old mice were then inoculated by intracerebral (i.c.) injection in the right brain hemisphere with a 26-gauge needle affixed to a Hamilton syringe sheathed by a wire guard allowing no more than a 4-mm penetrance into the skull cavity, as described previously (84). Mice received either 10^5 FFUs of ZIKV FG15 in PBS or PBS alone in a volume of 10 μ l. Survival and clinical signs were monitored daily for 6 days, and the mice were euthanized for brain collection. Another cohort of mice ($n = 7$ to 8 per strain) was similarly infected and monitored daily for 21 days to assess symptoms and survival.

(iii) WNV and RVFV infection. Groups of 8- to 12-week-old mice were inoculated i.p. with 10^3 FFUs of WNV strain IS-98-ST1 or 10^2 PFU of RVFV strain ZH548. Survival and clinical signs were monitored daily for up to 14 days (RVFV) or 21 days (WNV).

MEF isolation and infection. Mouse embryonic fibroblasts (MEFs) were isolated from individual fetuses from one or more genetically identical females at day 13.5 to 14.5 of gestation and cultured in DMEM supplemented with 10% FBS (Eurobio) and 1% penicillin-streptomycin (Gibco) at 37°C. MEFs were used until passage 2.

MEFs were plated at identical densities in culture dishes 24 h before infection. MEFs were infected with the ZIKV FG15 strain at an MOI of 5. After 2 h of incubation at 37°C, the inoculum was replaced with fresh medium. Supernatants were collected at 24, 48, and 72 h p.i. Titration was performed by FFA in Vero cells.

Focus-forming assay. Vero cells were seeded at 3×10^4 per well in 100 μ l complete medium (DMEM, 10% FBS) in 96-well plates. After overnight incubation at 37°C, the medium was replaced with 40 μ l of serial 10-fold dilutions of the samples, and 115 μ l of methylcellulose overlay was added 2 h later. After 40 h of incubation, the culture medium was removed and cells were fixed with 100 μ l/well of 4% paraformaldehyde for 20 min and permeabilized with a solution of 0.3% Triton X-100 and 5% FBS in PBS for 20 min. The cells were washed and incubated with a mouse MAb directed against ZIKV envelop protein (MAb 4G2, purified from the ATCC hybridoma) for 1 h at 37°C (1/250 in blocking buffer). The cells were further washed, incubated with a secondary antibody (Alexa Fluor 488-conjugated anti-mouse IgG; Invitrogen) for 45 min at 37°C, and washed. Infected cell foci were counted using an ImmunoSpot CTL analyzer, and viral titers were calculated from the average number of foci.

Viral genome quantification by RT-qPCR. Blood samples were centrifuged to recover plasma from which viral RNA was extracted with a QIAamp viral RNA minikit (Qiagen). Brain tissue samples were homogenized at 4°C in 1 ml of the TRIzol reagent (Life Technologies), using ceramic beads and an automated homogenizer (PreCellys). Total RNA was extracted according to the manufacturer's instructions. cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase (Life Technologies) in a Bio-Rad MyCycler thermocycler. ZIKV and DENV cDNAs were quantified by a TaqMan quantitative PCR (qPCR) in a Viia7 instrument (Life Technologies), using standard cycling conditions. The following primer sets adapted from previous works (85–87) were used to detect ZIKV and DENV RNA: for ZIKV FG15, forward primer 5'-CCG CTG CCC AAC ACA AG-3' and reverse primer 5'-CCA CTA ACG TTC TTT TGC AGA CAT-3' with the probe 5'-6FAM-AGC CTA CCT TGA CAA GCA ATC AGA CAC TCA A-MGB-3' (Life Technologies) (where 6FAM is 6-carboxyfluorescein); for ZIKV HD78788, forward primer 5'-AAA TAC ACA TAC CAA AAC AAA GTG GT-3' and reverse primer 5'-TCC ACT CCC TCT CTG GTC TTG-3' with the probe 5'-6FAM-CTC AGA CCA GCT GAA G-MGB-3' (Life Technologies); and for DENV-1 KDH0026A, forward primer 5'-GGA AGG AGA AGG ACT CCA CA-3' and reverse primer 5'-ATC CTT GTA TCC CAT CCG GCT-3' with the probe 5'-6FAM CTC AGA GAC ATA TCA AAG ATT CCA GGG-MGB-3' (Life Technologies). The viral load is expressed on a log₁₀ scale as the number of viral genome copies per milliliter (plasma samples) or per number of total RNA micrograms (brain samples) after comparison with a standard curve produced using serial 10-fold dilutions of a plasmid containing the corresponding fragment of ZIKV genome.

Western blot analysis. MEFs (5×10^6) were preincubated with 100 μ g IFNAR1-blocking antibody (MAb MAR1-5A3; BioXCell) for 7 h and then stimulated or not with 300 IU/ml mouse IFN- α (Miltenyi Biotec) for 15 min. The MEFs were detached and centrifuged at $300 \times g$ for 5 min, and the cell pellet was resuspended in cold PBS. The MEFs were then lysed into extraction buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 10% glycerol, 30 mM NaP, 50 mM sodium fluoride), containing protease inhibitor (cOmplete, EDTA free; Roche) and phosphatase inhibitors (phosStop Easy Pack; Roche) in which lysis was completed with 2.5 IU of nuclease (Benzonase; Sigma). The lysates were incubated on ice for 30 min, and the insoluble fraction was separated by centrifugation. Protein concentrations were determined by the Bradford assay, and equal amounts of protein were further used. Protein denaturation was performed in Laemmli buffer at 95°C for 5 min. After separation on a 12% polyacrylamide gel (Bio-Rad), the proteins were transferred on an Immobilon-Polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and incubated overnight with the following antibodies: anti-phospho-STAT1 Tyr701 (1/1,000; catalog number 9167; Cell Signaling), anti-total STAT1 N terminus (1/500; catalog number 610115; BD Biosciences), and anti- α -tubulin (1/8,000; catalog number T5168; Merck). The membranes were incubated for 1.5 h at room temperature with an anti-mouse or an anti-rabbit IgG horseradish peroxidase-linked secondary antibody (1/10,000 MAb NA931 and NA934V; Amersham), and the signals were visualized using autoradiography.

Histopathology. After necropsy, the brain was removed, fixed for 48 to 72 h in 10% neutral buffered formalin, and embedded in paraffin; 4- μ m-thick sections were stained in hematoxylin-eosin. The morphology of the microglial cells was assessed by immunohistochemistry using rabbit anti-Iba1 primary antibody (1:50 dilution; catalog number 01919741; Wako Chemical) as previously described (88). Sections were analyzed by a trained veterinary pathologist in a study with coded slides carried out in a blind manner. Quantification of the microglia reaction was carried out using automated detection of the Iba1 labeling signal and measurement of the labeled area on a low-magnification ($\times 2$) picture of a brain section of each mouse, using ImageJ software (<https://imagej.nih.gov/ij/>).

Genetic analysis. Broad-sense heritability was calculated as previously described (61).

The plasma viral load at days 2 and 6 p.i. and the plasma viral load decrease, measured for 159 mice of the 35 CC strains (average, 4.5 mice per strain), were used in quantitative trait locus (QTL) mapping using the *rqtl2* R package (89) and the GigaMUGA genotypes of the CC founders and CC strains available from <http://csbio.unc.edu/CCstatus/CCGenomes/#genotypes>. A genome scan was performed using the *scan1* function with a linear mixed model using a kinship matrix. Statistical significance levels were calculated from 1,000 permutations.

Genotype-phenotype associations for specific genes (*Ifnar1*, *Oas1b*) were tested by the Kruskal-Wallis test using the founder haplotype as the genotype.

Statistical analysis. Statistical analyses were performed using R software (v3.5.2). Kaplan-Meier survival curves were compared by the log-rank test. Two-way ANOVA was used for testing mouse strain and sex effects on the plasma viral load at day 2 p.i. (Fig. 2E). Student's *t* test was used to compare the viral loads in tissues, except when data showed heterogeneous variance between groups, in which case we used Kruskal-Wallis and Wilcoxon nonparametric tests. These tests were also used for assessing the mouse strain effect on the plasma viral load and on the plasma viral load decrease (Fig. 3). Pearson's coefficient was used for determination of the correlation between the plasma viral load at days 2 and 6 p.i. (Fig. 3B) and the correlation between measurements of the plasma viral load determined by FFA and RT-qPCR (Fig. 2D). Student's *t* test was used to compare the viral titers between the strains in *in vitro* experiments. *P* values of <0.05 were considered statistically significant.

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5.2. Identification of loci modulating the susceptibility to ZIKV in *Ifnar1*-deficient mice

In the previous part, we investigated how genome-wide variants could impact susceptibility to ZIKV infection in mice. We described that the susceptibility of *Ifnar1* knockout mice is influenced by their genetic background and that the broad genetic diversity of CC mice, treated by anti-IFNAR antibody, expressed phenotypes ranging from complete resistance to high susceptibility. Genetic analyses did not lead to the identification of genetic loci controlling the phenotypic variations in CC mice but rather revealed a genetic control by multiple genes with small effects. Dissecting the genetic architecture of susceptibility to ZIKV may be more efficiently achieved using dedicated intercrosses between mouse strains with contrasted phenotypes in response to ZIKV infection.

As *Ifnar1*-deficient mouse strains are permissive to ZIKV but have differential susceptibility to the infection according to their genetic backgrounds, we decided to use intercrossed *Ifnar1*-deficient mice to identify potential modifier genes. Here, the genetic analysis of a F2 population between B6-*Ifnar1* and 129-*Ifnar1* identified two recessive loci controlling the peak plasma viral load and the time to death after ZIKV infection, respectively.

5.2.1. Susceptibility to ZIKV infection in B6129(*Ifnar1*)F2 mice

We previously showed that C57BL/6J-*Ifnar1*^{-/-} (B6-*Ifnar1*) mice develop more rapid and severe clinical symptoms and higher mortality than 129S2/SvPas-*Ifnar1*^{-/-} (129-*Ifnar1*) mice after ZIKV infection. We also found that viral load persisted longer in B6-*Ifnar1* mice. These results reveal that these two *Ifnar1*-deficient strains clearly differ in their susceptibility to ZIKV (Manet *et al.* 2019).

To identify the genetic determinants and mechanisms controlling the differences of susceptibility between B6 and 129 inbred backgrounds, we generated a F2 population between B6-*Ifnar1* and 129-*Ifnar1* mice. 192 F2 mice were infected i.p. with 10⁷ FFUs of ZIKV FG15 strain.

We evaluated the severity of symptoms after ZIKV infection (average clinical score, with numerical values given as follows: 0, no symptom; 1, ruffled fur; 2, emaciation, hunched posture and/or hypo activity; 3, hind limb weakness, prostration and/or closed eyes; and 4, moribund or dead) as well as mouse survival. We previously showed that, in the same experimental conditions, the mortality rate of B6-*Ifnar1* mice was 100% compared to only 12.5% in 129-*Ifnar1* mice (Manet *et al.* 2019). In the F2 progeny, the majority of the mice displayed mild and short-lasting symptoms including moderate body weight loss, ruffled fur and diminished activity, while about a quarter suffered from prolonged disease (Figure 29). The overall mortality rate in the progeny was 18.2% over a 14 days period after infection, with the majority of susceptible mice dying from infection between days 6 and 8 p.i. (Figure 29).

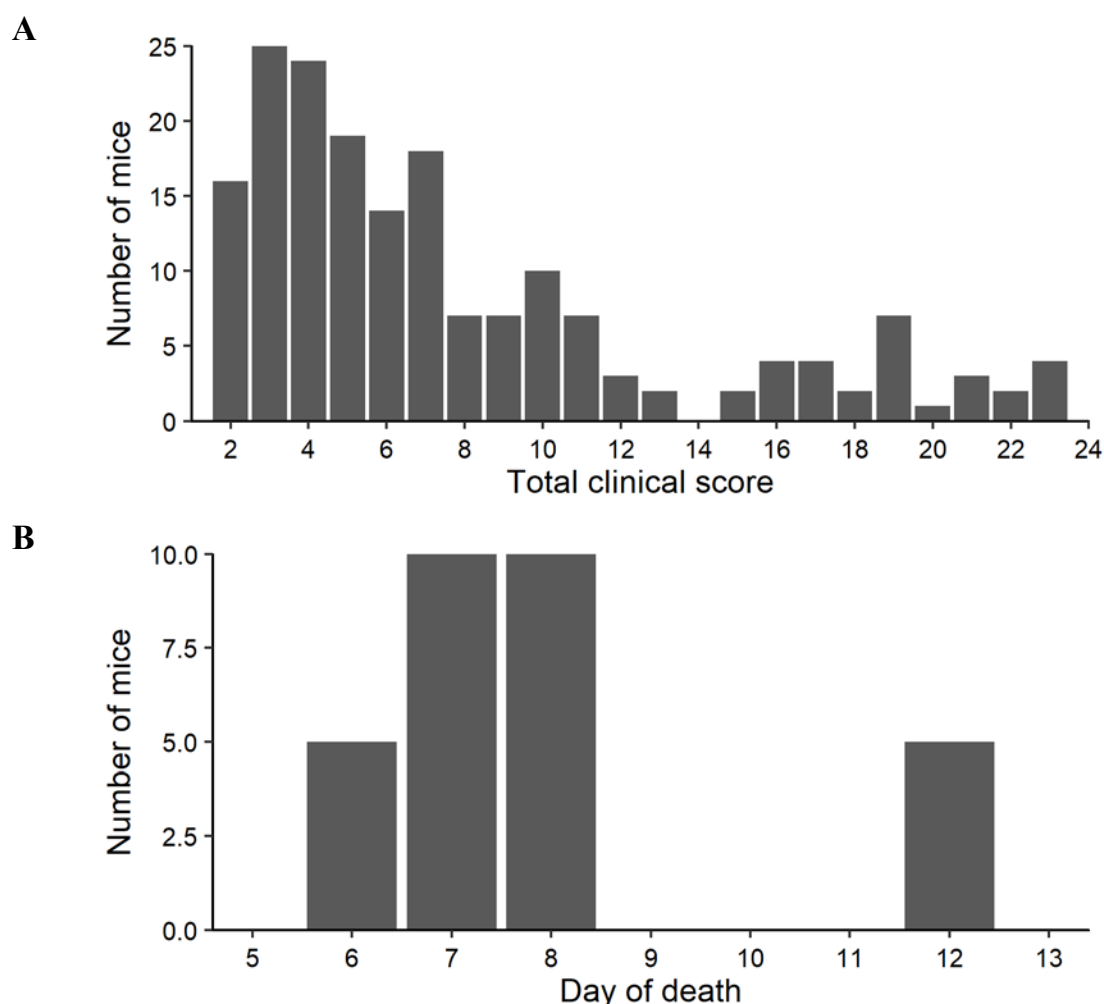


Figure 29. Distribution of the total clinical score and of the time of death in 192 B6129(*Ifnar1*)F2 mice.

Total clinical score was calculated as the sum of daily clinical scores between days 6 and 14 p.i. (clinical score with numerical values given as follows: 0, no symptom; 1, ruffled fur; 2, emaciation, hunched posture and/or hypo activity; 3, hind limb weakness, prostration and/or closed eyes; and 4, moribund or dead) (A), mouse survival was recorded over a 14 days period following infection, all surviving mice were euthanized at the end of the experiment (B).

We also measured the plasma viral load at days 2 and 6 p.i. F2 mice demonstrated large phenotypic variations, extending beyond the mean values of the parental strains, for both peak plasma viral load and decrease rate of plasma viral load (the difference of the \log_{10} plasma viral loads between days 2 and 6 p.i.) (Figure 30).

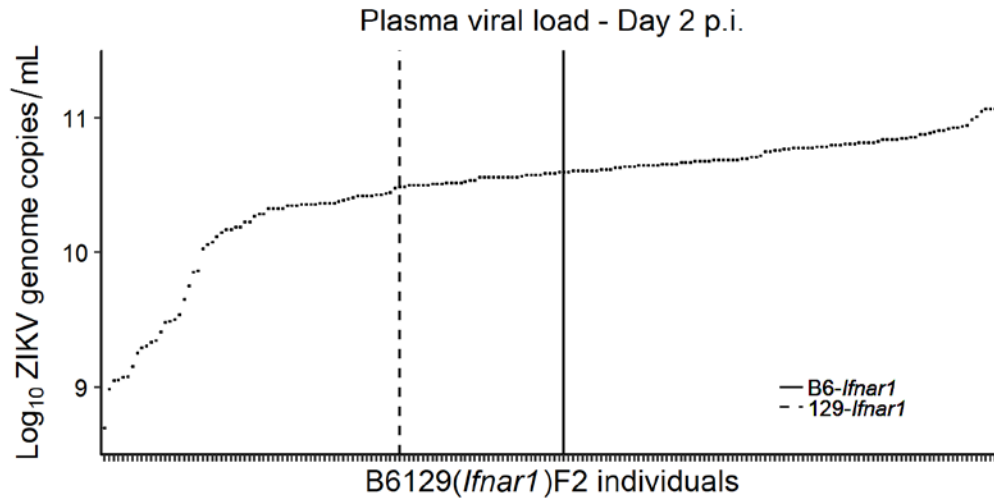
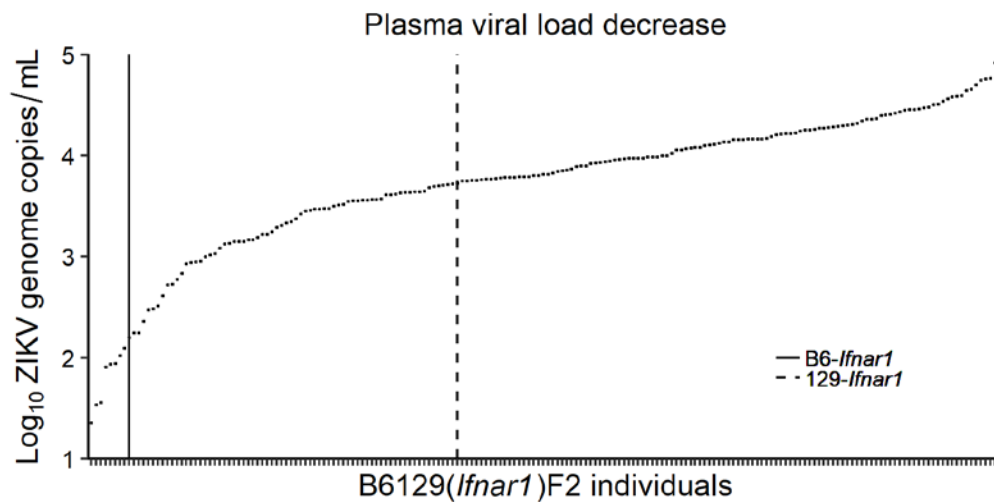
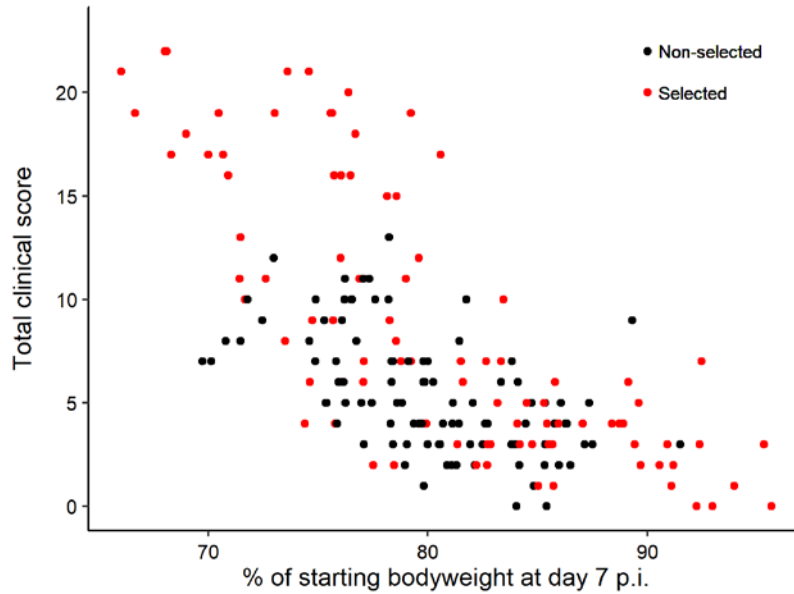
A**B**

Figure 30. Peak and rate of decrease of plasma Zika viral load in 192 B6129(*Irfnar1*)F2 mice. Peak plasma viral load measured at day 2 p.i. (A) and difference between plasma viral loads at days 2 and 6 p.i. (B), quantified by RT-qPCR. Data shown as dots with parental mean values as vertical lines.

Notably, we observed a strong correlation between the body weight loss at day 7 p.i. and the total clinical score, calculated as the sum of daily clinical scores between days 6 and 14 p.i. ($r^2 = -0.67$, $p = 1.3 \times 10^{-25}$); and between the peak and the rate of decrease of plasma viral loads ($r^2 = 0.66$, $p = 1.4 \times 10^{-25}$) (Figure 31). Based on these phenotypic distributions and correlations, we selected 94 out of 192 F2 mice for genome-wide genotyping and QTL analysis. This selection included all susceptible mice and mice with extreme values of peak plasma viral load, or decrease rate of plasma viral load. Selected F2 mice, depicted in red in Figure 31, thus displayed phenotypic values covering the maximal range for survival, clinical and virological parameters.

A



B

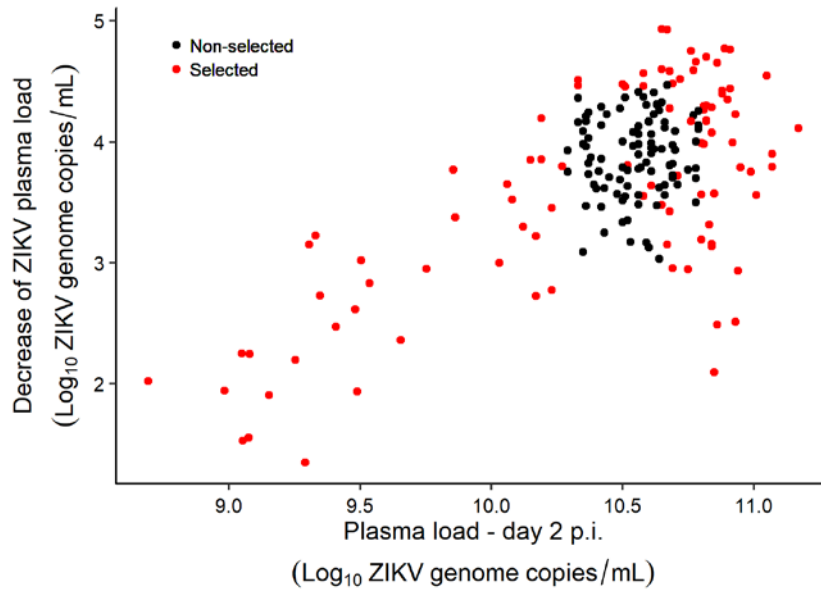


Figure 31. Correlations between clinical and virological phenotypic traits in the B6129(*Ifnar1*)F2 progeny.

Correlation plot between the total clinical score (sum of individual daily clinical scores) and the body weight loss at day 7 p.i. (shown as the percentage of starting body weight) (A) and between the peak plasma viral load at day 2 p.i. and the decrease rate of plasma viral load (B). F2 mice selected for genotyping are depicted as red dots.

5.2.2. Genetic mapping analysis reveals two significant QTLs controlling the susceptibility to ZIKV infection

To identify loci linked to the susceptibility to ZIKV infection, we performed a QTL mapping analysis in the 94 selected F2 mice. 2,762 informative markers were retained in the 9,914 SNPs of the MiniMUGA array and were evenly distributed across the chromosomes (Figure 32).

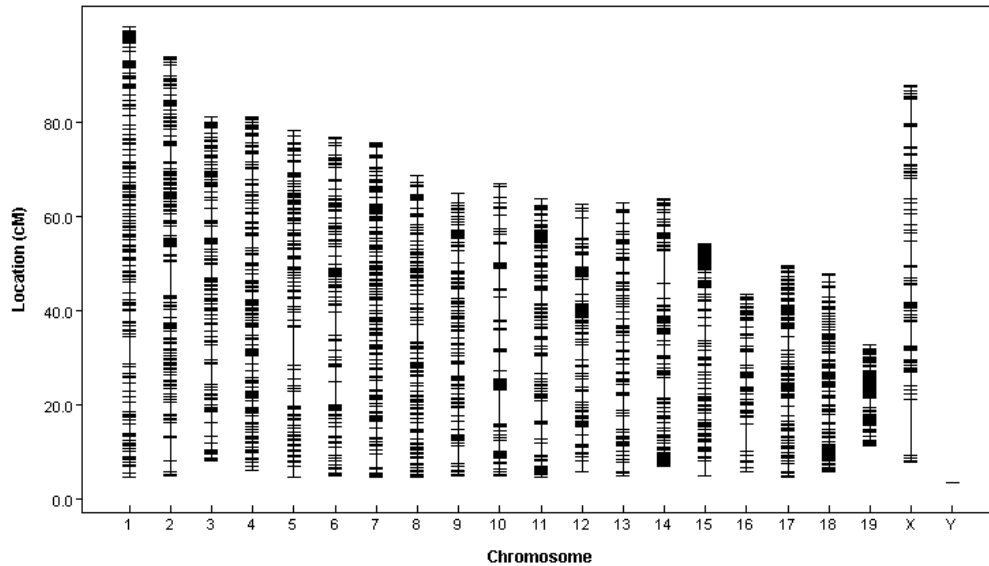


Figure 32. Marker density map.

Genetic location along the chromosomes (in cM) of informative markers used for QTL mapping in F2 mice.

Two QTLs were identified as significantly associated with the peak plasma viral load and with the time to death (Figure 33). For the peak plasma viral load at day 2 p.i., a QTL (LOD score = 4.33; $p < 0.05$) was found on chromosome 5 at position 84.66 Mb and explained 19.1% of the phenotypic variance. For the time to death phenotype, a QTL (LOD score = 3.69; $p < 0.05$) was identified on chromosome 12 at position 12.0 Mb. These loci were named ZIKV susceptibility locus-1 (*Zsl1*) and *Zsl2* respectively. QTL confidence intervals were calculated using a Bayesian credible interval method and are given in Table 7.

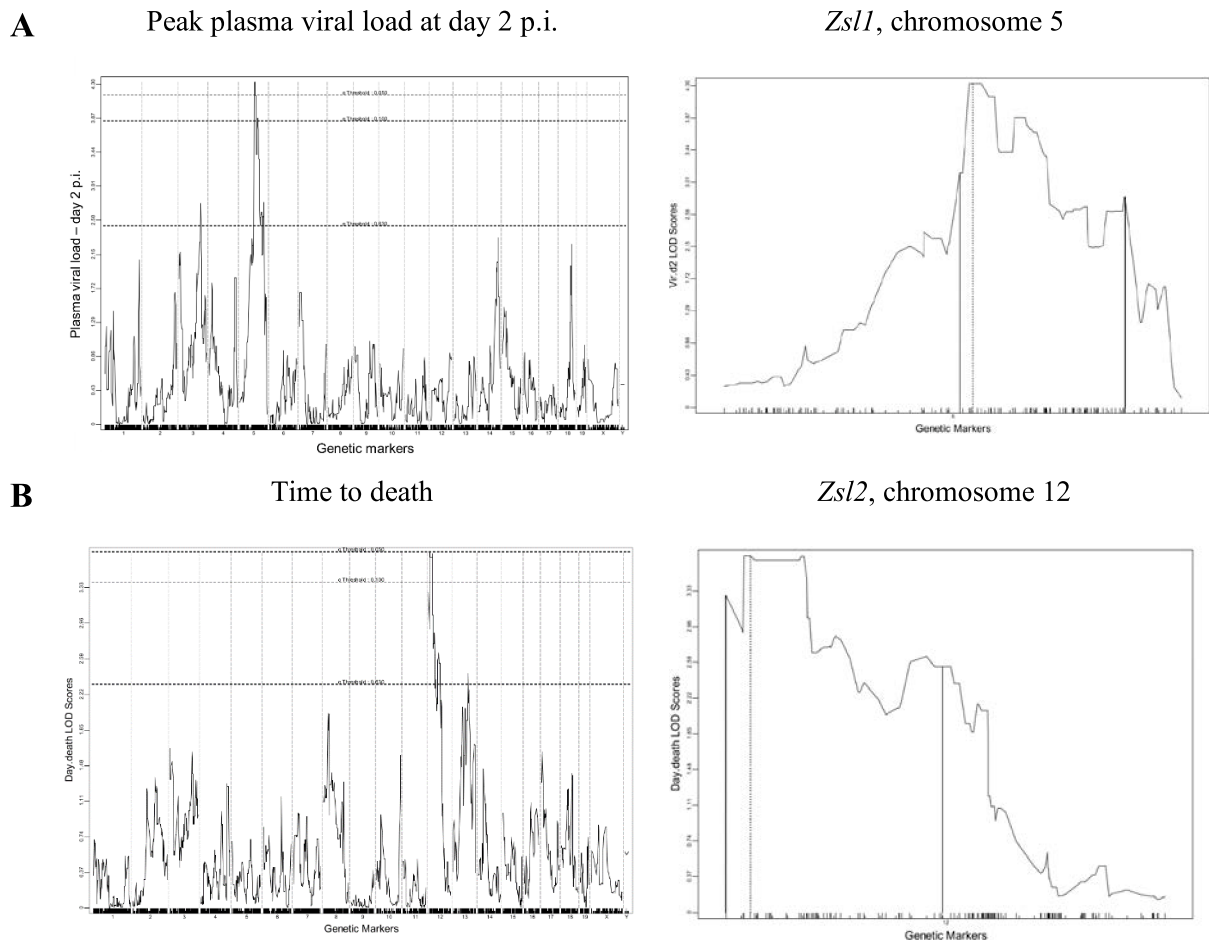


Figure 33. Significant QTLs controlling peak plasma viral load and time to death in 94 B6129(*Ifnar1*)F2 mice.

QTL mapping was performed on peak plasma viral load (A) and on time to death (B). Left panel : whole-genome scans. Right panel : genome scan of the chromosome containing the QTL. Horizontal dashed lines indicate genome-wide significance thresholds ($p = 0.63$, $p = 0.10$, $p = 0.05$) determined by permutation testing ($n=1000$). Vertical solid lines indicate 95% Bayesian confidence interval boundaries and the vertical dotted line indicates the peak of the QTLs.

QTL	Phenotype	Chr	Maximum LOD score	Peak position (Mb)	95% (Mb)	CI	PVE (%)	Number of genes in the interval
<i>Zs11</i>	Plasma viral load - day 2 p.i.	5	4.33	84.66	79.12-132.32		19.1	546
<i>Zs12</i>	Time to death	12	3.69	12.0	2.79-61.58		NA	230

Table 7. QTL summary.

Abbreviations : Chr, Chromosome ; cM, centimorgan ; CI, confidence interval; PVE, percentage of variance explained.

Two suggestive QTLs were identified as controlling the plasma viral load at day 6 p.i. and the mouse survival (Figure 34) on chromosomes 8 and 12 respectively. The QTL on chromosome 12 is largely overlapping between time to death and survival phenotypes, which could be expected.

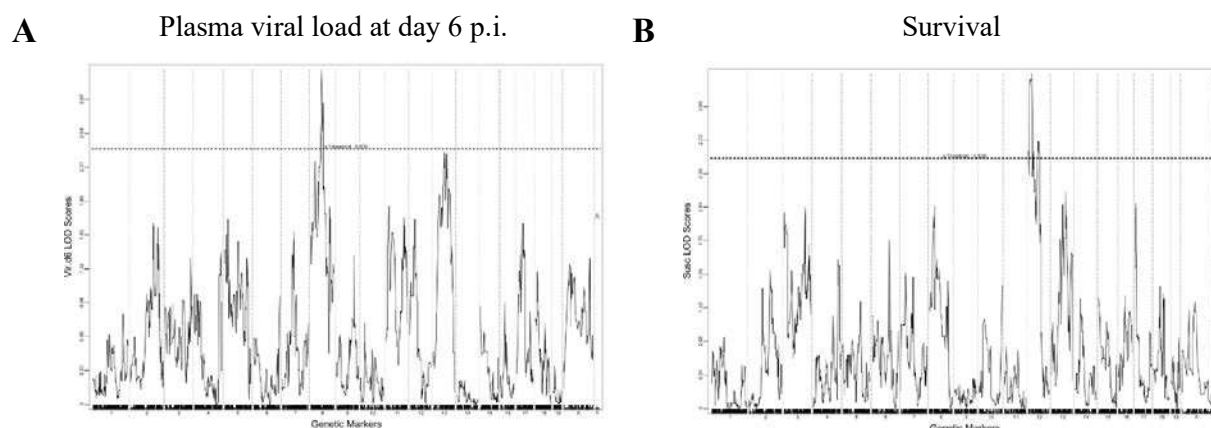


Figure 34. Suggestive QTLs controlling plasma viral load at day 6 p.i. and survival in 94 B6129(*Ifnar1*)F2 mice.

QTL mapping was performed on plasma viral load at day 6 p.i. (A) and on survival (B). Horizontal dashed line indicate genome-wide significance threshold ($p = 0.63$) determined by permutation testing ($n=1000$).

To determine the contribution of each allele to the QTLs, each locus effect was evaluated based on the nearest SNP from the QTL peak. Effect plots are presented in Figure 35, with A and B referring to B6 and 129 alleles, respectively. For *Zs11*, AA genotype was associated with a lower peak plasma viral load at day 2 p.i. compared with AB and BB genotypes. Likewise, for *Zs12*, the AA genotype was associated with early mortality compared to the two other genotypes. These results indicate that the A allele seems to act in a recessive way for both phenotypic traits.

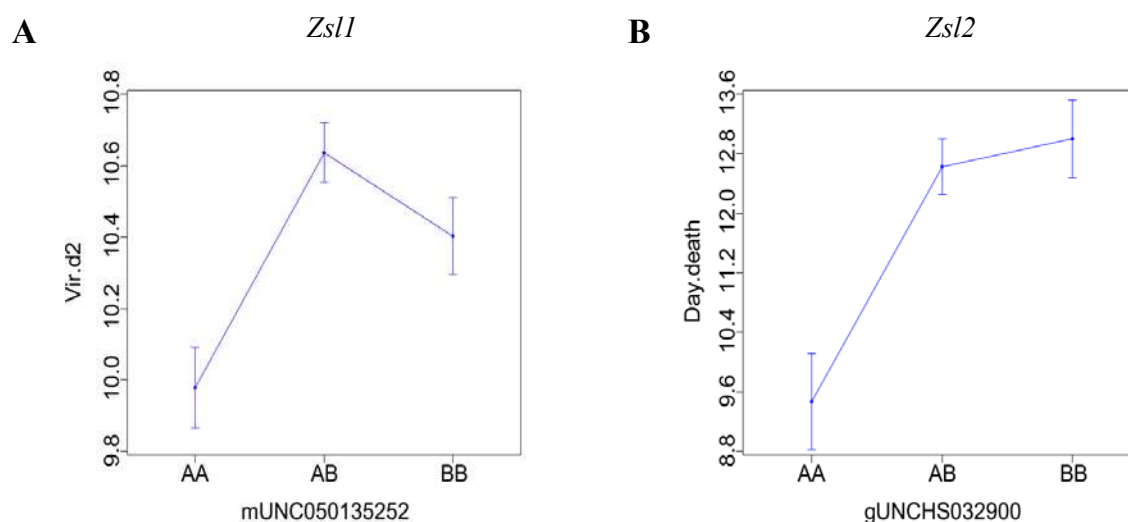


Figure 35. Allelic effects of *Zs11* and *Zs12* QTLs.

Allelic distribution of *Zs11* at marker mUNC050135252 (A) and *Zs12* at marker gUNCHS032900 (B). X-axis indicates the genotype of the SNP marker with A and B representing B6 and 129 alleles, respectively. Y-axis displays the phenotypic quantification as mean \pm SD with peak plasma viral load for *Zs11* and time to death for *Zs12*.

5.2.3. Discussion

In a previous study, we explored the role of host natural genetic variants on ZIKV susceptibility using genetically diverse mice (Manet *et al.* 2019). We showed that the genetic diversity in the CC panel enabled large phenotypic variations in the severity and pathology of ZIKV disease, though genetic analyses did not lead to the identification of genetic loci controlling the phenotypic variations in CC mice. We also previously showed that the susceptibility of *Ifnar1* knockout mice is largely influenced by their genetic background.

Ifnar1-deficient mice have been used extensively to study the mechanisms of ZIKV disease owing to their permissiveness to ZIKV replication and infection (Dowall *et al.* 2017; Lazear *et al.* 2016; Miner *et al.* 2016a; Rossi *et al.* 2016; Tripathi *et al.* 2017). While they have allowed the investigation of many aspects of ZIKV infection such as brain pathology and neuronal damage or vertical transmission and CZS, their constitutive defect in the type I IFN response could affect or limit the relevance of the findings to humans (Morrison and Diamond 2017). Besides, the type I IFN response plays a crucial role in the early immune response against viral pathogens, but in humans, ZIKV is able to antagonize this response at various steps of the signaling cascade (Beaver *et al.* 2018; Cumberworth *et al.* 2017). Identifying genes controlling the susceptibility to ZIKV in the context of an antagonized IFN response would thus be highly relevant to humans. Indeed, human genetic studies of dengue disease have identified a number of susceptibility genes with functions not only in the IFN response but also in the cellular and adaptive immune responses (Manet *et al.* 2018). Finally, genetic analysis in CC strains strongly suggested that, contrary to other viruses for which major host genetic determinants have been identified (*Oas1b* for WNV (Mashimo *et al.* 2002) or *Mx1* for IAV (Horisberger *et al.* 1983)), susceptibility to ZIKV in CC strains seems to be under the control of several genes with moderate effects. Genetic mapping analysis in *Ifnar1*-deficient mice would therefore be more likely to identify some of these small effect genes.

In the present study, we intercrossed B6-*Ifnar1* and 129-*Ifnar1* mice and identified two significant QTLs controlling the susceptibility to ZIKV. The first QTL, *Zs11* on chromosome 5, was associated with the peak plasma viral load at day 2 p.i., and the second QTL, *Zs12* on chromosome 12, controlled the time to death after infection. For both QTLs, mice homozygous for the B6 allele displayed lower phenotypic values compared with mice with a heterozygous or 129 homozygous genotype, indicating a recessive mode of inheritance of the B6 alleles and suggesting that those B6 alleles could be hypomorphic or carry a loss-of-function mutation. More specifically, mice homozygous for the B6 allele at *Zs12* showed early mortality, which is consistent with the highly susceptible phenotype of the parental strain B6-*Ifnar1*. However, a lower peak plasma viral load was unexpectedly observed in mice homozygous for the B6 allele at *Zs11* while this trait did not differ between the parental strains (Manet *et al.* 2019). B6 allele at *Zs11* confers a slightly decreased peak plasma viral load though the absolute value remains elevated, therefore the observed variation might not be influencing overall mouse survival. Consistently, results on the parental strains and on CC mice from the previous study also suggested that viral load alone could not reliably predict clinical outcome of ZIKV infection.

Although the confidence intervals (CIs) of *Zs11* and *Zs12* loci encompass large chromosomal segments (53 and 59 Mb, respectively), candidate genes can be considered in these regions based on their known functions in immune or inflammatory responses or on the phenotypic traits in the corresponding mouse models. For example, the gene list can be reduced by using a criterion for phenotype such as “abnormal immune system physiology” (Phenotype identification: MP:0001789 in MGI, <http://www.informatics.jax.org/>) as this phenotype term gathers several phenotypic traits related to immune and inflammatory responses or host response to infection, which are relevant in the case of a viral infection. Using this phenotypic trait as a selection criterion, gene list can be narrowed from 546 to 48 protein-coding genes in *Zs11* (Table 8) and from 230 to 22 in *Zs12* (Table 9).

Genome coordinates	Symbol	Gene name
88519809-88527891	<i>Jchain</i>	immunoglobulin joining chain
88765013-88783277	<i>Dck</i>	deoxycytidine kinase
89527429-89583740	<i>Npffr2</i>	neuropeptide FF receptor 2
90759360-90761624	<i>Cxcl5</i>	chemokine (C-X-C motif) ligand 5
90768518-90770063	<i>Ppbp</i>	pro-platelet basic protein
90794534-90803067	<i>Cxcl15</i>	chemokine (C-X-C motif) ligand 15
91074622-91093646	<i>Ereg</i>	epiregulin
91139599-91148432	<i>Areg</i>	amphiregulin
92321347-92328079	<i>Cxcl9</i>	chemokine (C-X-C motif) ligand 9
92346638-92348889	<i>Cxcl10</i>	chemokine (C-X-C motif) ligand 10
95956951-95961068	<i>Cxcl13</i>	chemokine (C-X-C motif) ligand 13
97884688-98030962	<i>Antxr2</i>	anthrax toxin receptor 2
99955935-99978938	<i>Hnrnpd</i>	heterogeneous nuclear ribonucleoprotein D
100553725-100572245	<i>Plac8</i>	placenta-specific 8
100679486-100719683	<i>Hpse</i>	heparanase
103425192-103598303	<i>Ptpn13</i>	protein tyrosine phosphatase, non-receptor type 13
103989765-104021796	<i>Hsd17b11</i>	hydroxysteroid (17-beta) dehydrogenase 11
104170712-104180127	<i>Dspp</i>	dentin sialophosphoprotein
104202613-104214102	<i>Dmp1</i>	dentin matrix protein 1
104435118-104441050	<i>Spp1</i>	secreted phosphoprotein 1
107716655-107726031	<i>Gfi1</i>	growth factor independent 1 transcription repressor
108660331-108684557	<i>Idua</i>	iduronidase, alpha-L
108669878-108675569	<i>Slc26a1</i>	solute carrier family 26 (sulfate transporter), member 1
109554709-109558993	<i>Crlf2</i>	cytokine receptor-like factor 2
110259135-110269899	<i>Pgam5</i>	phosphoglycerate mutase family member 5
113612354-113650426	<i>Cmkrl1</i>	chemokine-like receptor 1
113818536-113832644	<i>Selplg</i>	selectin, platelet (p-selectin) ligand
113842439-113908706	<i>Coro1c</i>	coronin, actin binding protein 1C
114130386-114139323	<i>Ung</i>	uracil DNA glycosylase
114380607-114421169	<i>Ube3b</i>	ubiquitin protein ligase E3B
114727408-114773522	<i>Git2</i>	GIT ArfGAP 2
114923240-114937915	<i>Oasl1</i>	2'-5' oligoadenylate synthetase-like 1
116013586-116024503	<i>Prkab1</i>	protein kinase, AMP-activated, beta 1 non-catalytic subunit
116408491-116422864	<i>Hspb8</i>	heat shock protein 8
117781032-117958840	<i>Nos1</i>	nitric oxide synthase 1, neuronal
120680203-120711669	<i>Dtx1</i>	deltex 1, E3 ubiquitin ligase
120812635-120824163	<i>Oasl1b</i>	2'-5' oligoadenylate synthetase 1B
121130533-121191397	<i>Ptpn11</i>	protein tyrosine phosphatase, non-receptor type 11
121371725-121385627	<i>Traf1</i>	TRAF type zinc finger domain containing 1
122206807-122242297	<i>Hvcn1</i>	hydrogen voltage-gated channel 1
122643911-122691432	<i>P2rx7</i>	purinergic receptor P2X, ligand-gated ion channel, 7
122707544-122729738	<i>P2rx4</i>	purinergic receptor P2X, ligand-gated ion channel 4
123015074-123030452	<i>Orail</i>	ORAI calcium release-activated calcium modulator 1
125277087-125341094	<i>Scarb1</i>	scavenger receptor class B, member 1
127595664-127632897	<i>Slc15a4</i>	solute carrier family 15, member 4
128984557-129008574	<i>Stx2</i>	syntaxin 2
129765558-129787253	<i>Psph</i>	phosphoserine phosphatase
130171798-130214342	<i>Rabgef1</i>	RAB guanine nucleotide exchange factor (GEF) 1

Table 8. Genes in *Zsll* associated with phenotypic trait “abnormal immune system physiology”. Protein coding genes and polymorphic pseudogenes retrieved in Mouse Genome Informatics (MGI: <http://www.informatics.jax.org/>) in chromosomal region 5: 79.12-132.32 Mb and using Phenotypes/Diseases criterion: MP:0001789.

Genome coordinates	Symbol	Gene name
3954951-3960642	<i>Pomc</i>	pro-opiomelanocortin-alpha
4862440-4874359	<i>Mfsd2b</i>	major facilitator superfamily domain containing 2B
8771396-8793716	<i>Sdc1</i>	syndecan 1
8947929-8972028	<i>Matn3</i>	matrilin 3
11265886-11319785	<i>Smc6</i>	structural maintenance of chromosomes 6
16894895-16987823	<i>Rock2</i>	Rho-associated coiled-coil containing protein kinase 2
21323509-21373632	<i>Adam17</i>	a disintegrin and metallopeptidase domain 17
24665833-24681813	<i>Cys1</i>	cystin 1
25093799-25096092	<i>Id2</i>	inhibitor of DNA binding 2
26442746-26456452	<i>Rsad2</i>	radical S-adenosyl methionine domain containing 2
28594173-28623377	<i>Colec11</i>	collectin sub-family member 11
29937956-30017658	<i>Pxdn</i>	peroxidasin
30893326-30911589	<i>Acp1</i>	acid phosphatase 1, soluble
31519827-31559969	<i>Slc26a4</i>	solute carrier family 26, member 4
32173473-32208659	<i>Pik3cg</i>	phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit gamma
32820335-32853369	<i>Nampt</i>	nicotinamide phosphoribosyltransferase
35497979-35534989	<i>Ahr</i>	aryl-hydrocarbon receptor
35992925-36004081	<i>Agr2</i>	anterior gradient 2
41024090-41955588	<i>Imp2l</i>	IMP2 inner mitochondrial membrane peptidase-like (S. cerevisiae)
53248677-54072175	<i>Npas3</i>	neuronal PAS domain protein 3
55280809-55303005	<i>Ppp2r3c</i>	protein phosphatase 2, regulatory subunit B", gamma
55489410-55492647	<i>Nfkb1a</i>	nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor, α

Table 9. Genes in *Zs12* associated with phenotypic trait “abnormal immune system physiology”. Protein coding genes and polymorphic pseudogenes retrieved in Mouse Genome Informatics (MGI: <http://www.informatics.jax.org/>) in chromosomal region 12: 2.79-61.58 Mb and using Phenotypes/Diseases criterion: MP:0001789.

Once narrowed down, these genes can be further examined based on more precise phenotypes in mouse models, on gene functions and on allelic variants segregating between the parental strains. The *Zs11* CI contains seven genes associated with decreased or increased susceptibility to viral infection: *Areg*, *Cxcl10*, *Jchain*, *Oas1b*, *Oas1l*, *Spp1*, *Trafd1*.

Amphiregulin, *Areg*, is a ligand of the EGF (epidermal growth factor) receptor and acts as an autocrine growth factor for a broad range of cell types. Interestingly, amphiregulin is expressed in innate lymphoid cells type 2 (ILC2) and in T-regulatory (Treg) cells; and amphiregulin deficiency in Treg cells has been shown to lead to severe influenza disease in mice (Arpaia *et al.* 2015).

Chemokine (C-X-C motif) ligand, *Cxcl* genes, may represent the main candidate genes in *Zs11*, among which *Cxcl10* is a pro-inflammatory cytokine involved in a variety of processes such as chemotaxis, differentiation, and activation of immune cells. *Cxcl10* knock-out mice display an impaired immune response to mouse hepatitis virus (MHV) infection associated with a decreased recruitment of T-cells in tissues (Dufour *et al.* 2002).

Immunoglobulin joining chain, *Jchain*, links the two monomer units of either IgM or IgA. Though fewer symptoms and a reduced mortality rate were observed in *Jchain*-deficient mice following herpes simplex type 2 infection, no difference in viral load was observed in these mice (Hendrickson *et al.* 2000). Thus, *Jchain* might be a less attractive candidate.

2'-5' oligoadenylate synthetase 1B, *Oas1b*, can be easily excluded from the candidate gene list as both B6 and 129-*Ifnar1* mouse strains carry a non-functional, truncated, allele. On the other hand,

2'-5' oligoadenylate synthetase-like 1, *Oas1l*, which does not have 2'-5'-OAS activity but binds dsRNA, displays a direct antiviral activity independently of the RNase L pathway (Eskildsen *et al.* 2003). However, *Oas1l* might not be the best candidate as it belongs to the ISG family and is most likely irrelevant in the context of viral infection in *Ifnar1*-deficient mice.

Secreted phosphoprotein 1, *Spp1*, acts as a cytokine by enhancing production of IFN- γ and IL-12 and reducing production of IL-10. *Spp1*-deficient mice display a severely impaired cell-mediated immune response after herpes simplex virus type 1 infection (Ashkar *et al.* 2000) while active *Spp1* was recently shown to facilitate WNV neuroinvasion to the brain (Paul *et al.* 2017).

TRAF type zinc finger domain containing 1, *Trafd1*, acts as a negative feedback regulator controlling excessive innate immune responses and may also negatively regulate the RLR pathway downstream of MAVS and upstream of NF-KB and IRF3 (Sanada *et al.* 2008). *Trafd1* is also an ISG, its implication in differential susceptibility of *Ifnar1*-deficient mice to ZIKV infection is thus questionable.

Finally, another interesting and potential candidate gene is *Ptpn13*, protein tyrosine phosphatase, non-receptor type 13, which has not been directly associated to an altered susceptibility to viral infection but is suspected to regulate the phosphoinositide 3-kinase (PI3K) pathway and to be an important negative regulator of the STAT signaling pathway (Nakahira *et al.* 2007).

One of the most obvious candidate gene in the *Zsl2* region is *Rsad2*, also known as Viperin, which has been shown to be an important host restriction factor of flaviviruses (Lindqvist *et al.* 2018; Vonderstein *et al.* 2018), including ZIKV (Panayiotou *et al.* 2018; Van der Hoek *et al.* 2017).

Pik3cg, phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit γ , is another potential candidate as *Pik3cg* knock-out mice were more susceptible to vaccinia virus infection and *Pik3cg* was shown to play a central role in the migration of effector CD8⁺ T cells (Martin *et al.* 2008).

Last but not least, *Id2*, inhibitor of DNA binding 2, is a transcription factor that represses the activity of E-box transcription factors, which are critical in the early development of B and T-cells. More precisely, lymphoid precursors that express the transcriptional repressor *Id2* can develop into ILCs and Natural killer (NK) cells. *Id2*-deficient mice actually lack ILCs and NK cells indicating that *Id2* is required for the development of ILCs at an early step (Artis and Spits 2015; Eberl *et al.* 2015). Unlike T-cells, ILCs do not express antigen receptors or undergo clonal selection when they are stimulated, but rather react quickly to infection or tissue injury and produce multiple cytokines to steer the immune response against the initial threat (Spits *et al.* 2016). In addition to ILCs being key players of the innate immune response, C57BL/6J mice have been shown to carry a hypomorphic allele of *Id2* compared to the *Id2* allele of 129S1/SvImJ mice (Zhang *et al.* 2014). *Id2* is thus an interesting candidate gene worth investigating.

Future work will aim at narrowing down these lists of candidate genes for *Zsl1* and *Zsl2*. One possible approach would be to use congenic strains to reduce the CI of the QTLs. While congenic strains are valuable tools for refining QTLs location, they require a lot of time to be produced and sometime fail to replicate the QTLs that were identified in a segregating population (Chevallier *et al.* 2013; Marquis *et al.* 2009). Refining the list of candidate genes could be achieved more efficiently by intersecting positional information derived from QTL analysis with in-depth immunophenotyping and

transcriptomic analyses in mock and ZIKV-infected mice from the parental strains, B6-*Ifnar1* and 129-*Ifnar1*, and in their F1 hybrids. As the immune response is critical in the context of a viral infection, it would be interesting to characterize the phenotype of these mice in terms of cytokines profile, for example using Bioplex or Luminex technologies to measure plasmatic levels of a multitude of inflammatory cytokines and by flow cytometry analyses of immune cell populations. Finally, using single-cell RNA sequencing could be an elegant way to combine immune cell phenotyping with gene expression analysis, allowing for unbiased clustering of immune cell populations including ILCs (Suffiotti *et al.* 2017).

Extensive analysis of these *Ifnar1*-deficient strains will most likely shed light on how genetic variants affect susceptibility as well as immune responses to ZIKV infection, and will provide comprehensive information on these widely-used mouse strains and on how to use them in the best way to model ZIKV or other flaviviral diseases.

5.3. Host genetics control of Zika virus replication in embryonic fibroblasts derived from Collaborative Cross mice

Article in preparation:

“Uncontrolled Zika viral replication in susceptible mouse cells results from a delayed and impaired induction of type I interferons.”

The final content of the article will be determined according to on-going and planned experiments, with the aim of identifying the molecular mechanisms. These experiments are presented after the article in the "On-going experiments and future directions" paragraph.

Notes:

For the sake of readability, figures and supplementary figures are included with their legends along the main text.

Material and methods are included in the previous section (4) and will be included in the final version of the article.

Title: Uncontrolled Zika viral replication in susceptible mouse cells results from a delayed and impaired induction of type I interferons.

INTRODUCTION

Zika virus (ZIKV) belongs to the *Flaviviridae* family, *Flavivirus* genus, which includes several other important pathogens such as Yellow Fever virus, Dengue virus, West-Nile virus and Japanese Encephalitis virus. ZIKV is a mosquito-transmitted virus, which was originally isolated in 1947 from a febrile Rhesus monkey in Uganda (Talero-Gutierrez *et al.* 2018). For over half a century, ZIKV was known to cause self-limiting febrile disease, with rare reported clinical cases (Baud *et al.* 2017). However, association with new syndromes, including encephalitis and Guillain-Barré syndrome in adults (Cao-Lormeau *et al.* 2016; Lannuzel *et al.* 2019), and congenital malformations in fetuses of infected pregnant women (Del Campo *et al.* 2017; Pomar *et al.* 2017; Walker *et al.* 2019), have begun to emerge since the 2016 Brazil ZIKV outbreak. Many factors are known to influence the clinical outcomes and the ability of the host to fight ZIKV infection. These factors include the viral strain and mutations, the route of infection, and the host physiological status as well as host genetic makeup (Liu *et al.* 2019; Rossi *et al.* 2018). Indeed, host genetics is increasingly being recognized as a key component in the host susceptibility to infection (Chapman and Hill 2012; Kenney *et al.* 2017). Notably, mouse and human genetic studies have led to the identification of host variants controlling the susceptibility to West-Nile and Dengue viruses (Manet *et al.* 2018).

The type I interferon (IFN) signaling pathway constitutes an early response to viral infections in mammals and is triggered by RIG-I-like and Toll-like recognition receptors. Once induced, type I (IFN- α and IFN- β) IFNs signal through their heterodimeric receptors (IFNAR1/IFNAR2), resulting in the expression of hundreds of IFN-stimulated genes (ISGs) with various antiviral and immunomodulatory functions (McNab *et al.* 2015). Many genes of the type I IFN cascade have been identified as host susceptibility or resistance factors to flaviviral infections, most of which have been identified by reverse genetic studies using specific knock-out mouse strains (Manet *et al.* 2018). For example, a constitutional deletion of *Ifnar1*, *Stat1* or *Stat2* has been shown to increase mouse susceptibility to ZIKV infection (Gorman *et al.* 2018; Kamiyama *et al.* 2017; Tripathi *et al.* 2017; Winkler and Peterson 2017). While these studies have contributed to our understanding of the role of the type I IFN response in ZIKV disease, they do not model the effects of natural variants that would be found in the natural population.

In a previous study, we explored the role of host natural genetic variants on ZIKV susceptibility using genetically diverse mice in an experimental setup where type I IFN response was blocked by a monoclonal antibody targeting the IFNAR1 receptor subunit (Manet *et al.* 2019). We showed that the genetic diversity in the Collaborative Cross (CC), a panel of recombinant inbred mice capturing approximately 90% of the natural genetic variants segregating in the mouse genome, enabled large phenotypic variations in the clinical severity of ZIKV disease, in the plasma viral load and in the severity of ZIKV-induced brain pathology. In this study, we also demonstrated that host genetic variants resulted in differences in the permissiveness of CC mouse cells to ZIKV replication.

In particular, we identified CC071 mice as being very susceptible, with high mortality, high peak plasma viral load and high viral replication in mouse embryonic fibroblasts (MEFs).

Here, we investigated the mechanisms driving the differences in ZIKV replication in MEFs from 11 CC strains. We showed that CC071 MEFs produced increasing numbers of infectious viral particles over 72h while this production decreased in all other strains tested. We further demonstrated that ZIKV infection and replication rates are increased in CC071 MEFs in late infection. Transcriptomic analysis on ZIKV-infected MEFs revealed delayed and weak induction of many innate immunity genes, including *Ifnb1* gene, in CC071 MEFs. *Ifnb1* induction was also impaired using Poly(I:C), a TLR3 agonist, demonstrating that CC071 are defective in the early events between virus detection and activation of *Ifnar1* transcription. Finally, genetic dissection using multiple CC strains suggested that this phenotype is not due to a single-gene deficiency but may result from defective interactions between components of the *Ifnb1* activation pathway.

RESULTS

Genetic background controls the production of Zika viral particles in MEFs

In our previous study, we have described large differences in the production of viral infectious particles between MEFs of the CC001 and CC071 strains, suggesting that increased replication rate in CC071 could contribute to its susceptible *in vivo* phenotype. To further evaluate the influence of host genetic factors on ZIKV replication rate *in vitro*, we measured the production of viral particles in MEFs derived from 9 additional CC strains and C57BL/6J strain. Cells were infected with ZIKV FG15 and ZIKV titer was measured in the supernatants 24, 48 and 72 hours after infection. For all time-points, ZIKV titer was characterized by large inter-strain variations spanning over 1 log₁₀ range (FIG 1), demonstrating a strong effect of host genes (Anova, 24h p.i.: $p=1.3 \times 10^{-10}$; 48h p.i.: $p=2.9 \times 10^{-13}$; 72h p.i.: $p=2.3 \times 10^{-22}$). In all CC strains, ZIKV titer increased between 24 and 48 hours p.i. and decreased between 48 and 72 hours p.i., except in CC071 MEFs which produced increasing amounts of viral particles, indicating that this strain has an outstanding susceptible phenotype.

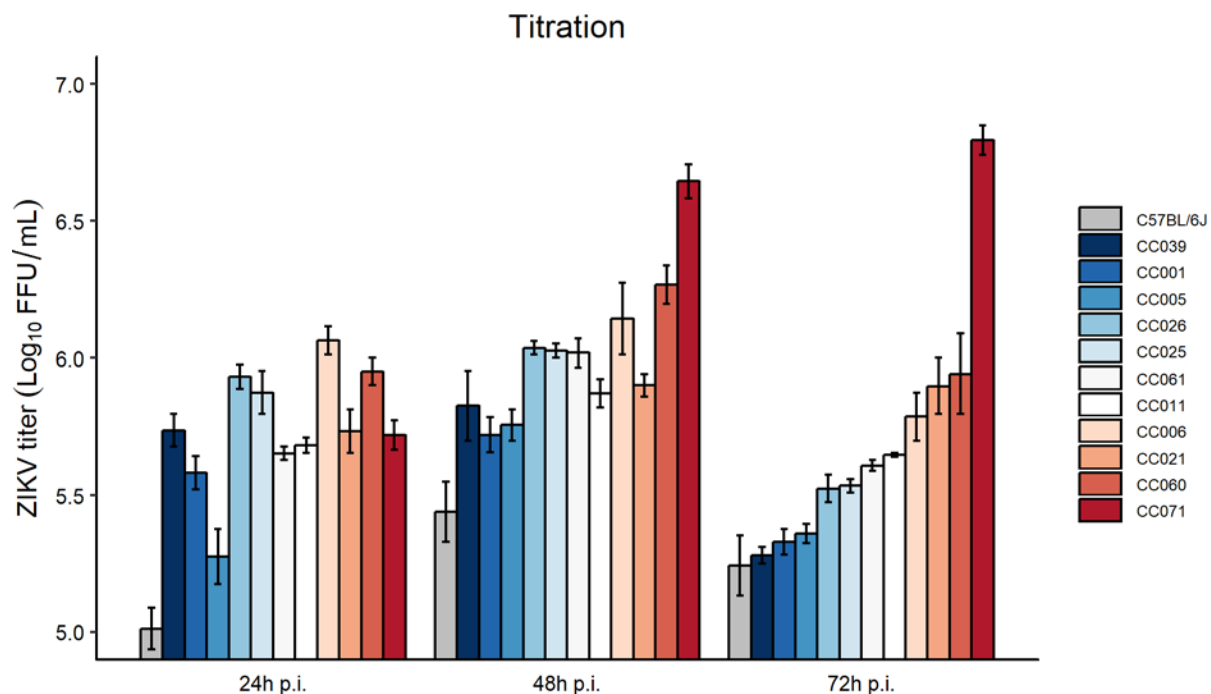


FIG 1 CC071 MEFs enable higher ZIKV titers 48 and 72 hours after infection compared with 10 other CC strains and C57BL/6J. MEFs were infected with ZIKV FG15 at MOI 5. ZIKV titer in the supernatant was quantified by focus-forming assay at 24, 48 and 72 hours p.i. Mean \pm SEM from 2 replicates. Strains were sorted according to the viral titer at 72 hours p.i.

ZIKV infection rate and replication are increased in CC071 MEFs in late infection

We further characterized ZIKV infection profile in MEFs from three strains including C57BL/6J and CC001 as “resistant” strains and CC071 as an outlier, susceptible, strain.

We first evaluated potential inter-strain differences in the binding capacity of ZIKV to its cellular receptors. There was no significant difference in ZIKV binding to the cell membrane of CC071 and C57BL/6J MEFs, which, on the other hand, was found to be moderately enhanced in CC001 compared to C57BL/6J cells (FIG 2A). We also assessed whether host genetic background could affect the viral entry into the cells, independently of viral binding to the cell surface. We did not detect significant variations in the capacity of ZIKV to enter C57BL/6J, CC001 or CC071 MEFs (FIG 2B).

We then measured ZIKV cellular infection rate and replication from 16 to 72 hours after infection. The number of ZIKV-infected cells was much higher in CC001 than in C57BL/6J MEFs and peaked at 24 hours p.i. before decreasing to reach low levels at 72 hours. In contrast, CC071 positive cells kept increasing at late time points, resulting in a significantly elevated infectivity rate at 72 hours p.i. (FIG 2C). ZIKV replication was overall correlated to the infectivity rate and led to higher levels of viral genome copies in CC071 MEFs 72 hours after infection (FIG 2D), consistently with our initial observations (FIG 1).

Overall, these results suggest that C57BL/6J and CC001 MEFs can efficiently control ZIKV infection, independently of early infection and replication rates, while CC071 MEFs cannot.

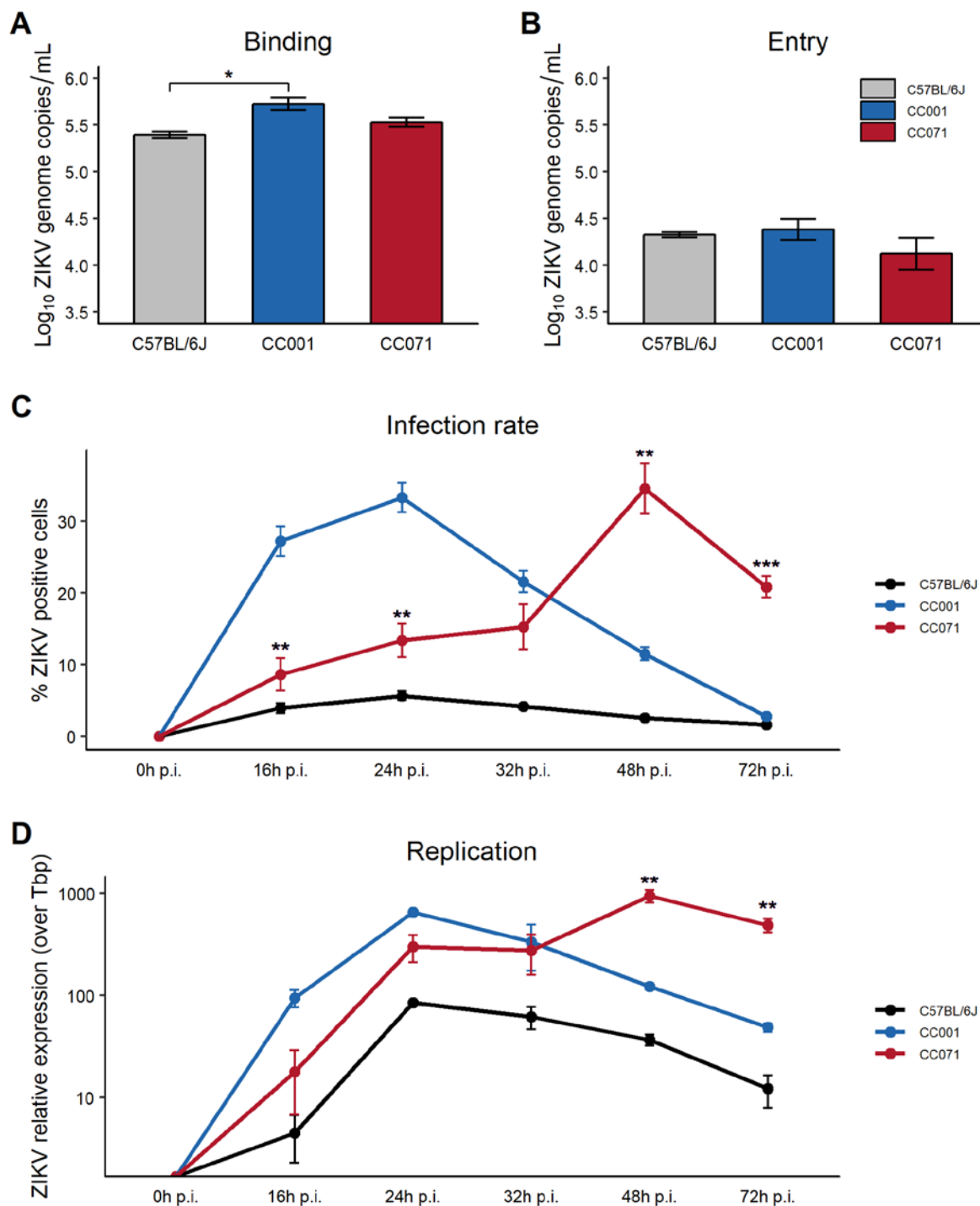


FIG 2 ZIKV infectivity and replication are increased in CC071 MEFs 48 and 72 hours after infection. MEFs were infected with ZIKV FG15 at MOI 5. (A) For viral attachment assay (binding), the cells were incubated with ZIKV at 4°C, washed and processed. (B) For viral entry assay, the cells were incubated with ZIKV at 4 °C before raising the temperature to 37°C, trypsinized, washed and processed. (C) For viral infectivity assay, cells were stained for ZIKV E protein at different time points after infection and the percentage of ZIKV-positive cells was determined by flow cytometry. (D) For intracellular viral replication assay, ZIKV RNA genome relative expression was determined by RT-qPCR on MEFs total RNA at different time points after infection by normalizing to *Tbp* housekeeping gene. In all graphs, results are mean \pm SEM of three biological replicates. (t tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; C and D: t tests between CC001 and CC071 strains).

Induction of *Ifnb1* expression is delayed in CC071 MEFs after ZIKV infection

Since type I IFN response is one of the first actors of the innate immune response required to stop viral replication and propagation, we hypothesized that it could be impaired in ZIKV-infected CC071 MEFs. We measured by RT-qPCR the expression of the *Ifnb1* gene after ZIKV infection in C57BL/6J, CC001 and CC071 MEFs. While *Ifnb1* was rapidly and strongly expressed in C57BL/6J and CC001 cells, its induction was notable in CC071 MEFs only from 48 hours p.i. (FIG 3). These data show that ZIKV-induced *Ifnb1* expression is delayed in CC071 MEFs and could explain the lowest capacity of CC071 MEFs to control the viral infection.

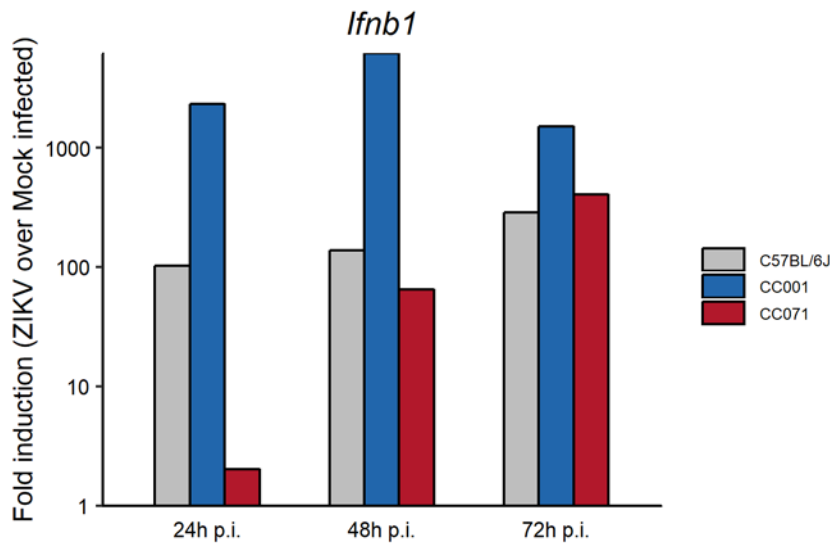
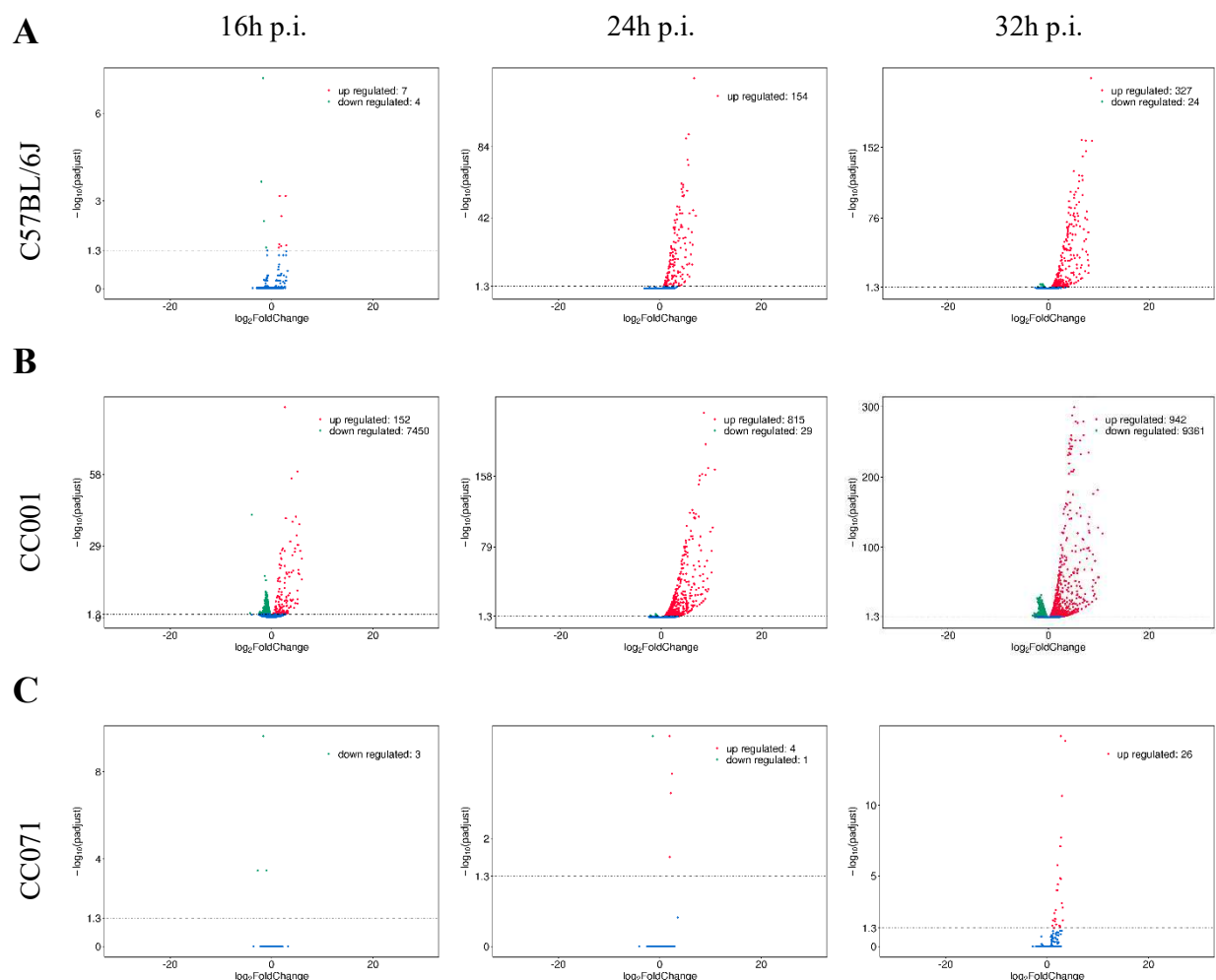


FIG 3 ZIKV-induced *Ifnb1* expression is delayed in CC071 MEFs. Levels of *Ifnb1* mRNA were determined by RT-qPCR at 24, 48 and 72 hours p.i. by normalizing to *Tbp* housekeeping gene. Results are expressed as fold induction relative to the non-infected (Mock) condition. Data representative of two independent experiments.

Transcriptome profiling of ZIKV-infected C57BL/6J, CC001 and CC071 MEFs

To gain deeper understanding of these inter-strain differences, we performed high throughput RNA sequencing on MEFs infected with ZIKV to characterize changes in gene expression. To appreciate the primary transcriptional events of the host response, we focused on early time points (16, 24 and 32 hours p.i.) after infection.

Using a FDR of 0.05 and \log_2 fold change >1 , the differential expression comparison of infected and mock-infected cells (3 biological replicates per strain) yielded strongly up-regulated genes (URG) in the three strains. The number of URG rose from 7 to 327 in C57BL/6J MEFs between 16 and 32 hours p.i., and between 152 and 942 in CC001 MEFs (SUPPL FIG 1). In contrast, only 3 to 26 URG were detected in CC071 MEFs. Of note, very large numbers of genes were found to be down-regulated in CC001 MEFs at 16 and 32 hours p.i. although the \log_2 fold change of expression was lower than that of URG (SUPPL FIG 1).



SUPPL FIG 1 Differentially expressed genes in MEFs from C57BL/6J, CC001 and CC071 at 16, 24 and 32 hours p.i. relative to non-infected MEFs. Volcano plot of DEGs illustrating significance cut-off set to \log_2 fold change > 1 . Results are the average of 3 biological replicates per strain.

We further restricted our analysis to URG. Within strain longitudinal comparison of the expression of individual URG revealed similar gene expression profiles in C57BL/6J and CC001 MEFs. In these two strains, the majority of early URG were up-regulated 24 and 32 hours p.i. (FIG 4A-B), whereas in CC071 cells, no URG was found at 16h and very few at 32h (FIG 4C), and the majority of URG at 32h were also up-regulated in C57BL/6J and CC001 MEFs (FIG 4D).

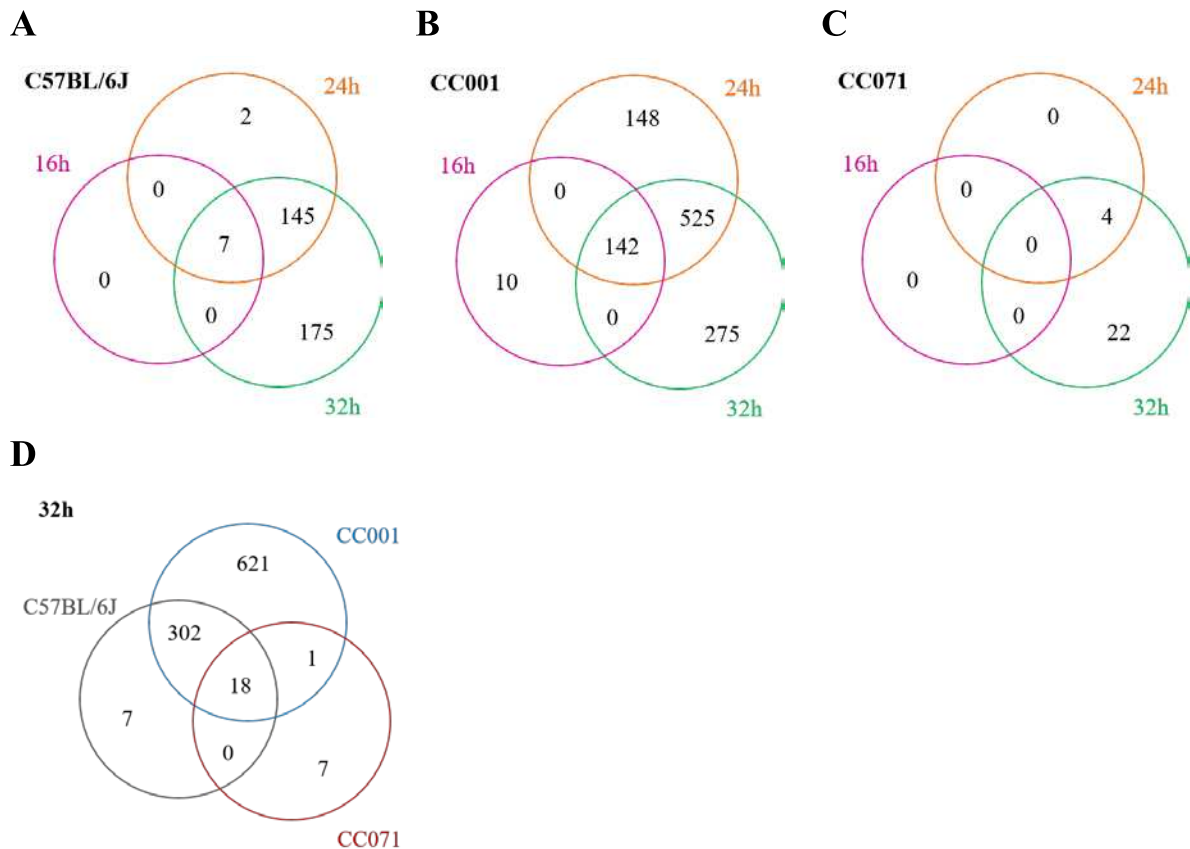
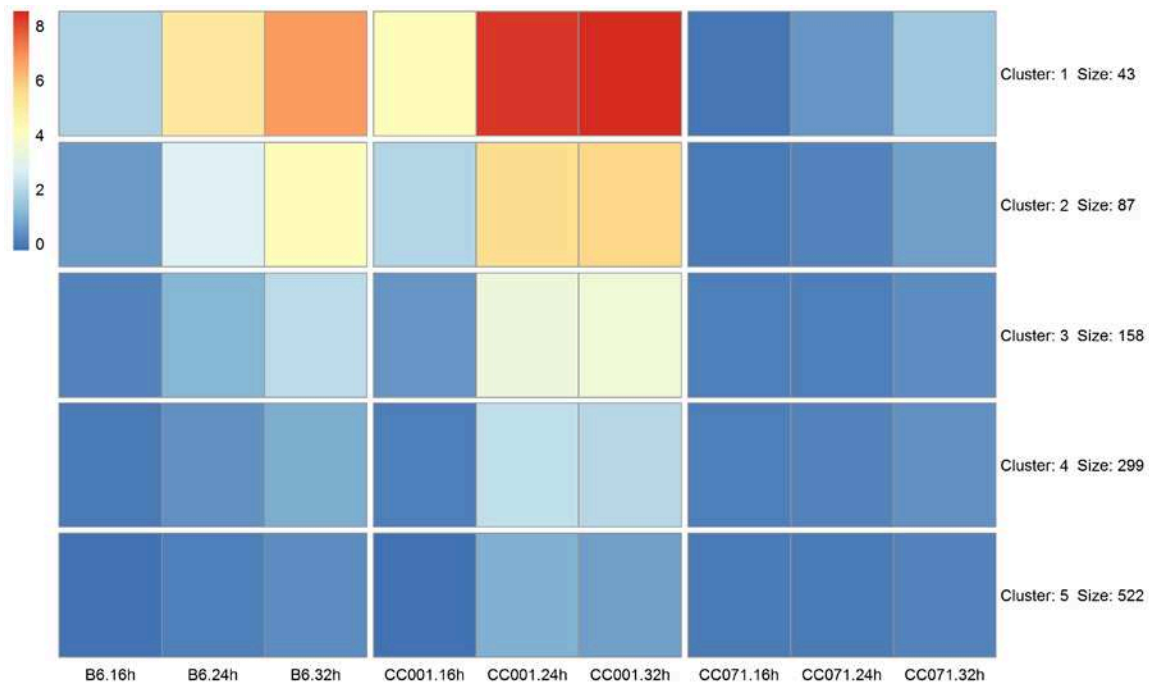


FIG 4 Transcriptome analysis of mock and ZIKV-infected MEFs from C57BL/6J, CC001 and CC071 at 16, 24 and 32 hours p.i. Venn diagrams indicate up-regulated genes between different time points of infection (A, B and C) and between mouse strains at 32 hours p.i. (D). Results are relative to the non-infected condition (FDR=0.05 and log2 fold-change > 1).

Induction of innate immunity genes is impaired and delayed in CC071 MEFs

Clustering analysis revealed at least two clusters of strongly URG in C57BL/6J and CC001 cells, which were only minimally up-regulated in CC071 MEFs (FIG 5A). Gene ontology (GO) enrichment analysis of cluster n°1 mainly pointed at biological processes related to the immune response, namely Innate immune response (GO:0045087), Response to type I IFN (GO:0034340), Cytokines production, signaling or response (GO:0001816, GO:0019221, GO:0034097) and Defense to virus (GO:0051607) (FIG 5B). The strongest URG in C57BL/6J and CC001 strains included *Mx1*, *Rsad2* (Viperin), *Oasl1*, *Ifit3*, *Ifit3b* and *Cxcl10*. *Ifnb1* gene was also strongly induced by ZIKV infection in C57BL/6J and CC001 but not CC071 cells (FIG 5B), consistently with our previous RT-qPCR findings (FIG 3).

A



B

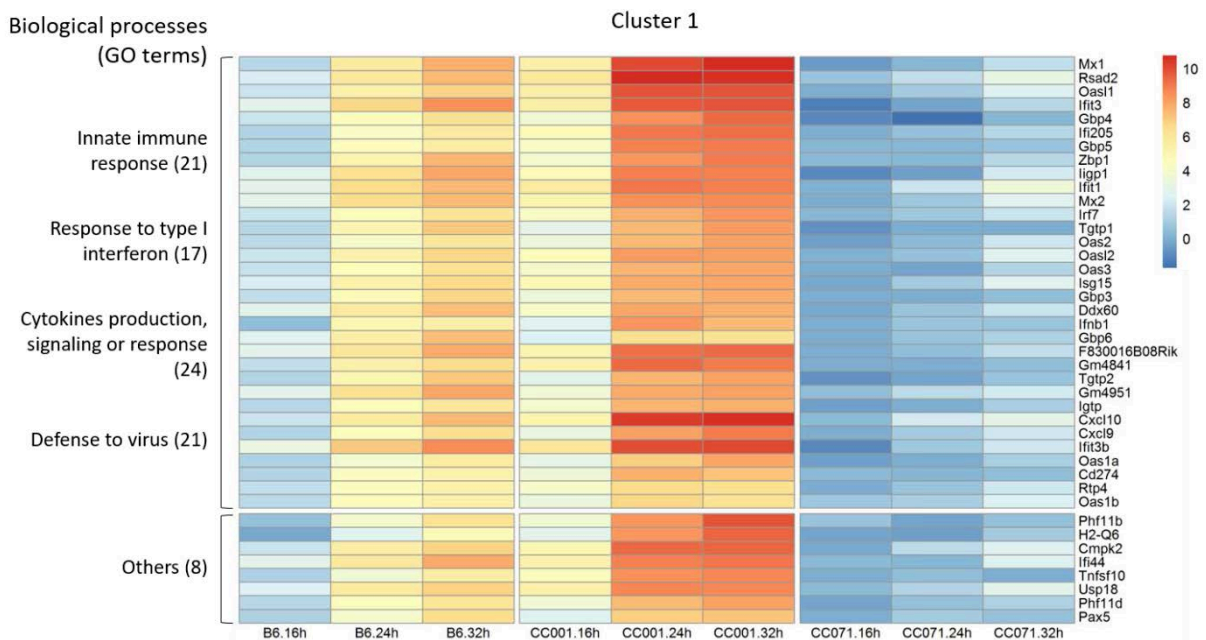
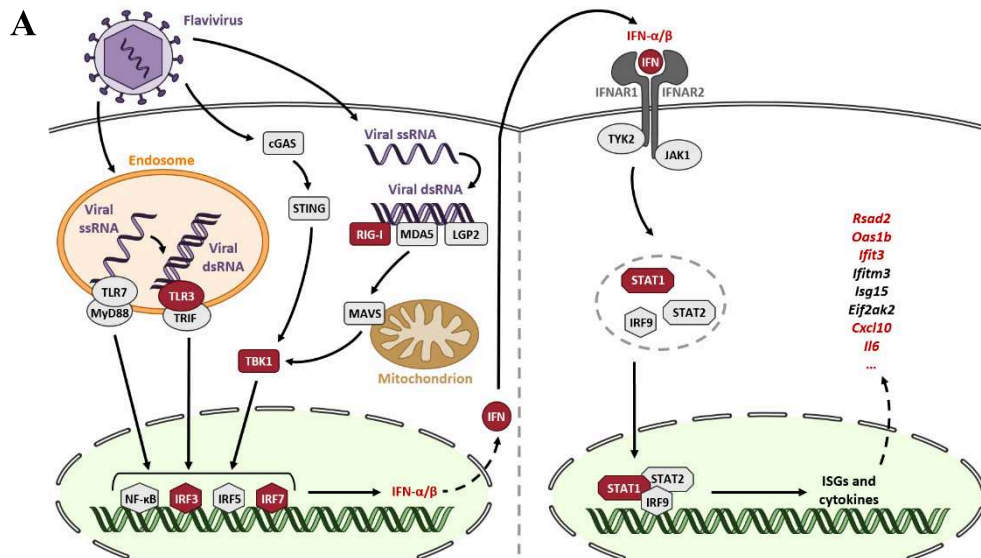


FIG 5 ZIKV infection rapidly leads to up-regulation of innate immunity genes in C57BL/6J and CC001 but not in CC071 MEFs. (A) Hierarchical clustering and heatmap reflecting gene expression changes of up-regulated genes. The color code shows the gene expression fold-change. (B) Heatmap of up-regulated genes in cluster 1, organized by biological processes (GO terms). The color code shows the normalized fold-change of the genes in each strain. Three biological replicates from each condition were collapsed and normalized to mock.

To evaluate more precisely how host genetic background influences the type I IFN transcriptional response, we looked at changes in expression of genes involved at different steps of this pathway, taking representative examples from viral sensing, signal transduction and induction of ISGs (FIG 6A). In C57BL/6J and CC001 cells, most genes were found to be up-regulated from 16 or 24 hours p.i. except for *Irf3* and *Tbk1* genes. In contrast, the only genes that were slightly up-regulated in CC071 were *Irf7*, *Rsad2*, *Oas1b* and *Cxcl10* (FIG 6B).

Overall, these results confirm that induction of *Ifnb1* expression is impaired and delayed in CC071 MEFs and is likely responsible for the subsequent delay in ISGs up-regulation.



B • B6 • CC001 • CC071

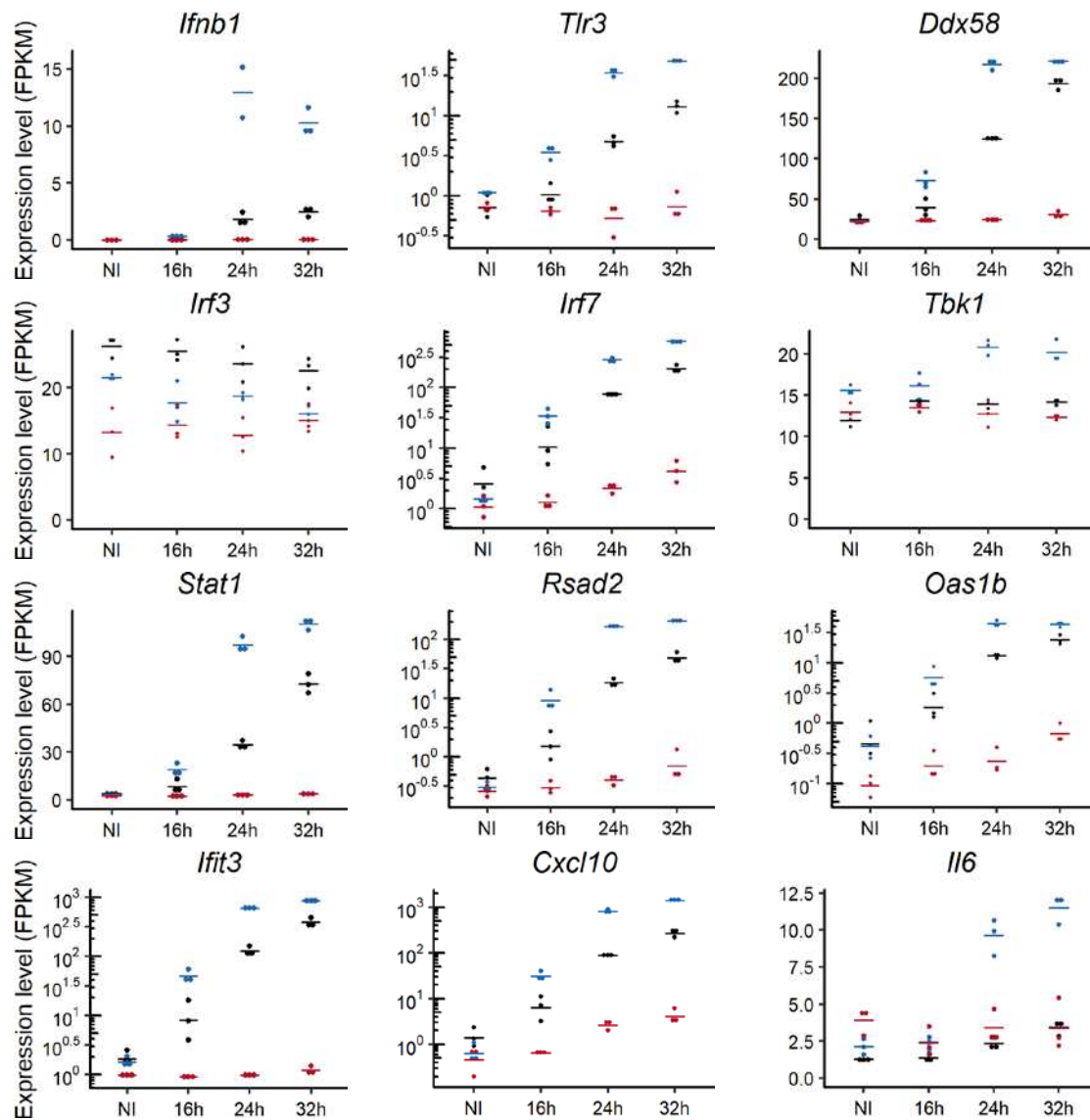


FIG 6 Induction of the expression of *Ifnb1* and further ISGs is delayed in CC071 MEFs. (A) Schematics of type I IFN response triggered by flavivirus infection (adapted from (Manet *et al.* 2018)). Changes in the expression of genes highlighted in red are illustrated in the following graphs. (B) Changes in mRNA expression levels of *Ifnb1*; viral sensors *Tlr3* and *Ddx58* (RIG-I); signal transducers *Irf3*, *Irf7*, *Tbk1* and *Stat1*; ISGs and cytokines *Rsad2* (Viperin), *Oas1b*, *Ifit3*, *Cxcl10* and *Il6* genes in non-infected (NI) and ZIKV-infected MEFs. FPKM, Fragments per kilobase of exon per million reads mapped.

CC071 MEFs normal response to IFN α stimulation confirms a defect in the induction of type I IFN response

To evaluate whether the induction of type I IFN was the main event responsible for the inability of CC071 MEFs to control ZIKV replication, we evaluated the capacity of CC071 MEFs to respond to type I IFN stimulation. We measured by RT-qPCR the induction of a few ISGs in MEFs treated with IFN α . While the expression of *Isg15*, *Ifitm3* and *Eif2ak2* (PKR) was dramatically lower in CC071 compared with C57BL/6J and CC001 infected cells (FIG 7A, data from the RNAseq experiment), similar expression levels were found in all three strains after stimulation with IFN α (FIG 7B). These data demonstrate that CC071 cells are able to respond to type I IFN stimulation and therefore confirm that their primary defect is in the induction of type I IFN.

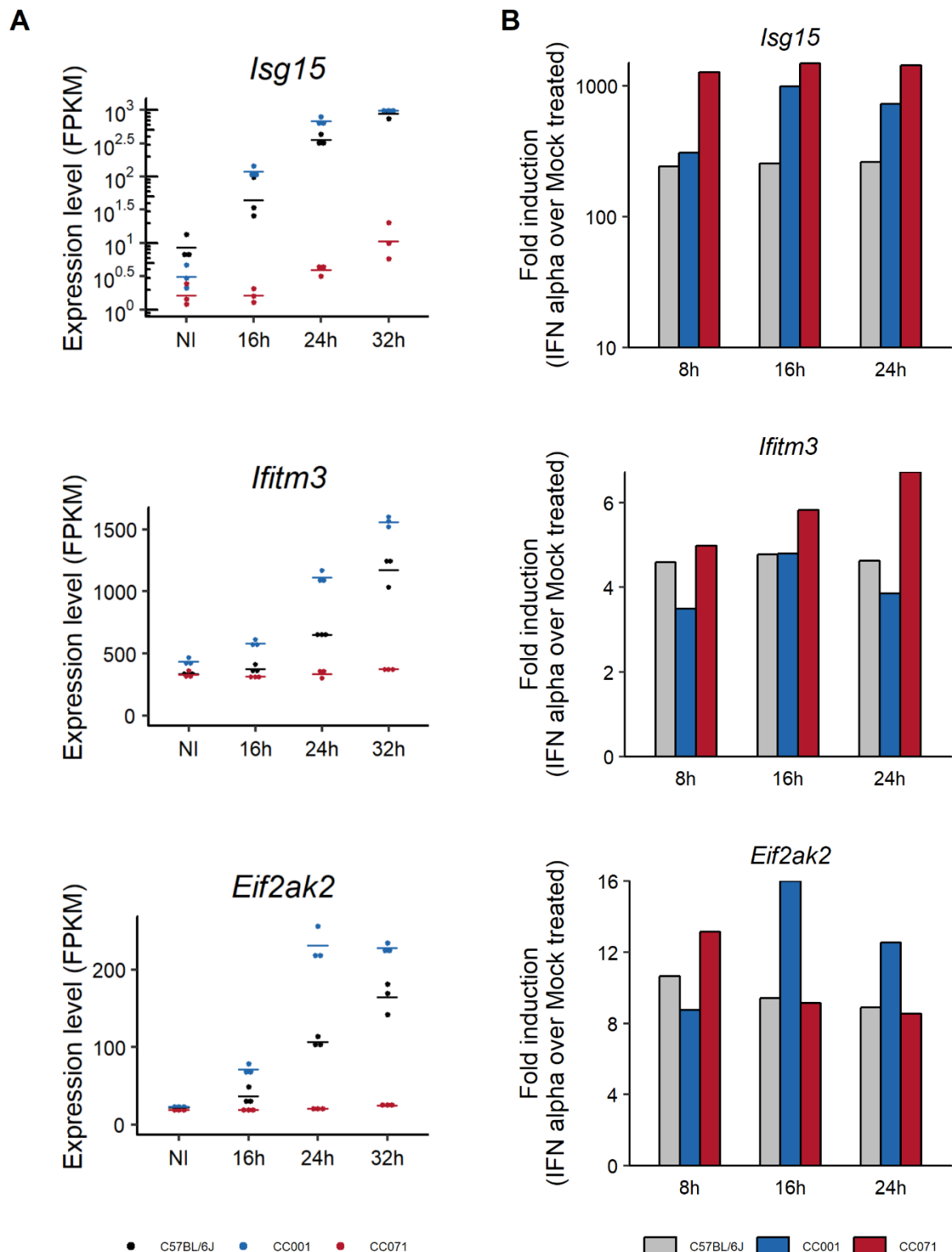


FIG 7 ISGs expression is impaired in ZIKV-infected but not in IFN α stimulated CC071 MEFs. (A) Changes in mRNA expression levels of ISGs *Isg15*, *Ifitm3* and *Eif2ak2* (PKR) in non-infected (NI) and ZIKV-infected MEFs. (B) MEFs were stimulated with IFN α and the expression of *Isg15*, *Ifitm3*, and *Eif2ak2* (PKR) mRNA were determined by RT-qPCR at 8, 16 and 24 hours by normalizing to *Tbp* housekeeping gene. Results are expressed as fold induction relative to the untreated (Mock) condition.

Impaired type I IFN induction in CC071 MEFs is not specific to ZIKV infection

To assess whether CC071 impairment of type I IFN induction resulted from a constitutive defect or from a specific virus-host interaction, we transfected MEFs with synthetic RNAs signaling through the same signaling pathways as ZIKV and measured the induction of *Ifnb1* expression. After transfection with poly(I:C), a TLR3 agonist, *Ifnb1* expression was delayed and less persistent in CC071 compared with C57BL/6J and CC001 MEFs (FIG 8A), whereas it was similar in all strains after transfection with 3p-hpRNA, a RIG-I agonist (FIG 8B).

These results suggest that the defect in type I IFN induction in CC071 MEFs is not specific to ZIKV infection (e.g. through the interaction with a viral protein) but rather point at a constitutive deficiency.

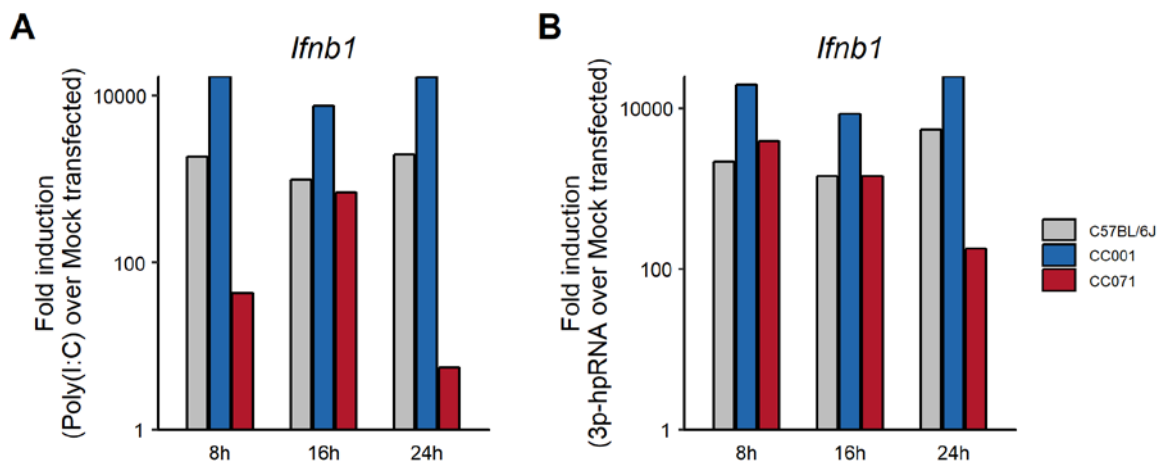


FIG 8 The induction of *Ifnb1* expression is delayed after specific-stimulation in CC071 MEFs. MEFs were transfected with either poly(I:C), a TLR3 agonist (A), or 3p-hpRNA, a RIG-I agonist (B), and the expression of *Ifnb1* gene was measured by RT-qPCR at 8, 16 and 24 hours by normalizing to *Tbp* housekeeping gene. Results are expressed as fold induction relative to the mock transfected condition.

Haplotype analysis of CC071 MEFs suggests defective interactions in the *Ifnb1* induction pathway

To determine which components of the *Ifnb1* induction pathway could be responsible for the CC071 phenotype, we compared the parental origin of 10 key player genes of this pathway (such as *Tbk1* or *Irf3*) in CC071 with that of 10 other CC strains which share one or the other of the CC071 allele (in green in TABLE 1). For each gene, we could match CC071 haplotype with at least one other CC strain, and yet none of these other CC strains reproduced CC071 phenotype (FIG 1). Additionally, no private variant in those genes was reported in the genomic sequence of CC071 (Srivastava *et al.* 2017). Together, these results suggest that the susceptibility to ZIKV in CC071 is not due to a single-gene deficiency but may rather result from defective interactions between molecular partners.

Gene	CC071	CC001	CC005	CC011	CC026	CC061	CC021	CC006	CC025	CC039	CC060
<i>Ddx58</i> (RIG-I)	HH	BB	EE	GG	CC	CC	HH	AA	HH	AD	EE
<i>Dhx58</i> (LGP2)	FF	EE	DD	EE	HH	BB	FF	DD	FF	FF	EE
<i>Ifih1</i> (MDA5)	GG	AA	DD	AA	HH	BB	GG	BB	AA	BB	CC
<i>Tlr3</i>	AA	AA	BB	HH	CC	EE	EE	AA	BB	FF	CC
<i>Mavs</i>	CC	AA	DD	AA	HH	BB	GG	CC	DD	HH	DD
<i>Tank</i>	GG	AA	DD	AA	HH	BB	GG	BB	AA	BB	CC
<i>Ticam1</i>	CC	AA	EE	EE	BB	DD	CC	DD	HH	AA	EE
<i>Tbk1</i>	HH	DD	GG	AA	HH	BB	AA	FF	AA	GG	BB
<i>Irf3</i>	FF	FF	HH	BB	BB	DD	CC	DD	EE	HH	BB
<i>Irf7</i>	HH	BB	DD	DD	DD	FF	HH	EE	DD	HH	DD
<i>Ifnb1</i>	HH	EE	DD	CC	CC	FF	HH	BB	HH	CC	DE

TABLE 1 Comparison of founder haplotypes carried by CC071 and other CC strains for the genes involved in the induction of *Ifnb1* expression. Green cells indicate matched haplotypes between CC071 and another CC strain. CC founder strains: AA, A/J; BB, C57BL/6J; CC, 129S1/SvImJ; DD, NOD/ShiLtJ; EE, NZO/HILtJ; FF, CAST/EiJ; GG, PWK/PhJ; HH, WSB/EiJ.

DISCUSSION

We have previously reported a significant difference in ZIKV replication rate between MEFs derived from resistant CC001 and highly susceptible CC071 mice (Manet *et al.* 2019). In order to increase our understanding of the host genetic control of ZIKV cellular replication, we extended these results to a total of 11 genetically different CC strains. Similarly to our *in vivo* study (Manet *et al.* 2019), we observed a strong effect of host genetic background on ZIKV replication *in vitro* (FIG 1). Interestingly, CC071 was the only strain with increasing viral titer in MEFs supernatant from 48 to 72 hours p.i., reinforcing our hypothesis that this enhanced viral replication could contribute to the highly susceptible phenotype of CC071 mice to ZIKV infection *in vivo*. However, our data show that mouse *in vivo* peak plasma viral load did not correlate strictly with viral replication in MEFs. Indeed, CC060 MEFs sustain relatively high levels of viral replication (FIG 1) while CC060 mice displayed the lowest peak plasma viral load among 35 CC strains (Manet *et al.* 2019). Conversely, CC005 MEFs produced low viral titers while CC005 mice showed high peak plasma viral load. This partial decorrelation between susceptibility to ZIKV and *in vitro* viral replication has never been reported before. It could be detected only because multiple inbred strains with large genetic diversity were investigated.

To gain insight into the mechanisms of CC071 extreme susceptibility, we compared CC071 with the more resistant C57BL/6J and CC001. We could rule out differences in the early events of ZIKV infection, specifically viral binding to the cell surface and viral entry into the cell (FIG 2). Early viral replication was not different between CC071 and both C57BL/6J and CC001 MEFs. However, ZIKV replication was significantly increased in CC071 cells 48 and 72 hours p.i., probably resulting in the increased cell infection rate at these late time points. Gene expression analysis revealed a delayed and weak induction of the type I IFN response in CC071 MEFs after ZIKV infection or after the chemical stimulation of TLR3, a sensor of dsRNA critically involved in the detection of infection by RNA viruses. However, CC071 MEFs were able to respond to type I IFN stimulation and to induce

the expression of ISGs. The comparison of CC071 with other CC strains sharing the same allele at major elements of the signaling cascade from TLR3 activation to *Ifnb1* induction did not allow us to identify one of these elements as responsible for the CC071 phenotype. This is in sharp contrast with other susceptible mouse models resulting from genetically engineered single-gene loss-of-function mutations (Manet *et al.* 2018). This leads us to hypothesize that the defective activation of *Ifnb1* could result from altered interactions between different components inherited from evolutionary distant parental strains (e.g. classical laboratory strain vs wild-derived strain). Further molecular studies will be required to characterize the precise molecular defect, in particular the phosphorylation and translocation to the nucleus of the transcription factor IRF3. IRF3 is a key transcription factor for the induction of type I IFN. It also directly modulates the expression of antiviral genes referred to as viral-stimulated genes (VSGs) (Green *et al.* 2018). We can speculate that in case of a deficiency in IRF3 signaling, some of these VSGs could be expressed with the same delay as *Ifnb1* in CC071 MEFs and could thus increase mouse susceptibility to ZIKV infection *in vivo*. We previously showed that CC071 mice have an increased susceptibility to several flaviviruses among which ZIKV, DENV and WNV (Manet *et al.* 2019). According to the results of the present study, we could also speculate that CC071 mice could be more susceptible to other viruses signaling through the same pathways.

Whatever the mechanism, the delayed and weak type I IFN response in CC071 provides a unique resource to study this critical component of innate immune response. The type I interferon (IFN) signaling pathway constitutes an early defense against viral infections and the kinetics of this response are often critical for the fast and efficient control of viral replication and dissemination (Fensterl *et al.* 2015; McNab *et al.* 2015). For example, the importance of a rapid IFN response has been demonstrated in mouse astrocytes infected with Tick-borne encephalitis virus (TBEV), which are able to control viral replication and infection in contrast to astrocytes derived from *Ifnar1*-deficient mice (Lindqvist *et al.* 2016). Unlike complete loss-of-function mutations of the *Ifna* or *Ifnb1* genes, CC071 exhibit a hypomorphic phenotype which may open the way to identifying compensatory mechanisms and pathways amenable to pharmacological modulation.

At the opposite of CC071, CC001 MEFs reacted with a very rapid and intense activation of *Ifnb1*, high viral replication early after infection as well as an elevated infection rate between 16 and 24 hours p.i. While the outcome of ZIKV infection in MEFs from CC001 and C57BL/6J was similar in terms of viral particles production at 72 hours p.i., CC001 and C57BL/6J infection profiles were quite different in the early time points and suggest a differential regulation of host responses to ZIKV infection between these two strains which both represent valuable models to study how cells are able to control viral replication. Crosses between resistant and susceptible strains like CC001 and CC071 could provide an efficient approach to dissect the complexity of host genetic control of ZIKV infection.

On-going experiments and future directions

Future work will aim at characterizing the molecular mechanisms leading to a delayed type I IFN response in CC071 MEFs. While the number of molecular players is relatively small in the targeted pathway, CC071 phenotype could result from deficiencies at different levels including transcriptional regulation and chromatin accessibility, translation and post-translational modifications or protein interactions, and combining various approaches will be necessary to dissect this complex network.

First, we will proceed to a deeper analysis of our transcriptomic data by looking at expression levels of different transcripts of the same gene between mouse strains, which could lead to the identification of splicing events affecting the downstream protein. In addition, in the present study transcripts were mapped to the C57BL/6J mouse reference genome, thus our analysis does not allow for discovery of new genes that could be present in CC001 or CC071 strains. Recent *de novo* genome assemblies for sixteen mouse inbred strains revealed that genes related to immunological processes, especially involved in innate immune responses, exhibit huge diversity among strains (Lilue *et al.* 2018). Aligning reads from our RNA sequencing experiment on CC001 and CC071 genomes might actually lead to new interesting findings.

A second approach will be to investigate protein levels and activation in mock and ZIKV-infected MEFs between strains. Several of the proteins leading to the induction of type I IFNs, such as IRF3, are basally expressed in cells but need to be phosphorylated to be activated (Schneider *et al.* 2014), which will be evaluated by immunofluorescence and Western Blot analyses. In addition, co-immunoprecipitation experiments could be performed to identify differences in protein-protein interactions between strains. Protein pull-down assays in CC001 and CC071 strains could also allow for the discovery of new interacting partners that would not be present or involved in the reference C57BL/6J strain. Finally, the evaluation of the binding of transcription factors could be evaluated by chromatin immunoprecipitation.

A third, complementary approach, will be to produce MEFs with various combinations of CC071 and CC001 alleles at the genes involved in the *Ifnb1* activation pathway, to assess their phenotype following ZIKV infection and to map the CC071 alleles associated with impaired *Ifnb1* activation. These MEFs will be produced from the progeny of a (CC001 x CC071)F1 x CC071 backcross.

Finally, studying CC071 phenotype in other cell types, such as immune or neuronal cells, will provide valuable information relative to ZIKV pathogenesis.

5.4. Influence of host genetic factors on ZIKV-induced type I IFN response in mouse primary neurons

We previously showed that ZIKV induces type I IFN response in MEFs, which is critical for the control of viral replication and infection. As ZIKV has the specificity of targeting neural cells, the team of Eliette Bonnefoy and our laboratory got interested in ZIKV-induced type I IFN response in neurons.

While type I IFNs are often necessary to restrict a viral infection, a finely tuned control of this innate immune response is required to avoid potential pathogenic effects. In the brain, abnormal induction of the type I IFN response has been associated with microcephaly, cognitive disorders and neurodegenerative diseases linked to pathological forms of the Tau protein (a major axonal component of neuronal cells) (Baruch *et al.* 2014; Crow and Manel 2015). We therefore sought to characterize the type I IFN response in mouse primary neurons infected with ZIKV by comparison with MEFs and by investigating the influence of host genetic factors.

5.4.1. ZIKV-induced type I IFN response is delayed in neurons compared with MEFs

We first compared the type I IFN response induced by ZIKV in MEFs and primary neurons isolated from C57BL/6J mice. Both cell types are obtained from mouse embryos. However, while MEFs can be frozen and cultured for multiple experiments, primary neurons are prepared freshly for each experiment from the dissected brain of E16.5 embryos. The expression of a dozen genes of the type I IFN pathway, and of ZIKV genome, was measured by RT-qPCR after purification of total RNA from infected and non-infected MEFs and primary neurons (three independent cultures).

The expression of *Ifnb1* and *Ifna4* was detected as soon as 26 hours p.i. in MEFs, reached its maximum level at 32 hours p.i. before decreasing from 48 to 72 hours p.i (Figure 36). In contrast, these two genes started to be expressed only from 48 hours after infection in primary neurons. These results indicate that the type I IFN response is delayed in primary neurons compared to MEFs. Consistent with these findings, the expression rates of other genes of the IFN pathway followed the kinetics profile of the type I IFNs expression. For example, the expression of *Irf7* was induced by ZIKV infection in MEFs from 32 hours p.i. whereas it increased in primary neurons between 48 and 64 hours p.i. (Figure 36). The same trend was observed for the *Isg15* and *Ddx58* (RIG-I) genes. Interestingly, while the expression of type I IFNs was delayed in primary neurons, it resulted in a delayed but stronger activation of *Stat1* and *Stat2* and certain ISGs such as *Oas1b* and *Eif2ak2* (PKR) in neurons compared with MEFs (Figure 36).

We observed that these differences in the kinetics of the IFN response between MEFs and neurons correlated with variations in viral replication. The number of ZIKV genome copies increased in MEFs from 26 to 32 hours p.i. and started declining from 48 to 72 hours p.i.; whereas it kept increasing between 17 and 72 hours p.i. in primary neurons, reaching much higher levels at late time points of infection (Figure 36). Finally, *Tnf* and *Il3* genes, encoding two inflammatory cytokines, were

expressed in MEFs but only *Tnf* expression was induced by ZIKV infection. By contrast, in primary neurons, *Tnf* was not detected but *Il3* was expressed at higher basal level compared with MEFs, though without induction by ZIKV infection (Figure 36). Overall, these results reveal a delayed type I IFN response, associated with an ineffective control of viral replication in neurons compared with MEFs.

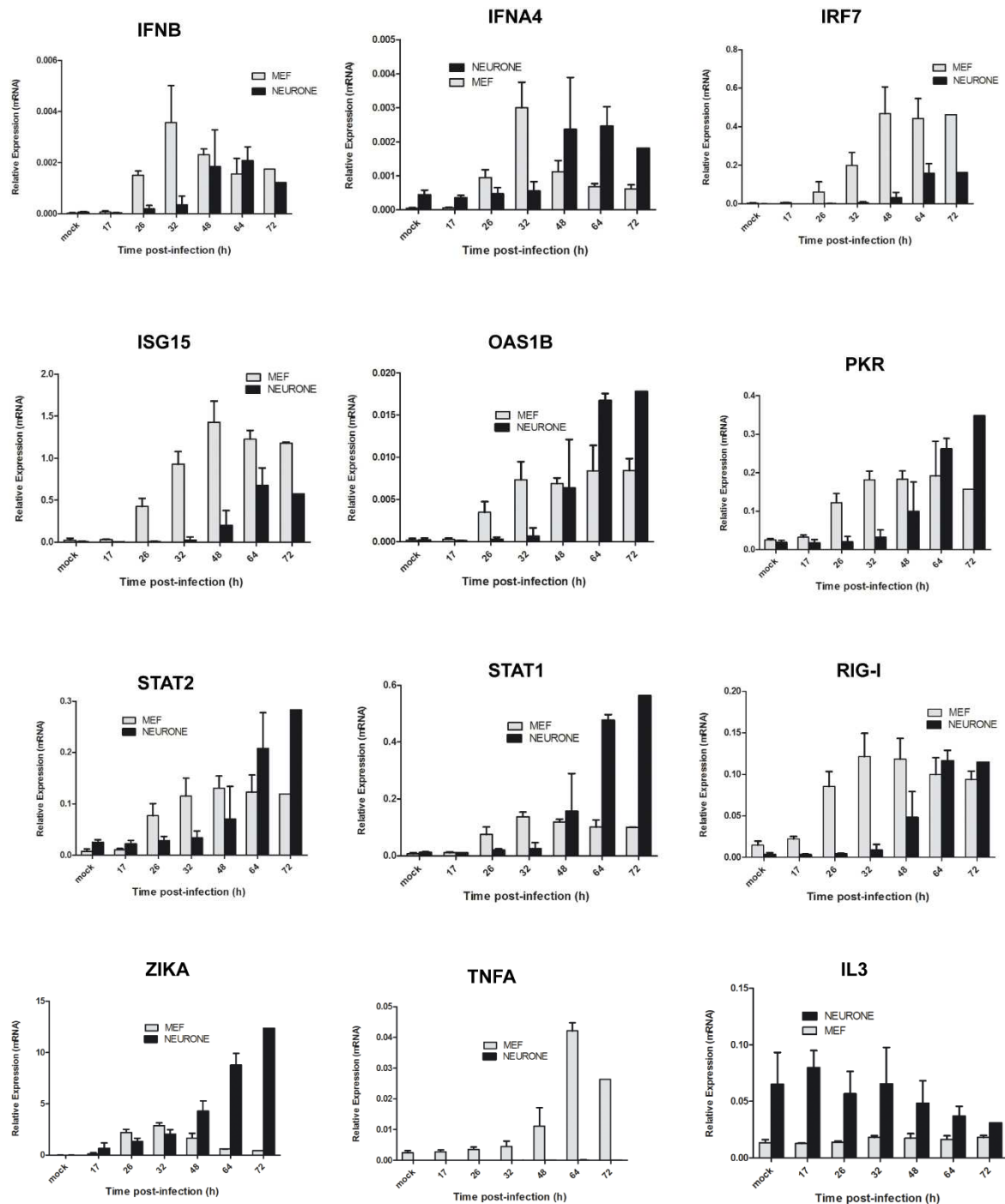


Figure 36. ZIKV-induced type I IFN response is delayed in C57BL/6J primary neurons compared to MEFs.

Gene expression was measured by RT-qPCR as described in the material and methods chapter. The relative level of mRNA was normalized to *Ribosomal protein large P0 (Rplp0)* endogenous control gene and plotted ($2^{-\Delta C_t}$). Results are mean \pm SEM from 3 independent experiments.

5.4.2. Host genetic determinants control ZIKV replication and induction of the type I IFN response in primary neurons derived from Collaborative Cross mice

We previously showed that host genetic factors have a strong influence on ZIKV replication in infected MEFs derived from CC mice. We specifically showed that a functional but delayed induction of the type I IFN response led to an increase of ZIKV replication in MEFs from susceptible CC071 mice.

We evaluated whether similar differences were conserved among cell types, and especially in ZIKV target cells such as neurons or neural progenitors. Thanks to our previous experience with MEFs and primary neurons derived from C57BL/6J, we characterized the influence of host genetic determinants on ZIKV replication and induction of type I IFN response in primary neurons from CC001 and CC071 mice using the same set of gene expression assays as previously. Notably, the two previous studies (parts 5.1 and 5.2) highlighted similar association between a delayed induction of type I IFNs and a lack of control of ZIKV replication, both between CC001 and CC071 MEFs and between C57BL/6J MEFs and primary neurons.

Interestingly, our preliminary results show that ZIKV replication was enhanced in CC071 compared with CC001 primary neurons at late time points of infection, similarly to what we showed in MEFs (Figure 37). In parallel, the expression kinetics of type I IFN genes in CC001 neurons was comparable with that of C57BL/6J neurons; however it was markedly different in CC071 neurons. Indeed, the expression of *Ifnb1* increased only from 64 hours p.i. and reached much higher levels at 72 hours p.i. in CC071 compared with CC001 and even C57BL/6J neurons. Expression of ISGs was consistent with these findings and were equally delayed in CC071 neurons. High expression levels of *Oas1b* and *Eif2ak2* (PKR) genes 72 hours p.i. were also consistent with a late but elevated expression of *Ifnb1* in CC071 neurons (Figure 37).

Both *Tnf* and *Il3* were expressed at low levels though both genes tended to have a higher basal expression in CC071 than in CC001 neurons. ZIKV infection did not seem to induce *Tnf* and *Il3* expression in neurons from CC001 and CC071, as observed in C57BL/6J neurons (Figure 37).

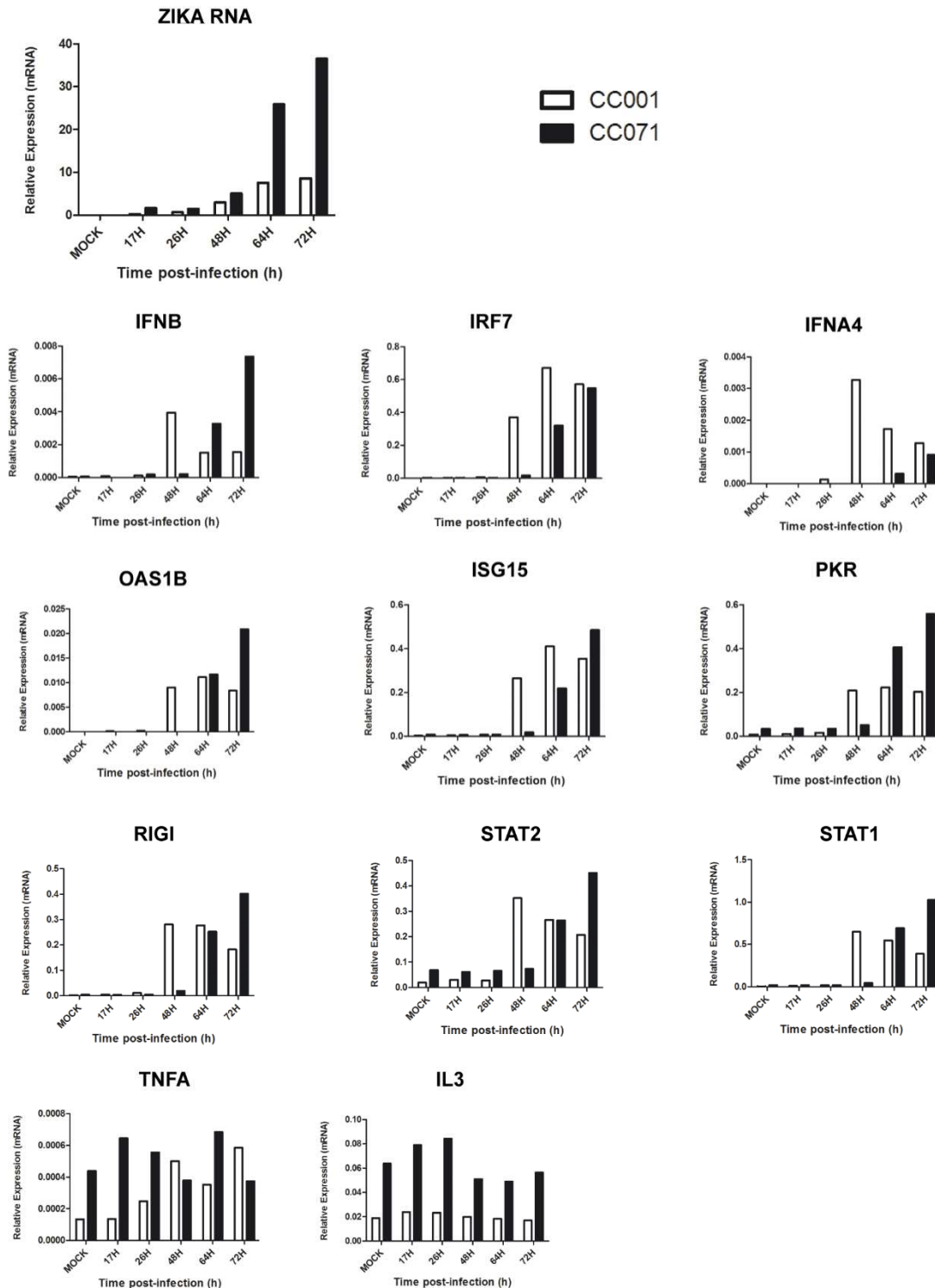


Figure 37. ZIKV-induced type I IFN response is delayed in mouse primary neurons derived from CC071 compared to CC001.

Gene expression was measured by RT-qPCR as described in the material and methods chapter. The relative level of mRNA was normalized to *Ribosomal protein large P0 (Rplp0)* endogenous control gene and plotted ($2^{-\Delta C_t}$). Results show data from one experiment.

5.4.3. Discussion

In this study, we report that ZIKV is able to infect both MEFs and mouse primary neurons though viral replication is stopped in MEFs but not in neurons, which accumulated Zika viral RNA until 72 hours p.i. We showed that the expression of *Ifnb1* gene and several ISGs, which partly constitute the type I IFN response, was delayed in neurons compared with MEFs. Finally, we highlighted the influence of host genetic background on the susceptibility of mouse primary neurons to ZIKV infection and showed that CC071 neurons displayed an extremely delayed but enhanced type I IFN response compared to C57BL/6J and CC001 neurons.

While a delayed type I IFN response can impair the ability of neurons to stop viral replication, a persistent late response can generate negative side effects, which could contribute to the development of neuropathological disorders (Baruch *et al.* 2014; Crow and Manel 2015). Parallel to a delayed type I IFN response, a strong and persistent expression of several ISGs was observed in primary neurons at late time points of infection compared with MEFs (Figure 36). One of these late up-regulated genes codes for PKR, a ds-RNA-dependent kinase known to inhibit translation and to activate NF-KB transcription factor. Aberrant activation of PKR has been associated with Alzheimer's disease (Ohno 2014) and more specifically with an increase of the activation of kinase GSK3b (Bose *et al.* 2011), which consecutively regulates β -catenin degradation and Tau phosphorylation.

We have obtained preliminary results indicating that ZIKV-infected neurons, but not MEFs, displayed increased levels of phosphorylated GSK3b protein associated with decreased levels of β -catenin protein (data not shown). These results also showed an enhancement of Tau protein phosphorylation alongside with a re-localization of total Tau protein from the axon to the dendrites (data not shown). As phosphorylated GSK3b kinase is one of the major protein responsible for the pathological phosphorylation of Tau protein (Krishnankutty *et al.* 2017), these results suggest that a persistent type I IFN response, such as the one induced by ZIKV in neurons, can lead to cellular damages associated with neurodegenerative disorders.

Interestingly, we have shown that ZIKV induces an extremely late, but enhanced, type I IFN response in CC071 primary neurons, with high levels of PKR gene expression (Figure 37). Future work will first aim at replicating the preliminary results obtained on CC001 and CC071 neurons, and then at investigating the levels of GSK3b and Tau proteins phosphorylation in CC071 strain and at confirming the pathological effects of the type I IFN response in neurons with a different genetic background.

Overall, this study will increase our understanding of how neurons respond to ZIKV infection, will provide novel data on ZIKV-induced neuropathologies and will describe new mouse models for the study of ZIKV neuropathogenesis.

6. DISCUSSION

When I started my PhD project, I was expecting to learn a lot about Zika, about genetics and about mice; and I was hoping to discover genes of susceptibility to ZIKV that would be useful in the fight against the disease in humans.

I did learn a lot about Zika, genetics and mice but I also learned a great deal about “Science”, some of its goals, methodologies and issues. My work on this project has generated valuable results and has also been an opportunity for reflection and personal advancement. Beyond further discussing my own results, I wish to illustrate these various aspects of my PhD through this general discussion, diving into the biology of ZIKV while taking a step back to get a bigger picture.

6.1. Host genetic diversity to learn about ZIKV pathogenesis

Zika virus and host genetics are at the center of my PhD project. So far, the intersection between these two fields of research remained poorly characterized as for its tangible nature, its biology and its significance. These knowledge gaps translated into several essential questions, which laid the ground for my PhD project:

- What is the importance of studying host genetics of susceptibility to ZIKV and what would be the benefits for humans?
- Which approaches can be used to efficiently study the influence of host genetics on the susceptibility to ZIKV infection?

These questions have been partially addressed in the main introduction and will be further discussed hereafter, with regards to the results that I have obtained in my PhD project.

6.1.1. Using genetically diverse mice to study host genetic control of susceptibility to ZIKV infection

As previously mentioned, different approaches have been used to study host genetics in mice, from the development of inbred and RI strains, transgenic mice, ENU-mutagenesis to GRPs. The mouse genetic diversity encompassed in these various tools can be exploited in many different ways, beyond and independently from the purpose of identifying QTLs. These mouse populations constitute homogeneous, genetically well-defined, permanent, shared and reproducible resources, which are instrumental to investigate the effects of host genetic diversity on complex, multifaceted diseases, to identify new models through new phenotypes and to explore pathophysiological mechanisms.

In particular, new advanced high-diversity mouse populations like the CC, the DO and their founder strains, provide a powerful platform to study complex diseases such as infections. As

proposed by Saul *et al.*, four modes of complex trait analysis can be used to investigate host genetics factors: (i) determination of trait heritability; (ii) correlation studies across traits; (iii) identification of strains with phenotypes of interest for focused mechanistic studies; and (iv) genetic mapping of complex traits (Saul *et al.* 2019).

Using both genetically-engineered *Ifnar1*^{-/-} mice and the CC has been fruitful for the study of ZIKV infection in my project. The results that I have obtained are discussed hereafter, both in the view of biological significance and as an illustration of the diverse methodological uses of mouse genetic diversity, their advantages and limits.

6.1.1.1. Phenotypic variations and correlations

One of the first findings of my studies was to reveal the phenotypic diversity of responses to ZIKV infection between genetically different mouse strains. While most studies describing potential animal models of ZIKV disease focused mainly on mouse immune status (Gorman *et al.* 2018; Lazear *et al.* 2016; Rossi *et al.* 2016; Tripathi *et al.* 2017; Winkler *et al.* 2017a), very few investigated the effect of whole-genome variants on the susceptibility to this virus (Snyder-Keller *et al.* 2019).

And yet, we found major differences in the susceptibility of two extensively used mouse models of ZIKV infection, the *Ifnar1*-deficient mice on B6 and 129 backgrounds. However, until now host genetic background was rarely mentioned as a potential variable in ZIKV studies (Dowall *et al.* 2017; Lazear *et al.* 2016) or in reviews on ZIKV animal models (Morrison and Diamond 2017; Winkler and Peterson 2017), which only alluded to variations in viral strain, dose and route of inoculation as well as the age of mice. Indeed, direct comparison of *Ifnar1*^{-/-} strain effect on response to ZIKV infection was hampered by the use of various experimental conditions between studies (Table 5). Our results demonstrate the major influence of mouse genetic background on complex diseases such as ZIKV infection. Our findings highlight the importance of describing precisely the mouse strain used in a study, which is not always perfectly implemented to date (Kamiyama *et al.* 2017; Liu *et al.* 2017). Additionally, our results are particularly important and relevant for all researchers using *Ifnar1*-deficient strains, especially in the context of studying viral infections, and attention should be drawn to the choice of the model and the interpretation of the findings.

Following on these results, we uncovered broad phenotypic diversity among strains of the CC after infection with ZIKV compared to similar mouse models described so far, notably C57BL/6J mice treated with the MAR1-5A3 anti-IFNAR mAb (Lazear *et al.* 2016; Smith *et al.* 2017; Zhao *et al.* 2016). Lazear *et al.* found no clinical disease or mortality in C57BL/6J treated with the anti-IFNAR mAb and infected with a recent ZIKV isolate of the Asian lineage (Lazear *et al.* 2016). In contrast, Smith *et al.* and Zhao *et al.* reported significant morbidity and mortality in those mice infected with ZIKV strains of the African lineage (Smith *et al.* 2017; Zhao *et al.* 2016). These studies suggested that the virus strain (African vs. Asian lineage) can affect susceptibility to infection in this model and highlighted the importance of replicating results with more than a single strain of ZIKV. In our study, we used a low-passage strain derived from a 2015 case of French Guyana (FG15) to increase relevance to human studies and confirmed our results using a strain of the African lineage. Infection with ZIKV FG15 of CC mice pre-treated with a single dose of MAR1-5A3 mAb resulted in moderate to very high levels of viral RNA in the blood, symptomatic ZIKV in a small proportion of CC strains

and mortality only in CC071. We reinforced our results on a few CC strains using a highly-virulent strain of the African lineage and confirmed the viral strain effect on susceptibility to ZIKV disease. But interestingly, we identified CC071 as a susceptible strain to both Asian and African strains of ZIKV, and at the opposite, CC001 as a resistant strain to both viral strains. While Smith *et al.* concluded from the 3 previous studies that lethality in MAR1-5A3 treated mice was essentially driven by the viral strain effect (Smith *et al.* 2017), we can now add that morbidity and mortality result from more complex interactions between viral parameters (strain, dose, route), mouse age and host genetic background. Finally, we also revealed major differences in ZIKV-induced brain lesions in our CC model, with pronounced neuroinflammation in CC005 and CC071 compared with CC001 mice. Unexpectedly, moderate to severe lesions were identified in the brain of CC005 mice infected with Asian or African ZIKV (data not shown) respectively, whereas CC005 did not display signs of disease or lethality. Smith *et al.* reported encephalitis lesions in their model and hypothesized that those pathologic changes could contribute to mouse morbidity and mortality (Smith *et al.* 2017). While this hypothesis remains valid, our results indicate that other factors could act jointly or independently to trigger mouse disease and death following ZIKV infection.

Comparison of models relying on *Ifnar1*^{-/-} mice and on wild-type mice treated with an anti-IFNAR mAb has been discussed in previous studies. Many researchers agree that *Ifnar1*^{-/-} mice are valuable models to study ZIKV basic pathogenesis but do not constitute an appropriate model for testing vaccines efficacy considering their deficient type I IFN response that can also impact B and T-cell priming and the adaptive immune response (Alves Dos Santos and Fink 2018; Lazear *et al.* 2016; Morrison and Diamond 2017; Smith *et al.* 2017). The MAR1-5A3-treated mouse model was already proposed as a potent alternative to *Ifnar1*-deficient mice as it allows for the induction of native immune responses (Lazear *et al.* 2016). Our results could expand the use of this model as we have identified a few strains displaying clinical ZIKV disease, which could represent an easy way of monitoring vaccine or treatment protective effect.

Like in many studies using the CC (Graham *et al.* 2015; Gralinski *et al.* 2015; Rasmussen *et al.* 2014; Zhang *et al.* 2018), the promise of broad phenotypic diversity was fulfilled in our project thanks to the millions of divergent SNPs segregating in this mouse population. Our CC screen allowed us to identify strains with extreme phenotype, which were not previously described, and which we used for further functional studies as discussed in the next paragraph. But every coin has two sides and while the CC exhibits phenotypic variations which better mimics the variations observed in human populations, its genetic diversity may impair the efficacy of some reagents commonly used in classical laboratory mice (Noll *et al.* 2019). In our study, validation of the efficacy of the MAR1-5A3 anti-IFNAR mAb was critical and allowed us to confidently interpret our results.

In addition to the discovery of many new phenotypes, the CC is an unequalled tool to perform correlations studies between traits. Indeed, the CC is a permanent collection of RI strains of genetically identical individuals and thus allows to produce and analyze data obtained from multiple mice from the same strains and from different laboratories (Noll *et al.* 2019; Saul *et al.* 2019). Comparison between traits allows for the identification of common mechanisms controlling correlated traits, as well as distinct mechanisms controlling dissociated phenotypes. For example, Ferris *et al.* reported reduced correlations between IAV titer and lung pathology, between IAV titer and

inflammation, and between clinical disease and pathology in pre-CC strains while those traits were correlated in the CC founder strains. These results brought new insight into IAV pathogenesis by dissociating phenotypes and allowed the authors to assess the relative contribution of inflammation and viral replication on disease outcome (Ferris *et al.* 2013). In our study, we showed that Zika plasma viral loads at days 2 and 6 p.i. were only moderately correlated and that clinical severity did not correlate with the intensity of brain histological lesions and neuroinflammation. These results allowed us to dissociate between phenotypic traits that were previously thought to be correlated and therefore provided evidence for distinct mechanisms and genetic determinants.

6.1.1.2. Functional studies on strains with extreme phenotypes

Studies using the CC often report considerable phenotypic diversity and thus lead to the identification of strains of interest with extreme phenotype or disease-specific characteristics. These exceptional strains can be used as new experimental models to study the pathogenesis of a complex disease. For instance, CC011 mice spontaneously develop a chronic proliferative colitis from the age of 20 weeks and have been characterized as a new model of human inflammatory bowel disease (Rogala *et al.* 2014). In another example, CC(032x013)F1 hybrid males have been shown to display weight loss, with prolonged virus detection in the brain and neuropathology after WNV infection. These mice are in fact able to control early infection in the spleen but sustain brain viral load for at least 2 months and thus constitute a new model of chronic WNV infection which was not previously observed in any other mouse strain (Graham *et al.* 2016).

In our study, we chose three strains exhibiting contrasted clinical and virological phenotypes to investigate ZIKV-induced brain pathology. We observed very different histopathological profiles of the brain in CC001, CC005 and CC071 strains. Moreover, by comparing systemic and intracranial infection routes, we could characterize the pathogenic effects of ZIKV on brain tissue, independently from systemic immune responses. Furthermore, we identified CC071 as a susceptible strain to several flaviviruses; this mouse strain could become a new model to investigate shared mechanisms of susceptibility to various viruses.

Functional studies can interrogate different levels of complexity, from molecular and cellular mechanisms to disease pathogenesis at the level of the whole organism. Investigating ZIKV infection in cells derived from the most susceptible strain, CC071, we demonstrated that a fast-acting IFN response is required for an efficient control of viral replication and infection whereas a late persistent IFN response could trigger deleterious consequences in neural cells in particular.

Correlation studies in CC strains can reveal traits dissociations and thus identify multivariate outlier strains (Saul *et al.* 2019). Each of these strains can be used as a new experimental model to study a specific phenotype and its mechanisms and thus multiplies the opportunities to discover variations in disease-relevant genes, pathways or traits.

6.1.1.3. Identification of genetic variants

While it has become only one of their purposes, GRPs were initially developed for the genetic mapping of new markers and complex traits (Taylor 1978; Williams and Auwerx 2015).

Having identified a sharp difference in susceptibility to ZIKV disease between B6 and 129-*Ifnar1* mice, we used a classical F2 intercross to identify modifier genes in *Ifnar1*-deficient mice. This approach was successful and allowed us to identify two loci controlling the susceptibility to ZIKV though with limitations inherent to the F2 intercross mapping strategy (Flint and Eskin 2012; Mott and Flint 2013). Indeed, the identified QTLs consisted in large chromosomal segments containing hundreds of genes. Our results nevertheless provide a strong evidence that genetic variants in these QTLs determine the response to ZIKV infection, but much remains to be done to identify solid candidate genes. While bioinformatics can help narrowing down these gene lists, additional experiments will be required to refine or validate candidate genes such as gene expression and functional analyses.

We used CC mice to map QTLs driving the susceptibility to ZIKV infection. The estimated 80% heritability of ZIKV plasma viral load, indicates a strong contribution of host genetics in the phenotypic variance of this trait and was a first encouraging step towards QTL mapping. However, using 35 different CC strains, our genetic analysis failed to identify QTLs reaching genome-wide significance threshold for the three tested traits.

The inability to detect QTLs driving susceptibility to ZIKV in CC mice could have several causes. First, the number of strains could be insufficient. A recent study determined that with 35 CC strains and an average of 5 mice per strain, we had 80% power of detecting a bi-allelic QTL explaining 30% or more of the phenotypic variance (Keele *et al.* 2019). However, while only one study reported significant QTLs controlling susceptibility to *Salmonella* Typhimurium infection using 35 CC strains (Zhang *et al.* 2018) all other studies on infectious diseases have detected QTLs using a much higher number of strains (pre-CC strains usually) (Durrant *et al.* 2011; Ferris *et al.* 2013; Gralinski *et al.* 2015; Vered *et al.* 2014). It is also possible that the outstanding phenotype of CC071 is controlled by genes which do not contribute to the rest of the phenotypic variation, introducing genetic heterogeneity which reduces QTL mapping power. An example is strain CC042 which displays extreme susceptibility to *Salmonella* Typhimurium under the control of at least two QTLs (Zhang *et al.* 2019) which were not detected when QTL mapping was performed on all CC strains together (Zhang *et al.* 2018). Thus, dissecting the genetic architecture of susceptibility to ZIKV in outlier strains like CC071 or CC001 will require complementary strategies, such as intercrosses (Noll *et al.* 2019). This strategy has several advantages: (i) reducing the genetic complexity (from 8 to 2 parental strains); (ii) improving the QTL mapping resolution using large numbers of F2 mice; and (iii) determining the mode of inheritance (dominant or recessive) of susceptibility alleles.

In addition to genetic mapping difficulties, we must also consider the restrictions of using mice to study ZIKV infection. As immunocompetent mice are refractory to ZIKV productive infection, the type I IFN response must be inhibited at least temporarily to allow for viral replication. While using the MAR1-5A3 mAb in targeted F2 crosses could be an efficient strategy, the cost of the reagent is certainly dissuasive. We have chosen a different approach and decided to use *Ifnar1*-deficient mice in two different intercrosses, the first between B6-*Ifnar1* and CC001 strains and the second between 129-*Ifnar1* and CC071 strains. *Ifnar1* homozygous F2 mice will be challenged and genotyped to achieve QTL mapping.

6.1.2. Benefits and applications for human studies

The results obtained throughout my PhD project have multiple benefits and applications for human studies on ZIKV and other flaviviruses.

We have described several new mouse strains as potential new models to investigate ZIKV pathogenesis. Investigating the genetic diversity of a large number of CC strains has significantly extended the range of phenotypes induced by ZIKV infection in mice and better model the clinical heterogeneity of human cases. Besides, correlation studies between phenotypic traits are not possible in human studies, many phenotypic traits are not accessible in patients and controls, and studies on genetically different individuals are often confounded by many factors. These new models thus better mimic the complexity observed in human populations while allowing for experimental control of most infection-related parameters and for dissection of inter-connected traits and mechanisms.

Moreover, data obtained from these new experimental models have increased our understanding on ZIKV pathogenesis. We have also identified potential candidate genes or pathways that modulate the susceptibility to ZIKV infection. Following on these leads, additional work could definitely yield more direct applications towards human studies. Strong candidates identified in our QTL intervals could be tested in human cohorts which are too small in size to provide the power required by GWAS. Our findings certainly open new avenues for research on ZIKV and other flaviviruses. Notably, the phenotypic variability of CC mice would allow studying the influence of host genetics on biological processes that are critical to understand ZIKV epidemiology and pathogenesis, such as viral transmission from the host to the vector, or the mechanisms of CZS in ZIKV-infected fetuses. CC mice also constitute an ideally suited platform to investigate shared and distinct mechanisms of susceptibility to various flaviviruses.

Finally, the description of common pathways or pathogenesis mechanisms between mice and humans could promote the discovery of new diagnostic biomarkers of disease severity, new host-directed drug targets and new strategies for vaccine development.

6.2. Host genetic diversity to improve animal models of infectious diseases

My PhD project required the use of mice as an experimental model to identify host susceptibility genes to ZIKV infection. Considering the high number of scientific publications relative to “mouse models of ZIKV infection” (Figure 38), it soon appeared to me as if the model was in itself a priority scientific goal. This ambiguity of the model being both the methodology and the purpose led me to question the definition of an experimental animal model, its implications and challenges; and to think about how my work on ZIKV put these notions in perspective.

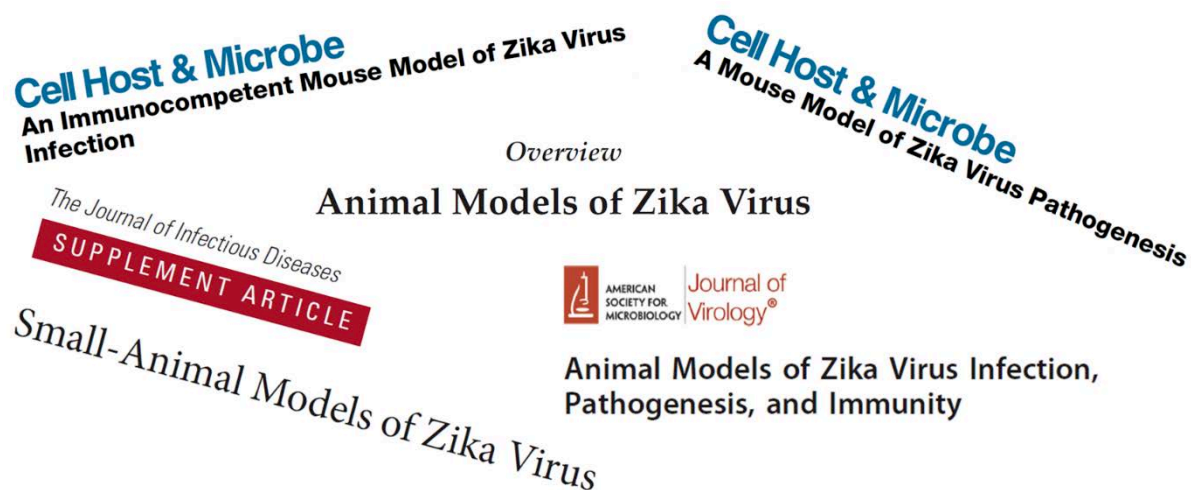


Figure 38. « Animal model of ZIKV infection », goal or method?

6.2.1. Are animal models relevant and required for research on human diseases?

Animals have long been used in biological research and in medicine to address various questions, from basic science to the development and assessment of vaccines and treatments. But there is concurrently an on-going debate in our societies over the validity and the requirement of animals as experimental models for human biology and medicine.

Animal models have made significant contributions to our understanding of fundamental biology and to the improvement of medicine over the past centuries. Most vaccines, which protect millions of people from fatal diseases, have been developed thanks to animal models. Insulin was used as a treatment for Type I diabetes for the first time in the dog in 1921 (Karamitsos 2011). Herceptin, a breast cancer drug that significantly reduces the death-rate in patients, was developed thanks to mice and macaques in the 1990's (Harries and Smith 2002). Many surgical and medical procedures have been designed and perfected in several animal species before being routinely used in humans, including blood transfusion, renal dialysis and organ transplant surgery. The vast majority of the Nobel Prizes awarded for Physiology or Medicine were dependent on research using animals,

including the prizes awarded in the past 30 years, highlighting the critical role of animal models in research.

Despite these major breakthroughs in medical research, it is equally true that studies performed in animal models are not always confirmed in human studies, and that the research findings from animal models cannot always be translated to humans (Hackam and Redelmeier 2006), especially for the development of new therapeutics. In addition to this low translation rate, animal models are now condemned by a few scientists for being liable for loss of opportunity in the field of new drug discovery (Akhtar 2015; Greek 2013).

Finally, the use of sentient animals in research for human purposes raises ethical issues and is often questioned by society. In the European Union, animal protection and welfare, especially in research, is strictly regulated, implemented and is under permanent consideration. I will not further discuss ethical aspects of animal welfare and protection in research in this discussion.

These conflicting reports led me to ask myself about the relevance and the requirement of animal models for research on human diseases.

Trying to answer this question, I wondered about the definition of what is an experimental model. For Gaston Bachelard, a scientific experimentation consists in provoking an observation with the goal of studying an event. In that purpose, scientists resort to instruments, which are to him, “only materialized theories” (*“les instruments ne sont que des théories matérialisées”*) (Bachelard 1934); meaning that the instrumentation, the model, is by itself an expression of a theory, a reflection of the scientific question. In line with this view, Georges Canguilhem later stated that “a model is nothing but its function” (*“un modèle n’est rien d’autre que sa fonction”*); meaning that the model gets its value only from its role in answering a question for which it was built (Canguilhem 1968). But from the first models in physics to the actual models in biology, the evolution of the experimental models has revealed the multiplicity behind the term “model” in science. Indeed, there have been several attempts of epistemologists to try to give a unique entity to the concept of experimental model in science, exercise which has proved so far very complex (Varenne 2008). In the meanwhile, some insights can be driven from the functions of the different types of models.

In the context of biology, it seems that we can draw two main functions of the animal models: (i) understand and explain a phenomenon, and (ii) predict the variations or the outcomes of a phenomenon. For example, in the first case, mice could be used as an experimental model to explain the neuropathogenesis of ZIKV while in the second case, mice could be used to predict the efficacy of a vaccine against ZIKV that will be later used in humans. In other words, we could use exploratory mouse models to study ZIKV infection or analogical mouse models of ZIKV infection. We can therefore distinguish the animal models used for fundamental research purposes from the animal models used as pre-clinical models for the evaluation of safety and efficacy of vaccines and therapeutics for human use. Interestingly, the expression “animal model of disease” is quite recent and started to be used commonly in the 1980’s, in the early days of animal transgenesis and when murine and human homologous mutations started to be characterized.

Consequently, the relevance of animal models to study human diseases appears tightly linked to their functions. In fundamental research, the relevance of an animal model is based on the adequacy

between the model and the scientific question. In other words, a potential inadequacy would indicate imperfections in the scientific conception or theory. In contrast, the relevance of pre-clinical animal models is based on the presumed similarity of responses between the model and the studied subject, in this case human beings. While the utility of animals used as experimental models to study the basic mechanisms of physiology and diseases is hardly ever questioned on the grounds of scientific relevance, part of the scientific community argues against the value of animals used as pre-clinical models because of the low translational rates in drug development (Greek 2013; Pound and Ritskes-Hoitinga 2018).

The use of animals in research has been justified partly by the fact that mammals share many biological and physiological features, and also because animals are affected by diseases such as those described in humans. These include common disorders such as diabetes, cancers, allergies but also infectious diseases as many pathogens target various host species. The disease etiology is often similar and so are the pathophysiological mechanisms, to such an extent that translational medicine is anchored in the daily veterinary practice: when a drug is not available for a given species, the law authorizes the veterinarian to use a drug developed for another species, including humans. Despite important similarities, there are genetic and environmental differences between humans and other mammals that result in physiological specificities. These differences, acknowledged by the scientific community, are better and better characterized; they are indeed at the center of the discipline of comparative medicine and, in this way, can lead to the discovery of novel pathogenic mechanisms and new therapies for humans and animals. These diverging characteristics also ground the argument that species differences render pre-clinical animal models invalid (Greek 2013; Pound and Ritskes-Hoitinga 2018).

It is indisputably true that mice, guinea pigs, macaques and any other animal species are no “experimental humans” and that only humans will develop human diseases. The species differences cannot be ignored or overcome; they must be better characterized and reduced when possible. In the case of ZIKV infection, a group has “humanized” the mouse STAT2 gene by genetic engineering to allow ZIKV to target STAT2 to degradation as in humans (Gorman *et al.* 2018). This model also required the use of a mouse-adapted ZIKV strain to achieve efficient viral replication. Several groups have developed “humanized mice” in which variable component of the mouse immune system have been replaced by their human counterparts (Di Santo and Apetrei 2017; Schmitt *et al.* 2018). Scientists who refute using pre-clinical animal models advocate for a “humanized” medicine through the use of “human-relevant” approaches (Greek 2013; Pound and Ritskes-Hoitinga 2018), including the use of human-based *in vitro* systems (human induced pluripotent stem cells (IPSCs), human “organs-on-chips” (Low and Tagle 2017)) and human trials for drug screening (Burt *et al.* 2016). While human experimentation raises serious ethical questions that need to be specifically and thoroughly addressed (Hill 2012), *in vitro* systems used as sole pre-clinical models generate data of limited value, just as animal models. Albeit not affected by species differences, they suffer from a strong discrepancy of entity; cells in culture, or even a micro-organ, cannot reproduce the integrated functioning of a whole living organism.

The search of a perfect pre-clinical model is, by nature, unfounded; a predictive model will always remain a model and, as good as it can be, will only provide a picture of the reality. It thus

appears to me that no reliable and valuable pre-clinical model should be ruled out, whether they are new exciting *in vitro* technologies or classical animal models.

In summary, in the absence of alternative methods, animal studies will remain essential to study human diseases, in basic science as well as in pre-clinical trials. In addition, with a precise characterization of the model, understanding of the validity issues, and proper critical interpretation of the results, relevance can be achieved. For that purpose, animal models have to be continuously improved.

6.2.2. How to improve animal models to study infectious diseases, notably ZIKV infection?

One way to improve the reliability of animal studies in biology and medicine is to systematically comply with high-standard methods, such as those employed in clinical trials (randomization, blinding, sample size calculation, eligibility and exclusion criteria, statistical methods, quality control etc.), in order to reduce the sources of bias (van der Worp *et al.* 2010).

Another possible improvement would be to further the analogy between the model and the human disease condition, taking into account the species differences. Indeed, many animal models only partially mimic the disease phenotype that is described in humans. The first reason is somehow historical and methodological. As I mentioned earlier, the use of the expression “animal model of human disease” is quite recent and became common after the characterization of murine and human homologous mutations. With the creation of many mouse strains carrying specific mutations, many new models have been defined primarily by their genetic characterization (e.g. a common mutation in a mouse strain and in human patients) and secondly by their phenotypes. This has led to a kind of abuse of the expression “mouse model of human disease” by academic scientists, suggesting a perfect similarity while it was often constructed as a purely experimental model, therefore frequently resulting in incomplete analogy between the model and the human condition. Precise and comprehensive phenotypic and mechanistic characterization is required to establish homologies between a mouse model and a human disease, rather than referring only to common molecular determinism.

The second reason is inherent to genetic and phenotypic variations within a given species. Laboratory mice have been established as highly homogeneous, inbred, strains, in which mice are homozygous at every locus of their genome. Most studies using mouse models usually refer to results obtained in a single mouse strain, i.e. in a single genetic background. On the other side, the genetic diversity in the human population is broad, and often leads, in combination with non-genetic factors, to polymorphic disease manifestations among patients. ZIKV infection is typically a polymorphic disease. While the majority of infected people do not display any signs of disease, some will present with a combination of flu-like symptoms, and finally a minority of individuals could suffer from severe complications of many kinds, such as uveitis, GBS, encephalitis or facial palsy (Kodati *et al.* 2017; Lannuzel *et al.* 2019; Munoz *et al.* 2017). One cannot expect to recapitulate all these variations in one single inbred strain of genetically identical mice.

Indeed, using a single mouse inbred strain to model complex infectious diseases very often results in a partial, not entirely satisfactory, model. For example, as mentioned previously, classical

mouse laboratory strains, such as BALB/c, do not fully recapitulate the features of EBOV infection in humans. While a mouse-adapted EBOV induces severe liver injury and lethal disease in those mice, the hallmarks of hemorrhagic syndrome (coagulopathies, vascular damages etc.) are not all observed in this model (Rasmussen *et al.* 2014). In another example, C57BL/6J mice do not capture certain clinical outcomes of WNV infection observed in humans. Indeed, people infected with WNV can develop encephalitis, which can be fatal or evolve towards a chronic disease. WNV has been shown to be neuroinvasive in C57BL/6J mice though chronic disease has not been described in this mouse strain (Graham *et al.* 2016). These examples illustrate well the fact that modeling a human disease with clinical heterogeneity between patients can be achieved if multiple genetic backgrounds are considered.

The results of my PhD project strongly support this conclusion: a unique mouse model will never take a full picture of ZIKV biology and disease. To go beyond this single model approach, I used genetically diverse mice to study ZIKV infection. Introducing genetic diversity in experimental animals is surely an appropriate means to identify more suitable models and provides unequalled opportunity to understand disease pathogenesis. As highlighted by our phenotyping and correlation studies, using a combination of models will be necessary as certain strains will be good models of ZIKV-induced brain pathology while others will be more suited for the investigation of ZIKV-induced immune responses. Importantly, models should be chosen primarily based on their relevance to answer the scientific question of the study. *Ifnar1*-deficient mice should not be ruled out for ZIKV studies solely on the principle that they do not reflect the immunological status of human individuals, but their use should be justified with regard to the scientific objectives. Finally, genetically diverse mice provides a fantastic opportunity to develop new experimental models that could be used as testing platforms, including in pre-clinical studies. As suggested by J. Nadeau and J. Auwerx: “Testing interventions and mutations in strains with different genetic backgrounds should become the standard, not the exception” (Nadeau and Auwerx 2019).

6.2.3. Host genetic diversity and the Collaborative Cross, the “Event Horizon Telescope” to capture black holes of human diseases

In April 2019, the first image of a supermassive black hole was released by the Event Horizon Telescope (EHT) international consortium (Akiyama *et al.* 2019). “We have seen what we thought was unseeable. We have seen and taken a picture of a black hole” mentioned S. Doleman, astrophysicist and EHT Director (Event Horizon Telescope). This event was a certainly a great achievement in the field of astrophysics. Drawing a conceptual comparison between this impressive collaborative project and the use of genetic diversity to study human diseases seemed to be an interesting opening.

The first obvious parallel that we can think of is the necessity to use more than a single device. Imaging a black hole from a single telescope would require a planet-sized radio dish which is obviously infeasible, but combining data simultaneously collected by 8 observatories across the globe was indeed an efficient strategy. Studying most infectious diseases in the global, genetically diverse, human population seems equally infeasible; but new mouse GRPs have successfully modeled high

levels of genetic diversity at a lower scale. These new experimental platforms could indeed represent a way to better apprehend the influence of host genetic diversity on common diseases.

Using an appropriate system is the first necessary step in such ambitious projects, but to achieve significance, these systems should be used at their maximum capacity. For the EHT project, several consecutive days with good weather conditions at 8 different locations on the globe were required for the observation of the black hole. In a different way, experimental conditions and procedures are created when using mouse GRPs and thus should be finely designed and controlled. Among the possibilities, using systems genetics approaches is a powerful strategy to get the most of mouse GRPs experiments. For a given experimental set-up, data should be collected across multiple biological scales, from genes and transcripts, to proteins and metabolites, and until phenotypic traits (Civelek and Lusis 2014).

Integration of multidimensional and heterogeneous data probably remains the most promising but also challenging processes. Petabytes of data were generated by the EHT project. Months of internationally conducted computational work were necessary to generate the first image of the M87 black hole and several teams worked both separately and collectively to warrant the reproducibility of the results. Efforts aiming at increasing international collaborations, data sharing and data integration between studies and species will be required to expand our capacities and to meet the demands of public health research.

Finally, the EHT really highlights the power of collaborative projects, and the “team effort” is deeply acknowledged by all of its members. Hopefully, similar collaborations between medical researchers will be as successful as this one; and optimistically, CC and other genetically diverse mice will allow capturing a significant amount of the biology underlying infectious and other human diseases.

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Title : Genetic control of susceptibility to Zika virus in the mouse using strains of the Collaborative Cross

Keywords : Zika virus, Mouse, Susceptibility to infectious diseases, Genetics of complex traits, Collaborative Cross, Innate immunity

Abstract :

Zika virus (ZIKV) is a mosquito-transmitted flavivirus responsible for worldwide epidemics and constitutes a major public health threat. The majority of ZIKV infections in humans are either asymptomatic or result in a mild febrile illness. However, some patients develop a more severe, sometimes life-threatening, form of the disease. Recent evidence showed that ZIKV infection can trigger Guillain-Barré syndrome and encephalitis in adults, as well as congenital malformations such as microcephaly. The severity of ZIKV disease in humans depends on many factors, likely including host genetic determinants.

We investigated how genome-wide variants could impact the susceptibility to ZIKV infection in mice. To this end, we used mouse strains of the Collaborative Cross (CC), a new genetic reference population encompassing a genetic diversity as broad as that of human populations.

First, we described that the susceptibility of *Ifnar1* (receptor to type I interferon) knockout mice is largely influenced by their genetic background. We then showed that the genetic diversity of CC mice, which IFNAR was blocked by anti-IFNAR antibody, expressed phenotypes ranging from complete resistance to severe symptoms and death with large variations in the peak and rate of decrease of plasma viral load, in brain viral load, in brain histopathology and in viral replication rate in infected cells. Differences of susceptibility between CC strains were correlated between Zika, Dengue and West Nile viruses. We identified highly susceptible and resistant CC strains as new models to investigate the mechanisms of human ZIKV disease and other flavivirus infections. Genetic analyses revealed that phenotypic variations were driven by multiple genes with small effects, reflecting the complexity of ZIKV disease susceptibility in human population. Notably, our results also ruled out a role of the *Oas1b* gene in the susceptibility to ZIKV.

In a second part, we searched for genes which modify the susceptibility of *Ifnar1* knockout mice in an F2 cross between C57BL/6J and 129S2/SvPas mice harboring the mutation. Genetic analysis revealed two Quantitative Trait Locus (QTL) controlling either the peak viremia or the mouse survival. Although these QTLs critical intervals contained hundreds of genes, data mining led us to identify a few candidate causal genes.

Then, we investigated how host genetic factors influence viral replication in infected cells using Mouse Embryonic Fibroblasts (MEFs) derived from a series of CC strains with contrasted phenotypes observed in response to ZIKV infection in vivo. MEFs from CC071 strain displayed unique features of increased viral replication rate in late infection. Using transcriptomic analysis, we demonstrated that the phenotype of CC071 infected MEFs resulted from a delayed induction of the type I interferon (IFN) response. Genetic analyses ruled out single gene deficiencies but rather suggested combined effects of multiple factors in the type I IFN induction signaling pathway.

Finally, we characterized the ZIKV-induced type I IFN response in MEFs and primary neurons derived from C57BL/6J mouse strain. Primary neurons were less capable than MEFs to control the viral replication due to a delayed IFN response. We later showed that host genetic factors also play a critical role in this context as ZIKV-infected CC071 primary neurons displayed an extreme phenotype compared to neurons from strains that are more resistant.

Altogether, our work has unraveled the role of host genes in the pathogeny of ZIKV infection and illustrates the potential of CC mouse strains for genetic studies and as new models of infectious diseases. Extensive analysis of CC strains with extreme phenotypes help us elucidate how genetic variants affect susceptibility as well as immune responses to flaviviral infection and will provide deeper understanding of the pathophysiology of human ZIKV disease.

Titre : Contrôle génétique de la sensibilité au virus Zika chez la souris à l'aide de lignées du Collaborative Cross

Mots-clés : Virus Zika, Souris, Sensibilité aux maladies infectieuses, Génétique des caractères complexes, Collaborative Cross, Immunité innée

Résumé :

Zika est un Flavivirus émergent transmis à l'Homme par piqûre de moustique. Il a récemment été à l'origine de plusieurs épidémies d'envergure mondiale et représente une menace pour la santé publique. L'infection Zika est souvent asymptomatique ou engendre un syndrome grippal bénin. Cependant, des complications sévères ont été associées au virus Zika, telles qu'un syndrome de Guillain-Barré ou des encéphalites chez l'adulte, ainsi que des malformations congénitales comme la microcéphalie. De nombreux facteurs sont susceptibles d'influencer la sensibilité d'un individu au virus Zika, y compris les variants génétiques de l'hôte.

Nous avons étudié le rôle des facteurs génétiques de l'hôte dans sa sensibilité à l'infection par le virus Zika. Pour cela, nous avons utilisé des lignées de souris du Collaborative Cross (CC), une population génétique de référence caractérisée par une diversité génétique aussi vaste que celle des populations humaines.

Nous avons d'abord montré que le fond génétique de souris déficientes pour le gène du récepteur à l'interféron de type I (*Ifnar1*) joue un rôle drastique dans leur sensibilité au virus Zika. La diversité génétique des souris CC, préalablement traitées par un anticorps bloquant le récepteur IFNAR, s'exprime par des phénotypes allant d'une résistance complète jusqu'à des formes sévères de la maladie. L'influence des facteurs génétiques de l'hôte s'exerce sur de nombreux paramètres tels que la virémie, la charge virale et les lésions pathologiques dans le cerveau, et enfin le taux de réplication dans les cellules infectées. Les différences de sensibilité entre lignées CC s'avèrent corrélées entre les Flavivirus Zika, Dengue et West-Nile. Nos analyses génétiques ont montré que de multiples gènes à effets faibles sous-tendent ces variations phénotypiques, reflétant la complexité de la sensibilité au virus Zika dans les populations humaines, et permettent d'exclure un rôle majeur du facteur de résistance *Oas1b*.

Nous avons ensuite cherché des gènes agissant comme modificateurs de la sensibilité chez des souris déficientes pour le gène *Ifnar1* dans un croisement F2 entre des souris C57BL/6J et 129S2/SvPas portant la mutation. L'analyse génétique a permis l'identification de deux QTLs (Quantitative Trait Locus), l'un contrôlant le pic de virémie et l'autre la survie. Une étude bio-informatique nous a permis d'identifier quelques gènes candidats.

Nous avons également étudié comment les facteurs génétiques de l'hôte impactent la réplication virale dans des fibroblastes embryonnaires murins (MEFs) dérivés d'une série de lignées de souris présentant des phénotypes contrastés en réponse à l'infection Zika. Nous avons identifié une augmentation de la réplication virale tardive dans les MEFs de la lignée CC071, résultant d'un retard à l'activation de la réponse interféron (IFN). Des analyses génétique et transcriptomique ont exclus des déficiences causées par des gènes uniques et ont favorisé l'hypothèse d'une combinatoire de gènes exerçant des effets faibles dans la voie d'induction de la réponse IFN.

Pour finir, nous avons caractérisé la réponse IFN induite par le virus Zika dans des neurones primaires murins. Cette étude a montré que la capacité des neurones primaires à limiter la réplication virale est moindre que celle des MEFs en raison d'un retard à l'induction de la réponse IFN. Enfin, les facteurs génétiques de l'hôte exercent un rôle critique dans ce contexte puisque les neurones primaires de CC071 présentent un phénotype extrême par comparaison avec des lignées plus résistantes.

Notre travail a mis en évidence le rôle des facteurs génétiques de l'hôte dans la pathogénie de l'infection Zika et illustre le potentiel des souris CC dans des études génétiques aussi bien qu'en tant que nouveaux modèles d'infection. Une analyse poussée des lignées aux phénotypes extrêmes permettra d'élucider les mécanismes génétiques de la sensibilité au virus Zika et améliorera notre compréhension de la maladie chez l'Homme.