The human immunoglobulin A Fc receptor (FcαRI) modulates adaptive immune responses in experimental colitis

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ABSTRACT
Ulcerative colitis is a state of chronic inflammation in the colon with unknown etiology, but characterized by massive influx of neutrophils in the inflamed tissue. It is furthermore hypothesized that aberrant T cell responses against commensal microorganisms play a role in the disease, which might be due to disruption of the epithelial barrier function. Immunoglobulin A (IgA), which is ample present in mucosal tissue, can bind to antigens that are present in the lamina propria, leading to the formation of IgA immune complexes. Subsequently, neutrophils are activated through the IgA Fc receptor FcαRI, hereby promoting immune responses. We investigated the role of IgA and FcαRI in the onset of inflammation by inducing experimental colitis in mice that either express human IgA (hIgA) or co-express human IgA and FcαRI (hIgA/FcαRI). The presence of FcαRI aggravated colitis, as reflected by decreased body weight of hIgA/FcαRI mice. Furthermore, increased expression of the Th17 cell-associated cytokines and chemokines CCL2, interleukin (IL)-1β, and IL-6 mRNA expression was observed in intestinal tissues from hIgA/FcαRI mice, which coincided with increased CD4⁺ T cell numbers. Enhanced release of pro-inflammatory cytokines and chemokines was also observed after cross-linking of FcαRI on human neutrophils. Thus, FcαRI on neutrophils likely plays a role in the induction of a Th17 cell response in experimental colitis.
**INTRODUCTION**

Ulcerative colitis (UC) is a state of chronic inflammation in the gastro-intestinal (GI) tract and constitutes together with Crohn’s Disease inflammatory bowel disease (IBD). With a yearly incidence of 1.2 to 20.3 cases per 100,000 persons per year, and a prevalence of 7.6 to 246.0 cases per 100,000 per year, the disease comprises a considerable health burden. Various risk factors have been identified, including diet, life style, smoking and genetic factors.\(^1\)

It is not yet known how aberrant immune responses are initiated in IBD, but initiation of inappropriate immune responses against commensal bacteria has been proposed.\(^2\) Experimental colitis can, for instance, not be induced in mice that are housed in a germ-free milieu, whereas disease develops rapidly after reconstitution with commensal bacteria\(^3\). Furthermore, defects in the mucosal barrier may play an important role because increased intestinal permeability was reported in IBD patients. Disruption of the epithelial barrier in several mouse models resulted in colitis.\(^2-4\)

Loss of barrier function allows access of luminal antigens to the tissue underlying the mucosal layer, such as the lamina propria (LP). Recognition of these antigens by dendritic cells (DCs) within the tissue induces the onset of the immune response. This results in a, for UC characteristic, massive influx of neutrophils, which causes tissue damage that can evolve into ulceration.\(^2\) Furthermore, aberrant responses of CD4\(^+\) T cells (also referred to as T helper or Th), that are important in maintaining mucosal immune homeostasis,\(^5,6\) are reported to play a major role in mediating and shaping pathological intestinal immune responses in IBD patients.\(^7-9\) Traditionally, UC was regarded to be mediated via Th2 cells.\(^10\) More recent a role of Th17 cells was reported, which are important in maintaining mucosal barrier function in the gut, but express cytokines that negatively affect regulator T cells (T-regs).\(^11,12\) In UC, IL-23 driven IL-17 expression is elevated and more Th17 cells are observed in the mucosa.\(^8,13\) Furthermore, different genes involved in the IL-23 - Th17 pathway have been associated with UC, including IL-23R, IL-12p40 and STAT3.\(^14\) Interestingly, crosstalk between Th17 cells and neutrophils has been demonstrated. Th17 cells release CXCL8 (IL-8), a potent neutrophil chemoattractant. Recruited and activated neutrophils in turn express the Th17 cell chemokines CCL2 and CCL20.\(^15\) This reciprocal chemotaxis can promote immune responses in inflamed tissue.

A defective epithelial barrier function, as seen in UC, may also result into increased antigen prevalence in the lamina propria, where these form complexes with immunoglobulin A (IgA). This is the most abundant antibody (Ab) in mucosal tissues, and more IgA is produced on daily basis (66 mg/kg/day) than all other isotypes combined.\(^16\) Two IgA isotypes are present in mucosal areas: dimeric IgA (dIgA) and secretory IgA (SIgA). Both isotypes originate from plasma cells in the LP as dIgA, but SIgA is formed during transepithelial transport of dIgA towards the lumen, via the polymeric Ig receptor (pIgR).\(^17\) A residual part of the pIgR remains attached to dIgA that is called the secretory component (SC), hereby forming SIgA. In the mucosa, SIgA exerts an antimicrobial role by binding antigens without inducing an immune response, as the SC prohibits binding to the prototypical IgA Fc receptor, Fc\(\alpha\)RI (CD89), by blocking the Fc binding site.\(^18\) However,
dIgA in tissue underlying the mucosa does bind FcαRI and subsequently can activate immune cells.\textsuperscript{19} Previous studies showed efficient phagocytosis and cell migration by neutrophils following stimulation with IgA.\textsuperscript{20,21} IgA immune complexes activate immune cells by cross-linking FcαRI, suggesting that IgA and FcαRI may be involved in the onset of inflammatory processes within mucosal tissues. Moreover, uptake of IgA by neutrophils has been observed in patients suffering from UC, supporting a role of IgA–FcαRI interactions in this condition.

\textit{In vivo} studies investigating the role of IgA and FcαRI have however long been restricted, since mice do not express an FcαRI homologue, and the murine IgA system differs from the human situation. To overcome this limitation, BALB/c mice expressing human IgA (hIgA)\textsuperscript{22} were crossed with BALB/c mice expressing FcαRI on neutrophils.\textsuperscript{23} This generated mice that produce human IgA, and co-express FcαRI on neutrophils (hIgA/FcαRI), which resembles the human situation. Experimental colitis was induced in these mice using dextran sulphate sodium (DSS).\textsuperscript{3,24} It was observed that the presence of FcαRI seriously aggravated inflammation and morbidity in the colon of these mice (Chapter 6).\textsuperscript{25} Furthermore, massive neutrophil recruitment towards the site of inflammation was observed, compared to mice that only expressed hIgA. Because neutrophils produce both pro- as well as anti-inflammatory cytokines that affect other components of the immune system,\textsuperscript{26} and we demonstrated the release of CCL2, CCL3 and CCL4 after cross-linking of FcαRI\textsuperscript{27} we now assessed the potential role of FcαRI in inducing adaptive immune responses in UC in more detail.

**MATERIAL AND METHODS**

**Cell isolation**

Primary neutrophils were isolated from peripheral blood, obtained from healthy donors using Lymphoprep (Axis-Shield, Oslo, Norway) density gradient centrifugation, after which erythrocytes were lysed in ammonium chloride buffer (155mM, 10 minutes, 4°C). Neutrophils were resuspended in RPMI 1640 (Gibco BRL, Paisley, UK) supplemented with glutamine and antibiotics. Blood samples were collected according to the guidelines of the Medical Ethical Committee of the VU University Medical Center (The Netherlands), in accordance with the Declaration of Helsinki. All donors gave informed consent.

**Coating sepharose beads**

Cyanogen bromide-activated sepharose beads (GE Healthcare Bio-Sciences, Uppsala, Sweden) were coated with 30 \(\mu\)g/ml bovine serum albumin (BSA) (Roche Diagnostics, Basel, Switzerland), or IgA (Cappel/MP Biomedicals, Santa Ana, CA), (o/n, 4°C) as previously described.\textsuperscript{21}

**Cytokine expression in neutrophils**

In order to produce investigate cytokine production after prolonged neutrophil stimulation, coated beads were embedded in 1% collagen gel (to prevent breakdown of chemokines by released neutrophil proteases), after which \(1\times10^6\) neutrophils were added. Neutrophil
supernatants were collected after indicated time points. Luminex fluorescent bead-based technology (Luminex, Austin, TX, USA) was used to measure and analyse cytokines in combination with the Bio-Plex system (Bio-Rad, Hercules, CA). Cytokine expression assays were performed according to the manufacturers’ guidelines.

**Animal experiments**
Mice were bred and maintained at the Central Animal Facility of the VU University (The Netherlands), under standard conditions. All experiments were performed according to institutional and national guidelines. The animal ethical committee of the VU University Medical Center approved all experiments.

**DSS induced colitis**
Male hIgA and hIgA/ FcαRI mice, aged between 12 and 14 weeks, were housed individually; *ad libitum* access of drinking water and food was provided. To achieve identical intestinal microbiota among the mice, bedding of all cages was mixed and evenly distributed. Mice received 1 % or 1.5 % DSS (45kD; TDB Consultancy AB, Uppsala, Sweden) in their drinking water for 5 days, followed by tap water until the end of the study. Fresh DSS solutions were prepared daily and mice were monitored every day to determine body weight and inspect general health conditions. Mice were sacrificed after 9 days.

**Cytokine expression in PCR**
Colon and small intestine tissue from mice was collected. RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) and chloroform and collected using isopropanol and ethanol. DSS was removed from RNA samples using the RNA cleanup protocol using the Mini RNeasy Kit (Qiagen, Venlo, the Netherlands), according to manufacturers guidelines. DNA was synthesized using MBI Fermentas Revertaid First strand cDNA synthesis Kit (Thermo Fischer Scientific, Waltham, MA), according to manufacturer’s guidelines, with a maximum input of 250ng RNA per reaction mix. Specific primers for cytokines and GapDH were used to determine cytokine expression in cDNA using 2x SybrGreen reaction mix (Invitrogen).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>GAC AAC TCA TCA AGA TTT TCA GCA</td>
<td>TTC ATG AGC CCT TCC ACA ATG</td>
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<tr>
<td>IFNγ</td>
<td>TAC TAC CTT CTT CAG CAA CAG C</td>
<td>AAT CAG CAG CGA CTC CTT TTC</td>
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<tr>
<td>CCL2 (MCP-1)</td>
<td>AGG CTG GAG AGC TAC AAC AGG A</td>
<td>TCT CAT TTG GTT CCG ATC CAG G</td>
</tr>
<tr>
<td>IL-1β</td>
<td>CTC GTG CTG TCG GAC CCA T</td>
<td>TGC CCG CTT TCA TTA CAC AGG A</td>
</tr>
<tr>
<td>TNFα</td>
<td>TGG AAC TGG CAG AAG AGG CAC T</td>
<td>CCA TAG AAC TGA TGA GAG GGA GGC</td>
</tr>
<tr>
<td>IL-6</td>
<td>GAG TTG TGC AAT GGC AAT TCT G</td>
<td>TGG TAG CAT CCA TCA TTT CTT TGT</td>
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</table>
Characterization of cell populations

hIgA and hIgA/FcαRI mice were sacrificed after 9 days. Blood was taken by cardiac puncture. Spleen, mesenteric lymph node (MLN), peripheral lymph nodes (PLN), small intestines (SI) and colons were removed. Spleen and MLN were dispersed through a metal strainer to generate single-cell suspensions. SI and colon were minced and enzymatically digested with Liberase™ (Roche, Penzberg, Germany) and DNase I (Roche) as described before, to create single cell suspensions. Single cell suspensions were stained (4°C, 30 minutes) with anti-mouse CD3, CD4, CD8 and B220 mAbs (eBioscience, San Diego, CA), washed (1300 RPM, 5 minutes) with 0.5% BSA in PBS and analyzed using flow cytometry (FACScalibur, BD biosciences, Franklin Lakes, NJ).

Statistical analysis

All analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Data are shown as mean ± standard deviation (SD). Statistical differences were determined using two-tailed unpaired Student’s t test (comparing 2 groups). Significance was accepted when p<0.05.

RESULTS AND DISCUSSION

Neutrophils are the first effector cells to arrive at any site of infection, clearing pathogens by means of phagocytosis, or degranulation of lytic enzymes. Furthermore, neutrophils have the ability to release neutrophil extracellular traps, which are web-like structures mainly consisting of DNA, elastase, histones and various other proteins. In this way extracellular micro-organisms are trapped, and dissemination is prevented. However, both antimicrobial factors and NETs are harmful to host cells. Excessive neutrophil activation can therefore lead to serious tissue damage, as is observed in UC. Furthermore, as neutrophils have the ability to secrete a plethora of cytokines and chemokines, they likely also play a role in the induction of adaptive immune responses. As was shown previously, DSS colitis in our mouse model is characterized by FcαRI-mediated neutrophil influx at inflamed sites of the intestines (Chapter 6). We

Figure 1. Using a Luminex multiplex cytokine assay, cytokine concentrations were measured in supernatants of neutrophils, which had been stimulated for 8 hours with HSA-, or IgA-coated beads that had been embedded in collagen gels.
previously demonstrated that in vitro FcαRI activation resulted in release of leukotriene B4 (LTB4), which is a potent neutrophil chemoattractant. We now investigated whether FcαRI-mediated neutrophil activation can contribute to induction of adaptive immune responses by measuring the release of chemokines and cytokines (Figure 1, and Table 1). Human neutrophils were added for 8 hours to collagen gels in which either HSA- or IgA-coated beads were embedded, after which supernatants were collected and presence of cytokines and chemokines was tested. IL-1β, IL-6, CXCL8 (IL-8), CCL2 (monocyte chemotactic protein 1, MCP-1), CCL3 (macrophage inflammatory protein 1α, MIP-1α), CCL4 (MIP-1β), and tumor necrosis factor (TNF)-α levels were increased in supernatants of neutrophils that had been added to IgA-bead containing collagen gels (Figure 1). A minimal increase was observed in granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) levels. After prolonged stimulation of neutrophils (24 and 48 hours) levels of IL-1β, IL-6 and CCL2 increased over time, whereas CXCL8, CCL3, CCL4 and TNFα concentrations decreased (Table 1). This suggests that the latter cytokines were mainly produced in early stages of neutrophil activation and have been degraded over time, explaining the lower observed concentrations. By contrast, IL1β, IL6 and CCL2 were likely produced continuously. Marginal production of G-CSF and GM-CSF was detected over time.

Next, we induced experimental colitis in mice using DSS in mice producing human IgA (hIgA) or co-expressing IgA and FcαRI (hIgA/FcαRI), as described before (Chapter 6). DSS was administered in the drinking water for 5 days, and mice were sacrificed at day 9, at which time point hIgA/FcαRI mice had lost more weight compared to hIgA mice, which is a strong indicator of more severe inflammation (Figure 2). Enhanced weight loss in hIgA/FcαRI mice was observed in experiments in which mice received 1%, and even more pronounced when 1.5% DSS was administered (Figure 2, right panel). hIgA/FcαRI mice furthermore, had an increased neutrophil influx in inflamed colon (Chapter 6), indicating

<table>
<thead>
<tr>
<th>Cytokine measured*</th>
<th>8h pg/ml (±SD)</th>
<th>24h pg/ml (±SD)</th>
<th>48h pg/ml (±SD)</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>9.57 (1.06)</td>
<td>10.62 (0.49)</td>
<td>14.76 (2.01)</td>
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<td>IL-6</td>
<td>77.15 (1.41)</td>
<td>106.25 (2.93)</td>
<td>145.42 (1.84)</td>
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<tr>
<td>CXCL8</td>
<td>3442.66 (861.12)</td>
<td>1024.16 (55.64)</td>
<td>2367.26 (59.15)</td>
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<td>G-CSF</td>
<td>4.50 (0.00)</td>
<td>4.27 (0.19)</td>
<td>5.02 (0.00)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>ND</td>
<td>ND</td>
<td>4.19 (1.51)</td>
</tr>
<tr>
<td>CCL2</td>
<td>15.18 (1.89)</td>
<td>18.17 (1.12)</td>
<td>35.87 (0.78)</td>
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<tr>
<td>CCL3</td>
<td>13.87 (0.43)</td>
<td>12.44 (0.65)</td>
<td>5.82 (0.41)</td>
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<tr>
<td>CCL4</td>
<td>235.58 (2.32)</td>
<td>188.89 (4.51)</td>
<td>62.64 (5.02)</td>
</tr>
<tr>
<td>TNFα</td>
<td>21.51 (3.65)</td>
<td>9.29 (0.00)</td>
<td>7.82 (0.69)</td>
</tr>
</tbody>
</table>

* Corrected for background, ND: Not detectable

Table 1. Cytokine expression by neutrophils determined after 8, 24 and 48 hours of stimulation with IgA-coated beads that had been embedded in collagen gels. Concentration is corrected for background by subtracting values obtained by stimulation with HSA-coated beads.

**Figure 2.** Body weight of hIgA versus hIgA/FcαRI mice in percentages at day 9, related to their initial weight at the start of the experiment. Data shown of two experiments. *P<0.05, **P<0.01
To investigate whether this process would lead to the induction of adaptive immune responses, mRNA expression of IFNγ, CCL2, IL-1β, TNFα and IL-6 was analyzed in colons and small intestines (Figure 3). No differences were observed in IFNγ or TNFα mRNA levels in hIgA mice versus hIgA/FcαRI mice. However, increased expression of CCL2, IL-1β and IL-6, was observed in both colons and small intestines of hIgA/FcαRI mice, compared to hIgA mice. CCL2 has been reported to originate from monocyte/macrophages. However,
Figure 4. Percentages of CD4^+^, CD8^+^ and B220^+^ cells were determined in colons, small intestines, spleens, blood, mesenteric lymph nodes (MLN) and peripheral lymph nodes (PLN) at day 9 after start of DSS treatment, using flow cytometry. *p<0.05.
FcαRI is not expressed on macrophages in tissues, although this can be upregulated during inflammation. When tissues were stained for FcαRI expression, only co-localization with the neutrophil marker GR-1 was observed, whereas F4/80+ cells (macrophage marker) did not express FcαRI (data not shown). Thus, increased expression of CCL2 in hIgA/FcαRI is likely due to production by neutrophils. It was previously demonstrated that supernatants from activated neutrophils recruited Th17 cells, which was reduced in the presence of anti-CCL2 antibodies. This indicated the occurrence of crosstalk between neutrophils and Th17 cells, which is further supported by enhanced production of IL-1β and IL-6 in hIgA/FcαRI mice, as these are cytokines involved in the generation of a Th17 response.

Thus, the presence of FcαRI and subsequent FcαRI-mediated neutrophil activation led to expression of Th17-associated chemokines and cytokines in hIgA/FcαRI mice, supporting the induction of adaptive immune responses. Therefore, differences in populations of cells of the adaptive immune system were studied next. Using flow cytometry, the percentage of CD4+, CD8+ and B220+ cells was determined in the colon, small intestine, spleen, mesenteric lymph nodes (MLN) and peripheral lymph nodes (PLN) (Figure 4). No differences in CD4+, CD8+ and B220+ cell populations were observed in blood, spleen, MLN or PLN of hIgA or hIgA/FcαRI mic+e (Figure 4). Furthermore, even though the percentage of CD3+ cells in either colon or small intestine was not different between both groups (data not shown), the percentage of CD4+ cells, was increased in hIgA/FcαRI mice compared to hIgA mice, whereas the CD8+ cell percentage was decreased. This supports the induction of CD4+ T cell responses, which has also been demonstrated in patients with UC, as both Th2 and Th17 cells have been reported to play an important role. Although we were not yet able to distinguish between different CD4+ T cell subsets, increased presence of CCL2, IL6 and IL-1β mRNA in small intestines and colons of hIgA/FcαRI mice is congruent with a Th17 response.

Additionally, we observed that the percentage of B220 positive B cells was increased in the small intestine of hIgA/FcαRI mice. Recently, it was demonstrated that B-cell Activating Factor (BAFF) and a proliferation-inducing ligand (APRIL), -which are cytokines essential for B cell development- can be produced by neutrophils. Thus, it is possible that neutrophil FcαRI may influence B cell development by secreting these factors (in addition to the abovementioned cytokines), although this still needs to be established. In this way, it is possible that a positive feedback loop is initiated between IgA-activated neutrophils and B cells, as local plasma cells, -derived from B cells-, are the primary source of IgA in the gut. In conclusion, we demonstrated that neutrophil stimulation via IgA and FcαRI induces release of chemokines and cytokines that are involved in the onset of both innate and adaptive immune responses. This may play an important role in ulcerative colitis, as increased expression of CCL2, IL1β and IL6 mRNA was observed in inflamed colons of hIgA/FcαRI mice, which was accompanied with enhanced percentages of CD4+ cells in the colon and small intestine. Additionally, a higher B cell percentage was observed in the small intestine, supporting that FcαRI on neutrophils is a potent inducer of adaptive immune responses during mucosal inflammation.
REFERENCES


FcαRI MODULATES ADAPTIVE IMMUNE RESPONSES IN COLITIS