SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

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

by

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The PhD Defense takes place at the **Lecture Hall of Department of Pediatrics**, Faculty of Medicine, University of Debrecen at, **11am, 17th November 2015**.

1. INTRODUCTION

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 infective microbes, dietary and metabolic products and diverse inorganic compounds. The intestine is therefore challenged with the daunting task of segregating the underlying tissues from potentially noxious and harmful endogenous and environmental molecules. The monolayer of intestinal epithelial cells (IECs) provides an important anatomical barrier to support appropriate segregation and protection from environmental challenges.

This study is aimed to create a simplified model mimicking the gut and studying the complexity of secreted chemokines by IECs under the influence of ATRA (All-trans retinoic acid), in an proinflammatory environment and their effect on myeloid cells and T lymphocytes.

1.1. Vitamin A and its effect on gut

The vitamin A (VA) metabolite retinoic acid (RA) is a key regulator of the cytokine transforming growth factor beta (TGF-β), which promotes T_{Reg} differentiation. VA also contributes to the formation of epithelial linings of mucosal surfaces, and its multifunctional metabolite RA acts as a critical driver of lymphocyte trafficking to the intestinal mucosa. ATRA induces the expression of the gut homing integrin α4β7 on myeloid cells and the chemokine receptor CCR9 on T-lymphocytes, while the lack of the αv or β8 integrin chains in DC impairs T_{Reg} functions and T_H17 responses *in vivo*. ATRA also modulates T_H17 effector T-lymphocyte differentiation in the gut, 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. VA deficiency has an effect on epithelial cell integrity and the composition of the gut microbiota. VA shows ’hormone-like’properties and described below are some of the effects of VA on IEC and gut homestasis.

1.2. Cytokines in maintaining gut homeostasis

Cytokines have been directly implicated in the pathogenesis of IBD in genetic and immunological studies, and they seem to have a crucial role in controlling intestinal inflammation and the associated clinical symptoms of IBD. The key role of cytokines is also highlighted by the fact that blockade of TNF-α is now commonly used as a standard therapy for IBD in the clinic. A combination of these IBD risk factors seems to initiate

alterations in epithelial barrier function thereby allowing the translocation of luminal antigens (for example, bacterial antigens from the commensal microbiota) into the bowel wall. Excessive cytokine responses to such environmental triggers cause subclinical or acute mucosal inflammation in a genetically susceptible host. In patients that fail to resolve acute intestinal inflammation, chronic intestinal inflammation develops that is induced by the uncontrolled activation of the mucosal immune system. In mucosal immune cells - such as Mf, T cells and the recently discovered subsets of innate lymphoid cells (ILCs) - seem to respond to microbial products or antigens from the commensal microbiota by producing cytokines that can promote chronic inflammation of the gastrointestinal tract. Loss-of-function mutations in the genes encoding IL-10 and IL-10R are associated with a very early-onset form of IBD that is characterized by severe intractable entero colitis in infants. Cytokine function in patients with IBD may be affected by the location and the type of inflammation, by immune cell plasticity, by different pathogenetic mechanisms or by the changing cytokine production patterns that occur during the course of the disease. These factors highlight the complexity of the mucosal cytokine network and suggest that anti-cytokine approaches that target a single pro-inflammatory cytokine will have major limitations in terms of offering an effective therapy for all clinical subgroups of IBD.

1.3. GM-CSF plays a significant role in mucosal immunology

The cytokine granulocyte-macrophage colonystimulating factor (GM-CSF or Csf2), is a key determinant of myeloid lineage differentiation and is required for the optimal function of tissue mononuclear phagocytes (MNPs), including Mf and DCs, thereby promoting host protection against environmental pathogens and vaccine responses. Despite the key role of Csf2 in promoting MNP survival, differentiation, and function, previous studies reported that mice lacking Csf2 or its receptor displayed only minor impairment in the development of spleen and lymph node DCs. Subsequent studies showing that Csf2 expression is increased in inflamed mice and that adoptively transferred monocytes generate DCs in the inflamed spleen but not in the steady-state spleen suggested that Csf2 is 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} differentiation has been established. 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 a crosstalk between IL-1β–secreting Mf and Csf2-producing ILC in the intestinal mucosa. Microbiota-driven IL-1β production by Mf promoted the release of Csf2 by ILC, which in turn acted on DCs and Mf, allowing for the maintenance of colonic T_{Reg} homeostasis. Ablation of Csf2 altered DC and Mf numbers and impaired their ability to produce regulatory factors such as RA and IL-10, which led to disrupted T_{Reg} homeostasis in the large intestine. Conversely, administration of Csf2 cytokine increases T_{Reg} frequency in the gut. Most notably, cell type–specific ablation of IL-1–dependent signaling in ILC abrogated oral tolerance to dietary antigens and compromised intestinal T_{Reg} homeostasis *in vivo*. Although the reduction in total T_{Reg} numbers was mostly observed in the large intestine, adoptive transfer studies in *Csf2−/−* mice revealed impaired T_{Reg} differentiation both in the small and large intestine, suggesting that Csf2- dependent immunoregulatory functions control T_{Reg} induction in both tissues establishing intestinal tolerance is critical for the prevention of intestinal diseases such as IBD. In a study of more than 300 patients with Crohn's disease, the presence of neutralizing antibodies to Csf2 in the serum correlated with ileal involvement and 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. Previous clinical trials of recombinant Csf2 in IBD have established patient benefit in terms of reduced disease severity and lower burden of corticosteroid use. Unpublished results of a larger trial of Csf2 in IBD has since failed to achieve primary clinical end points, but it remains likely that a subset of IBD patients with defective Csf2 production or function could benefit from this therapy. The uncovered key role for Csf2 in the maintenance of intestinal tolerance is consistent with previous studies showing that absence of Csf2 can also contribute to lupus-like disease, insulitis, and age-related glucose intolerance and further emphasizes the critical role of tissue-resident phagocytes in the maintenance of tissue integrity.

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

Mammalian antimicrobial peptides (AMPs) are members of a diverse array of protein families, all of which function 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 the microbial challenges faced by these tissues and the continuous threat of microbial invasion at these sites. The AMPs of the gut encompass

representatives of several distinct protein families. These include defensins, cathelicidins, C-type lectins, ribonucleases and psoriasin. Among the most abundant, diverse and highly expressed AMP families in the gut are the α-defensins. These include α-defensin 5 (DEFA5; also known as HD5) and DEFA6 (also known as HD6) in humans. The α-defensins are small peptides (~2-3 kDa) with a conserved three-dimensional structure that is characterized by an amphipathic arrangement of cationic and hydrophobic residues, resulting in a positively charged surface that is spatially separated from a neighbouring hydrophobic region. This unique arrangement promotes attraction of α-defensins to the negatively charged cell surface and insertion into the lipid-rich membrane. 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 have marked differences in their activity spectrum and expression patterns.

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

Colon enterocytes express β-defensins and cathelicidins. 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 and RNase. 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 glyco proteins 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 the epithelial surface.

2. 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-1β 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-1β 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 spectrometro based methodology for the semi quantitative determination of relative β-defensin expressions in Caco2 cells stimulated by IL-1β.

3. 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 (Nunclon, Rochester, NY) at 37°C in 10% and 5% CO_2, respectively. HT-29 cells were cultured in RPMI-1640 medium supplemented with 1% antibiotic-antimycotic solution and 10% fetal bovine serum in 5% CO_2. 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-1β or TNF-α) in combination with or without 10nmol 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-1β 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 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 ($3x10^5$) 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 four 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).

Pheripheral 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-CSFRα-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 Mk, CXCL7, CCL20 and CXCL16 were ordered from PeproTech, UK, CXCL8 and CXCL1 are from MiltenyiBiotec.

IL-17 and IFNγ 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 (10^6cells/well) in 10% RPMI medium for 2 days at 37°C in a humidified atmosphere containing 5% CO_2. PHA and Con 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 ΔCt 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 perfom 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 5 µg/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.

ELISA

Determination of β-defensin 2 protein levels form 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.

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 ^{o}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 C_{18} 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 ± SD. Two group differences were analyzed by Student's t-test. p value (two-tailed) less than 0.05 was considered statistically significant.

4. RESULTS

4.1. 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 human CEC in resting state and in an inflammatory milieu mimicked with TNF-α or IL-1β 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-1β and TNF-α were able to trigger the secretion of Midkine (Mk), CXCL16 and CXCL7 by CEC, but their expression could efficiently be down regulated by ATRA. However, the secretion of CXCL1, CXCL8 or CCL20 by IL-1β-stimulated CEC was not influenced by ATRA. Our results also revealed that the *in vitro* induced inflammatory milieu created by pro-inflammatory 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-1β and TNF-α was higher in the presence of ATRA.

4.1.1. 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-1β and TNF-α act as potent activators of CEC. 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-1β 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. Caco2 cells secrete detectable levels of CCL19, CCL21 and CCL22 constitutively similar results were obtained for HT-29 cells, but HCT116 secreted only CCL19 at detectable levels. 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 entero-invasive bacteria, but inhibition of the NF-κB pathway abolished CCL22 expression in response to pro-inflammatory stimuli. The chemokines CXCL7, CXCL16 and Mk with different functional activities were also constitutively secreted by resting CEC suggesting their role in the maintenance of epithelial cell homeostasis.

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

In our model system CEC were left untreated or stimulated by IL-1β or TNF-α in combination with or without ATRA for 6 hrs and the cell culture supernatants were subjected to chemokine array analysis. When Caco2 cells were activated by TNF-α or IL-1β, the secretion levels of the chemokines CXCL7, CXCL16 and Mk did not change significantly as compared to unstimulated cells. However, the expression of CXCL16 and Mk 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 could be induced only when IL-1β or TNF-α was combined with ATRA treatment demonstrating the dependence of its secretion on ATRA. The secretion levels of CCL20 and CXCL1 was not affected by ATRA and could be induced exclusively by IL-1β 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. 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. Even though HCT116 showed a similar overall pattern of chemokine secretion as the other CEC, trace amounts of NF-κB-dependent inflammatory chemokines (CCL2, CXCL2 and CXCL10), not observed in Caco2 and HT29 cells were detected indicating CEC type-dependent regulation of chemokine secretion.

4.1.3. 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. 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-1β, but this process could be down regulated by ATRA. To analyze whether the supernatant of IL-1β-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-1β 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 and Mf developed by GM-CSF toward CXCL1 and CXCL8 known to be involved in the chemotaxsis and migration of polymorphonuclear leukocytes to inflammatory sites as compared to CCL20. Interestingly, the supernatant of Caco2 cells pre-stimulated with TNF-α had no such effect on cell migration. 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. These results suggest that in an inflammatory environment ATRA also modulates the migratory potential of myeloid cells in a cell type-dependent manner.

4.1.4. 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, while the CX3CR1$^+$ resident Mf population samples the microenvironment by protruding dendrites. 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-1β, 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. These results also indicated that even in the presence of inflammatory stimuli (supernatant of IL-1β 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, and was decreased when the ATRA-conditioned CEC supernatant was added to Mf differentiated from monocytes with M-CSF and activated by IL-1β or TNF-α.

4.1.5. 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 IFNγ 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 or GM-CSF 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 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-1β 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 IFNγ-secreting cells could not be detected in the $CD4^+$ T-cell population. Thus ATRA is able to exert different effects on monocyte-derived myeloid cells differentiated upon co-culture with $CD4^+$ T cells. This may indicate a broad range of RA-mediated effects involved in shaping the gut microenvironment.

4.2. SRM based methodology for semi-quantitation of AMPs secreted by Caco2 cell supernatnat

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 for the

analysis of β-defensin 2, β-defensin 3 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 and 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 found 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 MIDAS Workflow Designer software (ABSciex).
The biological relevance of defensins is exerted only in their secretory form, so in the next step β-defensin 2 levels in the supernatant of Caco2 cells were analyzed. We could confirm the significant increase of β-defensin 2 levels in stimulated Caco2 supernatants using MRM and Western-blot. The level of other defensins were also examined, significant β-defensin 3 level increase could also be demonstrated upon IL-1β treatment. The level of α-defensins could be analyzed but we did not see significance among the different samples mainly because of unavailability of unique sequences between α-defensin 1, 2 and 3. The cells were stimulated with IL-1β for six hours and the intracellular and secreted defensins were analysed. The first step was the determination of β-defensin 2 mRNA level in control and stimulated cells with RT-qPCR. The results show that the β-defensin 2 mRNA level was significantly higher after IL-1β treatment. These data were confirmed at protein level as well by SRM and Western-blot.

5. DISCUSSION

The cytokines secreted at increased levels in patients with IBD have been identified as TNF-α and IL-1β, 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. The detailed analysis of chemokine expression induced by the supernatants of CEC pre-activated by IL-1β or TNF-α demonstrated that 1) the secretion of the CCL20, CXCL1 and CXCL8 chemokines could be induced only by IL-1β 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-

1β 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-1β 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. 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. The chemokine CCL20 exhibited unique features as it could be induced exclusively by the supernatant of IL-1β stimulated CEC that could completely be inhibited by ATRA. This chemokine expression is associated with IBD. Mk acts as a multifunctional cytokine and growth factor with bactericidal and fungicidal activity. 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. When CEC were stimulated by IL-1β 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 trans-migration. ATRA is also responsible for the homeostatic regulation of $CD11b^{+}CD103^{+}$ DC 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 i.e. the supernatant of activated CEC are able to support the acquisition of the gut phenotype of human DC. 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. In contrast, $CD103^{+}$ cells are able to migrate and can trigger adaptive immune responses by expressing gut homing receptors on T-cells.In our *in vitro* model with human CEC (Caco2, HT-29, 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 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 Treg differentiation. It has also been shown that Th17 cells can exhibit both anti- and pro-inflammatory properties depending on the cytokine signals received. These results altogether indicated that the detailed

characterization of the gut milieu under different conditions is of utmost imporatnce for understanding the complexity of regulation leading to the maintenance or loss of gut homeostasis. Other than the secretion of chemokines observed when Caco2 cells were stimulated with IL-1β 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 we analysed and their relative quantitation asserted. The 13 different AMPs analysed in Caco2 cell supernatants are 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-1β.Based on our developed method for measuring defensin concentrations, using the targeted mass spectrometric method, we have analyzed the levels of β-defensin 1, β-defensin 3 and β-defensin 4. The levels of β-defensin 1 did not show significant change after proinflammatory stimulus, being in accordance with previous data regarding the constitutive expression of β-defensin 1 which it cannot be further stimulated by proinflammatory cytokines. Elevated levels of β-defensin 3 were determined upon inflammatory stimulus, indicating the inducible feature of this defensin. The level of β-defensin 4 was not changed significantly upon IL-1β treatment, phenomenon described by other researchers as well. β-defensin 4 has a strong additive effect on β-defensin 3 but it seems that this effect does not require elevated β-defensin 4 expression. Considering that defensins exert their biological activities outside the cell and their secreted forms exhibit various biological activities, in the next step the levels of β-defensins 1-4 in the supernatants of Caco2 cells were examined. Using the developed SRM method the levels of β-defensin 2 in the supernatants of control and IL-1β stimulated Caco2 cells were analyzed. Both by the summed AUC and the light:heavy ratio indicated significantly higher β-defensin 2 levels in the supernatant of IL-1β treated Caco2 cells compared to the controls. With ELISA experiments a robust upregulation of β-defensin 2 levels in the supernatants of IL-1β treated Caco2 cells was observed in comparison to controls, and similar observation was made with dot blot. These results demonstrate the increased production and secretion of β-defensin 2 preferentially in inflammatory conditions. For further validation of the newly developed SRM-based method the levels of increasing amount of β-defensin 2 standard protein were determined using both ELISA and SRM assays.

6. SUMMARY

Retinoids and other derivatives of vitamin A are known to have important functions in regulating differentiation and proliferation of epithelial cells. We have tried to examine the immunological effects of ATRA in colon epithelial cell lines in combination with different pro-inflammatory activators. We chose the two most potent pro-inflammatory cytokines known IL-1β and TNF-α for studying the effect of ATRA. So I used the two cytokines in the presence or absence of ATRA as inducers on Caco2 colon epithelial cell lines for 6hr. We observed that ATRA was responsible for conferring the CD103 cell surface expression patterns on DC and Mf. In case of CX3CR1 surface expression, DC treated with ATRA conditioned CEC supernatant showed an increase in CX3CR1 expression but Mf showed a decrease in CX3CR1 expression with a similar treatment. The fact that ATRA has varied effect on different cells indicates the capability of ATRA to affect the final T-lymphocyte responses. In our *in vitro* system we observed that ATRA treated CEC supernatant can influence the DC which when co-cultured with CD4+ T lymphocytes, T_H17 cells were decreased while for Mf, the number of T_H17 cells was significantly increased.

Defensins represent an important group of AMP consisting of 16 – 50 amino acids, organized to a structurally conserved compact structure and 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 expression. Elevated levels β-defensin 3 levels 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. Caco2 human epithelial cells were challenged with IL-1β as an inflammatory stimulus and the levels of β-defensin 2 was analyzed in cell lysates and cell culture supernatants. The developed method was validated using qPCR, quantitative ELISA and Western blot. The gene and protein expression levels of β-defensin 2 analyzed were significantly higher in IL-1β treated samples compared to the unstimulated controls. Beside β-defensin 2, the levels of β-defensin 3, β-defensin 4 and α-defensin was examined as well and significantly higher levels of β-defensin 3 could be determined in the supernatants of activated Caco2 cells as well. Our results show that the targeted proteomics method developed here offers an alternative approach for the mass spectrometric analyses of some defensins.

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List of publications related to the dissertation

1. Kalló, G., **Chatterjee, A.**, Tóth, M., Rajnavölgyi, É., Csutak, A., Tőzsér, J., Csősz, É.: Relative quantification of human [béta]-defensins by an SRM - based proteomics approach.
Rapid Commun. Mass Spectrom. Article in press, 1-32, 2015.
IF:2.253 (2014)

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, 2015.
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List of other publications

3. Ganapathy, A., Pandey, N., Srisailapathy, C.R.S., Jalvi, R., Malhotra, V., Venkatappa, M., **Chatterjee, A.**, Sharma, M., Santhanam, R., Chadha, S., Ramesh, A., Agarwal, A.K., Rangasayee, R.R., Anand, A.: Non-Syndromic Hearing Impairment in India: High Allelic Heterogeneity among Mutations in TMPRSS3, TMC1, USHIC, CDH23 and TMIE. *PLoS One. 9* (1), 10 p., 2014.
DOI: http://dx.doi.org/10.1371/journal.pone.0084773
IF:3.234

4. **Chatterjee, A.**, Jalvi, R., Pandey, N., Rangasayee, R., Anand, A.: A novel locus DFNA59 for autosomal dominant nonsyndromic hearing loss maps at chromosome 11p14.2-q12.3. *Hum. Genet. 124* (6), 669-675, 2008.
DOI: http://dx.doi.org/10.1007/s00439-008-0596-3
IF:4.042

Total IF of journals (all publications): 12,765
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

18 September, 2015

7. Conferences and Presentations

'The induction and Expression of various genes in simulating viral infection in colonic epithelial cell lines'- Arunima Chatterjee and Eva Rajnavolgyi Poster Presentation at MetaHIT conference on Human Metagenomics, China, Shenzhen, 1-3 March, 2010.

'Expression and Induction of Intracellular Pattern Recognition Receptors in colonic epithelial cell lines'- Arunima Chatterjee and Eva Rajnavolgyi. Short talk at CROSSTALK Aberdeen Fall School October 19-23,2009, Scotland.

'The interplay of cytokines and signaling pathways induced by the intracellular sensors RIG-like helicases (RLH) and Nod Like Receptors (NLR) observed in gut epithelial cell lines'- Arunima Chatterjee and Eva Rajnavolgyi. Presentation at Marie Curie ITN Network Meeting, Debrecen, 2-4 August 2010, Hungary.

'Comparing the Effect of All trans Retinoic Acid and proinflammatory signals on colon epithelail cell lines'- Arunima Chatterjee and Eva Rajnavolgyi. Poster presentation at Lessons from Host Pathogen Interaction, Milano Italy, 28-29 April, 2011.

Factors affecting epithelial–immune cells cross-talk and the interplay of cytokines and signaling pathways induced by ATRA. Arunima ,Eva Rajnavolgyi,Francois Lefevre, HerveBlottier. Wageningen, Netherlands, CROSSTALK 4th Network Meeting, September 12-13th 2011.

Cytokine and chemokine mediated interplay of human epithelial and dendritic cells modulated by ATRA. Arunima Chatterjee, Eva Rajnavolgyi. Poster Presentation at Host-Microbes Cross-talk: From Animal models to Human patients, University of Oslo (Norway), 12-13th April,2012.

Studying the factors affecting intestinal epithelial-immune cell Crosstalk and the interplay of cytokines & signaling pathways induced by *All Trans Retinoic Acid* by a co-culture model. Arunima Chatterjee, Eva Rajnavolgyi. Presentation at 'The disruption of the war metaphore of Human-microbe interactions in the gastrointestinal tract' meeting held at Paris Descartes University at Paris on 3-4th September 2013.