CHAPTER 6

The human immunoglobulin A Fc receptor

FcαRI aggravates colitis

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ABSTRACT
Dimeric immunoglobulin A (dIgA) is produced by local plasma cells at mucosal sites. We previously identified a key role in mucosal defense as cross-linking of the IgA Fc receptor (FcαRI) by dIgA-antigen complexes led to neutrophil recruitment, which may eradicate impending infections when pathogens have crossed the epithelial barrier of the gut. Ulcerative colitis (UC) is a chronically remitting and relapsing disorder of the colon, characterized by large neutrophil infiltrates and severe inflammation. Because we recently demonstrated that neutrophils in colons of patients with UC phagocytosed IgA-complexes, we hypothesized that uptake of these complexes by FcαRI-expressing neutrophils contributes to the pathogenesis of UC. To investigate this notion, we generated new genetically-engineered mice, which produce human IgA and express human FcαRI on neutrophils (hIgA/FcαRI mice). Next, experimental colitis was induced with dextran sulphate sodium (DSS). We show that hIgA/FcαRI mice experienced significant weight loss and severe colitis compared to FcαRI-negative littermates. Importantly, as we recently demonstrated that blocking FcαRI abrogated IgA-induced neutrophil migration and tissue damage in ex vivo experiments, our results are the basis for development of novel therapies for patients with UC, which may significantly decrease morbidity and improve quality of life.
INTRODUCTION

IgA is the predominant antibody (Ab) class in mucosal areas, where it has a key role in mucosal defense.\textsuperscript{1-2} It is produced in the lamina propria by local plasma cells as dimeric molecules, which bind to the polymeric Ig receptor. It is then transported through epithelial cells and released into the lumen as secretory IgA (SIgA) that contains an additional molecule, which is referred to as secretory component (SC). SIgA serves as a mucosal protector, and represents a first line of defense against invading pathogens by forming an antiseptic coating of the mucosal wall.\textsuperscript{3-5} In addition, 1-3 mg/ml IgA is present in the circulation as monomeric molecules, and as such is the second prevalent Ab in serum.\textsuperscript{6,7} IgA is generally considered to act as a non-inflammatory antibody.\textsuperscript{8} SIgA is indeed a poor opsonin due to (partial) blockage of the binding site for the IgA Fc receptor Fc\alphaRI (CD89) by SC, precluding activation of myeloid immune cells.\textsuperscript{9} Additionally, it was demonstrated that monomeric serum IgA induces inhibitory signals.\textsuperscript{10,11} In contrast, cross-linking of Fc\alphaRI by IgA-immune complexes (containing either monomeric or dimeric IgA) initiates potent pro-inflammatory functions, including respiratory burst, release of cytokines, phagocytosis, and antigen presentation.\textsuperscript{12-15} We previously demonstrated that invading \textit{Escherichia coli} bacteria that are opsonized with serum IgA, are vigorously ingested by Fc\alphaRI-expressing phagocytes like neutrophils and Kupffer cells.\textsuperscript{16} Thus, cross-linking of Fc\alphaRI during infection with IgA-opsonized pathogens results in pro-inflammatory responses, whereas naturally occurring IgA (not complexed with an antigen) induces inhibitory signals through Fc\alphaRI to maintain immune system homeostasis.\textsuperscript{10,12} We furthermore recently identified a pro-inflammatory role for dIgA, as cross-linking of Fc\alphaRI by dIgA-antigen complexes led to neutrophil migration.\textsuperscript{14} Therefore, we proposed that after breach of the epithelial barrier invading pathogens are opsonized with dIgA, which will induce a self-contained positive feedback loop by recruitment of neutrophils until clearance of invading pathogens has been achieved. However, we additionally observed that neutrophils of patients with ulcerative colitis (UC) had taken up IgA complexes.\textsuperscript{14} As such, abnormal accumulation of IgA-antigen complexes in chronically diseased tissues may lead to continuous neutrophil activation and infiltration, resulting in serious tissue damage.

UC is a subtype of chronic inflammatory bowel disease with a largely unknown etiology that presents in susceptible hosts.\textsuperscript{17} Histological hallmarks of UC are invasion of crypt epithelium and lamina propria by neutrophils, disruption of the epithelial lining, and consequently, mucosal ulceration and crypt abscess formation in the bowel wall.\textsuperscript{17} In active UC, histological evidence of high-density neutrophil accumulation in the intestinal lumen correlates with epithelial injury and clinical disease activity.\textsuperscript{18} Since we demonstrated that dIgA complexes recruit neutrophils,\textsuperscript{14} we now investigated whether IgA-Fc\alphaRI interactions may contribute to the pathogenesis of this disease.
MATERIAL AND METHODS

Animals
We previously generated α1KI mice that produce human IgA, and FcαRI transgenic mice, in which receptor expression pattern, regulation, interaction with human IgA and FcαRI function mimics the human situation. Both mouse strains were of Balb/c background, and crossing of mice yielded two groups: hIgA mice, which only produce human IgA and hIgA/FcαRI mice, which produce human IgA and express FcαRI. Both mouse strains were of BALB/c background, therefore, wild type BALB/c mice and FcαRI Tg mice were used as additional controls. Mice were bred and maintained at the Central Animal Facility of the VU University, Amsterdam, The Netherlands under standard conditions with food and water ad libitum. All experiments were performed according to institutional and national guidelines. The animal ethical committee of the VU University Medical Center approved all experiments.

Flow cytometry and ELISA
BALB/c-wt (wt), BALB/c-FcαRI (FcαRI), BALB/c-hIgA (hIgA) and BALB/c-hIgA/FcαRI (hIgA/FcαRI) mice were sacrificed and blood was taken by cardiac puncture. Spleen, mesenteric lymph node (mLN), and colon were removed. Spleen and mLN were dispersed through a metal mesh to generate a single-cell suspension. Cells were stained (4°C, 30 minutes) with anti-mouse CD4, CD8, B220, GR-1 mAbs (eBioscience, San Diego, CA) and anti-human FcαRI mAb (BD, Franklin Lakes, NJ), washed (400 RCF, 5 minutes) with 0,5% BSA in PBS and analyzed by flow cytometry. Ig classes and subclasses were analyzed by ELISA with anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b, IgG3, IgE and anti-human IgA mAbs as described.

Histology and immunofluorescence
Colon, spleen and mLN cryosections (6 μm) were fixed in anhydrous acetone, air-dried and non-specific binding was blocked by incubation (15 minutes, RT) with 10% normal goat serum diluted in 0,5% BSA in PBS. Sections were stained with anti-mouse CD3 and anti-mouse B220 mAbs (eBiosciences) (1 hour, RT), and analyzed with a Leica DM6000 microscope (Leica Microsystems B.V., Rijswijk, The Netherlands). Colonic cryosections were stained with hematoxylin and eosin and analyzed with a Leica DM2500 microscope (Leica Microsystems B.V., Rijswijk, The Netherlands), or fluorescently labeled with anti-human FcαRI (BD), and anti-mouse GR-1 or F4/80 mAbs (eBiosciences) (1 hour, RT). The number of neutrophils was analyzed using analySIS Pro 3.2 (Soft Imaging System, GmbH, Munster, Germany).

DSS induced colitis
Male hIgA and hIgA/FcαRI mice between 12 and 14 weeks of age were housed individually with ad libitum access to drinking water and food. After DSS removal, animals were provided with HydroGel (ClearH₂O, Portland, ME) and in water soaked food pellets. To standardize intestinal microbiota colonization of mice, bedding of all cages was mixed and evenly distributed two weeks in advance. Mice received 2% or 1.5% DSS (45kD; TDB.
Consultancy AB, Uppsala, Sweden) in their drinking water for 5 days, which was prepared fresh daily. Mice were recorded daily for body weight and general health condition. Human endpoints were defined at > 20% of body weight loss and/or signs of severe discomfort (e.g. bloody diarrhea, hunched posture, immobilized behaviour). Body weight changes were calculated by dividing body weight on indicated days by body weight at day 0, and expressed in percentage. After sacrifice, small intestines and colons were removed, length was measured and photographs were taken (Lumix 10x optical zoom, Panasonic Benelux, 's-Hertogenbosch, The Netherlands). Samples of colons were taken and snap frozen in liquid nitrogen.

Statistical analysis
All analyses were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA). Data are shown as mean ± standard error of the mean (SEM). Statistical differences were determined using Mann Whitney test (comparing 2 groups), or ANOVA (comparing > 2 groups). Significance was accepted when P< 0.05.

RESULTS AND DISCUSSION
In vivo studies investigating the role of IgA and FcαRI have been restricted, since mice do not express an FcαRI homologue, and the murine IgA system differs from the human situation. To overcome this limitation, we generated a novel model by crossing mice that produce human IgA (hIgA) with transgenic mice expressing human FcαRI on neutrophils. This resulted in the generation of two groups of mice. In one group mice only produced hIgA, which were used as controls. In the second group mice produced both hIgA and expressed FcαRI (hIgA/FcαRI). First, we analyzed the composition of immune cells of this new mouse model. As additional controls BALB/c wild-type (neither producing hIgA nor expressing FcαRI) and FcαRI transgenic mice (not producing hIgA) were included. No differences were observed between percentages of CD4+ T cells, CD8+ T cells and GR-1+ (Ly6C/Ly6G) cells in the blood of all groups (Figure 1A, C). However, several B cell intrinsic and previously documented features of the hIgA mouse model were observed in both hIgA and hIgA/FcαRI mice in agreement with earlier observations. B cell compartments of both hIgA and hIgA/FcαRI mice were smaller in blood, spleen, mLN and colons compared to wild-type and FcαRI mice (Figure 1A, B). Furthermore, no IgM was produced and IgG levels were lower (Figure S1), but no differences were observed between hIgA and hIgA/FcαRI mice. Interestingly, FcαRI expression was lower on neutrophils in hIgA/FcαRI mice compared to FcαRI transgenic mice (not producing hIgA), supporting that the presence of hIgA modulates FcαRI expression (Figure 1D). This is in accordance with several patient studies in which it was suggested that increased presence of plasma IgA down-regulated FcαRI expression. The same concentration of hIgA was present in both hIgA and hIgA/FcαRI mice in blood, independent of FcαRI expression (Figure 1E). Furthermore, hIgA was detected in intestinal fluid, supporting that mouse pIgR transported hIgA over the epithelial barrier (Figure 1F). Thus, neither the number of immune cells nor antibody titers differed between hIgA and hIgA/FcαRI mice. As such, the only difference between both mouse groups was the presence of
Figure 1. Characterization of hIgA and hIgA/FcRI mice. (A) CD4⁺ T cells, CD8⁺ T cells and B220⁺ cells of blood, spleen and mesenteric lymphnode (mLN) of BALB/c wild-type (wt), FcRI transgenic, hIgA and hIgA/FcRI mice were analyzed by flow cytometry (% gated; percentages of total blood, spleen or mLN cells). (B) Representative images of immunostaining of CD3⁺ T cells (green fluorescence) and B220⁺ cells (red fluorescence) of colonic patches, spleen or mLN. Scale bar, 250 µm. (C, D) Percentages of GR-1⁺ cells (C) and expression of FcRI on neutrophils (D) in blood were analyzed by flow cytometry. (E, F) The concentration of serum hIgA (E) and intestinal fluid hIgA (F) was analyzed by ELISA. *** P < 0.0001 by one-way ANOVA (A), or Mann Whitney test (D).

Figure 2. FcRI aggravates colitis. (A, B) Individual daily changes in body weight (A) and survival curves (B) when hIgA mice (left panel) and hIgA/FcRI mice (right panel) were given 2% DSS for 5 days (†; sacrificed mice). (C, D) Individual daily changes (C) and mean daily changes (D) in body weight of hIgA and hIgA/FcRI mice when given 1.5% DSS. The relative percentage body weight at day 9 is shown in right panel in d. * P< 0.05, by Mann Whitney test, data are means ± SEM. The experiment was repeated three times yielding similar results.
FcαRI, which rendered it an excellent model to determine the contribution of FcαRI in intestinal inflammation.

To investigate whether FcαRI may play a role in the pathogenesis of UC, we induced experimental colitis by administering DSS in the drinking water of hIgA and hIgA/FcαRI mice for 5 days. When mice were given 2% DSS, both groups started to lose weight after day 4. However, after cessation of DSS 4 out of 5 hIgA mice recovered and gained body weight (Figure 2A, left panel). By contrast, 80% of the hIgA/FcαRI mice continued to lose body weight and had to be sacrificed (Figure 2A, right panel and Figure 2B), strongly supporting that FcαRI aggravated colitis. To prevent excessive loss of body weight, allowing the study of immunological processes, mice received 1.5% DSS in the next experiments. Although hIgA mice displayed a slight weight loss (Figure 2C, left panel and Fig 2D), hIgA/FcαRI mice significantly lost more body weight, indicating that the presence of IgA and FcαRI contributed to a more severe disease (Figure 2C, right panel and Figure 2D).

After 9 days, mice were sacrificed for macroscopic and microscopic analyses of the intestinal tracts. The small intestines of hIgA/FcαRI mice were significantly shortened compared to hIgA mice (Figure 3A, B and Figure S2A, B for healthy controls), which indicated that DSS administration also induced enteritis in hIgA/FcαRI mice, in addition to colitis. Shortening of the intestines is a characteristic of experimental colitis as inflammation induces fibrosis and a subsequent decrease in length.23 In hIgA/FcαRI mice length of small intestines furthermore correlated with weight loss (Figure 3C, right panel) compared to hIgA mice (Figure 3C, left panel), supporting that weight loss was representative of the severity of inflammation. Colon lengths of hIgA/FcαRI mice were also slightly decreased compared to hIgA mice, albeit not significantly different (Figure 3D and Figure S2D for healthy controls). However, whereas no apparent changes were observed in macroscopic or microscopic appearance of colons of hIgA mice, colons of hIgA/FcαRI mice were swollen and severely inflamed (Figure 3E, F and Figure S2C, E for healthy controls). Additionally, in contrast to healthy control colons (Figure S2C) and colons of hIgA mice (Figure 3E, left panel), solid fecal pellets could not be detected in colons of hIgA/FcαRI mice (Figure 3E, right panel).

Cryosections of the colon of hIgA/FcαRI mice showed extensive tissue damage of mucosa and submucosa and large immune cell infiltrates (Figure 3F and Figure S2E for healthy controls). Moreover, the number of GR-1+ cells in the colon of hIgA/FcαRI mice was significantly increased (Figure 3G). GR-1 is mostly known as a marker for mature neutrophils, although low to intermediate expression is present on myeloid-derived suppressor cells and subpopulations of monocytes and macrophages.24 As these latter populations also express F4/80 (macrophage marker), double stainings with GR-1 were performed to distinguish between cell populations. No co-localization of F4/80 with GR-1 or FcαRI was observed (Figure 3H, left and middle panel), supporting that the GR-1+ immune cell infiltrate consisted of neutrophils (Figure 3H, left panel). We previously demonstrated that cross-linking of neutrophil FcαRI by IgA-immune complexes induces release of leukotriene B4 (LTB4), which is a strong chemoattractant of neutrophils.14 FcαRI+ cells in inflamed colons of hIgA/FcαRI were also positive for GR-1 staining (Figure
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3H, right panel), strongly supporting that IgA-FcgRI interactions induces neutrophil migration.

Excessive or unchecked neutrophil recruitment leads to tissue damage, mainly due to the persistent release of harmful inflammatory cytokines, reactive oxygen species and proteases by the infiltrated cells. An association between colonic neutrophilia and progression of acute colitis was observed in humans, in addition to FcgRI

+ -neutrophil accumulation in rectal biopsies of patients with active UC relative to healthy controls, or patients in remission. Furthermore, we recently demonstrated that FcgRI contributed to tissue damage in Linear IgA Bullous Disease. This blistering disorder is characterized

Figure 3 (A-E). hIgA/FcgRI mice show more severe inflammation in DSS induced colitis. (A, B) Representative images (A) and length (B) of small intestines of hIgA and hIgA/FcgRI mice at day 9 when 1.5% DSS was administered in the drinking water. (C) Correlation between length of small intestines and body weight of hIgA mice (left panel) and hIgA/FcgRI mice (right panel). (D, E) Length (D) and representative images (E) of colons (3 mice) at day 9. Arrows; fecal pellets, arrow heads; swollen tissue.
Figure 3(F-H). hIgA/FcαRI mice show more severe inflammation in DSS induced colitis. (F) Representative histological images (H&E staining) of distal colons of hIgA (left panels) and hIgA/FcαRI (right panels) mice (3 mice). Scale bar, 1mm; higher magnification scale bar, 100µm. (G) The number of GR-1+ cells in cryosections of the distal colon of hIgA and hIgA/FcαRI mice. FOV; field of view. (H) Representative immuno-histological image of the distal colon of hIgA/FcαRI mice at day 9, stained for F4/80+ (macrophage marker; blue fluorescence), GR-1+ cells (green fluorescence) and FcαRI+ cells (red fluorescence), after 1.5% DSS had been administered in the drinking water. Scale bar; 100 µm.

* P< 0.05, ** P < 0.01 by Mann Whitney test.
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by large FcαRI⁺-neutrophil infiltrates and IgA autoantibodies directed against collagen XVII of the basement membrane of the skin. Importantly, blocking FcαRI-IgA interactions abrogated neutrophil migration and tissue damage ex vivo. In conclusion, FcαRI exacerbated experimental colitis in mice that express human IgA by recruiting neutrophils, which resulted in significant tissue damage. Novel therapeutic strategies in which FcαRI is blocked may therefore dampen the uncontrolled inflammatory processes in patients with UC and improve the quality of life.
REFERENCES


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**SUPPLEMENTARY INFORMATION**

**Figure S1. Concentration of serum Ig’s.** Serum murine IgM, IgG1, IgG2a, IgG2b, IgG3 and IgE of BALB/c wild-type (wt), FcaRI transgenic, hIgA and hIgA/FcaRI mice was analyzed by ELISA (n = 10 per group). ND; not detectable.

**Figure S2. Healthy hIgA and hIgA/FcaRI mice.** (A, B) Representative images (A) and length (B) of small intestines of healthy hIgA and hIgA/FcaRI control mice. (C, D) Representative macroscopic images (C) and length (D) of colons of healthy hIgA and hIgA/FcaRI control mice (E) Microscopic (H&E staining) images of colons of healthy hIgA and hIgA/FcaRI control mice. Scale bar; 1 mm, higher magnification scale bar; 100 µm.