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Intestinal epithelial cells instruct myeloid cell-mediated T-cell responses

Arunima Chatterjee

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INTESTINAL EPITHELIAL CELLS INSTRUCT MYELOID CELL-MEDIATED T-CELL RESPONSES

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THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

INTESTINAL EPITHELIAL CELLS INSTRUCT MYELOID CELL-MEDIATED T-CELL RESPONSES

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ABBREVIATIONS

AMP – Antimicrobial peptide
APC – Antigen presenting cells
ATRA – All trans retinoic acid
AUC – Area under the curve
CD – Crohn's disease
CEC – Colonic epithelial cell
DC – Dendritic cell
DSS – Dextran sulphate sodium
FOXP3 – Forkhead box protein 3
GALT – Gut associated lymphoid tissue
GM-CSF – Granulocyte-macrophage colony-stimulating factor
IBD – Inflammatory bowel disease
IEC – Intestinal epithelial cells
IFN γ – Interferon gamma
ILC – Innate lymphoid cells
LP – Lamina propria
LPS – Lipopolysaccharide
MAF – Macrophage activating factor
Mf – Macrophage
PPAR γ – Peroxisome proliferator-activated receptor gamma
PRR – Pattern recognition receptor
RALDH – Retinaldehyde dehydrogenase
RAR – Retinoic acid receptor
RARE – Retinoic acid response element
RBP – Retinol binding protein
RDH – Retinol dehydrogenase
ROR γ t – Receptor-related orphan receptor- γ t
RXR – Retinoid X receptor
SRM – Selected reaction monitoring
TLR – Toll like receptor
TNF- α – Tumour necrosis factor alpha
TGF- β – Transforming growth factor beta
VA – Vitamin A

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1. INTRODUCTION

The adult human intestinal tract is referred to as “physiologically inflamed” due to the presence of the enormous number of B- and T-lymphocytes as well as macrophages (Mf), dendritic cells (DC), eosinophils and mast cells. If all these immune cells were present in other tissues at such concentrations, they would be regarded as an abnormal chronic inflammatory cell infiltrate[1]. In such a scenario the main players that maintain gut homeostasis should be those mechanisms, which provide with tolerogenic signals exerted on specialized myeloid cells with antigen presenting function. The discovery showing that retinoic acid imprints the homing potential of leukocytes to the gut and also enhances the induction of regulatory T-cells highlighted the potential role for ATRA (all trans retinoic acid) in mucosal tolerance. It has also been shown that systemic immune responses at sites distal to the gut, such as autoimmune arthritis and experimental autoimmune encephalitis, can also be modulated through the gut microbiota [2] and thus lymphocyte trafficking through the gut is considered as a necessary “*rite of passage*” for effector T-cells. ATRA gradients have been shown to regulate naive CD4⁺ T-cell differentiation and cytokine responses in effector CD4⁺ T-cells. ATRA is essential for Th1 lineage stability and constrains the conversion to Th17 committed cells. Vitamin A deficient mice lack Th17 cells suggesting that endogenous ATRA signaling is required for the generation and/or the maintenance of Th17 cells. At *in vivo* settings ATRA is also required for Th2 responses and the administration of exogenous ATRA may exhibit opposing effects on T-cell polarization via inhibition of Th1 and Th17 responses observed in both *vitro* and *in vivo*. ATRA is also able to alter the sensitivity of cells to alternative cell fates through the regulation of cytokine receptors. Thus the dominant effects of ATRA may well be dependent on the instructive cytokine milieu. In such a model, ATRA may enhance or stabilize the actions of cytokines and the function of transcription factors that guide Th cell programming rather than directly specifying T-cell fate. In this way, synthesis of ATRA by APC (antigen presenting cells) provides an additional checkpoint for the stabilization of T-cell fate commitment [3].

This study is aimed to establish a simplified human *ex vivo* experimental model system mimicking the interplay of associated chemokines secreted by resting and stimulated colonic epithelial cell (CEC) under the influence of ATRA in a proinflammatory environment in the context of their effects on myeloid cells and T lymphocyte functionality. We also propose comparable and feasible methods for the cost effective and rapid detection and quantification β -defensin levels secreted by CECs acting as a first line of defence in the intestinal tract.

2. THEORITICAL BACKGROUND

The intestinal tract represents the largest mucosal surface of the human body exposed constantly to a myriad of environmental stimuli including components of beneficial and also infective microbes, dietary and metabolic products and diverse inorganic compounds. The intestine is preferentially challenged with the daunting task of segregating the underlying tissues from the potentially noxious and harmful endogenous and environmental molecular structures. Thus the monolayer of intestinal epithelial cells (IECs) provides an important anatomical barrier to support the appropriate segregation of innocent and harmful material and provides protection from various environmental challenges.

2.1. Vitamin A and its effects on the gut environment

The vitamin A (VA) metabolite ATRA is a key regulator of cytokine transforming growth factor beta (TGF- β) expression, which promotes regulatory T-cell (T_{reg}) differentiation[2]. VA also contributes to the formation of epithelial linings of mucosal surfaces[3] and its multifunctional metabolite ATRA [4] acts as a critical driver of lymphocyte trafficking to the intestinal mucosa[5]. ATRA also induces the expression of the gut homing integrin $\alpha 4\beta 7$ on the surface of myeloid cells and the chemokine receptor CCR9 on T-lymphocytes. On the contrary, the lack of αv or $\beta 8$ integrin chains in DC impairs T_{reg} functions and Th17 responses *in vivo*[6]. ATRA can modulate Th17 effector T-lymphocyte differentiation in the gut[7] however, the *in vivo* effects of ATRA in intestinal and extra-intestinal compartments results in controversial outcomes presumably due to targeting multiple cell types with diverse functional activities[8]. VA deficiency also has an impact on epithelial cell integrity and the composition of the gut microbiota[9]. VA shows '*hormone-like*' properties with some effects on IEC and gut homeostasis as described below.

2.1.1. Vitamin A metabolism

"A vitamin is a substance that makes you ill if you don't eat it." (Albert Szent-Gyorgyi, Nobel Prize in Physiology or Medicine, 1937). Vitamins (*vital amines*) involve essential organic compounds required in trace amounts in the diet, because they cannot be synthesized in sufficient quantities by an organism[10]. VA is usually acquired from the diet either as all-*trans* retinol, retinyl esters, or β -carotene. All-*trans* retinol is esterified to retinyl esters and stored in the liver, or it can associate with retinol binding protein (RBP), which transports retinol to target tissues. All-*trans* retinol is oxidized intracellularly to all-*trans* retinal by ubiquitously expressed retinol dehydrogenases (RDH), which belong to the short chain

dehydrogenase reductase (SDR) gene family as shown in Figure 1. At least two RDH are physiologically involved in this rate-limiting step: RDH1 and RDH10. The cytosolic retinal dehydrogenase enzymes (RALDHs) catalyze the irreversible oxidation of all-*trans* retinal to ATRA[11, 12]. ATRA exerts its effects mostly through binding to the heterodimers of nuclear RA receptors (RAR α , β , γ) and retinoid X receptors (RXR α , β , γ)[13], although some specific effects can be mediated via the peroxisome proliferator-activated receptors (PPAR β/γ)[14, 15]. RAR-RXR heterodimers are ligand-dependent transcription factors that bind to *cis*-acting DNA sequences referred to as ATRA response elements (RARE), located in the promoter region of RA target genes. Although RAR receptors are ubiquitously expressed, RAR β expression is markedly enhanced by ATRA. Termination of ATRA signaling is achieved through its catabolism into oxidized metabolites, such as 4-hydroxy ATRA and 4-oxo ATRA, by enzymes of the CYP26 family. Among these enzymes, CYP26A1 is directly upregulated by ATRA[12, 15]. While ATRA metabolites have been mostly considered to be biologically inert, there is evidence indicating that some of these metabolites retain the ability to signal through RAR[4]. However, the potential roles of ATRA metabolites in immune responses have not been described.

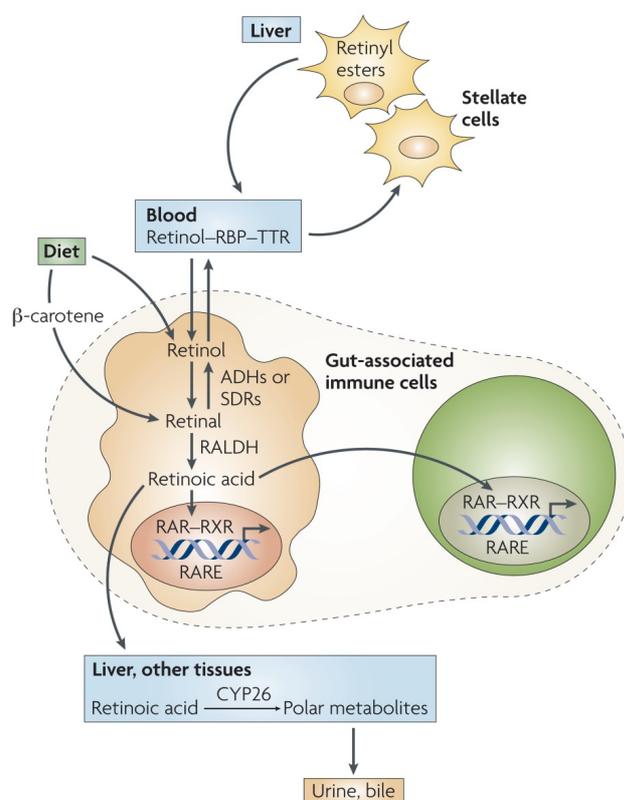


Figure 1: Vitamin A (retinol) is obtained from the diet and is transported in the blood as a complex with retinol-binding protein (RBP) and transthyretin (TTR). In the liver, retinol is esterified to retinyl esters and stored in stellate cells. In other tissues, including gut-associated immune cells, retinol is oxidized to retinal by alcohol dehydrogenases (ADHs) or short chain dehydrogenase/reductases (SDRs). Retinal is then oxidized to all-*trans*-retinoic acid in an irreversible reaction that is catalysed by retinal dehydrogenases (RALDHs). Retinoic acid acts on immune cells by binding to the retinoic acid receptor (RAR). Retinoic acid is catabolized in the liver and in other tissues by the enzyme CYP26 and its metabolites are eliminated in the bile and urine. *Nat Rev Immunol.* 2008 September ; 8(9): 685–698.

2.1.2. The affects of vitamin A on adaptive immune cell subsets

ATRA is known to enhance T-cell proliferation and cytotoxicity. Consistent with its *in vivo* role in T-cell functions cells deficient in VA show defects in CD4⁺ T-cell activity that could be due to the inavailability of ATRA to bind to RXR [16]. ATRA interferes also with B-cell proliferation and apoptosis, a process mediated by the binding of VA metabolites to the RAR receptors[17]. ATRA also modulates antigen presentation via exerting direct effects on DC activities leading to increased expression of matrix metalloproteinases able to increase the migration of tumour-infiltrating DCs to the draining lymph nodes thus boosting the potential of tumour-specific T-cell responses [18]. In the presence of inflammatory stimuli, such as TNF- α ATRA enhances DC maturation and antigen-presenting capacity, both mediated by RXR receptors. However, it should be noted that DCs pre-treated with ATRA can apparently store this metabolite, which when released could ultimately act directly on T-cells and other cells thus contributing to the final outcome of an immune response [19]. VA metabolites also modulate the Th1–Th2-cell balance and the differentiation of T_{reg} and Th17 cells as shown in Figure 2. VA deficiency correlates with decreased Th2-cell responses and conversely, VA supplementation blocks the production of Th1-cell cytokines *in vitro* and *in vivo* [5, 20]. In fact ATRA promotes Th2-cell differentiation by inducing *IL4* gene expression [21]. Moreover, ATRA blocks the expression of the Th1 master regulator T-bet and induces Th2-cell promoting transcription factors, such as GATA3 (GATA-binding protein 3), macrophage-activating factor (MAF) and signal transducer and activator of transcription 6 (STAT6). It has been proposed that ATRA exerts its Th2-cell-promoting effect indirectly through the modulation of APC as well as acts directly on T-cells to induce Th2-cell differentiation through RAR proteins[5].

Although transforming growth factor- β (TGF β) drives the generation of induced T_{reg} cells that express the transcription factor forkhead box protein 3 (FOXP3) in peripheral tissues, it was recently demonstrated that this process can significantly be enhanced by ATRA[2, 22] (Figure 2). DCs from the gut-associated lymphoid tissue (GALT) or in small intestinal lamina propria also enhance T_{reg}-cell differentiation in a ATRA-dependent manner [23, 24]. A recent study indicated that Mf but not DCs are responsible for inducing T_{reg} cells in the intestinal lamina propria[25], and that DCs are mainly involved in the induction of Th17 cells at this site[25, 26]. The reasons for these seemingly discrepant results are unclear. In addition to inducing FOXP3, ATRA also upregulates the gut-homing receptors on T_{reg} cells thus targeting these cells to the gut mucosa [22, 24].

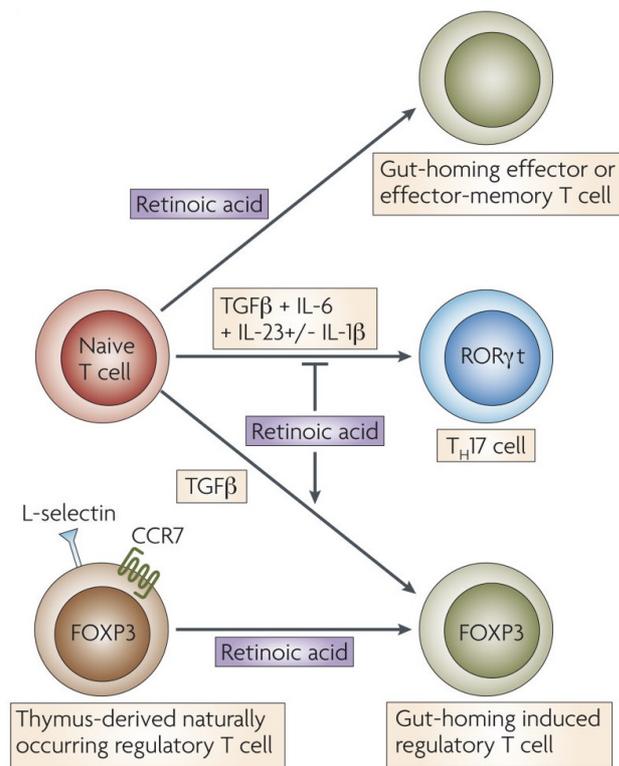


Figure 2: In addition to upregulating the expression of gut-homing receptors, retinoic acid has also been reported to promote T-helper-2 (Th2) cell differentiation. Moreover, retinoic acid blocks the differentiation of T helper 17 (Th17) cells and induces forkhead box protein 3 (FOXP3)⁺ regulatory T (T_{Reg}) cells in the presence of transforming growth factor-β (TGFβ) by reciprocally downregulating receptor-related orphan receptor-γt (RORγt) and inducing FOXP3 expression in T-cells, respectively. Retinoic acid also enhances the TGFβ-driven induction of T_{Reg} cells and induces gut-homing receptor expression in both naturally occurring and induced T_{Reg} cells. Th17-cell differentiation requires TGFβ, interleukin-6 (IL-6), IL-23 and, in humans, IL-1β. Cassani et al. *Mol Aspects Med.* 2012 Feb;33(1):63-76.

The differentiation of T_{reg} and Th17 cells is regulated reciprocally by cytokine signals [27]. Exposure of activated CD4⁺ T-cells to TGFβ alone induces T_{reg} cells, whereas the combination of TGFβ together with IL-6, IL-1β and IL-23 or IL-21 blocks FOXP3 induction and induces Th17-cell differentiation[28]. However, exposure of CD4⁺ T-cells to ATRA together with TGFβ and IL-6 negates the Th17-cell-promoting effect of IL-6 via enhancing the induction of T_{reg} cells and blocking the induction of receptor-related orphan receptor-γt (RORγt), a key transcription factor for Th17-cell differentiation[2]. It should be noted that this effect is only observed when ATRA is used over a given concentration[26], while low concentrations of ATRA seem to be necessary for Th17-cell differentiation. Thus ATRA has a dual role in maintaining immunological tolerance: it favours the induction of T_{reg} cells and simultaneously can block or enhance Th17-cell differentiation, depending on its concentration.

2.1.3. Vitamin A modulates lymphocyte homing

Although naive lymphocytes migrate preferentially through secondary lymphoid organs, effector and memory lymphocytes acquire ‘trafficking’ molecules that endow them with the capacity to migrate to selected extra lymphoid tissues and to sites of inflammation. The

gastrointestinal mucosa is the main body surface exposed to environmental antigens and is also the paradigmatic tissues for which tissue-specific adhesion and chemoattractant receptors (also known as homing receptors) have been characterized in detail. For example, effector and memory lymphocytes migrating to the small bowel require expression of the $\alpha 4\beta 7$ -integrin and CC-chemokine receptor 9 (CCR9).

It has also been demonstrated that the lymphoid microenvironment in which the lymphocytes are activated determines the set of homing receptors they acquire, thus T-cells activated in the GALT acquire gut-homing receptors[29]. In the lymphoid microenvironment, DCs are essential contributors of efficient T-cell activation. Several groups have shown that DCs from Peyer's patches and mesenteric lymph nodes are sufficient to induce the expression of $\alpha 4\beta 7$ -integrin and CCR9 supporting imprinting the gut-homing capacity of activated T- [30] and B-cells[26]. The means how GALT-resident DCs could imprint lymphocytes with a gut-homing phenotype was shown 25 years ago in rats suffering from both protein-caloric and VA deficiencies causing impaired migration of recently activated mesenteric lymphocytes to the small intestinal mucosa [31], while without VA deficiency it did not affect lymphocyte migration. Adoptive transfer experiments showed that impaired lymphocyte migration could be observed only when donor lymphocytes were from protein-caloric deficient and VA deficient rats and failed when wild-type cells were transferred to protein caloric-deficient and VA-deficient recipients. These results suggested that VA deficiency affected mostly the lymphocyte migratory capacity, but not target tissues[31]. The molecular basis for these observations was recently determined in a study showing that mice depleted of VA had decreased numbers of effector and memory T-cells in the gut mucosa, but not in other tissues [5]. The VA metabolite ATRA was sufficient to induce the expression of $\alpha 4\beta 7$ -integrin and the CCR9 chemokine by activated T-cells, even in the absence of DCs. Blocking ATRA receptors of the RAR family significantly decreased the induction of $\alpha 4\beta 7$ -integrin expression by T-cells in GALT-resident DCs, showing that ATRA is essential for the gut-imprinting capacity of the DCs. GALT-resident DCs, unlike DCs from other tissues, express RALDH enzymes, which are essential for ATRA biosynthesis. These results altogether indicate that ATRA plays a pivotal role for imprinting gut-homing T-cells [5].

Although VA deficiency decreases the number of T- and B-cells in the small bowel lamina propria[5] it does not affect lymphocyte migration to the colon[31]. Similarly, GALT resident DCs imprint T- and B-cells with homing capacity for the small bowel, but they do not induce colon-homing T-cells. Therefore, ATRA is neither necessary nor sufficient to imprint colon-homing lymphocytes. The molecular signals that are responsible for lymphocyte homing to

the colon and the reasons why T-cell migration to this compartment is controlled differently from homing to the small bowel are still to be determined.

2.2. The role of cytokines involved in the maintenance of gut homeostasis

Based on genetic and immunological studies cytokines have directly been implicated in the pathogenesis of IBD and their crucial role in controlling intestinal inflammation and the appearance of the associated clinical symptoms of IBD. The key role of cytokines in this process has also been highlighted by the blockade of TNF- α commonly used as a standard therapy for IBD and other inflammatory diseases[32].

A combination of IBD risk factors seems to initiate alterations in epithelial barrier function thereby allowing the translocation of luminal antigens including bacterial antigens from the commensal microbiota into the bowel wall. Excessive cytokine responses to such environmental triggers cause subclinical or acute mucosal inflammation in genetically susceptible hosts[32]. In patients that fail to resolve acute intestinal inflammation, chronic inflammation develops that is induced by the uncontrolled activation of the mucosal immune system. In mucosal immune cells such as Mf, DC and T-cells and the recently discovered subsets of innate lymphoid cells (ILCs) are able to respond to microbial products derived from the commensal microbiota by producing cytokines that promote chronic inflammation of the gastrointestinal tract.

Genome-wide association studies have identified several IBD susceptibility loci that contain genes encoding proteins involved in cytokine and chemokine receptor signalling, and T-helper cell responses. These involve various signalling molecules such as signal transducer and activator of transcription 1 (STAT1), STAT3, STAT4, CC-chemokine receptor 6 (CCR6), CC-chemokine ligand 2 (CCL2), CCL13, IL-12 receptor (IL-12R), IL-23R and Janus kinase 2 (JAK2). Further studies have identified IBD risk loci that contain genes encoding a plethora of cytokines: IL-2, IL-21, interferon- γ IL-10 and IL-27) highlighting a potentially major role for these cytokines in disease pathogenesis[33]. Loss-of-function mutations in the genes encoding IL-10 and IL-10R are associated with a very early-onset of IBD that is characterized by severe intractable enterocolitis in infants. Cytokine function in patients with IBD may be affected by the localization and type of inflammation, immune cell plasticity, different pathogenetic mechanisms or by changes in cytokine production patterns to occur in the course of the disease. The concerted action of these factors build up a complex mucosal cytokine networks. This mode of action suggests that anti-cytokine approaches targeting a single pro-inflammatory cytokine has major limitations in terms of effective therapy for the different

clinical subgroups of IBD[34]. In the following section we describe the major characteristics of the most potent proinflammatory cytokines involved in IBD i.e the IL-1 and TNF families.

2.2.1. The IL-1 family of cytokines and IBD

Lamina propria DCs and Mf are key APCs infiltrating the inflamed mucosa in IBD. In response to components of the commensal microbiota associated with the activation of signalling pathways triggered by various pattern recognition receptors (PRR), these cells produce large amounts of pro-inflammatory cytokines, such as IL-1 β , IL-6, IL-18 and TNF- α [35]. A significant decrease in the ratio of IL-1 receptor antagonist of IL-1 could be detected in the intestinal mucosa in patients with Crohn's disease and ulcerative colitis when compared to control subjects, indicating the activation of the IL-1 system in IBD. TNF- α but not IL-1 blockade was effective in the treatment of mice with chronic dextran sodium sulphate (DSS)-induced colitis. Collectively, these findings suggested that IL-1 has a prominent role in the initiation, rather than in the perpetuation of colonic inflammation. Importantly, blockade of the IL-1 family member IL-18, a cytokine preferentially induced in Mf and epithelial cells in patients with Crohn's disease exerted beneficial effects in several murine models of acute and chronic colitis[36]. Furthermore, deficiency of the IL-1 β -converting enzyme (ICE; also known as caspase 1), an enzyme that cleaves IL-1 β and IL-18 into active cytokines, protected mice from DSS-induced colitis suggesting that the blockade of IL-1 family members may be relevant for the therapy of chronic intestinal inflammation[37].

2.2.2. Membrane-bound and soluble TNF in IBD

TNF is produced as a transmembrane protein and soluble TNF is released by proteolytic cleavage by the TNF-converting metalloproteinase enzyme (TACE; also known as ADAM17). The production of both membrane-bound and soluble TNF by mononuclear cells in the lamina propria is markedly augmented in patients with IBD. In particular, CD14+ macrophages, adipocytes, fibroblasts and T-cells from patients with IBD have been shown to produce large amounts of TNF- α [38]. TNF may exert various pro-inflammatory functions in colitis upon binding to its receptors TNFR1 and TNFR2, followed by the intracellular activation of the transcription factor nuclear factor- κ B (NF- κ B). TNFR1 signalling may also cause cell death via the activation of receptor-interacting protein kinase 1 (RIPK1) and caspase 3 proteins.

The treatment of IBD with neutralizing antibodies against soluble and membrane-bound TNF (such as infliximab and adalimumab) has been validated as highly effective treatment options

via inducing T-cell apoptosis *in vivo*, whereas agents that preferentially block soluble TNF (for example etanercept) had no therapeutic effect[32]. Thus, the development of strategies that more specifically target the membrane-bound TNF–TNFR2 interaction is of potential interest for future therapy of IBD.

On the contrary, anti-cytokine therapies such as antibodies specific for TNF, IL-12 or IL-23 and the blockers of cytokine signalling such as tofacitinib seem to have beneficial clinical effects only in certain subgroups of patients[32]. This may reflect that cytokine networks in the inflamed mucosa are more complex than previously appreciated and are the subject of multiple layers in the regulation by microbial, genetic and immunological factors. A key feature of the mucosal cytokine network is its dynamic fluidity and ability to traverse spatial boundaries. Thus the blockade of a single cytokine in patients with IBD may lead to the development of alternative compensatory pro-inflammatory cytokine pathways. Furthermore, the pathological mechanisms driving mucosal inflammation may differ between patients and could explain why targeting of a single pro-inflammatory cytokine is not sufficient.

2.3. GM-CSF plays a significant role in the regulation of mucosal immunity

The cytokine granulocyte-macrophage colony stimulating factor (GM-CSF/Csf2) is a key determinant of myeloid lineage differentiation and is required for the optimal function of tissue resident mononuclear phagocytes (MNPs), including Mφ and DCs thereby promoting host protection against environmental pathogens. Despite the key role of Csf2 in promoting MNP survival, differentiation and function, previous studies reported that mice lacking Csf2 or its receptor display only minor impairment in the development of spleen and lymph node resident DCs. Studies showing that Csf2 expression is increased in inflamed tissue and adoptively transferred monocytes can generate DCs in inflamed but not in steady-state spleen suggested that Csf2 acts as a major proinflammatory cytokine that controls the differentiation of inflammatory but not steady-state DCs *in vivo*. These results are consistent with the contribution of Csf2 to the pathophysiology of numerous inflammatory and autoimmune diseases. The role of microbial commensals that colonize the large bowel to promote the induction of Foxp3⁺ T_{reg} cell differentiation has been established[39]. However the cellular cues that promote T_{reg} accumulation in response to gut commensals have only recently started to be unraveled. The gut microbiota promotes intestinal homeostasis by supporting the crosstalk between IL-1β-secreting Mφ and Csf2-producing ILC in the intestinal mucosa. Microbiota-driven IL-1β production by Mφ promotes the release of Csf2 by ILC, which in turn acts on DCs and Mφ, allowing the maintenance of colonic T_{reg} cell homeostasis. Ablation

of Csf2 alters DC and Mf numbers and impairs the ability to produce regulatory factors such as ATRA and IL-10, which lead to disrupted T_{reg} cell homeostasis in the large intestine. Conversely, administration of the Csf2 cytokine in mice increases T_{reg} cell frequency in the gut. Most notably, the cell type-specific ablation of IL-1-dependent signaling in ILC abrogates oral tolerance to dietary antigens and compromises intestinal T_{reg} homeostasis *in vivo*. Although the reduction in total T_{reg} cell numbers is mostly observed in the large intestine, adoptive transfer studies in *Csf2*^{-/-} mice revealed impaired T_{reg} cell differentiation both in the small and large intestine. These results suggest that Csf2-dependent immunoregulatory functions control T_{reg} cell induction in both tissues establishing intestinal tolerance being critical for the prevention of intestinal diseases such as IBD. A clinical study performed with more than 300 patients with Crohn's disease (CD) revealed that the level of neutralizing antibodies against Csf2 in the serum correlated with ileal involvement and in the development of penetrating pathology, whereas a more recent study identified reduced levels of Csf2 receptor (Csf2R) and impaired receptor activity in a mixed group of IBD patients[40]. Previous clinical trials with recombinant Csf2 in IBD patients have established patient benefit in terms of reduced disease severity and lower burden of corticosteroid use. Unpublished results of a large Csf2-based clinical trial in IBD failed to achieve primary clinical end points, but it remains likely that a subset of IBD patients with defective Csf2 production or function still could benefit from this therapy. Thus the key role for Csf2 in the maintenance of intestinal tolerance is consistent with previous studies showing that the absence of Csf2 can also contribute to lupus-like disease, insulinitis, and age-related glucose intolerance and emphasizes the critical role of tissue-resident phagocytes in the maintenance of tissue integrity. It is now well established that the gut commensal flora promotes immune homeostasis in the host. The commensal driven MNP-ILC-Csf2 axis acts as a key regulator of intestinal T-cell homeostasis in the mouse intestine. Disturbance of this regulation can radically alter MNP-associated effector functions resulting in impaired tolerance to dietary antigens (Figure 3).

2.4. Defensins are secreted by IEC as part of a first line of mucosal defence

Mammalian antimicrobial peptides (AMPs) are members of a diverse array of protein families, all having the potential to rapidly kill or inactivate microorganisms. The epithelial cells lining the gut, skin and respiratory tract produce a rich arsenal of AMPs, probably reflecting the complexity of microbial challenges these tissues are exposed to and the continuous threat of microbial invasion at these sites[41]. The AMPs of the gut encompass

representatives of several distinct protein families. These include defensins, cathelicidins, C-type lectins, ribonucleases and psoriasin[42].

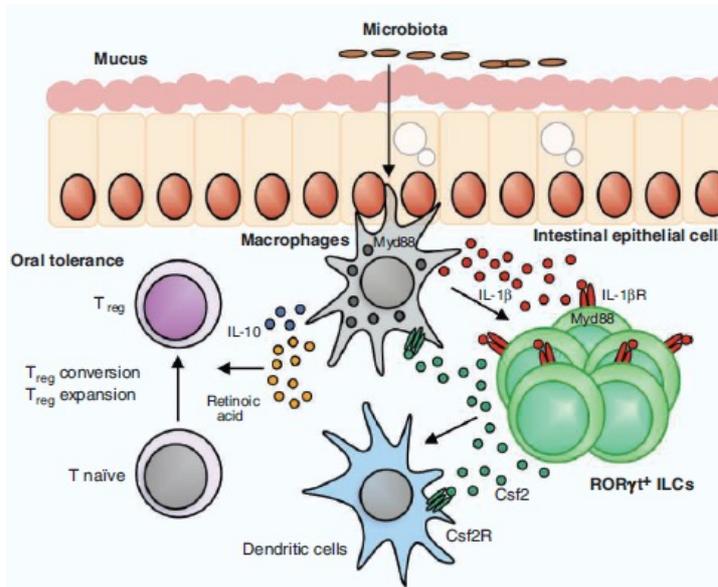


Figure 3: Innate Lymphoid Cells (ILC) translate microbial cues into immunoregulatory signals in the intestine. Microbial structures sensed by intestinal mononuclear phagocytes lead to IL-1 β release. IL-1 β engages IL-1receptor on ILC and promotes Csf2 release. ILC-derived Csf2 triggers DC and MNP production of regulatory molecules including RA and IL-10, which in turn promote the induction and expansion of T_{regs}. Csf2-primed DCs and MNPs promote T_{reg} homeostasis locally and also in mesenteric lymph nodes. Mortha et al. Science. 2014 Mar 28;343(6178):1249288.

Among the diverse, most abundant and highly expressed AMP families in the gut are the α -defensins. They are small peptides of \sim 2-3 kD with a conserved three-dimensional structure characterized by an amphipathic arrangement of cationic and hydrophobic residues[43], resulting in a positively charged surface that is spatially separated from a neighbouring hydrophobic region. This unique structure promotes the attraction of α -defensins to the negatively charged cell surface and insertion into lipid-rich membranes. In general, α -defensins have a broad spectrum of activity against both Gram-positive and Gram-negative bacteria, and in some cases are active against fungi, viruses and protozoa however, particular defensin species exhibit marked differences in their activity spectrum and expression patterns[44]. The human α -defensins include DEFA5/HD5 and DEFA6/HD6.

Under steady-state conditions epithelial cells produce the majority of AMPs on the body surface, although infiltrating immune cells during inflammation can also contribute to AMP production. The intestinal epithelial cell surface comprises distinct epithelial cell lineages each of which expresses a distinct group of AMPs. The enterocyte is the most abundant epithelial cell lineage of both the small and large intestine.

Colon enterocytes express β -defensins and cathelicidins[45]. Paneth cells are located at the base of crypts of Lieberkühn and are unique to the small intestine. Many AMPs are expressed

abundantly by Paneth cells including α -defensins[44] and RNase[46]. Goblet cells constitute a third epithelial cell lineage that is present in both the small and large intestine. A major function of goblet cells is to secrete mucin glycoproteins that assemble to form a thick gel-like mucus layer that overlies the epithelium and functions in part to concentrate secreted AMPs at or near to the epithelial surface[47].

2.4.1. Mechanism of AMP actions

Many AMPs target essential cell wall or cell membrane structures, which limits the ability of microorganisms to evolve resistance. Several AMPs act as enzymes that kill bacteria by carrying out an enzymatic attack on cell wall structures. Lysozyme and phospholipase A2 (PLA2), both highly expressed by Paneth cells, function through such a mechanism. Lysozyme hydrolyses the glycosidic bounds between the N-acetyl-glucosamine and N-acetyl-muramic acid that constitute the carbohydrate backbone of cell wall peptidoglycan, whereas secretory PLA2 (sPLA2) kills bacteria by hydrolysing bacterial membrane phospholipids[48]. Many AMPs kill bacteria through non-enzymatic disruption of the bacterial membrane.

In vertebrates defensins comprise a major family of membrane-disrupting peptides. The clusters of cationic residues of most defensins interact with the surface of the bacterial membrane through electrostatic interactions with negatively charged phospholipid groups. This interaction is followed by the formation of defensin pores in the bacterial membrane that disrupts membrane integrity and promote the lysis of the targeted microorganism[49].

Certain AMPs such as RNase7[50] and dermcidin[51] do not act through membrane disruption but function by metal chelation thus regulating the availability of essential trace elements such as Zn^{2+} and Mn^{2+} to augment other host defence mechanisms for microbial killing[52]. The other proteins have very low direct antimicrobial potency but are abundantly expressed in epithelia arguing for their substantial role in host defence. These findings altogether suggest that different AMP families use distinct molecular mechanisms to kill microorganisms. The use of diverse killing strategies presumably helps to limit the evolution of microbial resistance to multiple AMPs.

2.4.2. Chemotactic activity of AMPs

The α - and β -defensins have been shown to have chemotactic activity and the capacity to recruit leukocytes by direct or indirect mechanisms, which can directly modify the outcome of inflammatory responses. Human α -defensins (DEFA1/neutrophil defensin 1 and 2 and β -defensins/DEFB103) and BD4/DEFB104 have been reported to be chemotactic for monocytes

and Mf, and BD2/DEFB4A[53]. The chemotactic activities of the different AMP groups differ from each other. For example, human α -defensins selectively induce the migration of naive CD4+CD45RA+ and CD8+ human T-cells, but not CD4+CD45RO+ memory T-cells[54]. By contrast, β -defensins are chemotactic for immature DCs and CD4+CD45RO+ memory T-cells. The chemotactic effect of human defensins can be inhibited by antibodies specific for CC-chemokine receptor 6 (CCR6)[54].

2.4.3. Expression and functions of antimicrobial peptides and proteins

The expression, secretion and activity of most AMPs are tightly controlled. This is necessary to prevent the potential toxic effects of many of these proteins on mammalian cell membranes. The chemokine-like activities and immune-modulatory effects of many AMPs presumably require tight control of expression in order to avoid triggering of unnecessary inflammatory responses. Studies performed in germ-free mice have revealed that some intestinal AMPs are expressed independently of the microbiota, whereas others require bacterial signals for their expression. For example, the expression of most intestinal α -defensins requires the transcription factor TCF4 belonging to the WNT pathway, but is independent on the microbiota[55]. Similarly, the expression of lysozyme and certain members of the β -defensin family do not require microbial signals[46].

Other intestinal AMPs depend on microbial cues for full expression. Members of α -defensins show increased levels of expression in conventionally raised mice in comparison to germ-free mice[55]. Similarly, key members of the human β -defensin family, including BD2, are expressed under the control of bacterial signals. For example, bacterial flagellin has been shown to be relevant for the induction of BD2 expression[56].

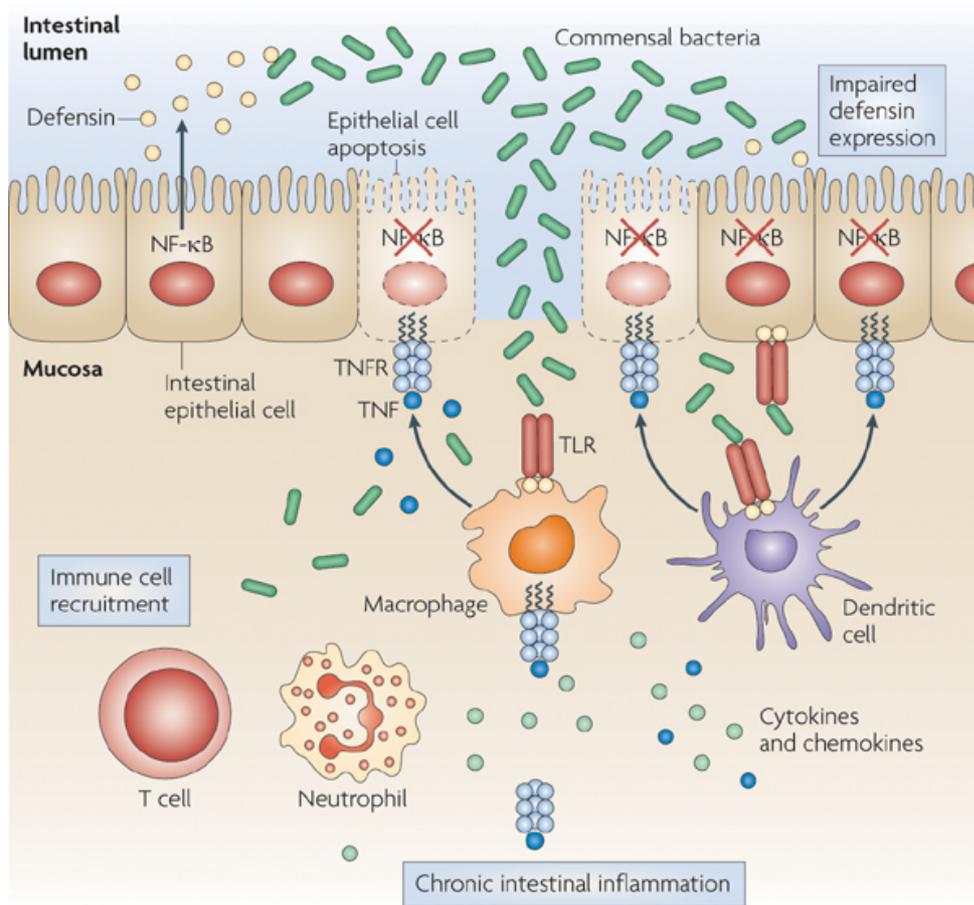
2.4.4. AMP secretion and IBD

Gut epithelial AMPs not only protect against pathogen colonization but also determine microbiota composition and limit the access of the microbiota to host tissues (Figure 4).

A key mechanism by which the mammalian intestine maintains homeostasis with its associated bacterial communities is to minimize contact between the bacteria and the intestinal epithelium[57]. This is accomplished in part by enhancing the physical barrier through the production and assembly of a thick mucus layer at the epithelial surface. Visualization of the spatial relationships between bacteria and the intestinal surface shows that the inner mucus layer remains relatively free of bacteria, whereas the outer mucus layer retains large numbers of bacteria[58]. Biochemical analysis of AMP localization has shown

that most AMP activity is confined to the mucus layer and is essentially absent from the luminal content[47]. Thus the mucus layer in addition to functioning as a physical barrier, limits bacterial penetration by forming a diffusion barrier that concentrates AMPs near to the epithelial cell surface. Given the crucial contributions of AMPs to the maintenance of homeostasis at body surfaces, dysregulation of AMP production and function can be associated with disease. Patients with IBD frequently exhibit increased numbers of epithelial cell surface-associated bacteria[59] suggesting a breakdown in immune mechanisms that normally limit direct contact between the microbiota and the intestinal mucosa. Several IBD risk alleles have been associated with altered intestinal epithelial AMP production. For example, genetic variants in the promoter region of the TCF4 and the NOD2 genes controlling α -defensin expression are associated with ileal Crohn's disease[60]. In these patients, severe intestinal inflammation is associated with decreased α -defensin expression[61]. These examples illustrate how genetic defects leading to decreased AMP production can be associated with intestinal inflammation and disease. However, it is important to note that in mice the defects in AMP production alone generally do not result in intestinal inflammation [57] suggesting that in humans IBD may require multiple genetic defects that target other immune mechanisms in addition to AMPs[62].

Body surfaces continuously face complex microbial challenges that include the maintenance of homeostasis in close contact with indigenous microorganisms and limiting exposure to pathogens. The intestine confronts these challenges by producing a complex arsenal of AMPs that directly kill microorganisms and can modulate immune responses. It is also becoming clear that endogenous AMPs are essential not only for protecting these sites from pathogenic microbial evasion but also for shaping the composition and location of indigenous microbial communities. To get a better insight to the importance of AMPs in regulating innate immune activities we have developed a targeted mass spectrometry method for the identification and quantitation of AMPs. In the present study the selected reaction monitoring (SRM) method was used in a semi-quantitative manner for the relative quantification of β -defensin 1, β -defensin 2, β -defensin 3 and β -defensin 4 derived from human Caco2 colonic epithelial cell lysates and culture supernatants.



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Figure 4: The intestinal epithelium has an essential role in the maintenance of immune homeostasis in the gut. By forming a mechanical barrier and expressing antimicrobial peptides (such as defensins), intestinal epithelial cells (IECs) prevent commensal bacteria from invading the gut mucosa (left hand side). Nuclear factor- κ B (NF- κ B) inhibition results in increased death of IECs and decreased expression of antimicrobial peptides, thereby compromising the epithelial barrier and allowing commensal bacteria to invade the colonic mucosa, which triggers severe chronic colitis in a MYD88- and tumour necrosis factor receptor 1 (TNFR1)-dependent manner (right hand side). TLR, Toll-like receptor. *Nature Reviews Immunology* **9**, 778-788 (November 2009)

3. AIMS OF THE STUDY

- Develop a human myeloid cell-based *in vitro* model system to mimic the response of colon epithelial cells (CEC) to inflammatory stimuli (IL-1 β or TNF- α) and the effect of ATRA on this inflammation.
- Identify, how the molecular information, collected by activated CEC supernatant could be translated to T-lymphocyte polarization.
- To demonstrate the role of ATRA, a metabolite of VA, in modulating the outcome of both innate and adaptive immune responses.
- Dissect the characteristics of DC- and Mf-mediated responses in the presence and absence of ATRA.
- To show that properly ‘educated’ myeloid cells are able to induce tolerance when co-cultured with autologous CD4+ T-cells.
- To test, whether GM-CSF deficiency in the gut could alter the phenotypic properties of DC and Mf considering that GM-CSF is able to enhance IL-1 β secretion of intestinal lymphoid cells and also has an effect on the number of T_{reg} cells.
- To demonstrate the expression of β -defensins at the protein level in Caco2 cell lysates and in the supernatant of these cells.
- To introduce a new mass spectrometry based methodology for the semi quantitative determination of relative β -defensin expressions in Caco2 cells stimulated by IL-1 β .

4. MATERIALS AND METHODS

Cell Culture of Caco2 colon epithelial cells

The human colorectal adenocarcinoma cell lines Caco2 is from ATCC-number HTB-37™ and HT-29 is from ATCC-number HTB-38™. The colorectal carcinoma cell line HCT116 was a generous gift from Dr. György Vereb, Department of Biophysics, University of Debrecen. Caco2 and HCT116 cells were cultured in RPMI-1640 medium supplemented with 1% antibiotic-antimycotic solution and 20% fetal bovine serum (GIBCO by Life Technologies, EU) in tissue culture flasks (Nunc, Rochester, NY) at 37°C in 10% and 5% CO₂, respectively. HT-29 cells were cultured in RPMI-1640 medium supplemented with 1% antibiotic-antimycotic solution and 10% fetal bovine serum in 5% CO₂. Cell culture medium was replaced every 2-3 days and the cells were passaged when sub-confluent.

Protein Array for Chemokine analyses

CEC of 70-80% confluency were plated overnight in RPMI supplemented with 10% FCS- followed by stimulation with 10ng/ml pro-inflammatory cytokines (IL-1 β or TNF- α) in combination with or without 10nmol/ml ATRA or left untreated for 1 hour. The cells were washed and replaced with fresh medium for 5 hr, when the supernatants were collected for chemokine analysis performed by a commercially available protein array (Proteome Profiler Arrays- ARY017, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Sample controls (transferrin R, gp130 and fibrinogen) included in the array allowed us the detection and quantitation of the secreted chemokines. Considering that ATRA dissolved in DMSO may have toxic effects on resting CEC, which could be further enhanced by activation with IL-1 β or TNF- α , we performed preliminary titration experiments to optimize the cell culture conditions by using 24h 7AAD-based viability assays performed by FACS analysis. These results indicated 98% viability of Caco2 and HT-29 cells in both the presence and absence of 10nmol/ml ATRA that was similar to those measured for untreated CEC.

***In vitro* cell migration and the chemotaxis assay**

Migration of three different groups of monocyte-derived cells, differentiated in GM-CSF+IL4, GM-CSF and M-CSF, were tested for cell migration to chemokines and cytokines secreted by Caco2, HT-29 and HCT116 cells. Monocytes (3×10^5) differentiated in the presence of the 3 different growth factors were placed on the upper chamber of a 5micron Corning transwell plate and the CEC supernatants were added to the lower chamber of the

transwell. After 24 h the monocyte-derived cells that migrated to the lower chamber were collected. 10,000 polystyrene beads (15micron) were added to each sample (Fluka Analytical, Germany) and the number of migrating cells was counted by FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed by the FlowJo software (Tree Star, Ashland, OR, USA).

Peripheral blood monocyte-derived cells

Leukocyte enriched buffy coats were obtained from healthy blood donors drawn at the Regional Blood Center of the Hungarian National Blood Transfusion Service (Debrecen, Hungary) in accordance with the written approval of the Director of the National Blood Transfusion Service and the Regional and Institutional Ethics Committee of the University of Debrecen, Medical and Health Science Center (Hungary). PBMCs were separated by a standard density gradient centrifugation with Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Monocytes were purified from PBMCs by positive selection using immunomagnetic cell separation with anti-CD14 microbeads, according to the manufacturer's instruction (MiltenyiBiotec, BergischGladbach, Germany). After separation on a VarioMACS magnet, 96–99% of the cells were CD14⁺ monocytes, as measured by flow cytometry. Monocytes were divided and cultured in 12-well tissue culture plates at a density of 2×10^6 cells/ml in 10% RPMI medium supplemented with three different growth factors: 80ng/ml GM-CSF (Gentaur Molecular Products, Brussels, Belgium), 100ng/ml IL-4 (PeproTech EC, London, UK), and M-CSF 50ng/ml (MACS, MiltenyiBiotec, Germany).

Peripheral blood lymphocytes and CD4⁺ T-cells

Autologous naive T-cells were separated from human blood mononuclear cells using the naive CD4⁺ T-cell isolation kit based on negative selection according to the manufacturer's instruction (MiltenyiBiotec).

Phenotypic characterization of myeloid cells by flow cytometry

Detection of the cell surface expression of monocyte-derived myeloid cells was performed by flow cytometry using anti-CD1a-PE, anti-CD209-PE, anti-CD14-PE, anti-CD83-PE, anti-CD103-PE, anti-CX3CR1-PE, anti-CCR7-PE (Beckman Coulter, Hialeah, FL, USA). The growth factor receptors were characterized by anti-GM-CSFR α -PE and anti-M-CSF R/CD115-PE (R&D Systems, USA) and isotype-matched control antibodies (BD PharMingen, San Diego, CA, USA). Fluorescence intensities were measured by FACS

Calibur (BD Biosciences, Franklin Lakes, NJ, USA), and data were analyzed by the FlowJo software (Tree Star, Ashland, OR, USA). The human chemokines MIP-1 α , CXCL7, CCL20 and CXCL16 were ordered from PeproTech, UK, CXCL8 and CXCL1 are from MiltenyiBiotec.

IL-17 and IFN γ ELISPOT assays

The monocyte-derived cells were cultured in GM-CSF+IL4, GM-CSF and M-CSF for 3 days along with the supernatant of unstimulated or cytokine activated Caco2 cells at 2×10^5 cells/well density. The cells were washed to remove all growth factors and supernatants and were co-cultured with naïve autologous CD4⁺ T-cells (10^6 cells/well) in 10% RPMI medium for 2 days at 37°C in a humidified atmosphere containing 5% CO₂. PHA (phytohaemagglutinin) and Con A (concanavalin A) activated T-cells were used as positive controls, untreated monocyte-derived cells, CD4⁺ T-cell co-cultures and CD4⁺ T-cells served as negative controls. Detection of cytokine-secreting T-cells was performed by the avidin-HRP system (NatuTec GmbH, Germany). Plates were analyzed by an ImmunoScan plate reader (CTL, Shaker Heights, OH, USA).

RT-qPCR

Total RNA was isolated by Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions and the total RNA content was quantitated by spectrophotometry (NanoDrop ND1000; Promega Biosciences, Madison, WI). cDNA was synthesized by using SuperScriptTM II Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative PCR was performed using 0.125 U Taq DNA polymerase (Fermentas, St. Leon-Rot, Germany) to detect double-stranded cDNA synthesis and Rox Reference Dye (Invitrogen) was used for normalization of the fluorescent reporter signal. Taqman Gene Expression Assay Hs00175474_m1 (Applied Biosystems) was used for the detection of β -defensin 2. The PCR cycling conditions involved an initial polymerase activation step for 5 minutes at 95°C, followed by 40 cycles of 12 s at 95°C for denaturation, 45 s at 60°C for annealing, and 15 s at 72°C for elongation. Relative expression levels were determined with StepOne Software v2.1 for StepOne Plus Real-Time PCR Systems (Applied Biosystems). The expression levels were calculated by the Δ Ct method using cyclophilin as a house keeping gene control.

Dot-blot analysis

40 µg protein of the Caco2 cell supernatants were spotted and dried to the PVDF membrane to perform the dot-blot analysis. The membranes were blocked with 5% milk powder containing TBS solution for one hour at room temperature. Anti β -defensin 2 IgG antibodies (Abcam, ab66072) were used at 5µg/ml concentration, and incubated for 24 hours at room temperature. The primary antibodies were washed out by repeated washing (three times for 5 minutes) with TBS. HRP-conjugated anti-mouse IgG antibodies (Amersham Biosciences, NA931V) were used as a secondary antibody in 1 ng/ml concentration, and after one hour incubation at room temperature the membranes were washed with TBS three times for five minutes. Visualization of the bands and dots were carried out with ECL reagent (Thermo Scientific) developed on a radiographic film.

Densitometry

Films were scanned using PharosFX Plus Molecular Imager (Bio-Rad) and the densitometry was carried out with Quantity One 4.6.7 software (Bio-Rad). Individual background subtraction was administrated in each case and the area under the intensity profile curve was calculated by the software.

ELISA

Determination of β -defensin 2 protein levels from 100 µl cell lysates and cell culture supernatants were performed by sandwich ELISA using the EK-072-37 kit (Phoenix Pharmaceuticals Inc) according to the provided protocol.

SRM-based targeted proteomic method development

In order to design specific SRM transitions for β -defensins we have utilized the amino acid sequences for β -defensins 1, 2, 3 and 4 available in the UniProt database (www.uniprot.org) followed by *in silico* trypsin digestion. Tryptic fragments with $\geq 95\%$ cleavage probability were selected for BLASTp analysis and the NCBI non-redundant protein sequence database was searched to determine the protein-specific, unique tryptic peptide sequences. Stable-isotope-labeled synthetic (SIL) crude peptides were obtained from JPT Peptide Technologies GmbH, Germany, while purified hBD2 SIL was obtained from PepsScanPresto, The Netherlands. The quality of the synthetic peptides was assessed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) conducted in our laboratory and the SRM spectra of singly charged y ions were recorded.

Sample preparation for mass spectrometry

The protein concentration of each sample was determined with Bradford method [30]. The proteins were denatured by the addition of 6 M urea and reduced using 10 mM dithiothreitol. The reduced samples were alkylated with 20 mM iodoacetamide and diluted with 25 mM ammonium bicarbonate to decrease the urea concentration to 1 M. Trypsin digestion was performed overnight at 37 °C by adding MS grade modified trypsin (ABSciex) in 1:25 enzyme: protein ratio. The digested peptides were lyophilized, dissolved in 1% formic acid. The samples were desalted with C₁₈ ZipTip tips (Millipore) and the eluates were lyophilized and redissolved in 1% formic acid.

Mass spectrometry analysis

SRM experiments were carried out on a 4000 QTRAP (ABSciex) mass spectrometer using NanoSpray II MicroIon Source and controlled by the Analyst 1.4.2 software (ABSciex). Carried out in collaboration with Proteomics Core Facility of University of Debrecen.

Statistical analysis

Statistical analysis was performed by one way analysis of variance (ANOVA) for multiple comparisons. Results are expressed as mean \pm SD. Two group differences were analyzed by Student's t-test. p value (two-tailed) less than 0.05 was considered statistically significant. The data for Mass spectrometry were statistically analyzed with the SigmaPlot 12.0 software (Systat Software Inc.) using student t-test. For the determination of correlation coefficient the linear regression method was used (Daniel W.W., Biostatistics: A foundation for analysis in the health sciences (Fifth edition), Wiley Series in probability and mathematical statistic-applied, 1991). The level of significance was set to 0.05.

5. RESULTS

5.1. The impact of ATRA on shaping human myeloid cell responses to epithelial cell-derived stimuli and on T-lymphocyte polarization

We designed *in vitro* experiments with resting human CEC and in an inflammatory milieu mimicked with TNF- α or IL-1 β stimulation in the presence or absence of ATRA. This was performed by monitoring the levels of secreted chemokines measured at the protein level and by investigating their impact on the phenotype and functional attributes of myeloid cells generated by different growth/differentiation factors. Considering that DC have the potential to instruct T-cells for inflammatory or regulatory directions, our final goal was to identify the impact of stimulated CEC-induced and DC-mediated effects on CD4⁺ effector T-lymphocyte responses.

5.1.1. Identification of chemokines secreted by resting and activated CEC

The single cell monolayer of CEC plays an essential role in the maintenance of gut homeostasis by supporting barrier function and defense against microbes preferentially through the secretion of chemokines. It is also well established that the pro-inflammatory cytokines IL-1 β and TNF- α act as potent activators of CEC[63]. In this study we applied a high throughput approach for identifying the chemokines secreted by CEC (Caco2, HT-29 and HCT116) in response to IL-1 β and TNF- α by using a commercially available Human Chemokine Array to quantify the relative levels of chemokines released by resting and activated CEC at the protein level. The sample controls provided (transferrin R, gp130 and fibrinogen) were used for calculating mean pixel densities of the respective dot blots. The results showed that resting Caco2 cells secrete detectable levels of CCL19, CCL21 and CCL22 constitutively (dots 2D, 9F, 8D in Figure 5A, summarized in Figure 5C). Similar results were obtained for HT-29 cells, but HCT116 secreted only CCL19 at detectable levels. Comparison of the relative cytokine levels secreted by the Caco2, HT-29 and HCT116 cell lines are summarized in Table 1. Considering that these CCL chemokines are known to attract myeloid cells, they may maintain a population of myeloid cells in the vicinity of CEC to support cellular interactions. It has previously been shown that MDC/CCL22 attracts T_H2 cytokine producing cells and its mRNA and protein expression is upregulated against enteroinvasive bacteria, but inhibition of the NF- κ B pathway abolished CCL22 expression in response to pro-inflammatory stimuli[1]. The chemokines CXCL7, CXCL16 and Mx with different functional activities were also constitutively secreted by resting CEC (dot 9C, 7F, 7D in Figure 5A) suggesting their role in the maintenance of epithelial cell homeostasis.

Table 1 : Relative expression of chemokines secreted by colonic epithelial cell lines

Caco2, HT-29 and HCT116 cells were treated with IL-1 β or TNF- α in the presence or absence of ATRA for 60 min. After removing the cell culture supernatant the cells were washed and replaced with fresh medium and the supernatants were collected after 5 hours and used for the Chemokine Array analysis according to the manufacturer's instruction to determine the variety of chemokines secreted by these cell lines. The values indicate the results of the densitometric analysis of dot blots and are normalized to the relevant sample control according to the manufacturer's instructions. For simplicity the value of 1 obtained after dividing with the sample control is referred to as 100.

		CCL19	CCL21	CCL22	Mk	CXCL16	CXCL7	CXCL1	CXCL8	CCL20
C	Caco2	30	30	30	90	70	40	-	-	-
	HT-29	40	30	40	80	90	60	-	-	-
	HCT-116	150	-	-	170	170	-	-	120	-
IL-1β	Caco2	-	-	-	100	80	-	120	140	30
	HT-29	-	-	-	80	90	-	100	90	30
	HCT-116	-	-	-	150	160	100	100	100	100
IL-1β+ATRA	Caco2	-	-	-	110	80	50	100	130	-
	HT-29	-	-	-	100	80	40	100	90	-
	HCT-116	-	-	-	160	140	90	110	110	-
TNF-α	Caco2	-	-	-	90	60	-	-	-	-
	HT-29	-	-	-	80	60	-	-	-	-
	HCT-116	-	-	-	150	150	-	10	10	-
TNF-α+ATRA	Caco2	-	-	-	90	60	-	-	-	-
	HT-29	-	-	-	90	80	20	-	-	-
	HCT-116	-	-	-	150	130	20	10	-	-
ATRA	Caco2	-	-	-	50	30	-	-	-	-
	HT-29	-	-	-	60	20	-	-	-	-
	HCT-116	40	20	30	80	80	-	-	-	-

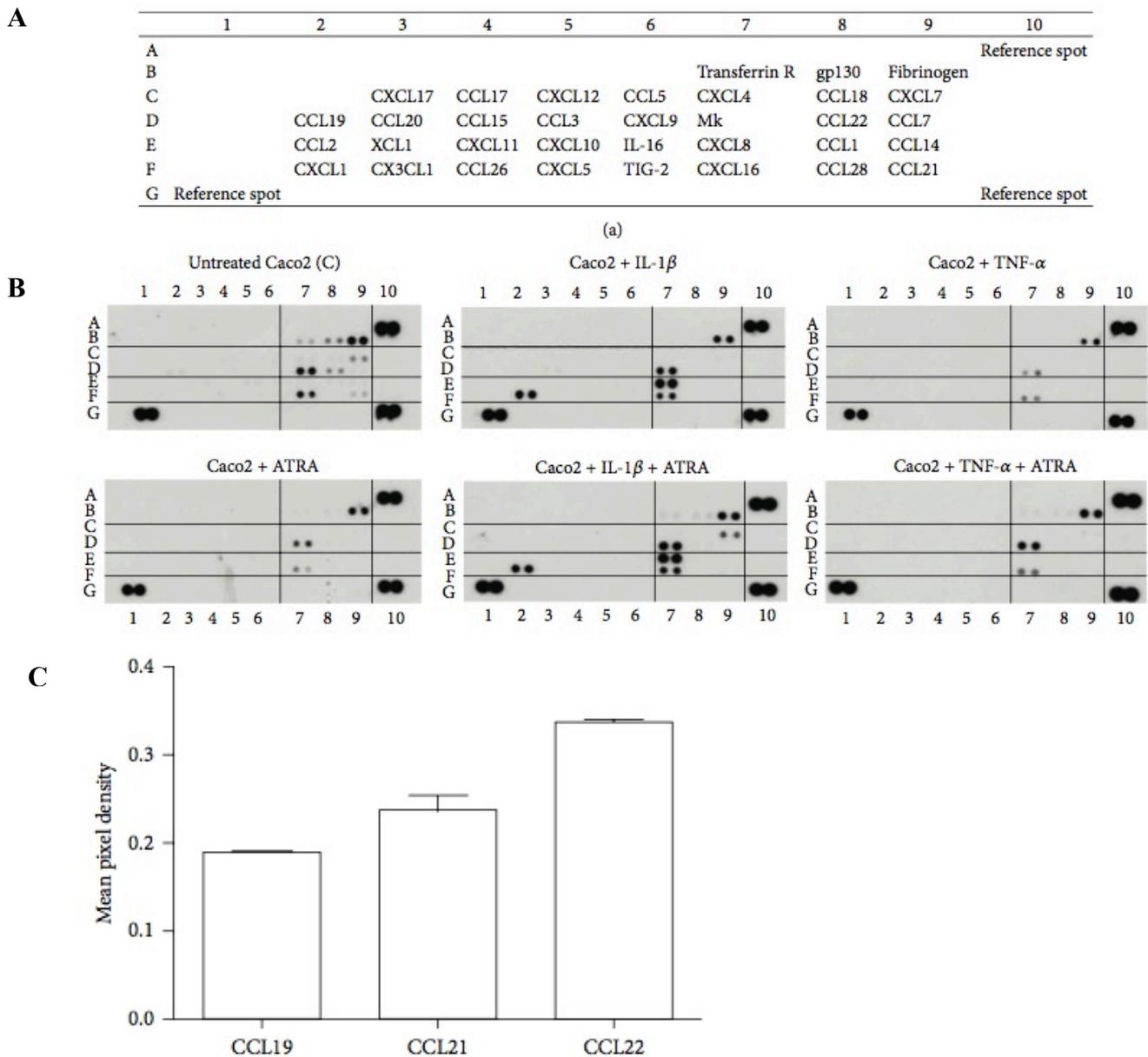


Figure 5: Chemokine secretion of human epithelial cells activated by IL-1 β or TNF- α in the presence or absence of ATRA: The relative levels of the Caco2 cell-derived chemokines were determined by calculating mean pixel densities of the individual blots normalized to sample control. The mean+SD of 4 independent measurements are shown. **A** – Localization of the chemokine probes in the membranes related to the the positive controls. **B** – Representative dot blots showing the relative expression of chemokines produced by untreated Caco2 cells without or with ATRA (upper and lower **panels 1**) as compared to Caco2 cells stimulated by IL-1 β (upper **panel 2**) and IL-1 β in the presence of ATRA (lower **panel 2**). Upper and lower **panels 3** correspond to Caco2 cells stimulated by TNF- α in the absence or presence of ATRA, respectively. **C** – Relative expression levels of CCL chemokines produced by unstimulated Caco2 cells.

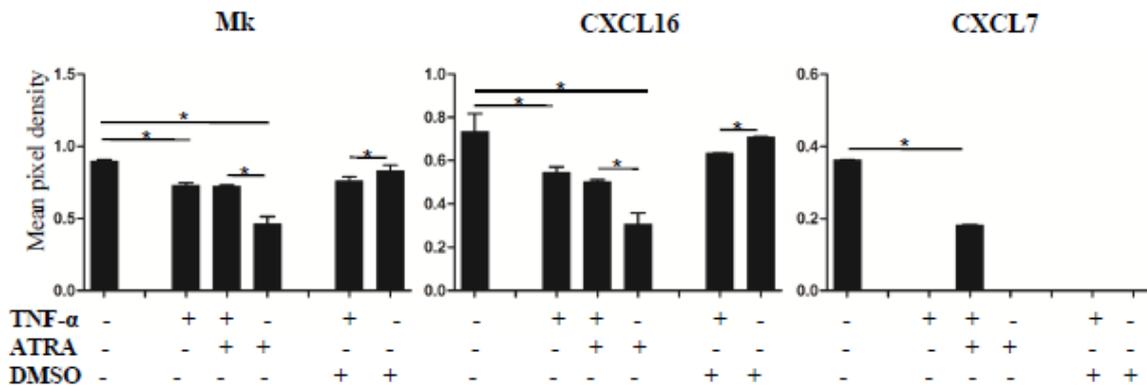
5.1.2. The effect of ATRA on the chemokine secretion by CEC pre-stimulated by IL-1 β or TNF- α

In our model system CEC were left untreated or stimulated by IL-1 β or TNF- α in combination with or without ATRA for 6 hrs and the cell culture supernatants were subjected to chemokine array analysis by calculating pixel densities using the relevant sample control (Figure 5). When Caco2 cells were activated by TNF- α or IL-1 β , the secretion levels of the chemokines CXCL7, CXCL16 and M κ did not change significantly as compared to unstimulated cells (dots 9C, 7F, 7D in Figure 5A, summarized in Figure 6A, 6B). However, the expression of CXCL16 and M κ was down regulated in the presence of ATRA suggesting that the secretion of these chemokines may contribute to the maintenance of epithelial cell homeostasis. However, under inflammatory conditions they do not mediate positive signals for DC. Remarkably, the secretion of CXCL7 (dot 9C in Figure 5A, summarized in Figure 6A and 6B) could be induced only when IL-1 β or TNF- α was combined with ATRA treatment demonstrating the dependence of its secretion on ATRA. DMSO, which is the standard solvent of ATRA also decreased CXCL7 secretion (Figure 6A and 6B). Depending on CEC sensitivity to DMSO, certain effects of this solvent have previously been reported, as inhibition of prostaglandin E2 production upon treatment of Caco2 cells with IL-1 β and attenuation of mRNA levels of IL-6, IL-1 α and IL-1 β [64]. These findings might be in line with inhibition of CXCL7 observed in our system. In the IEC-18 cell line IL-1 β induced increased mRNA levels of MCP-1/CCL2, MIP-1 α /CCL3, inducible NO synthase and RANTES/CCL5, which are upregulated in a NF- κ B dependent manner[65].

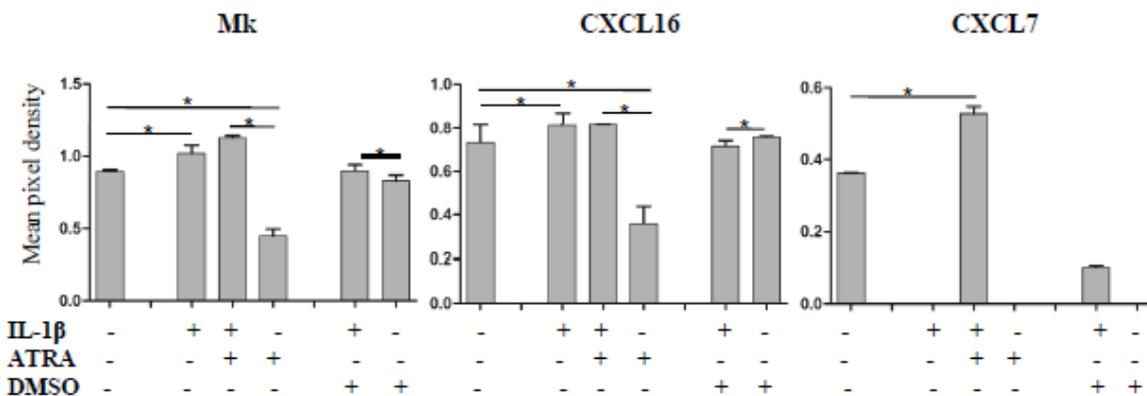
The secretion levels of CCL20 and CXCL1 was not affected by ATRA (dots 3D, 2F and 7E in Figure 5A, summarized in Figure 6C) and could be induced exclusively by IL-1 β but not by TNF- α . Surprisingly, CXCL8 secretion was upregulated not only by IL- β with or without ATRA, but also by DMSO used as a vehicle for ATRA (Figure 6C). DMSO induced strain on the actin cytoskeleton and integrins expressed by Caco2 cells were suggested to be transferred to actin-associated molecules like α -actinin-1, acting as a scaffold protein and interacted directly with ERK1/2 leading to phosphorylation and increased secretion of CXCL8. The *in vivo* relevance of this effect is underscored by the physical deformation of CEC during peristalsis and villous motility[66]. Along with IL-8/CXCL8 and GRO α /CXCL1, Caco2 cells also express MCP-1/CCL2 as a result of IL-1 stimulation, but our chemokine array-based method did not detect CCL2[67]. These results suggest that the expression of individual chemokines depends on the means of activation and also on ATRA, which can modulate the outcome of chemokine secretion, whereas the group of chemokines not affected by ATRA

indicates the complexity of chemokine-mediated regulation in the gut. Similar results were obtained for HT-29 and HCT116 CEC as summarised in Table1. [65, 68, 69].

A



B



C

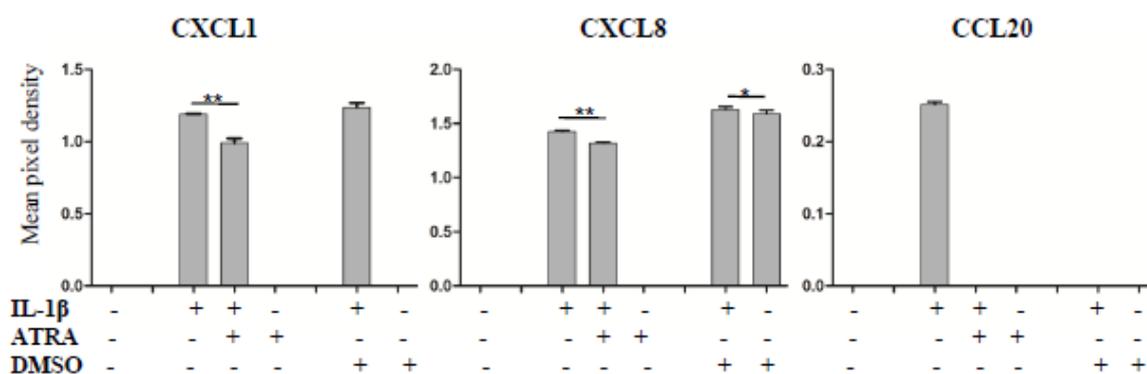


Figure 6: Effects of ATRA on the expression of chemokines in cytokine-stimulated Caco2 cells: Relative expression of chemokines in Caco2 cells, pre-stimulated by TNF- α or IL-1 β in the presence or absence of ATRA, was determined as described in Figure 5 and was compared to unstimulated cells cultured with or without ATRA. The possible contribution of DMSO used as a solvent control of ATRA was tested in Caco2 cells cultured in the presence of TNF- α or IL-1 β with or without DMSO. **A** – Expression of chemokines secreted by Caco2 cells pre-stimulated by TNF- α , in the presence or absence of ATRA. **B** – Expression of chemokines secreted by

*Caco2 cells pre-stimulated by IL-1 β in the presence or absence of ATRA. C – Chemokines induced exclusively by IL-1 β stimulation in Caco2 cells. *p<0.05 **p<0.01 Bar diagrams indicate mean \pm SD of 4 dot blots which was averaged after densitometry analysis and normalized with the 'sample control' provided with the kit.*

5.1.3. ATRA regulates the chemokine-dependent migration of myeloid cells generated by different hematopoietic growth/differentiation factors

Based on the results showing the inhibitory effect of ATRA on the secretion of some chemokines, we next sought to assess the chemokine-driven migratory potential of myeloid cells. We set up a transwell system and measured myeloid cell migration *in vitro* by using CCL19 and CCL21 chemokines as positive controls of cell recruitment. Myeloid cells differentiated from primary human monocytes by GM-CSF+IL4 or GM-CSF to DC exhibited detectable but low migratory potential as compared to cells mobilized by high concentration (200 ng/ml) of CCL19 and CCL21 chemokines (Figure 7A and 7B), while monocytes cultured in M-CSF gave rise to macrophages with undetectable migratory activity (data not shown). The highest migratory potential could be attributed to cells differentiated in the presence of GM-CSF or GM-CSF+IL4 and stimulated by the supernatant of Caco2 cells pre-activated by IL-1 β , but this process could be down regulated by ATRA. To analyze whether the supernatant of IL-1 β -stimulated CEC has a direct effect on the migration of DC differentiated by GM-CSF+IL4 or GM-CSF the cells were subjected to direct cell migration assays toward the chemokines exclusively secreted by IL-1 β used at pre-titrated concentrations (CXCL1 (1ng/ml), CXCL8 (100ng/ml) and CCL20 (50ng/ml). We observed the high migratory capacity of DC differentiated by GM-CSF+IL4 (Figure 7C) and Mf developed by GM-CSF (Figure 7D) toward CXCL1 and CXCL8 known to be involved in the chemotaxis and migration of polymorphonuclear leukocytes to inflammatory sites [70, 71] as compared to CCL20. Interestingly, the supernatant of Caco2 cells pre-stimulated with TNF- α had no such effect on cell migration (Figure 7A and Figure 7B). When the migratory potential of myeloid cells was related to the cell surface expression of CCR7 we found that that DC differentiated by GM-CSF in the presence of ATRA-conditioned CEC supernatant exhibited decreased CCR7 expression, while in cells differentiated in GM-CSF+IL4 it remained unchanged (Figure 7E). Similar results were obtained by using the HT-29 and HCT116 cell lines (data not shown). These results suggest that in an inflammatory environment ATRA also modulates the migratory potential of myeloid cells in a cell type-dependent manner.

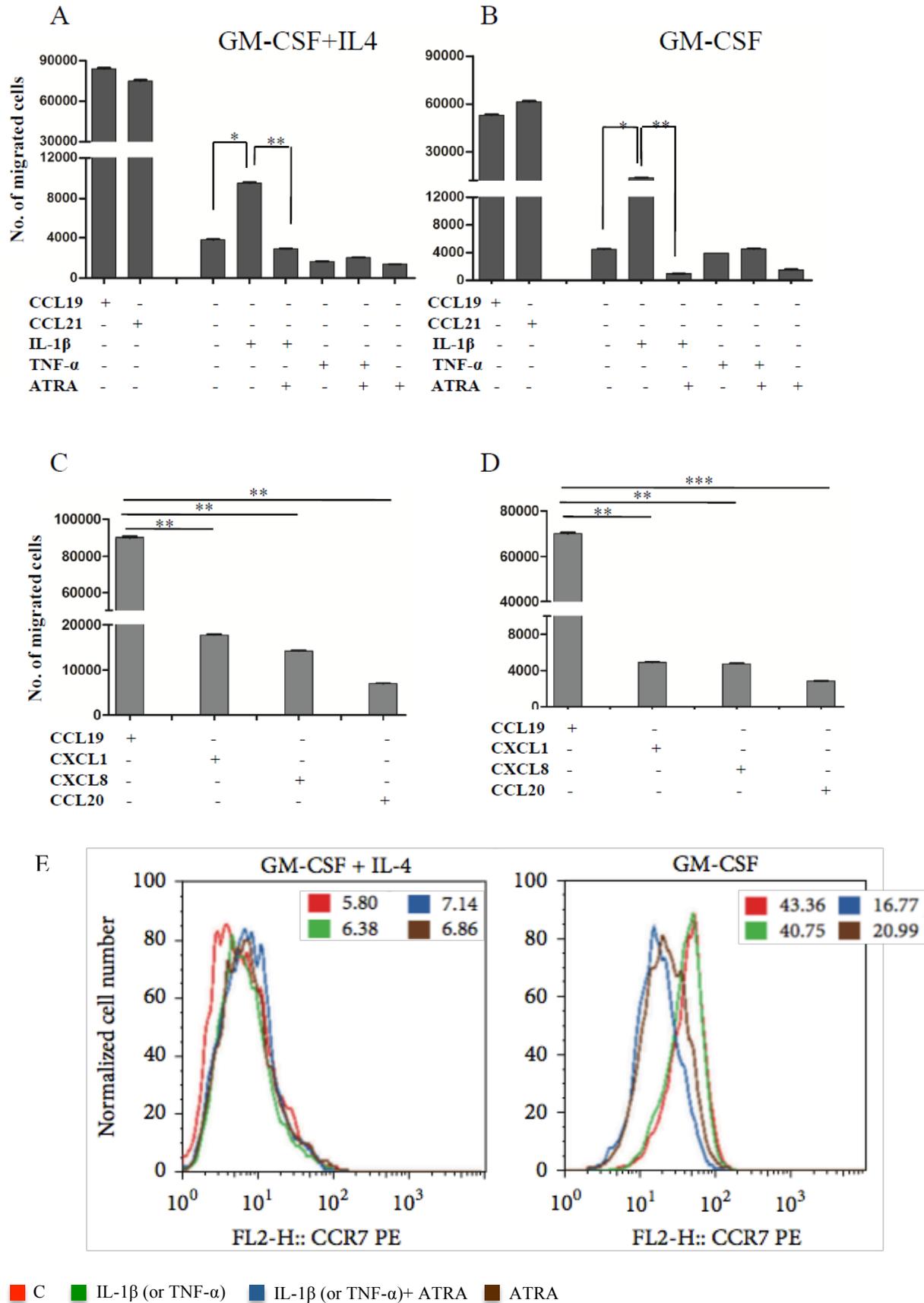


Figure 7: ATRA modifies the chemokine-dependent migration of in vitro differentiated myeloid cells: Caco2 cells were activated as described in Figure 5. The migratory potential of the monocyte-derived cells generated

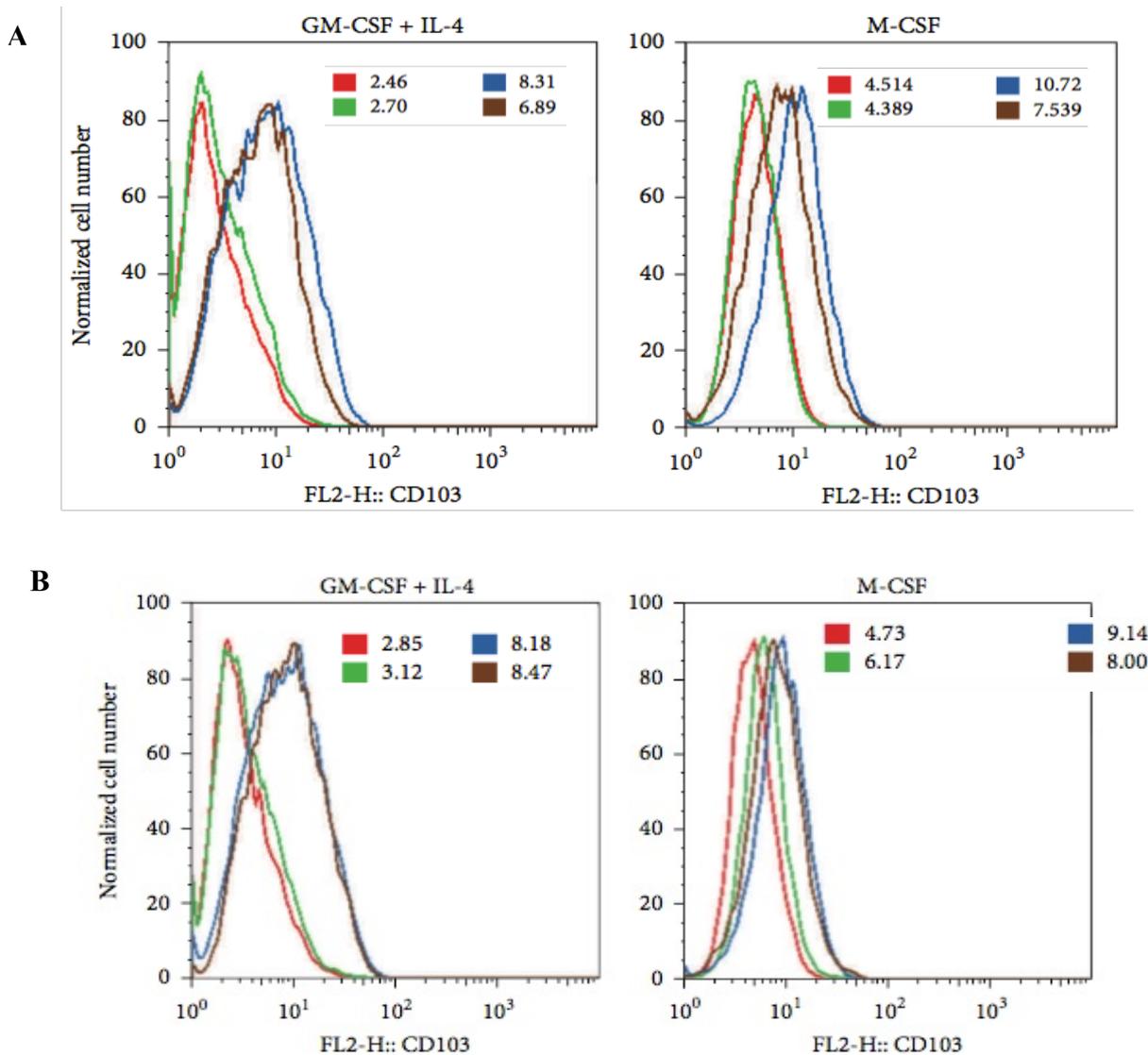
by GM-CSF+IL4 or GM-CSF was tested in transwell chambers. 3×10^5 cells were placed to the upper chamber and the Caco2 cell supernatants to the lower chamber of the transwell plate. After 24 hrs the number of cells, which migrated to the lower chamber in response to the Caco2 cell supernatants was counted by flow cytometry. The migratory potential of cells generated in the presence of GM-CSF+IL4 (A) or GM-CSF (B) is shown as compared to the migratory potential of CCL19 and CCL21 chemokines, used as positive controls. To determine the cause of high migration in response to IL-1 β treated CEC supernatant the GM-CSF+IL4 (C) and GM-CSF (D) differentiated monocytes were treated with CXCL1, CXCL8 and CCL20 chemokines which were secreted exclusively by CEC on IL-1 β treatment. Bar diagrams indicate mean \pm SD of 3 independent experiments * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ Another fraction of monocyte-derived cells, differentiated by GM-CSF+IL-4 or GM-CSF were also stimulated by the supernatant of IL-1 β pretreated CEC in the presence or absence of ATRA. The cell surface expression of CCR7 in the differentiated monocyte derived cells was measured by flow cytometry (E). Red line depicts untreated monocyte-derived cells, green is for monocyte-derived cells treated by the supernatant of Caco2 cells previously activated by IL-1 β , blue line is for monocyte-derived cells pre-treated with the supernatant of Caco2 cells activated by IL-1 β and ATRA, brown line corresponds to cells treated by the supernatant of unstimulated Caco2 cells in combination with ATRA. Results of 3 independent experiments are shown as mean \pm SD of MFI.

5.1.4. ATRA supports the development of migratory CD103⁺ myeloid cells

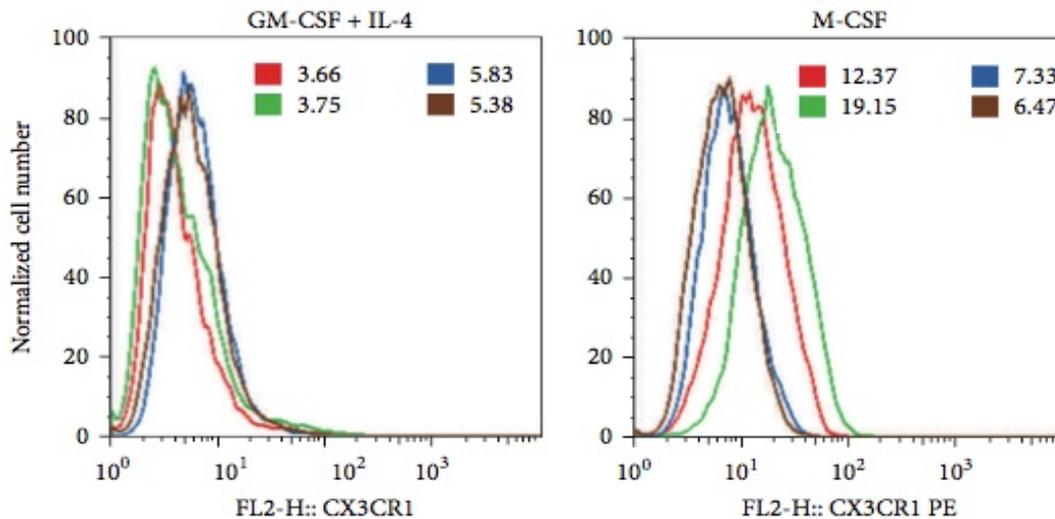
The dominant DC population of the gut is represented by CD103⁺ migratory cells that express the enzymes required for the metabolism of VA[4], while the CX3CR1⁺ resident Mf population samples the microenvironment by protruding dendrites[72]. To assess how efficiently we could manipulate the effects of ATRA on CEC, we differentiated blood-derived monocytes with GM-CSF+IL4 or M-CSF to generate DC and Mf, respectively followed by the stimulation of cells with the supernatant of activated Caco2 cells. The cell surface expression of CD103⁺ and CX3CR1⁺ measured by FACS analysis revealed that the presence of Caco2 cell supernatants obtained from ATRA+IL-1 β , ATRA+TNF- α or ATRA-pretreated CEC could increase the expression of the CD103 integrin in cells differentiated by GM-CSF+IL4 or M-CSF to obtain DC and Mf, respectively (Figure 8A, B). These results also indicated that even in the presence of inflammatory stimuli (supernatant of IL-1 β or TNF- α activated CEC) ATRA was able to promote the development of CD103⁺ myeloid cells. In a similar experimental system, the frequency of CX3CR1⁺ cells generated by GM-CSF+IL4 and stimulated by cytokine-activated CEC supernatant was also increased in case the cell culture was conditioned by ATRA. In contrast to this finding, the expression of CX3CR1 remained unchanged in cells generated by GM-CSF (data not shown), and was decreased when the ATRA-conditioned CEC supernatant was added to Mf differentiated from monocytes with M-CSF and activated by IL-1 β or TNF- α (Figure 8C and 8D). Similar results were obtained with HT-29 and HCT116 cells

(data not shown). To confirm that the level of ATRA could be reconstituted in CEC after the washing procedure, we also added ATRA directly to DC and detected increased expression of CD103. To rule out the effect of other chemokines in the development of CD103⁺ cells, the chemokines secreted by activated CEC were added directly to monocytes at pre-titrated concentrations (Mk 10ng/ml, CXCL16 100ng/ml, CXCL7 10ng/ml, CXCL1 1ng/ml, CXCL8 100ng/ml and CCL20 50ng/ml) along with the differentiating growth factors GM-CSF+IL4 or M-CSF and the expression of CD103 was measured by FACS. When the *in vitro* generated myeloid cells were treated directly with the different chemokines in the absence of ATRA, no phenotypic changes of DC and Mf could be detected, showing their direct dependence on ATRA.

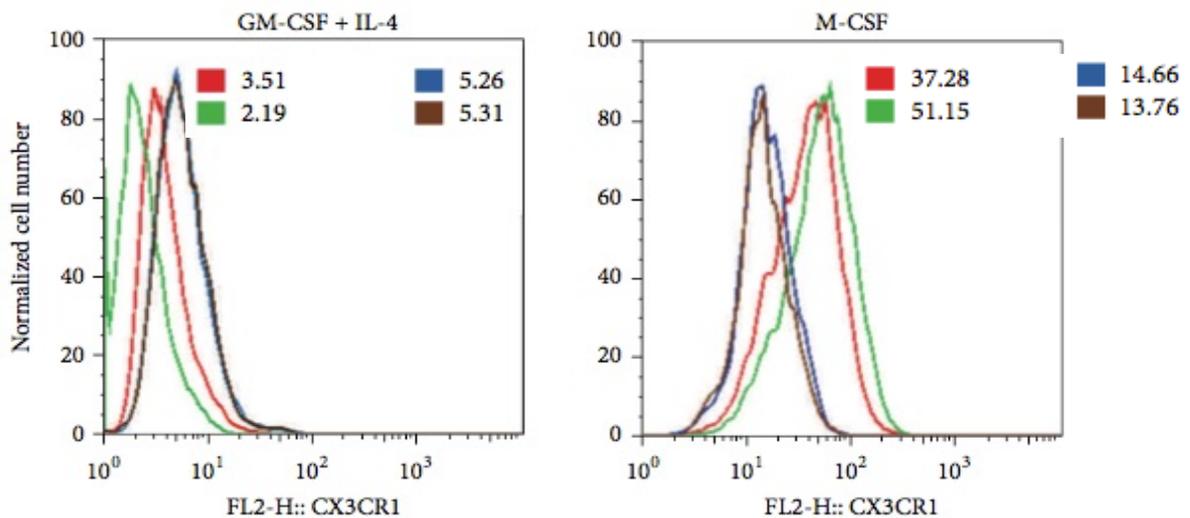
■ C ■ IL-1 β (or TNF- α) ■ IL-1 β (or TNF- α)+ ATRA ■ ATRA



C



D



■ C
 ■ IL-1β (or TNF-α)
 ■ IL-1β (or TNF-α)+ ATRA
 ■ ATRA

Figure 8: ATRA promotes the expression of CD103⁺ cells in in vitro differentiated myeloid cells: Monocytes were differentiated in the presence of GM-CSF+IL4 or M-CSF, respectively. The differentiated cells were treated by the supernatants of Caco2 cells pre-activated by IL-1β or TNF-α in the presence or absence of ATRA. The phenotype of the myeloid cells was characterized by measuring the cell surface expression of α4β7/CD103 and CX3CR1 on day 3 of in vitro myeloid cell differentiation by flow cytometry. Histograms show the cell surface expression of CD103 in cells differentiated by GM-CSF+IL-4 (or M-CSF) and stimulated by the supernatants of Caco2 cells pre-treated by IL-1β (A) or TNF-α (B) and the cell surface expression of CX3CR1 in cells differentiated by M-CSF (or GM-CSF+IL-4) and stimulated by the supernatants of Caco2 cells pre-stimulated by IL-1β (C) or TNF-α (D). MFI of a typical measurement out of 3-5 independent experiments is shown. Red line depicts untreated cells, green is for cells pretreated with the supernatant of Caco2 cells activated by IL-1β or TNF-α, blue is for myeloid cells pre-treated with the supernatant of Caco2 cells activated by IL-1β or TNF-α in combination with ATRA, brown corresponds to cells treated by the supernatant of unstimulated Caco2 cells and ATRA.

5.1.5. ATRA promotes development of CD14, GM-CSF receptor expression and autocrine secretion of IL-1 β in GM-CSF

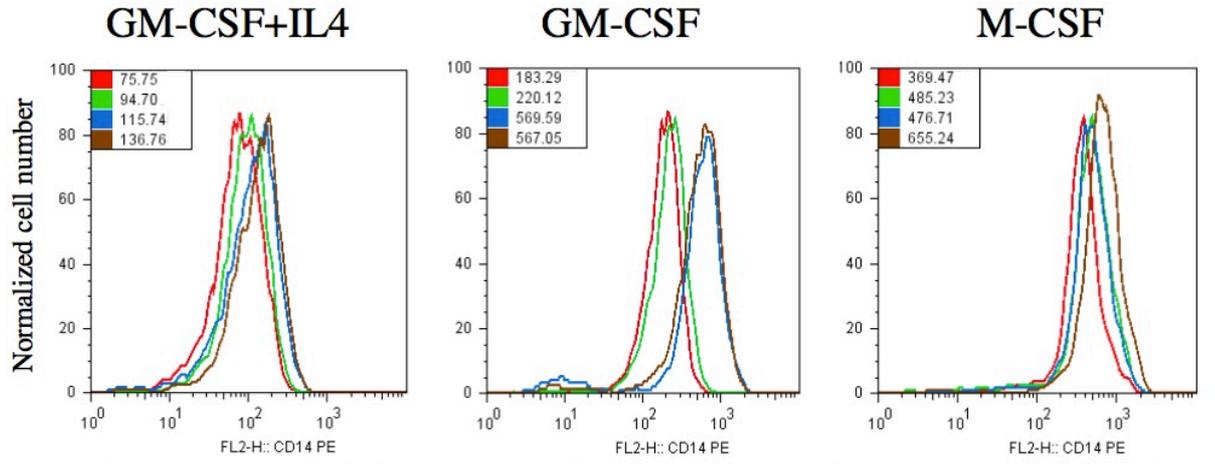
Inhibited expression of the LPS-binding receptor CD14 is associated with increased susceptibility to gastroenteritis and ulcerative colitis (UC)[90] so we used FACS analysis to study the effect of ATRA on CD14 surface expression. We observed the suppression of CD14 expression in myeloid cells generated with GM-CSF+IL-4 and GM-CSF but those matured in M-CSF did not show much effect on CD14 surface expression by ATRA (Figure 9A). The expression of GM-CSF receptor is higher in healthy individuals than in UC or Crohn's Disease (CD) patients[40], we observed that ATRA enhances the receptor expression in GM-CSF and M-CSF matured myeloid cells in comparison to untreated myeloid cells (Figure 9B). The expression of M-CSFR (Figure 9B) was shown to be enhanced by IL-1 β stimulated CEC supernatant and the number of Th17 cells was increased when CD4⁺ T-cells were co-incubated with Mf educated by the supernatant of CEC pre-activated by IL-1 β in the presence of physiological concentration of ATRA (Figure 10C). Mf in the gut with the help of IL-1 β and GM-CSF produce ATRA and IL-10 for maintainance of gut homeostasis[12] so we measured the IL-1 β re-secretion by ELISA. Caco2 cells were treated with IL-1 β in the presence of ATRA for 1hr. The culture medium of Caco2 was removed, replaced with fresh medium and the cells were cultured for an additional 5hrs. The supernatant was collected and added to monocytes differentiating in GM-CSF+IL4 or GM-CSF cells for 2hrs. The myeloid cells were washed and replaced with medium and after 24hrs the supernatant was used for IL-1 β ELISA. The autocrine secretion of IL-1 β by monocyte-derived cells was observed exclusively when the monocytes were generated in the presence of GM-CSF (Figure 9C).

Figure 9: ATRA promotes CD14⁺, GM-CSF receptor expression and autocrine secretion of IL-1 β in GM-CSF myeloid cells: Monocytes were differentiated in the presence of GM-CSF+IL4, GM-CSF or M-CSF, respectively. The differentiated cells and phenotype of the myeloid cells was characterized as mentined in Figure 4. Histograms show the cell surface expression of CD14 (A) and receptor expression (B) in cells differentiated by GM-CSF+IL-4, GM-CSF or M-CSF respectively. MFI of 3-5 independent experiments is shown. Red line depicts untreated cells, green is for cells pretreated with the supernatant of Caco2 cells activated by IL-1 β or TNF- α , blue is for myeloid cells pre-treated with the supernatant of Caco2 cells activated by IL-1 β or TNF- α in combination with ATRA, brown corresponds to cells treated by the supernatant of unstimulated Caco2 cells and ATRA. (C) Caco2 cells were treated with IL-1 β in the presence of ATRA for 1hr. The culture medium of Caco2 was removed, replaced with fresh medium and the cells were cultured for an additional 5hrs. The supernatant was collected and added to monocytes differentiating in GM-CSF+IL4 or GM-CSF. The autocrine secretion of IL-1 β induced by the activated Caco2 supernatant was measured by ELISA. The autocrine secretion of IL-1 β by

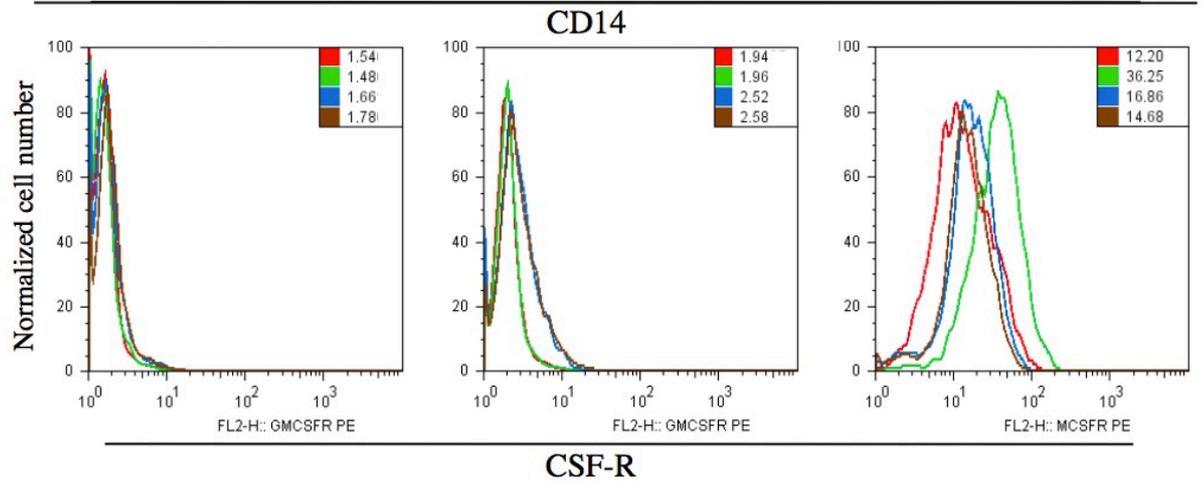
monocyte-derived cells was observed exclusively when the monocytes were generated in the presence of GM-CSF. Bar diagrams indicate mean \pm SD of 3 independent experiments * p <0.05 ** p <0.01

■ C ■ IL-1 β (or TNF- α) ■ IL-1 β (or TNF- α)+ ATRA ■ ATRA

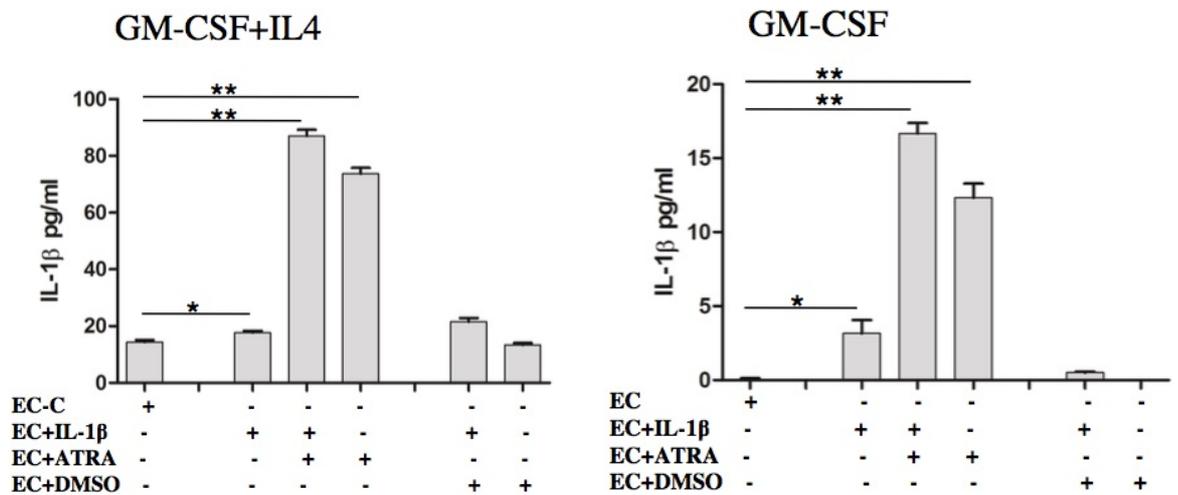
A



B



C



5.1.6. Translation of the molecular information collected by CEC-stimulated myeloid cells to CD4⁺ T-lymphocytes

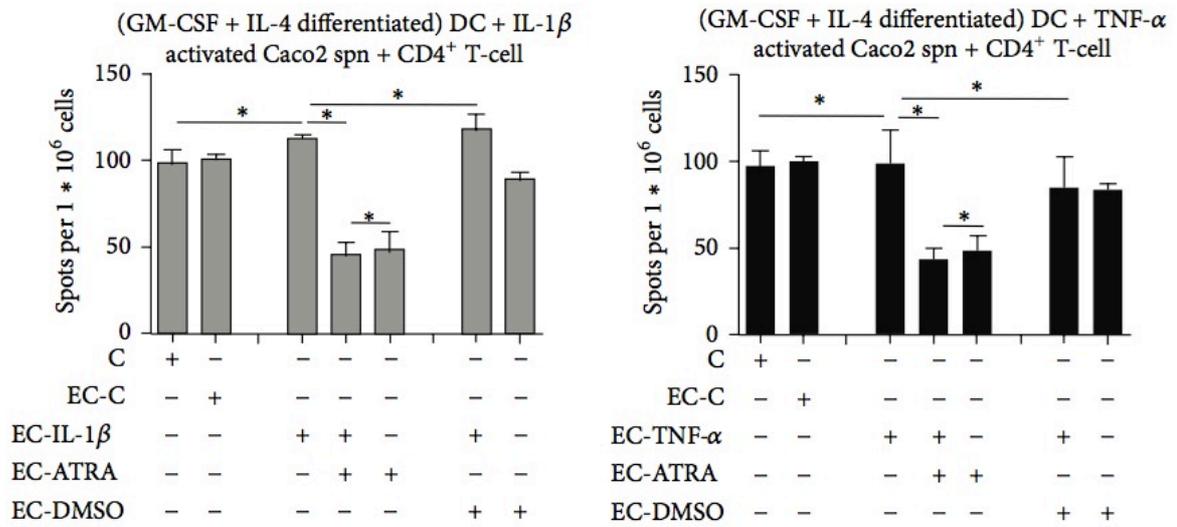
Considering the sensitivity of myeloid cells to pro-inflammatory signals provided by activated CEC and the modulatory effects of ATRA, we set out to test whether DC and Mf as antigen presenting cells could activate and polarize T-lymphocytes. To test this scenario, myeloid cells differentiated by GM-CSF+IL4, GM-CSF and M-CSF respectively, were activated by supernatants of cytokine-activated Caco2 cells followed by co-culturing them with autologous CD4⁺ T-lymphocytes, and the number of IL-17 and IFN γ cytokine producing T-cells was detected by ELISPOT assays. We found that CD4⁺ T-cells co-cultured with myeloid cells differentiated from monocytes to DC with GM-CSF+IL4 (Figure 10A) or GM-CSF (Figure 10B) and 'educated' by the supernatants of activated Caco2 cells in combination with ATRA resulted in significant suppression of IL-17 producing cell numbers. In contrast, monocyte-derived cell generated by M-CSF (Figure 10C) and pre-treated in a similar manner before co-culture with CD4⁺ T-cells, the number of IL-17 cytokine secreting cells was significantly increased indicating that DC and Mf exhibit different T-cell polarizing activities. Although slight differences could be observed in the magnitude of T-cell responses provoked by CEC supernatants activated by IL-1 β or TNF- α , the T-lymphocyte responses were polarized to the T_H17 direction in both cases independent on the pro-inflammatory cytokine used for CEC stimulation underpinning the role of DC-mediated inflammatory signals in driving CD4⁺ T-lymphocyte responses. Under similar culture conditions IFN γ -secreting cells could not be detected in the CD4⁺ T-cell population. Similar 'education' of myeloid cells by ATRA-conditioning was observed also for HT-29 and HCT116 cell lines (data not shown).

Figure 10: The effect of gut epithelial cell-mediated myeloid cell stimulation on the activation and polarization of T-lymphocytes:

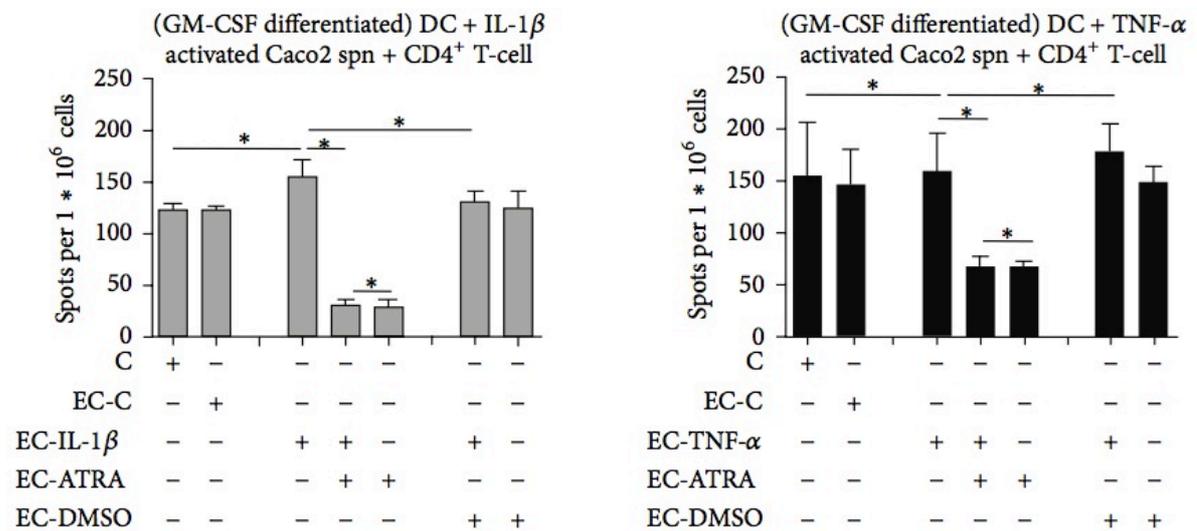
To assess the effects of stimulated myeloid cells on effector T-cell polarization the supernatants of activated Caco2 cells were added to myeloid cells differentiated by GM-CSF+IL4, GM-CSF or M-CSF for 3 days and the washed cells were co-cultured in fresh medium with autologous CD4⁺ T-lymphocytes for another 2 days. The activation and polarization of CD4⁺ T-cells co-incubated with myeloid cells generated by GM-CSF+IL4 (A), GM-CSF (B) or M-CSF (C) was detected by the IL-17 ELISPOT assay. In the figure legends, C stands for unstimulated myeloid cells co-incubated with CD4⁺ T-cells, EC-C for monocyte-derived cells 'educated' by resting Caco2 cell supernatant followed by co-incubation with CD4⁺ T-cells, EC-IL-1 β , EC-TNF- α , EC-ATRA, EC-DMSO correspond to cultures containing monocyte-derived cells 'educated' with the supernatants of Caco2 cells pretreated with IL-1 β or TNF- α in the presence or absence of ATRA, or with DMSO used as solvent

control, followed by co-incubation with CD4⁺ T-cells. **p*<0.05 Mean±SD of the number of IL-17 secreting cells measured in 4 independent experiments are shown.

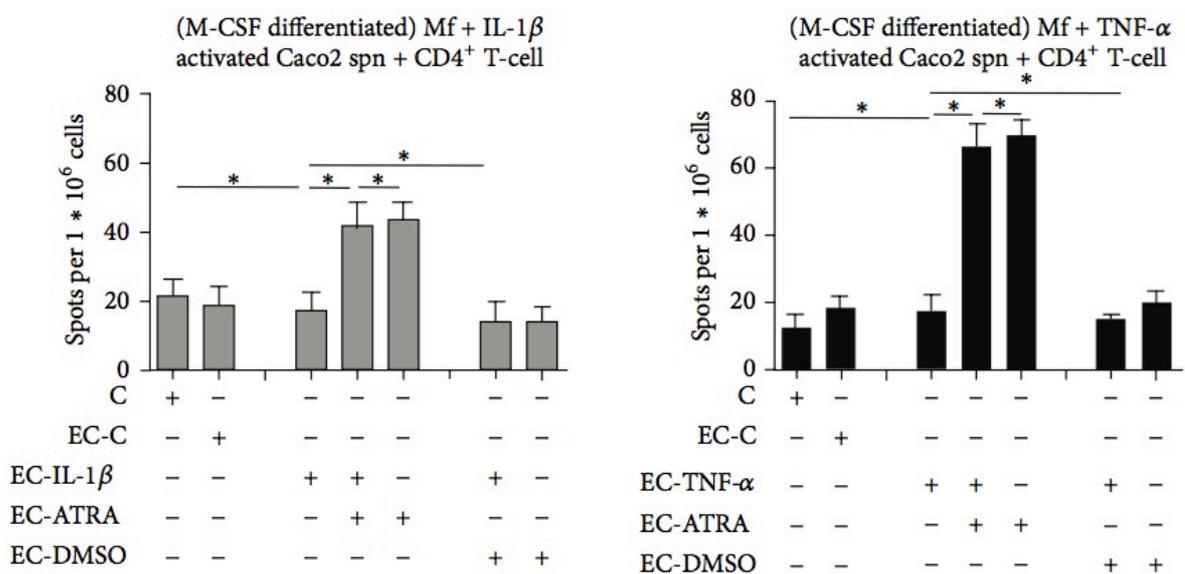
A



B



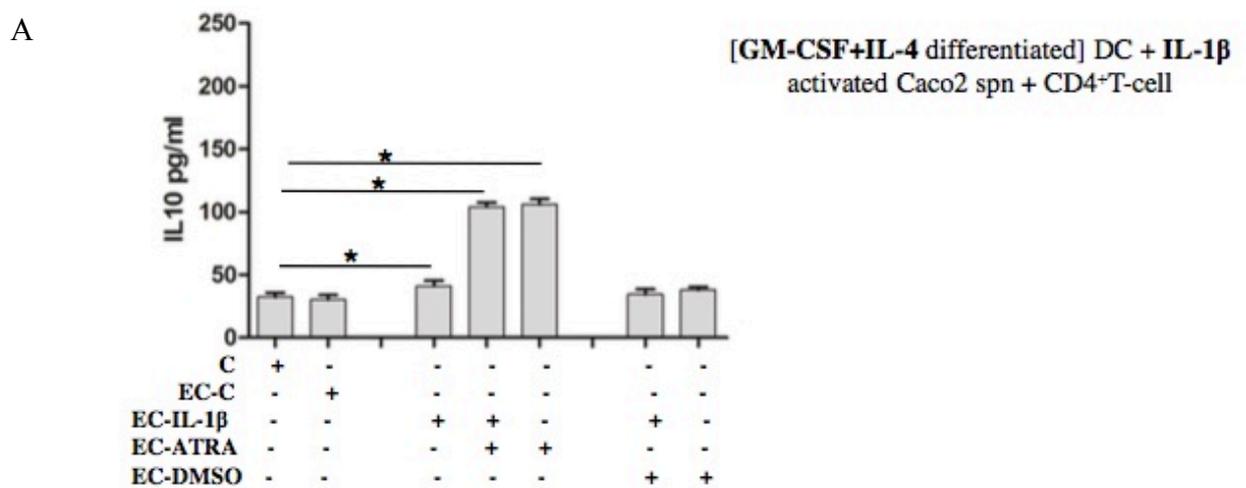
C

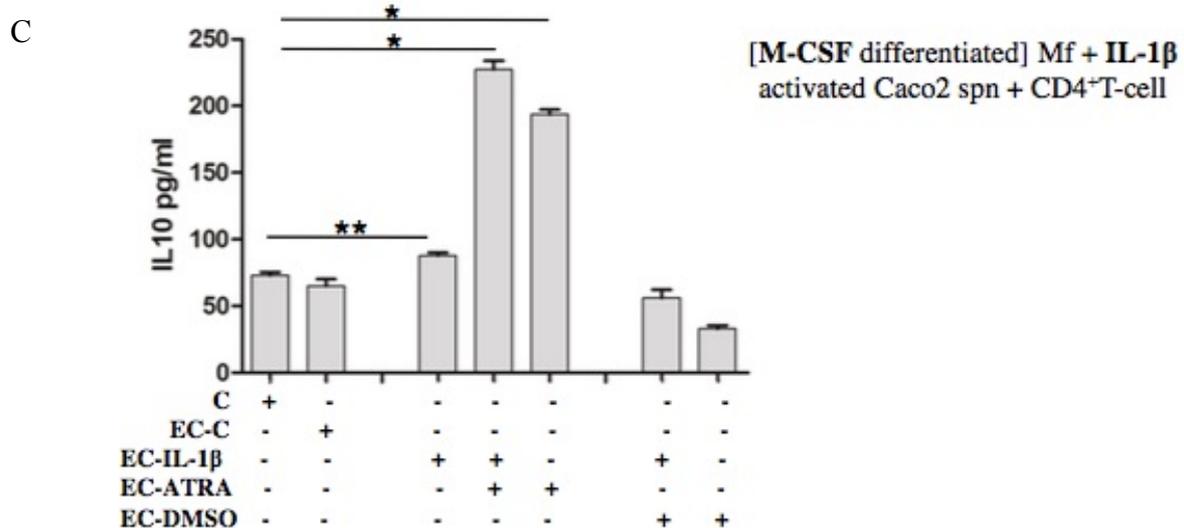
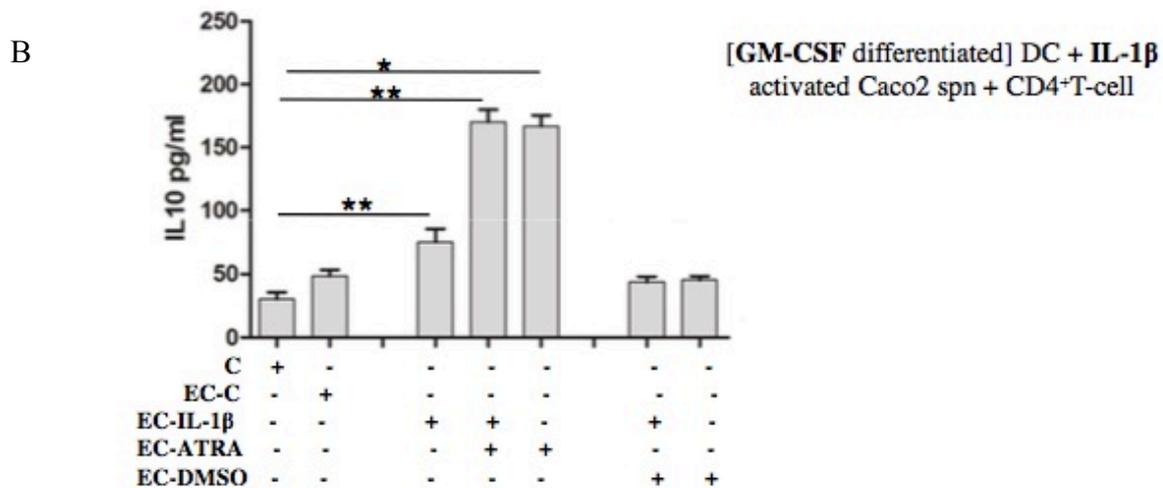


5.1.7. CD4⁺ T-lymphocytes secrete IL-10 after coincubation with 'educated' myeloid cells

Th17 cells can exhibit both anti- and pro-inflammatory properties depending on the cytokine signals received[95] confirmed by our results showing suppression of IL-17 secretion in GM-CSF+IL-4 and GM-CSF matured myeloid cells whereas IL-17 secretion was enhanced in M-CSF mature myeloid cells. Taking into account the limitations of an *in vitro* model system and the fact that T_{reg} cells are attributed to maintenance of homeostasis in the gut by secretion of IL-10, we attempted to observe IL-10 secretion by CD4⁺ T cells using ELISA. Irrespective of the fact whether CD4⁺ T cells were incubated with 'educated' myeloid cells generated in GM-CSF+IL-4 (Figure 11A), GM-CSF (Figure 11B) or in M-CSF (Figure 11C), IL-10 was secreted by all. This ascertains the fact the DC and Mf play a role in maintaining gut homeostasis by secreting IL-10 in GM-CSF dependent manner.

Figure 11: Secretion of IL-10 by CD4⁺T-cells after co-culturing them with myeloid cells generated in (A) GM-CSF+IL-4, (B) GM-CSF or (C) M-CSF 'educated' by pretreatment with ATRA conditioned Caco2 supernatant. In the figure legends, C stands for unstimulated myeloid cells co-incubated with CD4⁺ T-cells, EC-C for monocyte-derived cells 'educated' by resting Caco2 supernatant followed by co-incubation with CD4⁺ T-cells, EC-IL-1 β , EC-TNF- α , EC-ATRA, EC-DMSO correspond to cultures containing monocyte-derived cells 'educated' with the supernatants of CEC pretreated with IL-1 β or TNF- α in the presence or absence of ATRA, or with DMSO used as solvent control, followed by co-incubation with CD4⁺ T-cells. Bar diagrams indicate mean \pm SD of 3 independent experiments * p <0.05 ** p <0.01





5.2. Development of an SRM based method for the semi-quantitative measurement of AMPs secreted by Caco2 cells

Our aim was to develop a specific and sensitive targeted method for the quantification of β - and α -defensins. We developed an LC-coupled SRM based mass spectrometry method[73] for the analysis of β -defensin 1-4, and α -defensins. In order to design specific SRM transitions for each protein, we have utilized the human defensin amino acid sequences from the UniProt database followed by *in silico* trypsin digestion. By using the NCBI BLASTp program we have searched for the unique tryptic sequences in each protein. In case of the α -defensins we did not find unique sequences for each protein instead we have found a common sequence for α -defensin 1, -2 and -3. Using these protein specific sequences we have designed SRM transitions using the Skyline MacCoss lab software (www.brendanx-uw1.gs.washington.edu).

The biological relevance of defensins is exerted only in their secretory form, so in the next step β -defensin 1-4 levels in the cell lysate and supernatant of Caco2 cells were analyzed by SRM Mass Spectrometry. A significant increase in β -defensin 2 and β -defensin 3 levels could be demonstrated upon IL-1 β treatment, while no significant change was observed for β -defensin 1 and 4 between untreated and IL-1 β stimulated samples. To further validate the methodology developed, β -defensin 2 levels in stimulated Caco2 cell lysate was confirmed through RT-qPCR expression analysis. Caco2 cell lysate and supernatants were subjected to ELISA, Dot blot and Western-blot analysis to obtain secretory protein data. The level of α -defensins could also be analyzed but we did not see significant changes among the different samples due to the unavailability of unique sequences for α -defensin 1, 2 and 3 (Figure 12, 13).

5.2.1 Determination of β defensin 2 expression and secretion in Caco2 cells

The cells were stimulated with IL-1 β for six hours and the intracellular and secreted defensins were analysed. As a first step β -defensin 2 mRNA levels were determined in control and stimulated cells with RT-qPCR (Figure 12 A). The result of three independent experiments normalized to cyclophilin demonstrated that β -defensin 2 mRNA levels were significantly higher after IL-1 β treatment. These data were confirmed at the protein level as well by Dot blots, western-blot and ELISA respectively (Figure 12 B, C, D, E). These results also indicated elevated levels of β -defensin 2 in IL-1 β treated cells as compared to unstimulated controls. These results show that the gene and protein expression levels of β -defensin 2 were elevated after inflammatory stimuli providing with further evidence for the inducible feature of β -defensin 2 during inflammation. According to our results a sensitive, specific alternative quantification method could be developed for the determination of human defensins.

5.2.2. β and α Defensin level analysis in cell culture supernatant

This SRM based approach has multiplex feature, the levels of β -defensin 2, β -defensin 3 and α -defensins can be analysed from tiny amounts of test sample thus giving an aid for researchers studying these host defence mechanisms. Using the developed SRM method we have analyzed the levels of β -defensin 2, β -defensin 3 and α -defensins in the cell culture supernatants (Figure 13. A, B). As expected we observed significantly higher levels of β -defensin 2 in IL-1 β treated cell supernatants. The level of β -defensin 3 also showed significant elevation in the supernatant of stimulated cells, while no significant difference could be observed in α -defensin levels.

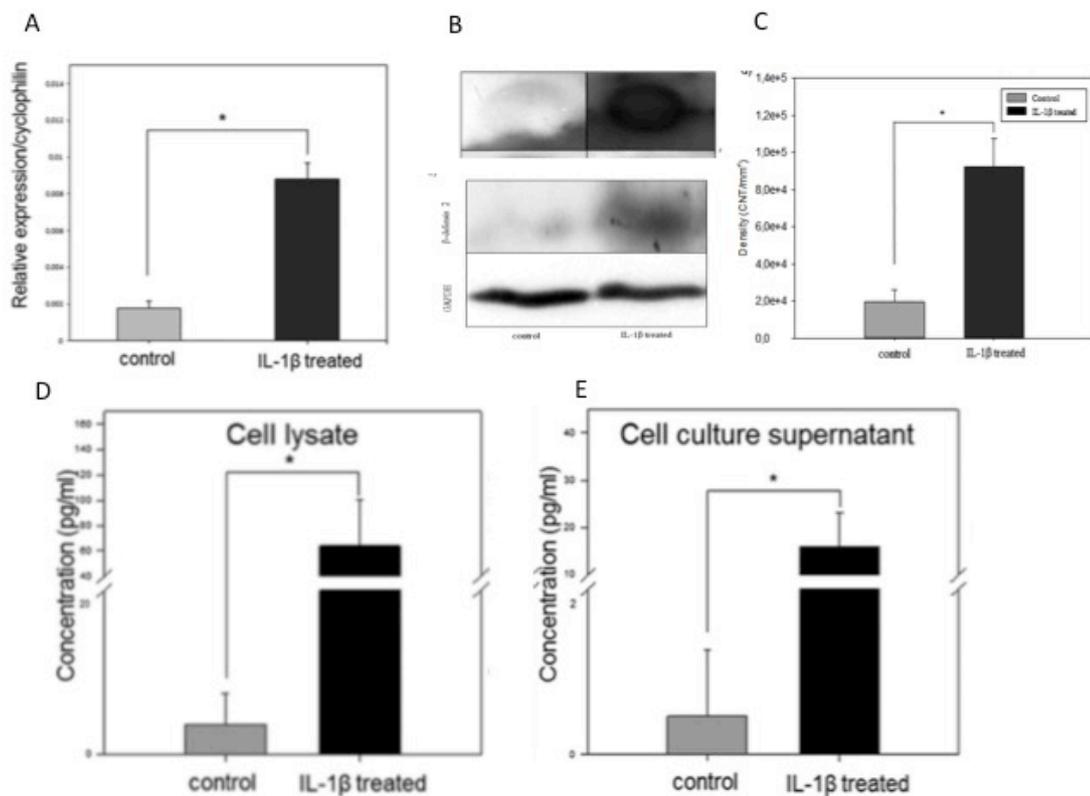


Figure 12: Quantification of β -defensin 2 in Caco2 cell lysate and supernatant: (A) qPCR data for β -defensin 2 normalized to cyclophilin ($n=3$). (B) Determination of β -defensin 2 levels in untreated Caco2 cells and IL-1 β stimulated Caco2 cells by Dot blot and Western blot (representative figures out of 3 independent experiments). (C) Densitometric examination of the dot-blot results ($n=3$). The grey bars represent the control group while the black bars the IL1 β treated group showing the mean values for three independent experiments. The standard error and the significance is shown, * indicate $p < 0,05$. ELISA of β -defensin 2 in (D) Caco2 cell lysate and (E) Caco2 cell supernatants. The grey bars indicate the control group, the black bars refer to the IL-1 β treated group; * indicate $p < 0,05$.

A possible explanation of these data could be the unavailability of unique sequences for α -defensin 1, -2 and -3 as far as the α -defensin amino acid sequences show high similarity making impossible the SRM design for unique peptides. Indeed, the commercially available antibodies are not specific for α -defensin 1, -2 or -3 and to our best knowledge they recognise all three α -defensins. These results further confirmed the role of β -defensin expression and secretion by colonic epithelial cells in inflammation. In summary, we have presented a targeted proteomics method for quantifying human α - and β -defensin levels as an alternative approach for classical antibody based defensin quantifications. As compared to the ELISA method the multiplex feature of this approach has the advantage of running parallel analyses

from the same sample, having relevance in cell biology and medical samples where the amounts to be studied can be very small.

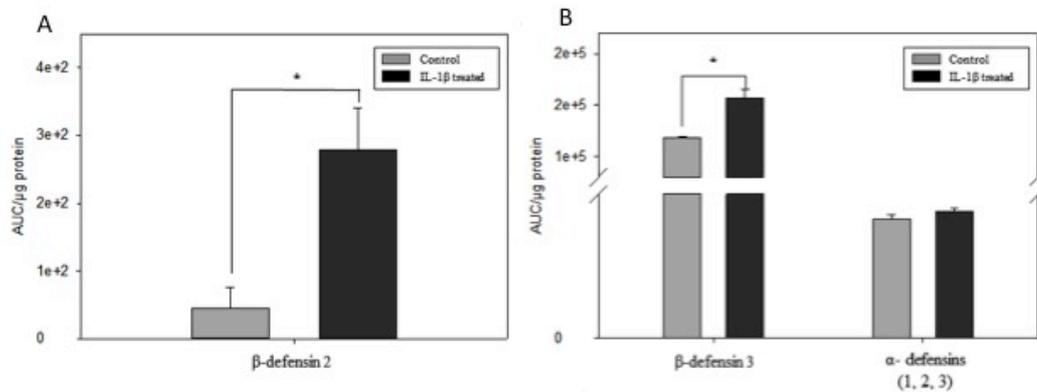


Figure 13: Determination of β defensin 2, 3 and α defensin levels in cell culture supernatant.

A) Determination of β -defensin 2 levels by targeted proteomics analysis ($n=3$). **B)** Determination of β -defensin 3 and α -defensins (1, 2, 3) level by targeted proteomics analysis ($n=3$). According to our results a sensitive and specific alternative quantification method for human defensins was developed. This SRM based analysis has multiplex features, the level of β -defensin 2, β -defensin 3 and α -defensins can be examined from tiny amounts of the given sample providing an aid for researchers studying innate defence mechanisms.

6. DISCUSSION

The cytokines secreted at increased levels in patients with IBD have been identified as TNF- α and IL-1 β , but the complete spectrum of chemokines and chemokine receptors involved in these regulatory networks has not been analyzed in detail. We designed an *in vitro* experimental system to study the effects and the interplay of cytokines, chemokines and RA in resting CEC and under inflammatory conditions for identifying the possible outcomes of myeloid cell-induced T-cell collaboration. We observed that unstimulated CEC secrete CCL chemokines with the potential to attract DC and Mf thus ensuring continuous contact with CEC to support LP homeostasis [73, 74]. It has previously been observed that TNF- α and IL-1 β do not induce the secretion of CCL22 in monocytes, macrophages, and B cells from human peripheral blood *in vitro*, but prolonged (12hr) treatment could induce the production of MDC/CCL22 protein by cultured human intestinal epithelial cells[1]. The detailed analysis of chemokine expression induced by the supernatants of CEC pre-activated by IL-1 β or TNF- α demonstrated that 1) the secretion of the CCL20, CXCL1 and CXCL8 chemokines could be induced only by IL-1 β and was not affected by ATRA, 2) constitutive expression of the chemokines Mk, CXCL16 and CXCL7 was not modified by the supernatant of activated CEC, 3) ATRA down regulated the expression of Mk and CXCL16 and 4) the secretion of CXCL7 could be induced by both IL-1 β and TNF- α in the presence of ATRA. Consistent with previous results we also observed in our *in vitro* model that CXCL8 and CXCL1 are secreted upon activation of CEC by IL-1 β but not by TNF- α showing that IL-1 family cytokines may exert dichotomous or opposing effects in maintaining gut homeostasis or inducing intestinal inflammation[75]. When Caco2 cells were treated with TNF- α in combination with *Clostridium difficile* toxin A, TNF- α itself did not influence the secretion of CXCL8 [69]. The chemokine CCL20 exhibited unique features as it could be induced exclusively by the supernatant of IL-1 β stimulated CEC that could completely be inhibited by ATRA. This chemokine is highly specific for its receptor CCR6 that is expressed by intestinal CEC and in human lymphoid tissues[76-78] and its expression is associated with IBD[79, 80]. The importance of the CCR6-CCL20 axis was also verified in CCR6 double negative mice showing decreased intestinal M-cell numbers and low IgA secretion upon rotavirus infection[81, 82]. Mk acts as a multifunctional cytokine and growth factor with bactericidal and fungicidal activity. Upregulation of Mk expression was also detected in the rat large intestine during DSS-induced colitis and was shown to activate CD4+ T-cells[83] leading to enhanced mucosal restitution during the repair process of colitis[84]. Ligation of the CXCR6 receptor by its CXCL16 ligand results in the activation of the MAP-kinase pathway observed

in patients with Crohn's disease and was associated with clinical benefits and rapid ulcer healing[85]. When CEC were stimulated by IL-1 β or TNF- α , the secretion levels of M α , CXCL16 and CXCL7 remained constant while the physiological concentrations of ATRA could decrease the secretion of these chemokines significantly. CXCL7 was also shown to promote neutrophil adhesion and trans-migration[86].

GM-CSF is a critical factor for DC development and expansion *in vivo*, it induces DC differentiation from monocytes and its absence can reduce the number of migratory DC[87, 88]. Based on this information and to further characterize the cell types involved, we generated myeloid cells with characteristic phenotypic properties by differentiating them in the presence of various growth factors (Table 2).

Table 2: Expression of CD1a, DC-SIGN and CD14 cell surface molecules on monocyte-derived cells generated in the presence of the colony stimulating factors GM-CSF+IL4, GM-CSF or M-CSF

Peripheral blood-derived monocytes were differentiated in the presence of GM-CSF+IL-4, GM-CSF or M-CSF in RPMI + 10% FCS for 3 days and the expression of the cell surface markers CD1a, DC-SIGN/CD209 and CD14 was monitored by flow cytometry using fluorescent labeled specific antibodies. The expression levels of CD1a, DC-SIGN and CD14 is indicated as mean fluorescence intensity (MFI).

	CD1a	DC SIGN	CD14
GMCSF+IL4	200.88	32.6	75.63
GMCSF	66.31	10.5	183.29
MCSF	6.01	4.3	369.47

We observed that the myeloid cells differentiated with ATRA exerted cell type-specific modulatory effects on their phenotype shown by the increased expression of CD14, GM-CSF and M-CSF receptors, while decreasing CCR7 expression and inhibiting memory T-cell migration to secondary lymphoid organs[89]. The role of ATRA in this process was confirmed by *in vitro* migration assays detecting moderate cell migration towards the supernatant of activated CEC containing a selected set of chemokines induced by activated CEC. ATRA inhibited the migration of myeloid cells differentiated in the presence of various growth factors even when they were stimulated by the supernatant of IL-1 β -activated Caco2 cells. These results suggest that ATRA is a potent regulator of the tolerogenic microenvironment in the gut acting at least partially via modulating chemokine responses. Despite slight differences in the levels of CEC-derived chemokine secretion, the overall effects on modulating the myeloid cell phenotype, the migratory potential and the DC- and M α -mediated polarization of CD4⁺ T-lymphocytes was comparable for the tested CEC that

involved the Caco2, HT-29 and HCT116 cell lines suggesting a common regulatory mechanism. Microbial signals sensed by Mf in the colon are dependent on GM-CSF and result in increased IL-1 β secretion. In the absence of microbes decreased IL-1 β secretion of mice lead to low GM-CSF levels in the gut, while DC and Mf support the generation of T_{regs} by producing ATRA and IL-10 in the presence of TGF- β . The production of these regulators was drastically decreased in the absence of GM-CSF, as shown in *Csf2*^{-/-} mice [91].

ATRA is also responsible for the homeostatic regulation of CD11b⁺CD103⁺ DC[92] and under inflammatory conditions its production is increased to keep the local environment under check. When the chemokines were added directly to DC to test their effects on CD103 expression, DC could not acquire CD103 surface expression in the absence of ATRA. Based on these results we suggest that human monocyte-derived CD103⁺ cells, induced by GM-CSF+IL4 or GM-CSF together with appropriate activation signals, such as the supernatant of activated CEC were able to support the acquisition of the gut phenotype in human DC. This was confirmed by experiments with blood-derived CD1c⁺ DC with a similar outcome (unpublished results) indicating that in the human system both cell types can acquire the capability to support CD103⁺ myeloid cell development. Differentiation of the CX3CR1⁺ population in the presence of GM-CSF+IL4 was also promoted by ATRA but it was inhibited in Mf. These results are in line with previous results showing that CX3CR1⁺ cells are inefficient in synthesizing ATRA and exhibit poor T-cell stimulatory capacity *in vitro* and *in vivo* when injected into intestinal lymphatics[93]. In contrast, CD103⁺ cells are able to migrate and can trigger adaptive immune responses by expressing gut homing receptors on T-cells[93].

The decreased number of T_{reg} cell as compared to Th17 cells have been indicated in Crohn's patients and the number of T_{regs} was influenced by ATRA and IL-10 in the gut environment[69]. Bone marrow-derived DC could be 'educated' by direct contact with murine epithelial MODE-K cells and the development of surface expression of CD103⁺ was also observed, which is involved directly in the development of T_{reg} differentiation [94].

In our *in vitro* model with human CEC (Caco2, HT-29, HCT116) and monocyte-derived DC, the development of T_{regs} was not observed. However, we were able to demonstrate the inhibition of Th17 cell numbers when autologous T-cells were co-cultured with 'ATRA educated' DC. We also observed the enhanced secretion of IL-10 by CD4⁺ T-cells after co-culturing them with ATRA-conditioned DC and Mf indicating suboptimal conditions for T_{reg} differentiation. It has also been shown that Th17 cells can exhibit both anti- and pro-inflammatory properties depending on the cytokine signals received[95]. These results

altogether indicated that the detailed characterization of the gut milieu under different conditions is of utmost importance for understanding the complexity of regulation leading to the maintenance or loss of gut homeostasis. Hence we can conclude that the pro-inflammatory cytokines IL-1 β and TNF- α , associated with inflammatory bowel diseases, exert different effects on gut epithelial cells monitored by the secretion of different combinations of chemokines in CEC. The metabolite ATRA, produced by both epithelial and myeloid cells, has the potential to down modulate some, but not all chemokine responses to support a tolerogenic microenvironment in the gut environment. Myeloid cells differentiated by GM-CSF+IL4 or GM-CSF (DC) and M-CSF (Mf) respond differently to activated epithelial cell-mediated stimuli and to ATRA. The signals provided by activated epithelial cells for DC and Mf can be translated to T-lymphocytes, but the number of Th17 producing cells is modulated differentially in DC and Mf indicating the complementary role of these myeloid cell types in regulating adaptive effector T-cell polarization.

Other than the secretion of chemokines observed when Caco2 cells were stimulated with IL-1 β and TNF- α we also wanted to analyse the secretion of defensin levels in cell culture supernatants of Caco2. We utilized the human AMP amino acid sequences from the UniProt database followed by *in silico* trypsin digestion and using the NCBI BLASTp program we have searched for the unique tryptic sequences in each protein. With this method, 13 different AMPs could be analysed and their relative quantitation was performed. The 13 different AMPs analysed in Caco2 cell supernatants involved ADM, ADM2, Urocortin, Leptin, β defensin 1, β defensin 2, β defensin 3, β defensin 4, dermicidin, RNase7, Protein S1000A7, BPI, α defensins. Unique *in silico* tryptic digests for the 3 different alpha defensins were not found hence the significance could not be determined. Out of this 13 AMPs, β defensin 1-4 and α defensins were detected in Caco2 cell lysate and supernatant when stimulated with IL-1 β .

Based on our developed method for measuring defensin concentrations by using the targeted mass spectrometric method, we have analyzed the levels of β -defensin 1, β -defensin 2, β -defensin 3 and β -defensin 4. β -defensin 2 expression and secretion was also confirmed with the help of RT-qPCR, dot blot, Western blot and ELISA. The levels of β -defensin 1 did not show significant change after proinflammatory stimulation being in accordance with previous data regarding the constitutive expression of β -defensin 1 which cannot be further stimulated by proinflammatory cytokines[96]. Elevated levels of β -defensin 2 and β -defensin 3 were determined upon inflammatory signals indicating the inducible feature of this defensin[97].

The level of β -defensin 4 was not changed significantly upon IL-1 β treatment, a phenomenon described by others[96]. β -defensin 4 has a strong additive effect on β -defensin 3 but this effect does not require elevated β -defensin 4 expression[98]. Thus we can conclude that the semi-quantitative targeted proteomics method developed in this study is appropriate for the relative quantification of human β -defensin 1-4 levels in biological samples as an alternative method for the classical antibody based defensin detection and quantification approaches. As compared to the ELISA and Western blot results, the multiplex feature of this approach has the ultimate advantage of determining multiple analytes from the same sample, having relevance in cell biology and medical applications, where the amount of the sample to be studied is limited.

7. SUMMARY

Retinoids and other derivatives of vitamin A are known to bear important functions in regulating differentiation and proliferation of epithelial cells (EC). We studied the effects of ATRA on colonic EC in combination with different pro-inflammatory activators. We selected the two most potent known pro-inflammatory cytokines IL-1 β and TNF- α for studying the effects of ATRA. When we used these cytokines in the presence or absence of ATRA as inducers of Caco2 colonic EC cell lines we observed that ATRA was responsible for conferring the CD103 cell surface expression on DC and Mf after 6 hrs. In case of CX3CR1 cell surface expression, DC treated with ATRA-conditioned CEC supernatant showed an increase in CX3CR1 expression, whereas in Mf decreased CX3CR1 expression was observed after similar treatment. The fact that ATRA exerted different effects on different cells indicates the capability of ATRA to affect the final outcome of T-lymphocyte responses. In our *in vitro* system we observed that ATRA treated CEC supernatant can influence the outcome of DC responses when co-cultured with CD4⁺ T lymphocytes, as the balance of Th17 cells was reduced, while in Mf the number of Th17 cells was significantly increased.

Defensins represent an important group of AMP consisting of 16 – 50 amino acids, organized to a structurally conserved compact organization associated with multiple functions in order to act as a first line of defense mechanism. The three subfamilies of defensins (α , β , θ) differ in their peptide length, location of disulphide bonds, their precursor structures and in the site of protein expression. Elevated levels β -defensin 3 in colonic mucosa of patients with ulcerative colitis suggest their role in the inflammatory response. In this study we have developed a targeted proteomics based method for the determination of defensin levels in cell lysates and cell culture supernatants. Human Caco2 epithelial cells were challenged with IL-1 β as an inflammatory stimulus and the levels of β -defensin 2 was analyzed in cell lysates and in cell culture supernatants. The developed method was validated by using qPCR, quantitative ELISA and Western blot. The gene and protein expression levels of β -defensin 2 were significantly higher in the IL-1 β treated samples as compared to the unstimulated controls. Beside β -defensin 2, the levels of β -defensin 3, β -defensin 4 and α -defensin was also measured and showed significantly higher levels in the supernatants of activated Caco2 cells. Our results show that the targeted proteomics method developed here offers an alternative approach for the mass spectrometric analyses of a selected set of defensins.

8. SUMMARY IN FRENCH

Résumé du premier manuscrit : Facteurs influençant le dialogue entre les cellules épithéliales et immunes : Interactions entre les cytokines et les voies de signalisation induites par l'ATRA. Nous avons choisi les deux plus puissantes cytokines pro-inflammatoires connues, l'IL1 β et le TNF α pour étudier les effets de l'ATRA. Nous avons utilisé ces deux cytokines, en présence ou en absence d'ATRA, pour stimuler pendant 6 h la lignée épithéliale de colon Caco2. Nous avons constaté que l'ATRA induisait l'expression de CD103 à la surface des cellules dendritiques (DC) et des macrophages (Mf). Quand l'expression du CX3CR1 était observé à la surface des cellules, les DC traitées avec un surnageant conditionné de CEC traitées par l'ATRA montraient une augmentation de l'expression de CX3CR1 alors que les Mfprésentaient un diminution de l'expression de CX3CR1 suite à un traitement similaire. Le fait que l'ATRA ait un effet variable sur différents types cellulairesmontrel'aptitude de l'ATRA à influencer les réponses lymphocytaires T finales. Dans notre système in vitro, nous avons constaté que les surnageants de CEC traitées par l'ATRA peuvent influencer les DC qui, lorsqu'elles sont cultivées avec de lymphocytes T CD4⁺, induisent moins de cellules Th17 alors que dans la même situation, les Mf conduisent à un nombre accru de cellules Th17.

Résumé du second manuscrit (*en collaboration avec le Dr. Éva Csősz, Department of Biochemistry and Molecular Biology, Proteomics Core Facility University of Debrecen*) : Quantification relative des β -défensines humaines dans les cellules épithéliales de colon par une approche protéomique basée sur la SRM ciblée. Dans cette étude, nous avons développé une méthode basée sur la protéomique ciblée pour déterminer les niveaux de défensines dans les lysats cellulaires et les surnageants de culture. La lignée épithéliale humaine Caco2 a été soumise à un stimulus inflammatoire constitué par l'IL-1 β et les niveaux de β -défensine 2 ont été analysés dans les lysats cellulaires et les surnageants de culture. La méthode a été validée par qPCR, ELISA et Western blot quantitatifs. Les niveaux d'expression du gène de la β -défensine 2 ainsi que de la protéine étaient significativement plus élevés dans les échantillons traités par l'IL-1 β comparés aux contrôles non stimulés. Les niveaux de β -défensine 2, de β -défensine 3, de β -défensine 4 et d' α -défensine ont également été étudiés et des niveaux significativement plus élevés de β -défensine 3 ont été détectés dans les surnageants de cellules Caco2 activées. Nos résultats montrent que la méthode de protéomique ciblée développée ici fournit une approche alternative aux analyses de spectrométrie de masse de certaines défensines.

Compiled by Dr. François Lefèvre, INRA, France.

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List of publications related to the dissertation

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2. **Chatterjee, A.**, Gogolák, P., Blottière, H.M., Rajnavölgyi, É.: The Impact of ATRA on Shaping Human Myeloid Cell Responses to Epithelial Cell-Derived Stimuli and on T-Lymphocyte Polarization. *Mediat. Inflamm.* 2015, 1-14, 2014.
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10. KEYWORDS

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Research Article

The Impact of ATRA on Shaping Human Myeloid Cell Responses to Epithelial Cell-Derived Stimuli and on T-Lymphocyte Polarization

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Vitamin A plays an essential role in the maintenance of gut homeostasis but its interplay with chemokines has not been explored so far. Using an *in vitro* model system we studied the effects of human colonic epithelial cells (Caco2, HT-29, and HCT116) derived inflammatory stimuli on monocyte-derived dendritic cells and macrophages. Unstimulated Caco2 and HT-29 cells secreted CCL19, CCL21, and CCL22 chemokines, which could attract dendritic cells and macrophages and induced CCR7 receptor up-regulation by retinoic-acid resulting in dendritic cell migration. The chemokines Mx, CXCL16, and CXCL7 were secreted by all the 3 cell lines tested, and upon stimulation by IL-1 β or TNF- α this effect was inhibited by ATRA but had no impact on CXCL1, CXCL8, and CCL20 secretion in response to IL-1 β . In the presence of ATRA the supernatants of these cells induced CD103 expression on monocyte-derived dendritic cells and when conditioned by ATRA and cocultured with CD4⁺ T-lymphocytes they reduced the proportion of Th17 T-cells. However, in the macrophage-T-cell cocultures the number of these effector T-cells was increased. Thus cytokine-activated colonic epithelial cells trigger the secretion of distinct combinations of chemokines depending on the proinflammatory stimulus and are controlled by retinoic acid, which also governs dendritic cell and macrophage responses.

1. Introduction

The adult human intestine is referred to as “physiologically inflamed” due to the presence of enormous number of B- and T-lymphocytes, as well as macrophages (Mf), dendritic cells (DC), eosinophils, and mast cells. If all these immune cells were present in other tissues at such concentrations, they would be regarded as an abnormal chronic inflammatory cell infiltrate [1]. In such a scenario, the main players maintaining gut homeostasis should be those mechanisms that provide tolerogenic signals for specialized myeloid cells with antigen presenting function.

The vitamin A (VitA) metabolite retinoic acid (RA) is a key regulator of the cytokine TGF- β , which promotes Treg differentiation [2]. VitA also contributes to the formation of

epithelial linings of mucosal surfaces [3], and its multifunctional metabolite RA [4] acts as a critical driver of lymphocyte trafficking to the intestinal mucosa [5]. All-trans retinoic acid (ATRA) induces the expression of the gut homing integrin $\alpha 4 \beta 7$ on myeloid cells and the chemokine receptor CCR9 on T-lymphocytes, while the lack of the αv or $\beta 8$ integrin chains in DC impairs Treg functions and Th17 responses *in vivo* [6]. ATRA also modulates Th17 effector T-lymphocyte differentiation in the gut [7]; however, the *in vivo* effects of ATRA in intestinal and extraintestinal compartments result in controversial outcomes presumably due to targeting multiple cell types with diverse functional activities [8]. VitA deficiency has an effect on epithelial cell integrity and the composition of the gut microbiota [9].

A single layer of colonic epithelial cells (CEC) forms the first line of defense against luminal pathogens. It communicates with other immune cells by direct contacts and by secreting an array of cytokines and chemokines. Chemokines represent low-molecular-weight proteins with pleiotropic effects on the recruitment and activation of leukocytes at inflammatory sites [10]. The dominant cell populations in the gut involve CX3CR1⁺ Mf, which directly sense luminal content by their extended membrane protrusions across the epithelium [11], and migratory CD103⁺ DC with tolerogenic potential. Apart from chemokines, colony-stimulating factor (CSF-2/GM-CSF) in the gut is a multifunctional cytokine that has an impact on DC and Mf numbers and can impair the ability of immune cells to produce regulatory factors such as RA and IL-10 and thus may lead to disrupted Treg homeostasis in the large intestine [12]. It also acts as an important regulator of human DC homeostasis by promoting *in vivo* expansion and differentiation from hematopoietic progenitors and monocytes [13]. Under steady state conditions, the low number of gut migratory DC is critically dependent on GM-CSF, but its level is dramatically increased during infection or inflammation and supports the development of DC precursors such as monocytes and inflammatory migratory DC thus modulating the composition of the DC pool [14].

Cytokines have been shown to be the causative factor and outcome of IBD pathogenesis. The major conclusive result has been shown by improvement in the IBD symptoms by blocking TNF- α . And a decrease in the IL-1 receptor antagonist in comparison to IL-1 was observed in IBD patients [15]. These data confirm that both TNF- α and IL-1 β are able to trigger inflammatory conditions such as those observed in Crohn's disease (CD) or ulcerative colitis (UC) but the comparison of their effects at molecular and functional levels in context of the human intestinal microenvironment has not been elucidated so far. Despite similarities in the functional and regulatory mechanisms in human and mouse, major differences have been observed in their cytokine secretion [16] and mucus layer organization [17].

Based on these data and to overcome the discrepancies between the human and mouse systems, we designed experiments with human CEC in resting state and in an inflammatory milieu mimicked with TNF- α or IL-1 β stimulation in the presence or absence of ATRA. This was performed by monitoring the levels of secreted chemokines measured at the protein level and by investigating their impact on the phenotype and functional attributes of myeloid cells generated by different growth/differentiation factors. Considering that DC have the potential to instruct T-cells for inflammatory or regulatory directions, our final goal was to identify the impact of stimulated CEC-induced and DC-mediated effects on CD4⁺ effector T-lymphocyte responses. We could detect the secretion of CCL19, CCL21, and CCL22 chemokines by unstimulated CEC, which has not been shown before. We also observed that both IL-1 β and TNF- α were able to trigger the secretion of Midkine (Mk), CXCL16, and CXCL7 by CEC, but their expression could efficiently be downregulated by ATRA. However, the secretion of CXCL1, CXCL8, or CCL20 by IL-1 β -stimulated CEC was not influenced by ATRA. Our

results also revealed that the *in vitro* induced inflammatory milieu created by proinflammatory chemokines was sufficient to increase the migratory potential of DC driven by GM-CSF but not by the other growth factors, and ATRA could further potentiate this effect. Furthermore, the molecular information collected by CEC and transmitted to DC could be translated to T-lymphocytes, which responded to CEC-initiated and DC-mediated stimulation by mounting Th17 responses. All these steps seemed to be under the control of ATRA as the response of CEC to both IL-1 β and TNF- α was higher in the presence of ATRA.

2. Materials and Methods

2.1. Cell Culture of Caco2 Colon Epithelial Cells. The human colorectal adenocarcinoma cell line Caco2 is from ATCC-number HTB-37 and HT-29 is from ATCC-number HTB-38. The colorectal carcinoma cell line HCT116 was a generous gift from Dr. György Vereb, Department of Biophysics, University of Debrecen. Caco2 and HCT116 cells were cultured in RPMI-1640 medium supplemented with 1% antibiotic-antimycotic solution and 20% fetal bovine serum (GIBCO by Life Technologies, EU) in tissue culture flasks (Nunclon, Rochester, NY) at 37°C in 10% and 5% CO₂, respectively. HT-29 cells were cultured in RPMI-1640 medium supplemented with 1% antibiotic-antimycotic solution and 10% fetal bovine serum in 5% CO₂. Cell culture medium was replaced every 2-3 days and the cells were passaged when subconfluent.

2.2. Protein Array for Chemokine Analyses. CEC of 70–80% confluency were plated overnight in RPMI supplemented with 10% FCS—followed by stimulation with 10 ng/mL proinflammatory cytokines (IL-1 β or TNF- α) in combination with or without 10 nmol ATRA or left untreated for 1 hour. The cells were washed and replaced with fresh medium for 5 hr, when the supernatants were collected for chemokine analysis performed by a commercially available protein array (Proteome Profiler Arrays—ARY017, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Sample controls (transferrin R, gp130, and fibrinogen) included in the array allowed us the detection and quantitation of the secreted chemokines. Considering that ATRA dissolved in DMSO may have toxic effects on resting CEC, which could be further enhanced by activation with IL-1 β or TNF- α , we performed preliminary titration experiments to optimize the cell culture conditions by using 24 h 7AAD-based viability assays performed by FACS analysis. These results indicated 98% viability of Caco2 and HT-29 cells in both the presence and absence of 10 nmol ATRA that was similar to those measured for untreated CEC.

2.3. In Vitro Cell Migration and the Chemotaxis Assay. Migration of three different groups of monocyte-derived cells, differentiated in GM-CSF+IL-4, GM-CSF, and M-CSF, was tested for cell migration to chemokines and cytokines secreted by Caco2, HT-29, and HCT116 cells. Monocytes (3×10^5) differentiated in the presence of the 3 different growth factors were placed on the upper chamber of a 5-micron Corning transwell plate and the CEC supernatants

were added to the lower chamber of the transwell. After 24 h the monocyte-derived cells that migrated to the lower chamber were collected. 10,000 polystyrene beads (15 micron) were added to each sample (Fluka Analytical, Germany) and the number of migrating cells was counted by FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed by the FlowJo software (Tree Star, Ashland, OR, USA).

2.4. Peripheral Blood Monocyte-Derived Cells. Leukocyte enriched buffy coats were obtained from healthy blood donors drawn at the Regional Blood Center of the Hungarian National Blood Transfusion Service (Debrecen, Hungary) in accordance with the written approval of the Director of the National Blood Transfusion Service and the Regional and Institutional Ethics Committee of the University of Debrecen, Medical and Health Science Center (Hungary). PBMCs were separated by a standard density gradient centrifugation with Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Monocytes were purified from PBMCs by positive selection using immunomagnetic cell separation with anti-CD14 microbeads, according to the manufacturer's instruction (Miltenyi Biotec, Bergisch Gladbach, Germany). After separation on a VarioMACS magnet, 96–99% of the cells were CD14⁺ monocytes, as measured by flow cytometry. Monocytes were divided and cultured in 12-well tissue culture plates at a density of 2×10^6 cells/mL in 10% RPMI medium supplemented with four different growth factors: 80 ng/ml GM-CSF (Gentaur Molecular Products, Brussels, Belgium), 100 ng/mL IL-4 (PeproTech EC, London, UK), and M-CSF 50 ng/mL (MACS, Miltenyi Biotec, Germany).

2.5. Peripheral Blood Lymphocytes and CD4⁺ T-Cells. Autologous naive T-cells were separated from human blood mononuclear cells using the naive CD4⁺ T-cell isolation kit based on negative selection according to the manufacturer's instruction (Miltenyi Biotec).

2.6. Phenotypic Characterization of Myeloid Cells by Flow Cytometry. Detection of the cell surface expression of monocyte-derived myeloid cells was performed by flow cytometry using anti-CD1a-PE, anti-CD209-PE, anti-CD14-PE, anti-CD83-PE, anti-CD103-PE, anti-CX3CR1-PE, and anti-CCR7-PE (Beckman Coulter, Hialeah, FL, USA). The growth factor receptors were characterized by anti-GM-CSFR α -PE and anti-M-CSF R/CD115-PE (R&D Systems, USA) and isotype-matched control antibodies (BD Pharmingen, San Diego, CA, USA). Fluorescence intensities were measured by FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA), and data were analyzed by the FlowJo software (Tree Star, Ashland, OR, USA). The human chemokines M_k, CXCL7, CCL20, and CXCL16 were ordered from PeproTech, UK; CXCL8 and CXCL1 are from Miltenyi Biotec.

2.7. IL-17 and IFN γ ELISPOT Assays. The monocyte-derived cells were cultured in GM-CSF+IL-4, GM-CSF, and M-CSF for 3 days along with the supernatant of unstimulated or cytokine activated Caco2 cells at 2×10^5 cells/well density. The cells were washed to remove all growth factors and

supernatants and were cocultured with naïve autologous CD4⁺ T cells (10^6 cells/well) in 10% RPMI medium for 2 days at 37°C in a humidified atmosphere containing 5% CO₂. PHA and Con A activated T cells were used as positive controls. Negative controls involved CD4⁺ T-cells and untreated monocyte-derived cells cocultured with CD4⁺ T-cell. Detection of cytokine-secreting T cells was performed by the avidin-HRP system (NatuTec GmbH, Germany). Plates were analyzed by an ImmunoScan plate reader (CTL, Shaker Heights, OH, USA).

2.8. Statistical Analysis. Statistical analysis was performed by one-way analysis of variance (ANOVA) for multiple comparisons. Results are expressed as mean \pm SD. Two group differences were analyzed by Student's *t*-test. *P* value (two-tailed) less than 0.05 was considered statistically significant.

3. Results

3.1. Identification of Chemokines Secreted by Resting and Activated CEC. The single cell monolayer of CEC plays an essential role in the maintenance of gut homeostasis by supporting barrier function and defense against microbes preferentially through the secretion of chemokines [18]. It is also well established that the proinflammatory cytokines IL-1 β and TNF- α act as potent activators of CEC [19]. In this study we applied a high throughput approach for identifying the chemokines secreted by CEC (Caco2, HT-29, and HCT116) in response to IL-1 β and TNF- α by using a commercially available Human Chemokine Array to quantify the relative levels of chemokines released by resting and activated CEC at the protein level. The sample controls provided (transferrin R, gp130, and fibrinogen) were used for calculating mean pixel densities of the respective dot blots. The results showed that resting Caco2 cells secrete detectable levels of CCL19, CCL21, and CCL22 constitutively (dots 2D, 9F, and 8D in Figure 1(a), summarized in Figure 1(c)). Similar results were obtained for HT-29 cells, but HCT116 secreted only CCL19 at detectable levels. Comparison of the relative cytokine levels secreted by the Caco2, HT-29, and HCT116 cell lines are summarized in Table 1. Considering that these CCL chemokines are known to attract myeloid cells, they may maintain a population of myeloid cells in the vicinity of CEC to support cellular interactions. It has previously been shown that MDC/CCL22 attracts Th2 cytokine producing cells and its mRNA and protein expression is upregulated against enteroinvasive bacteria, but inhibition of the NF- κ B pathway abolished CCL22 expression in response to proinflammatory stimuli [1]. The chemokines CXCL7, CXCL16, and M_k with different functional activities were also constitutively secreted by resting CEC (dot 9C, 7F, and 7D in Figure 1(a)) suggesting their role in the maintenance of epithelial cell homeostasis.

3.2. The Effect of ATRA on the Chemokine Secretion by CEC Prestimulated by IL-1 β or TNF- α . In our model system, CEC were left untreated or stimulated by IL-1 β or TNF- α in combination with or without ATRA for 6 hr and the cell culture supernatants were subjected to chemokine array analysis by calculating pixel densities using the relevant

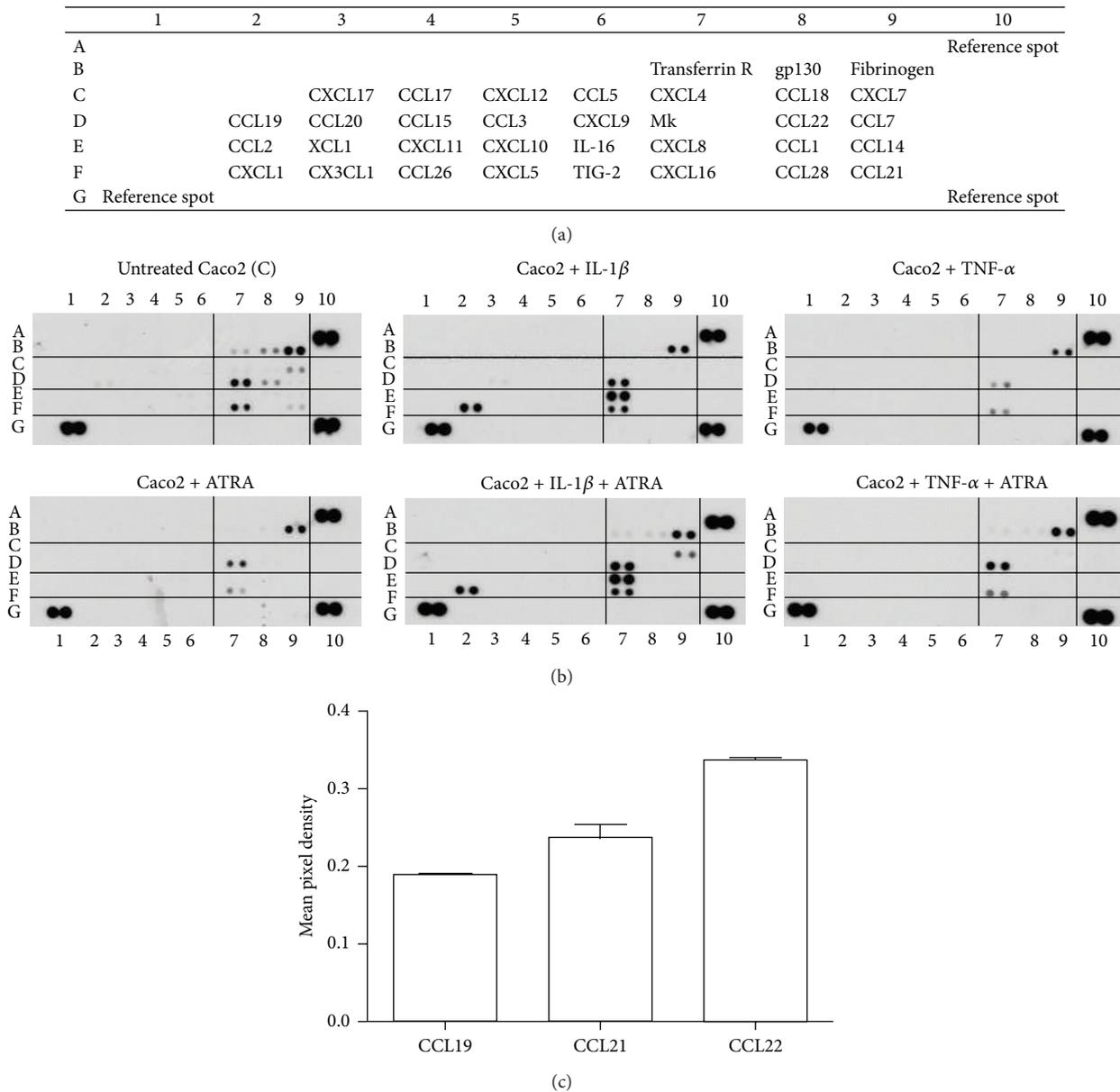


FIGURE 1: Chemokine secretion of human epithelial cells activated by IL-1 β or TNF- α in the presence or absence of ATRA. Caco2 cells were activated by 10 ng/mL IL-1 β or TNF- α in the presence or absence of 10 nmol ATRA. After 1 hr of activation, the supernatants were removed and the cells were washed and cultured in fresh medium. After 5 hr the supernatants of nontreated and activated Caco2 cells were collected and the relative levels of chemokines were determined by a Proteome Profiler Array used according to the manufacturer's instructions. The relative levels of the Caco2 cell-derived chemokines were determined by calculating mean pixel densities of the individual blots normalized to sample control fibrinogen. The mean \pm SD of 4 independent measurements is shown. (a) Localization of the chemokine probes in the membranes related to the positive controls and the reference spots. (b) Representative dot blots showing the relative expression of chemokines produced by untreated Caco2 cells without or with ATRA (upper and lower panels 1) as compared to Caco2 cells stimulated by IL-1 β (upper panel 2) and IL-1 β in the presence of ATRA (lower panel 2). Upper and lower panels 3 correspond to Caco2 cells stimulated by TNF- α in the absence or presence of ATRA, respectively. (c) Relative expression levels of CCL chemokines produced by unstimulated Caco2 cells.

sample control (Figure 1). When Caco2 cells were activated by TNF- α or IL-1 β , the secretion levels of the chemokines CXCL7, CXCL16, and Mk did not change significantly as compared to unstimulated cells (dots 9C, 7F, and 7D in Figure 1(a), summarized in Figures 2(a) and 2(b)). However, the expression of CXCL16 and Mk was downregulated in

the presence of ATRA suggesting that the secretion of these chemokines may contribute to the maintenance of epithelial cell homeostasis. However, under inflammatory conditions they do not mediate positive signals for DC. Remarkably, the secretion of CXCL7 (dot 9C in Figure 1(a), summarized in Figures 2(a) and 2(b)) could be induced only when IL-1 β or

TABLE 1: Relative expression of chemokines secreted by colon epithelial cell lines. Caco2, HT-29, and HCT116 cells were treated with IL-1 β or TNF- α in the presence or absence of ATRA for 60 min. After removing the cell culture supernatant the cells were washed and replaced with fresh medium and the supernatants were collected after 5 hours and used for the Chemokine Array analysis according to the manufacturer's instruction to determine the variety of chemokines secreted by these cell lines. The values indicate the results of the densitometric analysis of dot blots and are normalized to the relevant sample control according to the manufacturer's instructions. For simplicity the value of 1 obtained after dividing with the sample control is referred to as 100.

		CCL19	CCL21	CCL22	Mk	CXCL16	CXCL7	CXCL1	CXCL8	CCL20
C	Caco2	30	30	30	90	70	40	—	—	—
	HT-29	40	30	40	80	90	60	—	—	—
	HCT-116	150	—	—	170	170	—	—	120	—
IL-1 β	Caco2	—	—	—	100	80	—	120	140	30
	HT-29	—	—	—	80	90	—	100	90	30
	HCT-116	—	—	—	150	160	100	100	100	100
IL-1 β + ATRA	Caco2	—	—	—	110	80	50	100	130	—
	HT-29	—	—	—	100	80	40	100	90	—
	HCT-116	—	—	—	160	140	90	110	110	—
TNF- α	Caco2	—	—	—	90	60	—	—	—	—
	HT-29	—	—	—	80	60	—	—	—	—
	HCT-116	—	—	—	150	150	—	10	10	—
TNF- α + ATRA	Caco2	—	—	—	90	60	—	—	—	—
	HT-29	—	—	—	90	80	20	—	—	—
	HCT-116	—	—	—	150	130	20	10	—	—
ATRA	Caco2	—	—	—	50	30	—	—	—	—
	HT-29	—	—	—	60	20	—	—	—	—
	HCT-116	40	20	30	80	80	—	—	—	—

TNF- α was combined with ATRA treatment demonstrating the dependence of its secretion on ATRA. DMSO, which is the standard solvent of ATRA, also decreased CXCL7 secretion (Figures 2(a) and 2(b)). Depending on CEC sensitivity to DMSO, certain effects of this solvent have previously been reported, as inhibition of prostaglandin E2 production upon treatment of Caco2 cells with IL-1 β and attenuation of mRNA levels of IL6, IL-1 α , and IL-1 β [20]. These findings might be in line with inhibition of CXCL7 observed in our system. In the IEC-18 cell line IL-1 β induced increased mRNA levels of MCP-1/CCL2, MIP-1 α /CCL3, inducible NO synthase, and RANTES/CCL5, which are upregulated in a NF- κ B dependent manner [21].

The secretion levels of CCL20 and CXCL1 were not affected by ATRA (dots 3D, 2E, and 7E in Figure 1(a), summarized in Figure 2(c)) and could be induced exclusively by IL-1 β but not by TNF- α . Surprisingly, CXCL8 secretion was upregulated not only by IL- β with or without ATRA, but also by DMSO used as a vehicle for ATRA (Figure 2(c)). DMSO induced strain on the actin cytoskeleton and integrins expressed by Caco2 cells was suggested to be transferred to actin-associated molecules like α -actinin-1, acting as a scaffold protein and interacted directly with ERK1/2 leading to phosphorylation and increased secretion of CXCL8. The *in vivo* relevance of this effect is underscored by the physical deformation of CEC during peristalsis and villous motility [22]. Along with IL-8/CXCL8 and GRO α /CXCL1, Caco2 cells also express MCP-1/CCL2 as a result of IL-1 stimulation, but our chemokine array-based method did not detect CCL2 [23]. These results suggest that the expression of individual chemokines depends on the means of activation and also

on ATRA, which can modulate the outcome of chemokine secretion, whereas the group of chemokines not affected by ATRA indicates the complexity of chemokine-mediated regulation in the gut. Similar results were obtained for HT-29 and HCT116 CEC as summarized in Table 1. Even though HCT116 showed a similar overall pattern of chemokine secretion as the other CEC, trace amounts of NF- κ B-dependent inflammatory chemokines (CCL2, CXCL2, and CXCL10), not observed in Caco2 and HT29 cells, were detected indicating CEC type-dependent regulation of chemokine secretion [10, 21, 24].

3.3. ATRA Regulates the Chemokine-Dependent Migration of Myeloid Cells Generated by Different Hematopoietic Growth/Differentiation Factors. Based on the results showing the inhibitory effect of ATRA on the secretion of some chemokines, we next sought to assess the chemokine-driven migratory potential of myeloid cells. We set up a transwell system and measured myeloid cell migration *in vitro* by using CCL19 and CCL21 chemokines as positive controls of cell recruitment. Myeloid cells differentiated from primary human monocytes by GM-CSF+IL-4 or GM-CSF to DC exhibited detectable but low migratory potential as compared to cells mobilized by high concentration (200 ng/mL) of CCL19 and CCL21 chemokines (Figures 3(a) and 3(b)), while monocytes cultured in M-CSF gave rise to macrophages with undetectable migratory activity (data not shown). The highest migratory potential could be attributed to cells differentiated in the presence of GM-CSF or GM-CSF+IL-4 and stimulated by the supernatant of Caco2 cells preactivated by IL-1 β , but this process could be downregulated by ATRA. To analyze

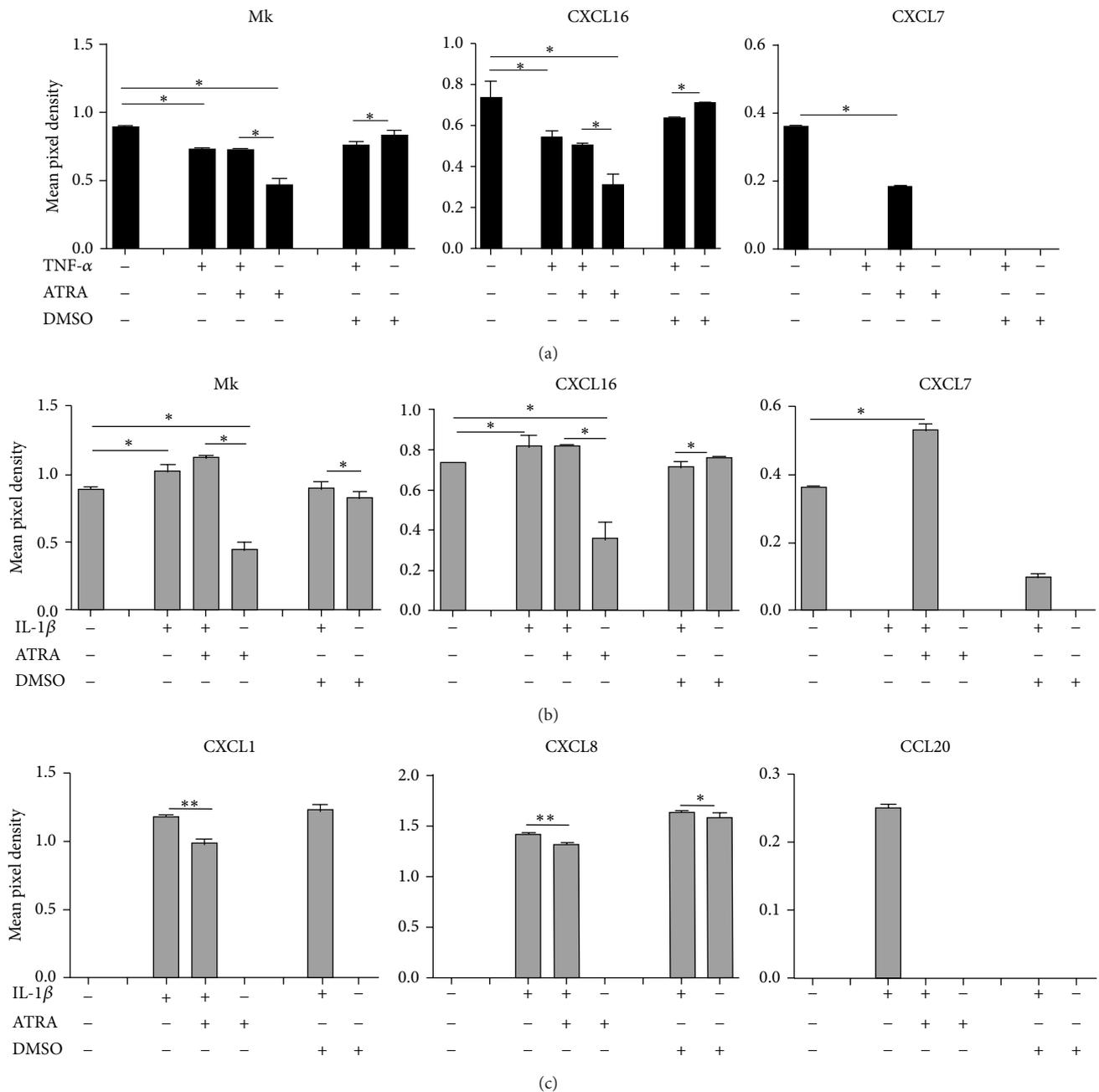


FIGURE 2: Effects of ATRA on the expression of chemokines in cytokine-stimulated Caco2 cells. Relative expression of chemokines in Caco2 cells, prestimulated by TNF- α or IL-1 β in the presence or absence of ATRA, was determined as described in Figure 1 and was compared to unstimulated cells cultured with or without ATRA. The possible contribution of DMSO used as a solvent control of ATRA was tested in Caco2 cells cultured in the presence of TNF- α or IL-1 β with or without DMSO. (a) Expression of chemokines secreted by Caco2 cells prestimulated by TNF- α , in the presence or absence of ATRA. (b) Expression of chemokines secreted by Caco2 cells prestimulated by IL-1 β in the presence or absence of ATRA. (c) Chemokines induced exclusively by IL-1 β stimulation in Caco2 cells. * $P < 0.05$, ** $P < 0.01$. Bar diagrams indicate mean \pm SD of 4 dot blots which was averaged after densitometry analysis and normalized with the “sample control” provided with the kit.

whether the supernatant of IL-1 β -stimulated CEC has a direct effect on the migration of DC differentiated by GM-CSF+IL-4 or GM-CSF, the cells were subjected to direct cell migration assays toward the chemokines exclusively secreted by IL-1 β used at pretitrated concentrations (CXCL1 (1 ng/mL), CXCL8

(100 ng/mL), and CCL20 (50 ng/mL)). We observed the high migratory capacity of DC differentiated by GM-CSF+IL-4 (Figure 3(c)) and Mf developed by GM-CSF (Figure 3(d)) toward CXCL1 and CXCL8 known to be involved in the chemotaxis and migration of polymorphonuclear leukocytes

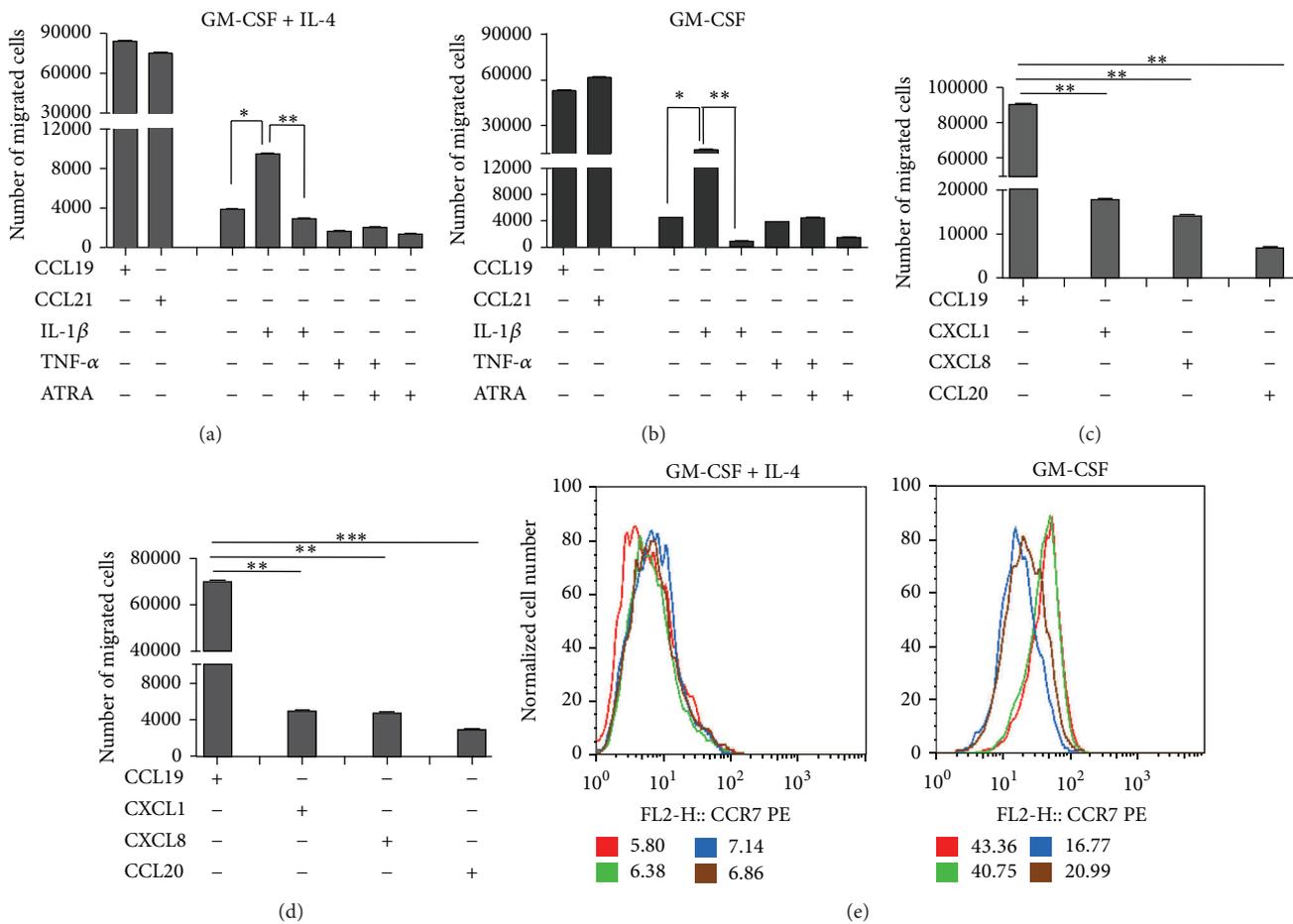


FIGURE 3: ATRA modifies the chemokine-dependent migration of *in vitro* differentiated myeloid cells. Caco2 cells were activated as described in Figure 1. The migratory potential of the monocyte-derived cells generated by GM-CSF+IL-4 or GM-CSF was tested in transwell chambers. 3×10^5 cells were placed on the upper chamber and the Caco2 cell supernatants on the lower chamber of the transwell plate. After 24 hr, the number of cells, which migrated to the lower chamber in response to the Caco2 cell supernatants, was counted by flow cytometry. The migratory potential of cells generated in the presence of GM-CSF+IL-4 (a) or GM-CSF (b) is shown as compared to the migratory potential of CCL19 and CCL21 chemokines, used as positive controls. To determine the cause of high migration in response to IL-1 β treated CEC supernatant, the GM-CSF+IL-4 (c) and GM-CSF (d) differentiated monocytes were treated with CXCL1, CXCL8, and CCL20 chemokines which were secreted exclusively by CEC on IL-1 β treatment. Bar diagrams indicate mean \pm SD of 3 independent experiments * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Another fraction of monocyte-derived cells, differentiated by GM-CSF+IL-4 or GM-CSF, was also stimulated by the supernatant of IL-1 β pretreated CEC in the presence or absence of ATRA. The cell surface expression of CCR7 in the differentiated monocyte derived cells was measured by flow cytometry (e). Red line depicts untreated monocyte-derived cells, green is for monocyte-derived cells treated by the supernatant of Caco2 cells previously activated by IL-1 β , blue line is for monocyte-derived cells pretreated with the supernatant of Caco2 cells activated by IL-1 β and ATRA, and brown line corresponds to cells treated by the supernatant of unstimulated Caco2 cells in combination with ATRA. Results of 3 independent experiments are shown as mean \pm SD of MFI.

to inflammatory sites [25, 26] as compared to CCL20. Interestingly, the supernatant of Caco2 cells prestimulated with TNF- α had no such effect on cell migration (Figures 3(a) and 3(b)). When the migratory potential of myeloid cells was related to the cell surface expression of CCR7 we found that DC differentiated by GM-CSF in the presence of ATRA-conditioned CEC supernatant exhibited decreased CCR7 expression, while in cells differentiated in GM-CSF+IL-4 it remained unchanged (Figure 3(e)). Similar results were obtained by using the HT-29 and HCT116 cell lines (data not shown). These results suggest that in an inflammatory environment ATRA also modulates the migratory potential of myeloid cells in a cell type-dependent manner.

3.4. ATRA Supports the Development of Migratory CD103⁺ Myeloid Cells. The dominant DC population of the gut is represented by CD103⁺ migratory cells that express the enzymes required for the metabolism of VitA [4], while the CX3CR1⁺ resident Mf population samples the microenvironment by protruding dendrites [11]. To assess how efficiently we could manipulate the effects of ATRA on CEC, we differentiated blood-derived monocytes with GM-CSF+IL-4 or M-CSF to generate DC and Mf, respectively, followed by the stimulation of cells with the supernatant of activated Caco2 cells. The cell surface expression of CD103⁺ and CX3CR1⁺ measured by FACS analysis revealed that the presence of Caco2 cell supernatants obtained from ATRA+IL-1 β , ATRA+TNF- α , or

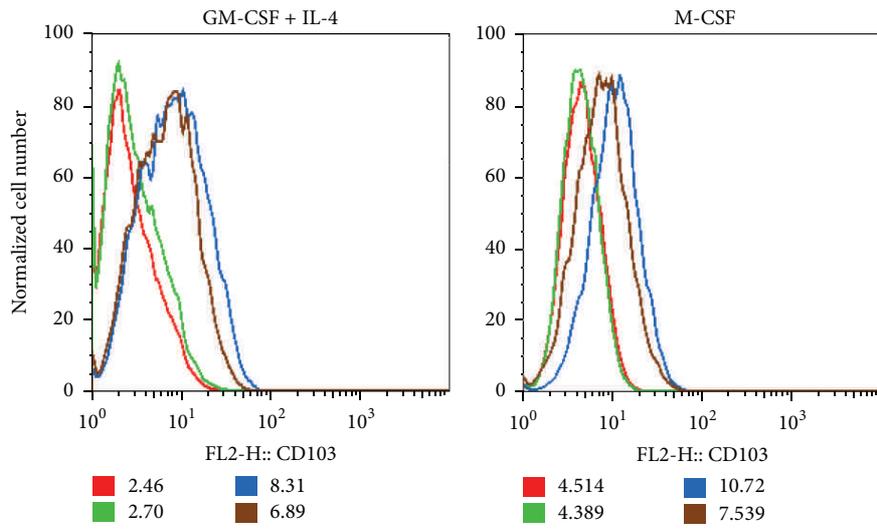
ATRA-pretreated CEC could increase the expression of the CD103 integrin in cells differentiated by GM-CSF+IL-4 or M-CSF to obtain DC and Mf, respectively (Figures 4(a) and 4(b)). These results also indicated that even in the presence of inflammatory stimuli (supernatant of IL-1 β or TNF- α activated CEC) ATRA was able to promote the development of CD103⁺ myeloid cells. In a similar experimental system, the frequency of CX3CR1⁺ cells generated by GM-CSF+IL-4 and stimulated by cytokine-activated CEC supernatant was also increased in case the cell culture was conditioned by ATRA. In contrast to this finding, the expression of CX3CR1 remained unchanged in cells generated by GM-CSF (data not shown) and was decreased when the ATRA-conditioned CEC supernatant was added to Mf differentiated from monocytes with M-CSF and activated by IL-1 β or TNF- α (Figures 4(c) and 4(d)). Similar results were obtained with HT-29 and HCT116 cells (data not shown). To confirm that the level of ATRA could be reconstituted in CEC after the washing procedure, we also added ATRA directly to DC and detected increased expression of CD103. To rule out the effect of other chemokines in the development of CD103⁺ cells, the chemokines secreted by activated CEC were added directly to monocytes at pretitrated concentrations (Mk 10 ng/mL, CXCL16 100 ng/mL, CXCL7 10 ng/mL, CXCL1 1 ng/mL, CXCL8 100 ng/mL, and CCL20 50 ng/mL) along with the differentiating growth factors GM-CSF+IL-4 or M-CSF and the expression of CD103 was measured by FACS. When the *in vitro* generated myeloid cells were treated directly with the different chemokines in the absence of ATRA, no phenotypic changes of DC and Mf could be detected (see Supplementary Figure 4 in Supplementary Material available online at <http://dx.doi.org/10.1155/2015/579830>), showing their direct dependence on ATRA.

3.5. Translation of the Molecular Information Collected by CEC-Stimulated Myeloid Cells to CD4⁺ T-Lymphocytes. Considering the sensitivity of myeloid cells to proinflammatory signals provided by activated CEC and the modulatory effects of ATRA, we set out to test whether DC and Mf as antigen presenting cells could activate and polarize T-lymphocytes. To test this scenario, myeloid cells differentiated by GM-CSF+IL-4, GM-CSF, and M-CSF, respectively, were activated by supernatants of cytokine-activated Caco2 cells followed by coculturing them with autologous CD4⁺ T-lymphocytes, and the number of IL-17 and IFN γ cytokine producing T-cells was detected by ELISPOT assays. We found that CD4⁺ T cells cocultured with myeloid cells differentiated from monocytes to DC with GM-CSF+IL-4 (Figure 5(a)) or GM-CSF (Figure 5(b)) and “educated” by the supernatants of activated Caco2 cells in combination with ATRA resulted in significant suppression of IL-17 producing cell numbers. In contrast, monocyte-derived cells generated by M-CSF pretreated with Caco2 cell supernatant (Figure 5(c)) and subsequently cocultured with CD4⁺ T-cells, the number of IL-17 cytokine secreting cells was increased significantly indicating that DC and Mf exhibit different T-cell polarizing activities. Although slight differences could be observed in the magnitude of T-cell responses provoked by CEC supernatants activated by IL-1 β or TNF- α , the T-lymphocyte responses were polarized

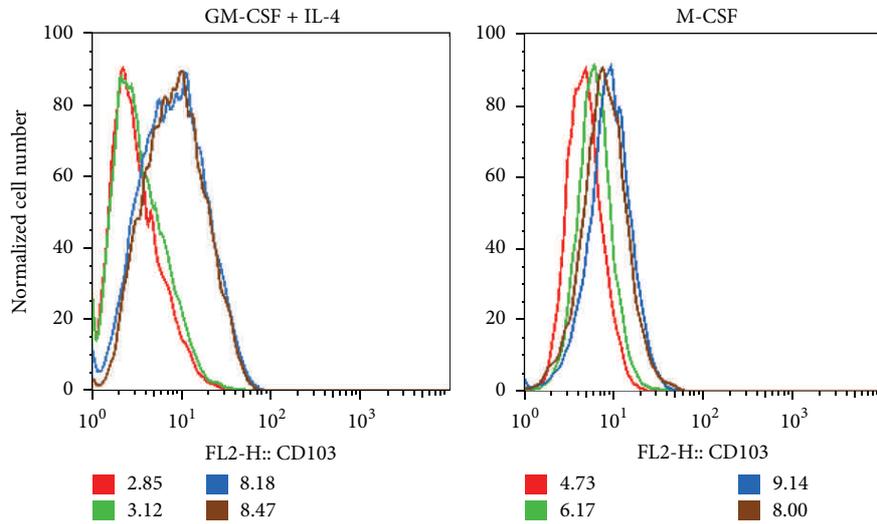
to the Th17 direction in both cases independent of the proinflammatory cytokine used for CEC stimulation underpinning the role of DC-mediated inflammatory signals in driving CD4⁺ T-lymphocyte responses. Under similar culture conditions IFN γ -secreting cells could not be detected in the CD4⁺ T-cell population. Similar “education” of myeloid cells by ATRA-conditioning was observed also for HT-29 and HCT116 cell lines (data not shown). Thus, ATRA is able to exert different effects on monocyte-derived myeloid cells differentiated upon coculturing with CD4⁺ T cells. This may indicate a broad range of RA-mediated effects involved in shaping the gut microenvironment.

4. Discussion

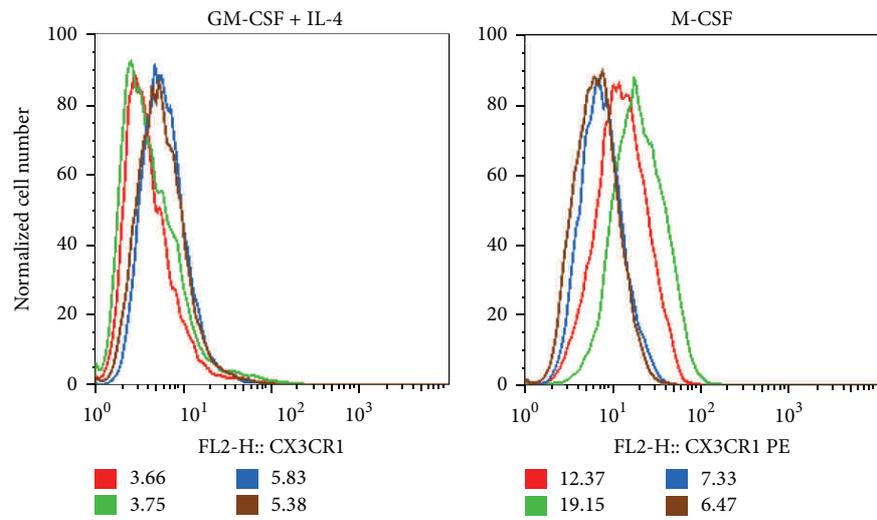
The cytokines secreted at increased levels in patients with IBD have been identified as TNF- α and IL-1 β , but the complete spectrum of chemokines and chemokine receptors involved in these regulatory networks has not been analyzed in detail. We designed an *in vitro* experimental system to study the effects and the interplay of cytokines, chemokines, and RA in resting CEC and under inflammatory conditions for identifying the possible outcomes of myeloid cell-induced T-cell collaboration (Supplementary Figure 1). We observed that unstimulated CEC secrete CCL chemokines with the potential to attract DC and Mf thus ensuring continuous contact with CEC to support LP homeostasis [27, 28]. It has previously been observed that TNF- α and IL-1 β do not induce the secretion of CCL22 in monocytes, macrophages, and B cells from human peripheral blood *in vitro*, but prolonged (12 hr) treatment could induce production of MDC/CCL22 protein by cultured human intestinal epithelial cells [1]. The detailed analysis of chemokine expression induced by the supernatants of CEC preactivated by IL-1 β or TNF- α demonstrated that (1) the secretion of the CCL20, CXCL1, and CXCL8 chemokines could be induced only by IL-1 β and was not affected by ATRA, (2) constitutive expression of the chemokines Mk, CXCL16, and CXCL7 was not modified by the supernatant of activated CEC, (3) ATRA downregulated the expression of Mk and CXCL16, and (4) the secretion of CXCL7 could be induced by both IL-1 β and TNF- α in the presence of ATRA. Consistent with previous results, we also observed in our *in vitro* model that CXCL8 and CXCL1 are secreted upon activation of CEC by IL-1 β but not by TNF- α showing that IL-1 family cytokines may exert dichotomous or opposing effects in maintaining gut homeostasis or inducing intestinal inflammation [29]. When Caco2 cells were treated with TNF- α in combination with *Clostridium difficile* toxin A, TNF- α itself did not influence the secretion of CXCL8 [10]. The chemokine CCL20 exhibited unique features as it could be induced exclusively by the supernatant of IL-1 β stimulated CEC that could completely be inhibited by ATRA. This chemokine is highly specific for its receptor CCR6, that is, expressed by intestinal CEC and in human lymphoid tissues [30–32] and its expression is associated with IBD [33, 34]. The importance of the CCR6-CCL20 axis was also verified in CCR6 double negative mice showing decreased intestinal M-cell numbers and low IgA secretion upon rotavirus infection [35, 36]. Mk acts as a



(a)

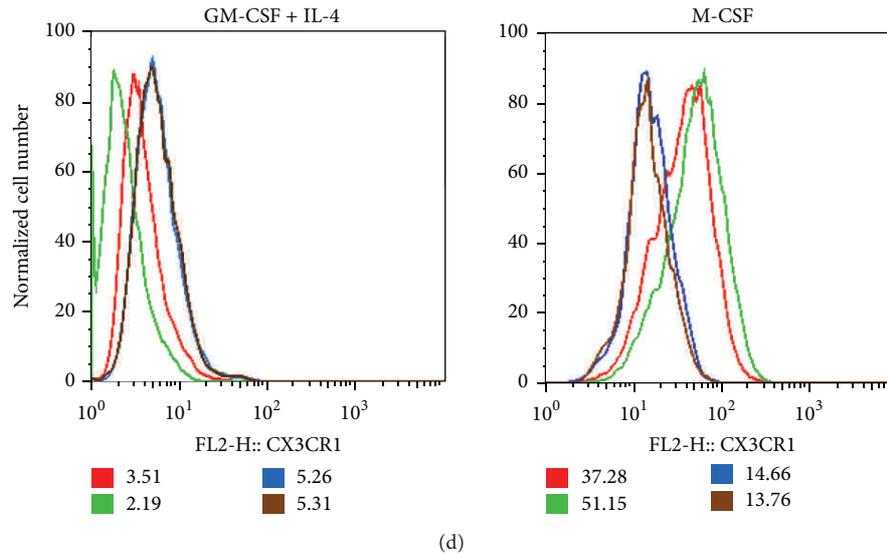


(b)



(c)

FIGURE 4: Continued.



(d)

FIGURE 4: ATRA promotes the expression of CD103⁺ cells in *in vitro* differentiated myeloid cells. Monocytes were differentiated in the presence of GM-CSF+IL-4 or M-CSF, respectively. The differentiated cells were treated by the supernatants of Caco2 cells preactivated by IL-1 β or TNF- α in the presence or absence of ATRA. The phenotype of the myeloid cells was characterized by measuring the cell surface expression of $\alpha 4\beta 7$ /CD103 and CX3CR1 on day 3 of *in vitro* myeloid cell differentiation by flow cytometry. Histograms show the cell surface expression of CD103 in cells differentiated by GM-CSF+IL-4 (or M-CSF) and stimulated by the supernatants of Caco2 cells pretreated by IL-1 β (a) or TNF- α (b) and the cell surface expression of CX3CR1 in cells differentiated by M-CSF (or GM-CSF+IL-4) and stimulated by the supernatants of Caco2 cells prestimulated by IL-1 β or TNF- α (d). MFI of a typical measurement out of 3–5 independent experiments is shown. Red line depicts untreated cells, green is for cells pretreated with the supernatant of Caco2 cells activated by IL-1 β or TNF- α , blue is for myeloid cells pretreated with the supernatant of Caco2 cells activated by IL-1 β or TNF- α in combination with ATRA, and brown corresponds to cells treated by the supernatant of unstimulated Caco2 cells and ATRA.

multifunctional cytokine and growth factor with bactericidal and fungicidal activity. Upregulation of Mk expression was also detected in the rat large intestine during DSS-induced colitis and was shown to activate CD4⁺ T cells [37] leading to enhanced mucosal restitution during the repair process of colitis [38]. Ligation of the CXCR6 receptor by its CXCL16 ligand results in the activation of the MAP-kinase pathway observed in patients with Crohn's disease and was associated with clinical benefits and rapid ulcer healing [39]. When CEC were stimulated by IL-1 β or TNF- α , the secretion levels of Mk, CXCL16, and CXCL7 remained constant while the physiological concentrations of ATRA could decrease the secretion of these chemokines significantly. CXCL7 was also shown to promote neutrophil adhesion and transmigration [40].

GM-CSF is a critical factor for DC development and expansion *in vivo*; it induces DC differentiation from monocytes and its absence can reduce the number of migratory DC [41, 42].

Based on this information and to further characterize the cell types involved, we generated myeloid cells with characteristic phenotypic properties by differentiating them in the presence of various growth factors (Table 2). We observed that the myeloid cells differentiated with ATRA exerted cell type-specific modulatory effects on their phenotype shown by the increased expression of CD14, GM-CSF, and M-CSF receptors, while decreasing CCR7 expression and inhibiting memory T-cell migration to secondary lymphoid organs

TABLE 2: Expression of CD1a, DC-SIGN, and CD14 cell surface molecules on monocyte-derived cells generated in the presence of the colony stimulating factors GM-CSF+IL-4, GM-CSF, or M-CSF. Peripheral blood-derived monocytes were differentiated in the presence of GM-CSF+IL-4, GM-CSF, or M-CSF in RPMI + 10% FCS for 3 days and the expression of the cell surface markers CD1a, DC-SIGN/CD209, and CD14 was monitored by flow cytometry using fluorescent labeled specific antibodies. The expression levels of CD1a, DC-SIGN, and CD14 are indicated as mean fluorescence intensity (MFI).

	CD1a	DC SIGN	CD14
GMCSF+IL-4	200.88	32.6	75.63
GMCSF	66.31	10.5	183.29
MCSF	6.01	4.3	369.47

[43] (Supplementary Figure 2). The role of ATRA in this process was confirmed by *in vitro* migration assays detecting moderate cell migration towards the supernatant of activated CEC containing a selected set of chemokines induced by activated CEC. ATRA inhibited the migration of myeloid cells differentiated in the presence of various growth factors even when they were stimulated by the supernatant of IL-1 β -activated Caco2 cells. These results suggest that ATRA is a potent regulator of the tolerogenic microenvironment in the gut acting at least partially via modulating chemokine

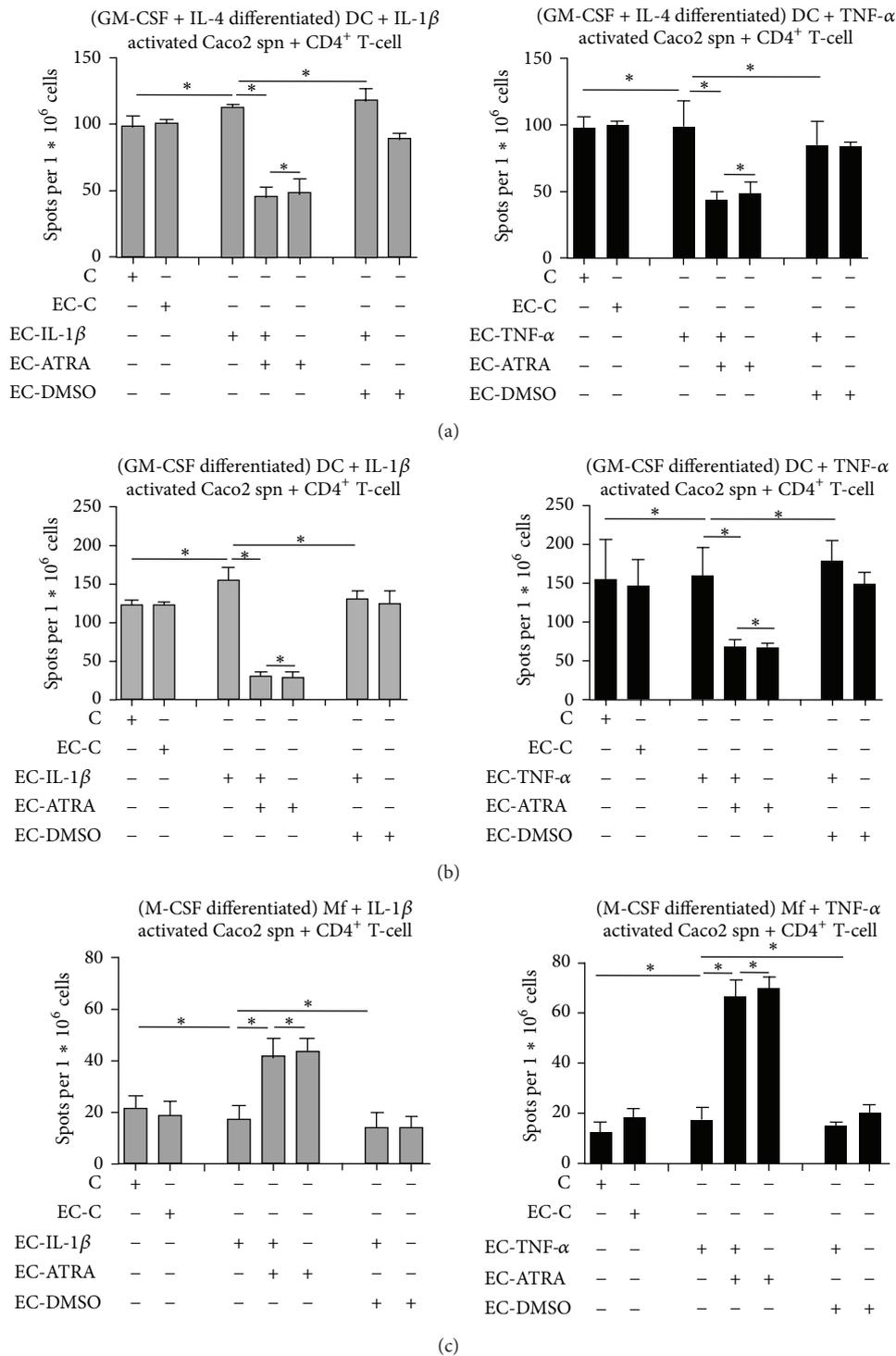


FIGURE 5: The effect of gut epithelial cell-mediated myeloid cell stimulation on the activation and polarization of T-lymphocytes. To assess the effects of stimulated myeloid cells on effector T-cell polarization, the supernatants of activated Caco2 cells were added to myeloid cells differentiated by GM-CSF+IL-4, GM-CSF, or M-CSF for 3 days and the washed cells were cocultured in fresh medium with autologous CD4⁺ T-lymphocytes for another 2 days. The activation and polarization of CD4⁺ T-cells c-incubated with myeloid cells generated by GM-CSF+IL-4 (a), GM-CSF (b), or M-CSF (c) was detected by the IL-17 ELISPOT assay. In the figure legends, C stands for unstimulated myeloid cells coincubated with CD4⁺ T cells, EC-C for monocyte-derived cells “educated” by resting Caco2 cell supernatant followed by coincubation with CD4⁺ T cells, and EC-IL-1 β , EC-TNF- α , EC-ATRA, and EC-DMSO correspond to cultures containing monocyte-derived cells “educated” with the supernatants of Caco2 cells pretreated with IL-1 β or TNF- α in the presence or absence of ATRA, or with DMSO used as solvent control, followed by coincubation with CD4⁺ T cells. * *P* < 0.05. Mean \pm SD of the number of IL-17 secreting cells measured in 4 independent experiments is shown.

responses. Despite slight differences in the levels of CEC-derived chemokine secretion, the overall effects on modulating the myeloid cell phenotype, the migratory potential, and the DC- and Mf-mediated polarization of CD4⁺ T-lymphocytes were comparable for the tested CEC that involved the Caco2, HT-29, and HCT116 cell lines suggesting a common regulatory mechanism.

Inhibited expression of the LPS-binding receptor CD14 (Supplementary Figure 2(a)) was shown to be associated with increased susceptibility to gastroenteritis and UC [44] and the expression of GM-CSF receptor was shown to be higher in healthy individuals than in UC or CD patients [45]. Microbial signals sensed by Mf in the colon are dependent on GM-CSF and result in increased IL-1 β secretion. In the absence of microbes, decreased IL-1 β secretion of mice led to low GM-CSF levels in the gut, while DC and Mf support the generation of Tregs by producing RA and IL-10 in the presence of TGF- β . The production of these regulators was drastically decreased in the absence of GM-CSF, as shown in *Csf2*^{-/-} mice [12]. In accordance with these findings we also observed the secretion of IL-1 β when monocyte-derived cells generated in GM-CSF were treated with the supernatant of Caco2 cells conditioned by ATRA (Supplementary Figure 3). In this setting ATRA also decreased GM-CSF receptor expression (Supplementary Figure 2(b)) which could contribute to keep the overall concentration of IL-1 β in the gut environment to controllable limits. ATRA is also responsible for the homeostatic regulation of CD11b⁺CD103⁺ DC [46] and under inflammatory conditions its production is increased to keep the local environment under check. When the chemokines were added directly to DC to test their effects on CD103 expression, DC could not acquire CD103 surface expression in the absence of ATRA (Supplementary Figure 4). Based on these results, we suggest that human monocyte-derived CD103⁺ cells, induced by GM-CSF+IL-4 or GM-CSF together with appropriate activation signals, that is, the supernatant of activated CEC, are able to support the acquisition of the gut phenotype of human DC. To confirm this finding, we also performed experiments with blood-derived CD1c⁺ DC and obtained a similar outcome (unpublished results) indicating that in the human system both cell types can acquire the capability to support CD103⁺ myeloid cell development. Differentiation of the CX3CR1⁺ population in the presence of GM-CSF+IL-4 was also promoted by ATRA but it was inhibited in Mf. These results are in line with previous results showing that CX3CR1⁺ cells are inefficient in synthesizing ATRA and exhibit poor T-cell stimulatory capacity *in vitro* and *in vivo* when injected into intestinal lymphatics [47]. In contrast, CD103⁺ cells are able to migrate and can trigger adaptive immune responses by expressing gut homing receptors on T-cells [47].

The expression of M-CSFR (Supplementary Figure 2(b)) was shown to be enhanced by IL-1 β stimulated CEC supernatant and the number of Th17 cells was increased when CD4⁺ T cells were cocultured with Mf educated by the supernatant of CEC preactivated by IL-1 β in the presence of physiological concentration of ATRA. The decreased number of Treg cell as compared to Th17 cells has been indicated in Crohn's patients and the number of Tregs was influenced by

RA and IL-10 in the gut environment [10]. Bone marrow-derived DC could be "educated" by direct contact with murine epithelial MODE-K cells and the development of surface expression of CD103⁺ was observed, which is involved directly in the development of Treg differentiation [48]. In our *in vitro* model with human CEC (Caco2, HT-29, and HCT116) and monocyte-derived DC, the development of Tregs was not observed. However, we were able to demonstrate the inhibition of Th17 cell numbers when autologous T-cells were cocultured with "ATRA educated" DC. We also observed the enhanced secretion of IL-10 by CD4⁺T-cells after coculturing them with ATRA-conditioned DC and Mf (Supplementary Figure 5) indicating suboptimal conditions for Treg differentiation. It has also been shown that Th17 cells can exhibit both anti- and proinflammatory properties depending on the cytokine signals received [49]. These results altogether indicated that the detailed characterization of the gut milieu under different conditions is of utmost importance for understanding the complexity of regulation leading to the maintenance or loss of gut homeostasis.

5. Conclusion

The proinflammatory cytokines IL-1 β and TNF- α , associated with inflammatory bowel diseases, exert different effects on gut epithelial cells monitored by the secretion of different combinations of chemokines in CEC. The metabolite RA, produced by both epithelial and myeloid cells, has the potential to downmodulate some, but not all chemokine responses to support a tolerogenic microenvironment in the gut environment. Myeloid cells differentiated by GM-CSF+IL-4 or GM-CSF (DC) and M-CSF (Mf) respond differently to activated epithelial cell-mediated stimuli and to RA. The signals provided by activated epithelial cells for DC and Mf can be translated to T-lymphocytes, but the number of Th17 producing cells is modulated differentially in DC and Mf indicating the complementary role of these myeloid cell types in regulating adaptive effector T-cell polarization.

Conflict of Interests

The authors declare no financial or commercial conflict of interests.

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Relative quantification of human β -defensins by a proteomics approach based on selected reaction monitoring

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RATIONALE: A targeted proteomics method based on selected reaction monitoring (SRM) is a relevant approach for the analysis of multiple analytes in biological samples. Defensins are phylogenetically conserved small antimicrobial peptides contributing to innate host defense and exhibiting low immunogenicity, resistance to proteolysis and a broad range of antimicrobial activities. The goal of the present study was to develop and optimize SRM-based targeted proteomics methods for the detection of human β -defensins 1–4 in various biological fluids.

METHODS: An SRM-based targeted proteomics method was developed and validated for the detection of human β -defensins 1–4. The supernatants of resting and IL-1 β -stimulated Caco2, HT-29 and SW-1116 colonic epithelial cells (CEC), cell lysates of CECs and tear samples of human healthy individuals were analyzed and the feasibility of the developed method was validated by ELISA and dot-blot analysis complemented by RT-qPCR.

RESULTS: Our results demonstrate that the developed SRM method offers an alternative approach for the cost-effective and rapid analysis of human β -defensins in samples with biological relevance.

CONCLUSIONS: A semi-quantitative targeted mass spectrometry method was developed and validated for the relative quantification of β -defensins 1–4 in cell culture supernatants and body fluid analyses. Copyright © 2015 John Wiley & Sons, Ltd.

AUC, area under the curve; AMP, antimicrobial peptide; CD, Crohn's disease; CEC, colonic epithelial cells; hBD, human β -defensin; IBD, irritable bowel disease; MRM, Multiple Reaction Monitoring; PTP, proteotypic peptide; SELDI, Surface Enhanced Laser Desorption Ionization; SIL, stable isotope labelled peptide; SRM, Selected Reaction Monitoring; UC, ulcerative colitis

Defensins represent a phylogenetically ancient and structurally conserved group of antimicrobial peptides (AMP) consisting of 16–50 amino acids. They exhibit multiple functional activities and act in a coordinated manner to provide a first line of defense against various pathogens.^[1] The α and β families of human defensins differ in their precursor structures, peptide length and the site of secretion. In humans, four β -defensin types are expressed referred to as β -defensin 1–4 (hBD1–4).^[1–4] Based on the UniProt database, β -defensins 5 and 6 also exist but no protein data have been published so far. The β sheets of these molecules create a compact tertiary structure referred to as the β -defensin fold.^[5]

Due to this unique organization human defensins are resistant to proteolysis and can exert their effects even under harsh environmental conditions.^[6] In epithelial cells β -defensins have been considered as potential AMPs providing a protective barrier against Gram-negative bacteria and *Candida* species.^[7] Another important function of β -defensins is their chemotactic activity resulting in the recruitment of various immune cells towards the site of infection or inflammation.^[8] Elevated levels of human β -defensin-2 (hBD2) was observed in patients with irritable bowel disease (IBD) and in ulcerative colitis (UC) indicating an activated immune system in these diseases. Increased levels of hBD2 was also detected in stool samples of the inflamed digestive tract,^[9] whereas increased hBD3 levels were described in the colonic mucosa of patients with UC.^[10] hBD4 functions as a synergistic partner of lysozyme-C and together with hBD3 exerts additive effects^[11] suggesting its protective role against different types of pathogens. Human β -defensin molecules are present in various body fluids such as saliva,^[12] sweat^[13] and tears,^[14] while hBD1 was also detected in the urogenital tract.^[15]

The large mucosal surface of the intestinal epithelium acts as an important physical and chemical barrier and confers a first line of defense against pathogenic bacteria, viruses and other microorganisms.^[16,17] In the small intestine hBD1 was described as a constitutively expressed AMP, the expression

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of which is not regulated by proinflammatory cytokines.^[46] However, the level of hBD4 is upregulated as a result of bacterial infection but not by classical proinflammatory signals such as IL-1 β .^[11] On the contrary, hBD2 and hBD3 were described as inducible AMPs, which respond to stimulation by elevated proinflammatory cytokine secretion.^[16–18] Inflammatory bowel diseases such as UC and CD are associated with increased levels of inducible β -defensins^[19] leading to epithelial cell proliferation, immune cell migration and enhanced production of proinflammatory cytokines.^[20] In addition to their antimicrobial and immune modulatory effects, some defensins have been identified as cancer-associated molecules with anti-tumor effects.^[21]

Basal tear fluid contains proteins, lipids, metabolites, salts and organic molecules.^[22] Studies aimed at characterizing the tear proteome have been performed and more than 1500 tear proteins have been identified.^[23,24] Literature data suggest that changes in tear proteome composition can be of diagnostic value in some diseases such as diabetic retinopathy,^[25,26] keratoconus,^[27] blepharitis,^[28] dry-eye^[29] and Sjögren's syndrome.^[30] Changes in tear hBD levels have also been reported after ocular surgery^[31] and in patients with pterygium.^[32]

Targeted proteomics preferentially relies on the SRM/MRM scan mode of triple quadrupole-containing mass spectrometers. In the course of sample analysis the first quadrupole transmits ions with a specified m/z , which is fragmented in the second quadrupole functioning as a collision cell, while the third quadrupole transmits a single specified fragment resulting in a signal when the precursor ion and its selected fragment are present at the same time.^[33,34] SRM-based methods ensure high specificity on one hand and quantitative data on the other hand, since the AUC of the specific signal corresponds to the amount of the compound entering the mass spectrometer. When functioning in a semi-quantitative setup, the targeted approach allows the relative quantification of specified analytes,^[35] but absolute quantification may also be possible.^[36] The usual requirement for absolute quantification includes at least three protein-specific sequences (proteotypic peptides, PTPs) with five transitions for each PTP,^[37] while in case of relative quantification one peptide with two transitions could be satisfactory.

Identification of β -defensins can be performed by gene expression profiling,^[38] ELISA, Western blot^[39–41] or SELDI.^[42,43] To our best knowledge the targeted proteomics approach has not been used so far for the detection of human β -defensins. Although the classical antibody-based methods are widely used for the quantitative or semi-quantitative analyses of proteins and peptides, the dynamic range in the quantitative settings can be a potential limiting factor and the determination of more than one analyte as well as lack of suitable antigens may restrict the utilization of this method. The SRM-based methods also offer multiplexing, but are more cost-effective than the classical antibody-based techniques. Another advantage of the targeted methods is their high flexibility, the possibility to design and validate SRM transitions together with the simultaneous analysis of multiple proteins.^[44,45] However, the limitation of the SRM-based approach could be the restricted availability of PTPs and the possible co-elution of interfering compounds.^[37,46]

In order to establish a cost-effective and flexible multiplex method for the analysis of the most common β -defensins, we have developed an SRM-based method that could be applied for the determination of hBD1–4 levels in different biological samples.

EXPERIMENTAL

Cell culture

In this study, we have used the human HT-29, SW-1116 and Caco2 colonic epithelial cell lines (CECs) derived from various types of human colorectal adenocarcinomas. The tested cell lines are adherent and resemble the features of the human colon. The HT-29 and SW-1116 cells were grown in RPMI medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μ g/mL streptomycin and 2 mM L-glutamine at 5% CO₂ and 37 °C. Caco2 cells were grown in 20% FCS containing RPMI medium supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, 2 mM L-glutamine and 1% non-essential amino acids at 5% CO₂ and 37 °C. Resting CECs (5×10^5 /mL) were stimulated with 10 ng/mL IL-1 β for 1 h followed by the removal of the supernatant that was replaced by fresh medium and the cells were incubated for 5 h before harvesting. The cell culture supernatants were collected without disturbing the cellular monolayer and trypsin-EDTA was added to collect the cells. The detached cells were washed twice with phosphate-buffered saline (PBS) in order to avoid contamination by the cell culture supernatant, and lysis buffer (50 mM Tris-HCl, pH 8.3, 1 mM EDTA, 17 mM β -mercaptoethanol, 0.5% Triton X) was added to collect the cell lysates.

ELISA

Determination of hBD2 protein levels in 100 μ L cell lysate or in cell culture supernatants was performed by sandwich ELISA in three biological replicates using the EK-072-37 kit (Phoenix Pharmaceuticals Inc.) according to the suggested protocol. Determination of hBD2 levels in tear samples was performed in a volume corresponding to 5 μ g total tear protein.

Development of the SRM-based targeted proteomics method

Amino acid sequences of hBD1–4 were retrieved from the UniProt database^[47] (Accession numbers: P60022, O15263, P81534, Q8WTQ1) and were subjected to analysis by the PeptideCutter software.^[48] Tryptic fragments with $\geq 95\%$ cleavage probability were selected for BLASTp analysis^[49] and the NCBI non-redundant protein sequence database was searched to determine the protein-specific, unique tryptic peptide sequences. Design of the SRM transitions was performed by the Skyline software.^[50] Stable-isotope-labeled synthetic (SIL) crude peptides were obtained from JPT Peptide Technologies GmbH, Germany, while purified hBD2 SIL was obtained from PepscanPresto, The Netherlands. The quality of the synthetic peptides was assessed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) conducted in our laboratory (data not shown) and the SRM spectra of singly charged y ions were recorded.

Sample preparation for mass spectrometry

Protein concentration of the tested samples was determined with the Bradford method.^[51] The proteins were denatured with 6 M urea and were reduced with 10 mM dithiothreitol. The reduced samples were alkylated with 20 mM iodoacetamide and diluted with 25 mM ammonium bicarbonate to decrease the urea concentration to 1 M. Trypsin digestion was performed at 37 °C overnight by adding MS-grade modified trypsin (ABSciex) in 1:25 enzyme-to-protein ratio. The digested peptides were lyophilized and dissolved in 1% formic acid. The samples were desalted with C18 ZipTip (Millipore), lyophilized and re-dissolved in 1% formic acid. Cell lysates and supernatants from each cell line were processed together on the same day, while in the case of the tears, samples belonging to each set were digested and analyzed in parallel on the same day.

Mass spectrometry analysis

SRM-based analysis of biological fluids was carried out on a 4000 QTRAP (ABSciex) mass spectrometer using a NanoSpray II MicroIon source and was controlled by the Analyst 1.4.2 software (ABSciex). The spray voltage was 2800 V, the ion source gas was 50 psi, the curtain gas was 10 psi and the source temperature was 110 °C. The cycle time was 1.15 s, which provides near 30 data points/peak. Chromatographic separation was performed with an EasyLC II system (Bruker). Desalting was performed on a

Zorbax 300SB-C18 column (5 × 0.3 mm, 5 μ m pore size; Agilent) followed by separation on a Zorbax 300SB-C18 analytical column (150 mm × 75 μ m 3.5 μ m pore size; Agilent). Solvent A was 0.1% formic acid in LC water, solvent B was acetonitrile containing 0.1% formic acid. The flow rate was 300 nL/min, and a 30 min water/acetonitrile gradient combined with a continuous increase of solvent B from 0% to 100% was applied. Sample blocking was carried out by a randomly selected control and a randomly selected IL1 β -treated sample that were paired and digested together and analyzed one after the other using the same conditions. In case of the cell culture supernatants a randomly selected control-IL1 β -treated sample pair followed by two other randomly selected control-IL1 β -treated pairs were analyzed (three replicates). The same procedure was applied for cell lysates and tear samples.

The CaCo2 cells and tears were analyzed in two experimental settings. In the first set of experiments purified non-labeled synthetic peptides were utilized for hBD1, 3 and 4 and a purified SIL peptide was utilized for hBD2. In the second set of the experiments crude SIL peptides for hBD1–4 were spiked into the CaCo2 cell lysate and the supernatant samples.

Data analysis

The SRM data were analyzed by using the Skyline software.^[50] The endogenous/SIL peptide ratios were calculated by the software. The Skyline data are publicly available through the Panorama web site.^[52]

Table 1. SRM transition parameters of representative proteotypic peptides for hBD1, hBD2, hBD3 and hBD4

Protein	Proteotypic peptide	Q1 (<i>m/z</i>)	Q3 (<i>m/z</i>)	Ion	Dwell time (ms)	DP	CE
hBD1	IQGTCYR	420.705	727.319	y6	20	61.8	19.7
		420.705	599.261	y5	20	61.8	19.7
		420.705	542.239	y4	20	61.8	19.7
		420.705	441.191	y3	20	61.8	19.7
		420.705	338.182	y2	20	61.8	19.7
hBD2	GIGDPVTCLK	522.773	945.507	y9	20	69.2	25.5
		522.773	832.423	y8	20	69.2	25.5
		522.773	775.401	y7	20	69.2	25.5
		522.773	660.374	y6	20	69.2	25.5
		522.773	563.322	y5	20	69.2	25.5
		522.773	464.253	y4	20	69.2	25.5
		522.773	363.206	y3	20	69.2	25.5
		522.773	260.196	y2	20	69.2	25.5
hBD3	GIINTLQK	464.776	829.514	y7	20	65.0	22.2
		464.776	716.430	y6	20	65.0	22.2
		464.776	603.346	y5	20	65.0	22.2
		464.776	489.303	y4	20	65.0	22.2
		464.776	388.255	y3	20	65.0	22.2
hBD4	ICGYGTAR	449.216	784.341	y7	20	63.9	21.3
		449.216	624.310	y6	20	63.9	21.3
		449.216	567.288	y5	20	63.9	21.3
		449.216	404.225	y4	20	63.9	21.3
		449.216	347.203	y3	20	63.9	21.3
		449.216	246.156	y2	20	63.9	21.3

Amino acids in *italics* represent carbamidomethylated cysteines.

DP: de-clustering potential, CE: collision energy

Table 2. Linear dynamic range of the hBD SIL peptides in cell culture supernatant and tear matrix. The values represent the dilution factors

Protein	Peptide	Linear dynamic range in cell culture supernatant	Linear dynamic range in tears
hBD1	IQGTCYR	250x–5x	500x–25x
hBD2	GIGDPVTCLK	250x–10x	500x–10x
hBD3	GIINTLQK	250x–5x	250x–25x
hBD4	ICGYGTAR	250x–5x	500x–25x

For the calculation of the linear dynamic range the log AUC values were plotted against the log concentration values and logistic regression was applied for curve calculation. The linear dynamic range was defined based on these curves.

Statistical analysis was performed with the SigmaPlot 12.0 software (Systat Software Inc.) using Student's *t*-test. Correlation coefficients were determined by the linear regression method;^[53] the level of significance was set to $p \leq 0.05$.

RESULTS AND DISCUSSION

In order to better understand the role of β -defensins in innate defense mechanisms we sought to develop an SRM-based targeted proteomics approach for the determination of the relative levels of hBD1–4. The amino acid sequence of these proteins was retrieved from the UniProt database, subjected to the PeptideCutter software, and the tryptic fragments with $\geq 95\%$ cleavage probability were selected for BLASTp analysis. The results demonstrated the presence of one peptide specific for each hBD including hBD1, -2 and -3, respectively. Thus hBD1 could be identified by the IQGTCYR sequence, hBD2 by GIGDPVTCLK and hBD3 by the GIINTLQK peptide underscoring the conserved structure of these small molecules (Table 1).

As an exception, four specific peptide sequences could be identified for hBD4 (LVLLLAISLLLYQDLVPR, ICGYGTAR, CPNTYACCLR, WDESLNLR). In order to select from the four available peptides a prediction using the CONSeQuence software^[54] was applied. Based on our previous experiments and literature data,^[55] from the peptides ICGYGTAR and WDESLNLR offered by the software the ICGYGTAR sequence was selected for the SRM design.

Optimization of SRM parameters and determination of the linear dynamic range for hBD peptides

SRM transitions for the selected β -defensin peptides were designed by using Skyline^[50] and were tested with SIL peptides. Signals for all transitions of γ ions were recorded and examined manually. All transitions showing co-elution in case of each individual peptide were selected and used for further analyses (Table 1). To determine the linear dynamic range increasing amounts of stable-isotope-labeled (SIL) synthetic hBD peptides in the dilution range of 1000- to 5-fold were spiked either into the cell culture supernatant or to the tear fluid. Using the developed SRM method 1000-fold dilution (approximately 75 fmol) of the tested hBD peptides could be detected, but the linear dynamic range of the peptides was different in the studied matrices (Table 2).

In the case of the SIL hBD1 peptide, the linear range in the cell culture supernatant matrix was at 250- to 5-fold dilution (approximately 300 fmol to 15 pmol), while in tears it was in the range of 500- to 25-fold dilution (approximately 150 fmol to 3 pmol). The linear range of the SIL hBD2 peptide in the cell culture supernatant and tears was between 250- to 10-fold dilution (approximately 300 fmol to 7.5 pmol) and 500- to 10-fold dilution (approximately 150 fmol to 7.5 pmol), respectively. The SRM analyses of the SIL hBD3 peptide showed that the linear range in the cell culture supernatant matrix was at the 250- to 5-fold dilution range (approximately 300 fmol to 15 pmol) and in tears between 250- to 25-fold dilution (approximately 300 fmol to 3 pmol). The linear range of the selected SIL hBD4 peptide was observed between 250- to 5-fold dilution (approximately 300 fmol to 15 pmol) both in the cell culture supernatants and the tear matrix. Based on these results, the designed SRM method exhibited a broad dynamic range and it allowed the analysis within a wide range of changes often observed in biological systems.

Determination of hBD1, hBD3 and hBD4 levels in human colonic epithelial cells

Considering that β -defensins are produced in the cytosol but exert their biological activities outside the cell, where their secreted forms can exhibit various biological activities, we measured the levels of hBD1, hBD3 and hBD4 both in cell lysates and cell culture supernatants of three different CEC lines. SIL peptides were spiked either into the epithelial cell lysates or the supernatants right before the analysis (Supplementary Fig. S1, see Supporting Information). Upon IL-1 β stimulation the level of hBD1 did not change significantly in SW-1116 and HT-29 cells; however, significant decrease was detected only in the Caco2 cell lysates (Fig. 1(a)). Considering the secreted hBD1 levels the decrease was significant in stimulated HT-29 cells but not in SW-1116 CECs (Fig. 1(d)).^[56,57]

Changes in the endogenous:SIL peptide ratio showed significantly higher hBD3 levels in all stimulated cells as compared to the cell lysates of their unstimulated counterparts (Fig. 1(c)), and these changes could also be detected at the level of secreted hBD3 (Fig. 1(e)). As a result of stimulation the level of intracellular hBD4 did not change significantly and a similar tendency has been observed as shown for hBD1 (Fig. 1(c)). The secreted form of hBD4 changed significantly only in the HT-29 cell culture supernatants, while in SW-1116 and Caco2 cells hBD4 levels followed the pattern of hBD1 (Fig. 1(f)). García *et al.* have shown that hBD4 collaborates with hBD3,^[11] but this functional interplay likely does not require elevated hBD4 expression. It seems that hBD1 and hBD4 exhibited similar

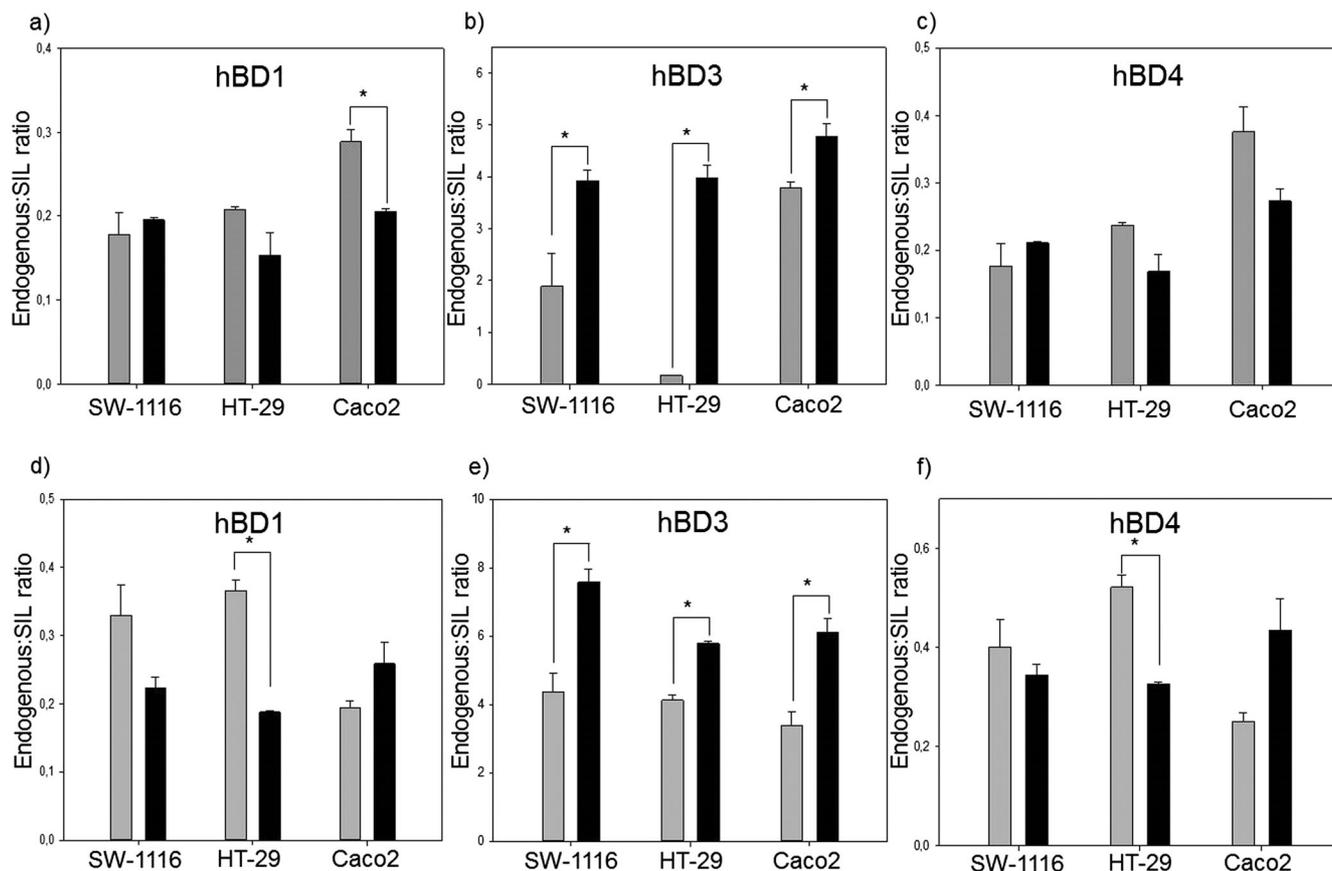


Figure 1. Determination of hBD1, hBD3 and hBD4 levels in colonic epithelial cells. (a) Determination of β -defensin 1 IQGTCYR levels in cell lysates. (b) Determination of β -defensin 3 GIINTLQK levels in cell lysates. (c) Determination of β -defensin 4 ICGYGTAR levels in cell lysates. (d) Determination of secreted β -defensin 1 IQGTCYR levels in cell culture supernatants. (e) Determination of secreted β -defensin 3 GIINTLQK levels in cell culture supernatants. (f) Determination of secreted β -defensin 4 ICGYGTAR levels in cell culture supernatants. The bars represent the mean values of three independent experiments with the standard error of the means, the grey bars show the values for the control group and the black bars correspond to the IL-1 β -treated group; * indicates $p < 0.05$.

kinetics when challenged by inflammatory stimuli such as IL-1 β . As a conclusion, our data demonstrate the feasibility of the SRM-based method in the comparative analysis of hBD levels and provide further evidence for the inducible feature of hBD3 in an inflammatory environment.^[17,56]

Validation of SRM data measured for hBD2 with ELISA and RT-qPCR

To further validate the results obtained for hBD2 by the SRM approach, the mRNA and protein levels of IL-1 β -stimulated and control CaCo2 cells was determined by the SRM, RT-qPCR and ELISA methods (Fig. 2 and Supplementary Fig. S2, see Supporting Information). The CaCo2 cells responded to the IL-1 β stimulation with increased hBD2 levels both in the cytosol and in the cell culture supernatant as indicated by the SRM-based method (Figs. 2(a) and 2(b)). The RT-qPCR data indicated higher hBD2 mRNA levels in IL-1 β -stimulated cells than in controls (Fig. 2(c)). In line with previous results the concentration of intracellular and secreted hBD2, determined by ELISA, also showed significantly higher hBD2 levels when the cells were stimulated by IL-1 β (Figs. 2(d) and 2(e)). Similar results were obtained with dot-blot analysis showing robust upregulation of secreted hBD2 in the supernatants of

IL-1 β -activated CaCo2 cells (Supplementary Fig. S2, see Supporting Information) demonstrating the activation-induced secretion of hBD2 under inflammatory conditions.^[58–61] It is important to note that despite the similar tendency of the changes determined with ELISA and SRM, the ELISA method resulted in higher changes as compared to those determined by using SRM. This may be due to the amplification steps making that technique more sensitive than the SRM approach. Comparison of the magnitude of changes obtained with SRM, RT-qPCR and ELISA revealed the ELISA method as the most sensitive method, while the RT-qPCR- and SRM-based assays provided changes with similar magnitudes. In a next step we also compared the efficacy of the ELISA and the newly developed SRM method for the analysis of hBD2 levels derived from the same samples (Fig. 2(f)). The correlation coefficient of the data obtained by the SRM and ELISA techniques (0.937) indicated that these methods provide comparable results. Based on these results the developed SRM approach can be evaluated as an alternative quantification method that is cheaper than the antibody-based techniques. It could be especially useful in those cases when the determination of more than one hBD is needed and the sample volume does not permit the administration of consecutive ELISA measurements.

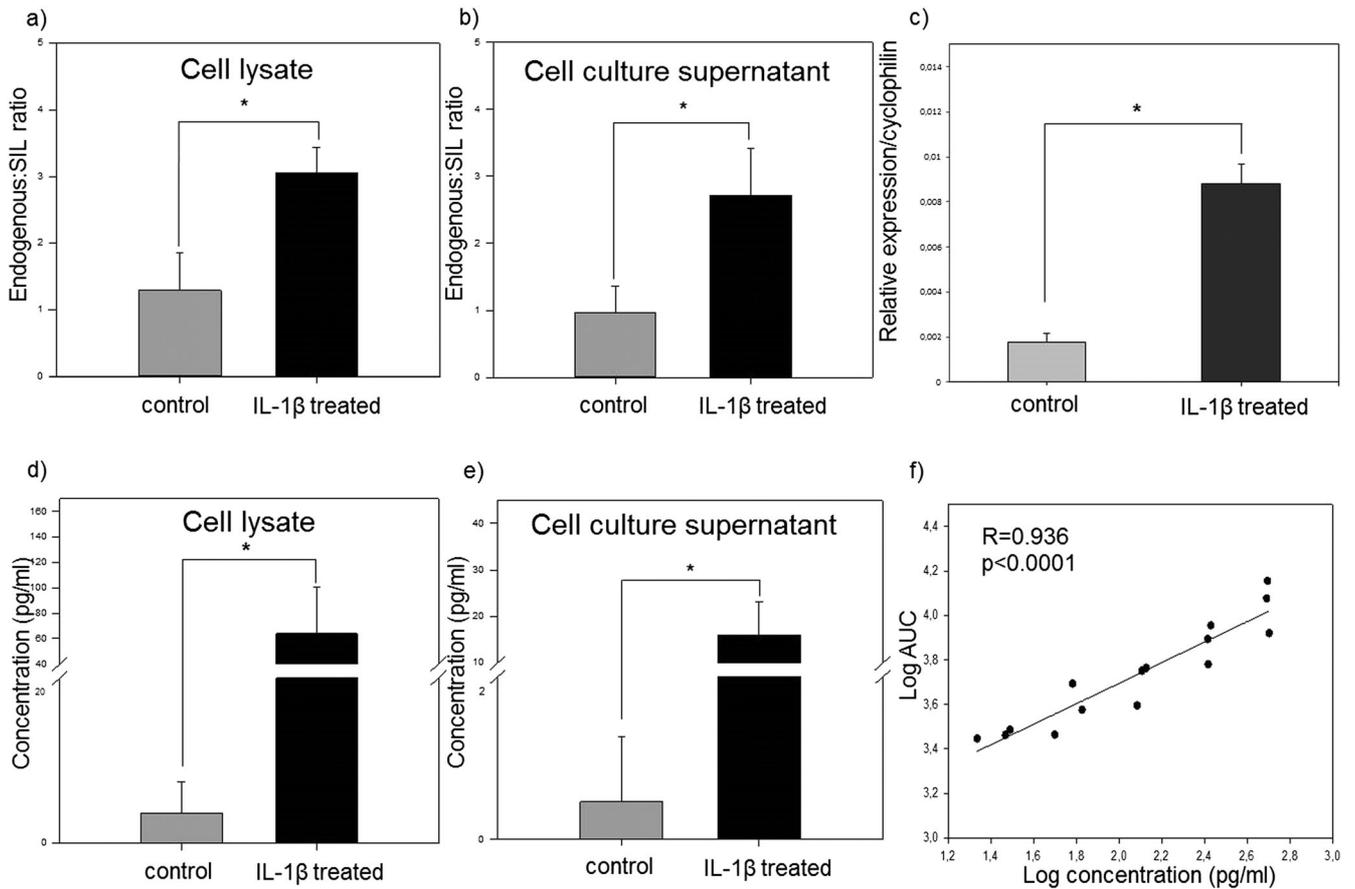


Figure 2. Analysis of hBD2 levels in Caco2 cell culture. (a) Determination of β -defensin 2 GIGDPVTCLK levels in Caco2 cell lysates. (b) Determination of secreted β -defensin 2 GIGDPVTCLK levels in Caco2 cell culture supernatants. (c) qPCR data for β -defensin 2 mRNA levels normalized to cyclophilin. (d) Determination of β -defensin 2 protein concentrations (pg/mL) in cell lysate using ELISA. (e) Determination of secreted β -defensin 2 protein concentrations (pg/mL) in cell culture supernatant with ELISA. The bars represent the mean values of three independent experiments with the standard error of the means; the grey bars show the values for the control group and the black bars correspond to the IL-1 β -treated group; * indicates $p < 0.05$. (f) Correlation analysis of the ELISA and SRM methods. The x axis contains the log concentration (pg/mL) of hBD2-containing samples determined using ELISA and the y axis contains the log area under the curve (AUC) values determined for the same samples using the SRM method. The correlation coefficient (R) and p value are indicated.

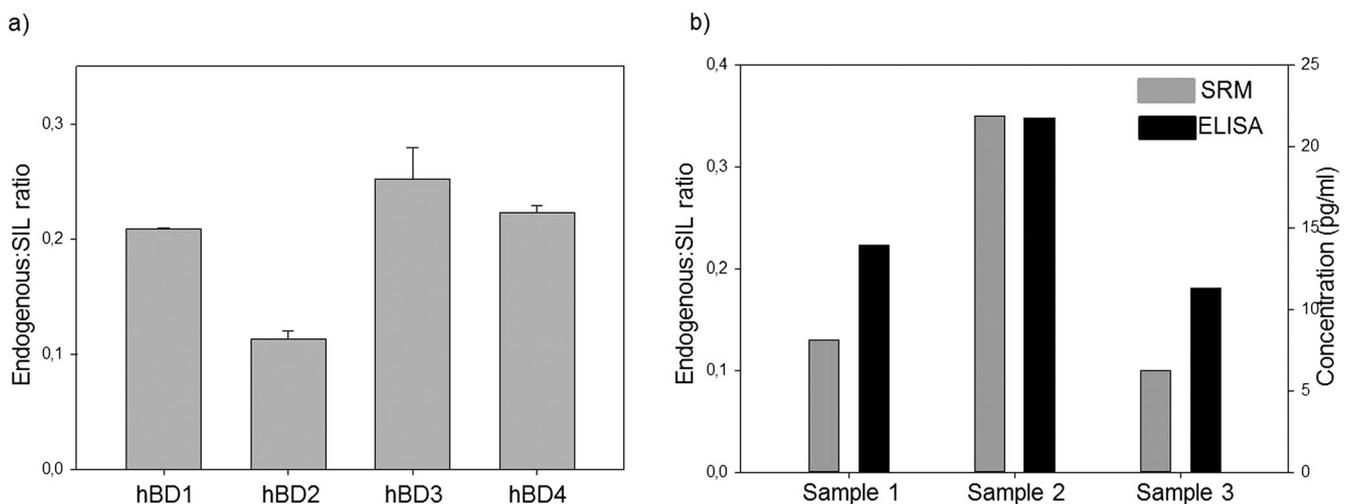


Figure 3. Analysis of β -defensin levels in tears. (a) Examination of β -defensin 1–4 levels in tears. The bars represent the mean values of three independent experiments with the standard error of means. (b) Comparison of tear β -defensin 2 amounts determined by ELISA (black bars) and SRM (grey bars).

Analysis of β -defensin levels in tears

Most of the body fluids contain a wide array of antimicrobial peptides as a part of the innate immune defense mechanisms. In this context the major function of β -defensins is to rapidly kill and/or inactivate microorganisms.^[62] AMPs also differ in the spatial area of secretion and this type of distribution depends on the actual environmental challenges, which could have an impact on the efficacy of combating against a large variety of microorganisms. In order to assess whether the newly developed SRM method could also be utilized for the analysis of biological fluids, such as human tears, we collected tear samples from healthy volunteers and analyzed them. The quantification of hBD1, hBD2, hBD3 and hBD4 peptides was performed by the SRM method (Fig. 3(a)) and the SRM analyses of hBD2 were confirmed by ELISA (Fig. 3(b) and Supplementary Fig. S3, see Supporting Information). The level of hBD2 was the lowest and the level of hBD3 was the highest, while the levels of hBD1 and hBD4 were almost the same, highlighting the non-inducible feature of hBD1 and hBD4.^[11,46] The β -defensin levels in tear samples were found to be lower than those detected in the supernatants of CECs indicating differences in the tissue-specific expression of β -defensins.^[63] The data indicate that the developed SRM method is suitable for the relative quantification of β -defensins also in body fluids typically of low sample volume such as tears, although the antibody-based method seems to be more sensitive than the developed MS-based method.

Analysis of accuracy

For the determination of accuracy of the developed SRM method the coefficients of variance (CVs) were calculated. Based on literature data no consensus CV value is available for validating the accuracy of datasets. The most widely used recommendations involve CV <20%^[64,65] or CV <25%.^[66,67] This type of analysis showed that the CV between the biological replicates of all SRM experiments was below 20%. Considering that CVs for SRM analyses were acceptable, we concluded that the developed and validated SRM method provides an accurate approach for the relative quantification of hBDs in different biological samples.

CONCLUSIONS

In this study we present a semi-quantitative targeted proteomics method appropriate for the relative quantification of hBD1–4 levels in biological samples as an alternative approach to the classical antibody-based detection and quantification systems. The ultimate advantage of the newly developed SRM method compared to ELISA or Western blot relies on its multiplex feature that allows a cost-effective simultaneous analysis of multiple analytes derived from the same sample, having relevance for cell biology and medical applications, where the amount of the sample to be studied is limited. The inducible feature of hBD2 and hBD3 confirmed at the mRNA and protein levels in the case of hBD2 together with the utility of the developed SRM-based targeted proteomics method for semi-quantitative analysis of hBD1–4 in cell lysates, culture supernatants and tear samples is highlighted.

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