Chapter 5

Blocking FcαRI as a therapeutic target for resolving IgA autoantibody-induced inflammation and tissue damage

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Abstract

Immunoglobulin A (IgA) is generally considered a non-inflammatory regulator of mucosal immunity. Conversely, we previously demonstrated that IgA is a potent proinflammatory stimulus for neutrophils after crosslinking of the Fc receptor FcαRI \textit{in vitro}. The role of FcαRI in IgA-mediated diseases is, however, poorly understood, mostly due to the lack of suitable mouse models. We now established a novel linear IgA bullous disease (LABD) mouse model using genetically modified mice that produce human IgA and express human FcαRI. Intravital microscopy demonstrated that injection of anti-collagen XVII IgA, which are also present in patients, resulted in neutrophil activation and extravasation from the blood vessels into skin tissue. Moreover, continued exposure to anti-collagen XVII IgA led to massive neutrophil accumulation, severe tissue damage and blister formation. Importantly, treatment with anti-FcαRI monoclonal antibodies not only prevented thickening of ear tissue as measure of inflammation, but was also able to resolve existing inflammation and tissue damage. Collectively, our data reveal a novel role of neutrophil FcαRI in IgA autoantibody-mediated disease and identify FcαRI as promising new therapeutic target to resolve chronic inflammation and tissue damage due to unrestrained neutrophil activation in patients.
Introduction

Immunoglobulin A (IgA) is the dominant antibody subclass present in mucosal areas and plays an important role in the mucosal immune system. A delicate balance between tolerating harmless antigens and commensals versus maintaining robust protection against pathogens is necessary to maintain homeostasis. IgA is in general considered as a non-inflammatory antibody. This holds true for secretory IgA that mainly serves as an antiseptic coating at the mucosa by, amongst others, neutralizing bacterial toxins as well as preventing adherence and invasion of microorganisms. Additionally, binding of monomeric serum IgA to FcαRI induces inhibitory signals, which plays an anti-inflammatory role under physiological conditions. However, crosslinking of the IgA Fc receptor FcαRI by either dimeric or serum IgA immune complexes effectively activates FcαRI-expressing immune cells, resulting in proinflammatory responses in vitro.

Especially neutrophils, which express FcαRI, are potently activated by IgA complexes. Neutrophils are the most abundant circulating leukocytes in humans, and play a fundamental role in innate immune responses. Crosslinking of FcαRI on neutrophils by IgA immune complexes induces phagocytosis, the production of reactive oxygen species (ROS), and release of cytokines as well as neutrophil extracellular traps (NETs). Furthermore, FcαRI crosslinking initiates the release of the potent neutrophil chemoattractant leukotriene B4 (LTB4) and concomitant neutrophil migration in vitro.

IgA autoantibodies are found in several chronic inflammatory or autoimmune diseases. Their contribution in inflammation is however poorly understood as evidence for a pathogenic role of neutrophil FcαRI/IgA interactions is limited. This is mostly due to the absence of suitable mouse models, as mice lack expression of a FcαRI homologue, which severely hampered investigating the in vivo role of FcαRI in IgA-mediated disease. A role for FcαRI in IgA nephropathy (IgAN) was supported in a transgenic mouse model, in which human FcαRI was expressed on monocytes/macrophages under the CD11b promotor. These mice developed disease with macrophage infiltration in damaged glomeruli, due to deposits of soluble FcαRI/IgA complexes. In accordance, soluble FcαRI complexed with IgA was also found in the serum and deposits in the kidney of IgAN patients. IgA autoantibodies may play a pathogenic role in celiac disease. Patients with celiac disease develop anti-tissue transglutaminase (tTG) IgA autoantibodies in response to gluten exposure. The gastrointestinal tract of patients with celiac disease is mostly infiltrated by mononuclear cells. The skin manifestation of celiac disease, referred to as dermatitis herpetiformis, is characterized by the presence of IgA autoantibodies against tTG that are cross-reactive with epidermal TG, resulting in granular IgA deposits at the dermal-epidermal junction. Interestingly, the immune cell infiltrate in dermatitis herpetiformis consists of neutrophils, suggesting that these may play a role in the pathogenesis of this disease.

Accumulation of neutrophils is also observed in ulcerative colitis, which is a variant of chronic inflammatory bowel disease. We previously demonstrated massive accumulation of FcαRI-expressing neutrophils and intracellular presence of IgA in neutrophils in colons of patients with ulcerative colitis. Additionally, we recently showed that IgA immune complexes present in plasma and synovial fluid of rheumatoid arthritis patients activated...
neutrophils and induced the release of NETs via FcαRI in vitro \(^\text{15}\). Similarly, serum of patients with the autoimmune skin disorder Linear IgA Bullous Disease (LABD), containing IgA autoantibodies against collagen XVII, induced neutrophil-mediated tissue damage in vitro \(^\text{16,17}\). These observations suggest that neutrophils play a detrimental role in autoimmune diseases in which IgA autoantibodies are present. To investigate the role of neutrophil FcαRI in IgA-mediated disease in vivo, we developed a novel LABD mouse model, which closely resembles the human disease. Our results provide the first evidence for a critical role of neutrophil FcαRI and IgA autoantibody interactions, resulting in massive accumulation of neutrophils and severe tissue damage in vivo. Importantly, we furthermore demonstrated that blocking FcαRI significantly reduced existing disease, emphasizing the therapeutic potential of anti-FcαRI monoclonal antibodies in IgA-mediated inflammation.

**Materials and Methods**

**Mice**

Transgenic mice that express human FcαRI on neutrophils \(^\text{18}\) were backcrossed onto BALB/c background. Mice were further crossed with either mice expressing enhanced green fluorescent protein (LysEGFP) on neutrophils \(^\text{19}\) for intravital imaging experiments, or with mice that were hIgA knock-in \(^\text{20}\). Obtained LysEGFP, FcαRI/LysEGFP, hIgA, and FcαRI/hIgA BALB/c mice were used bred and housed at the Central Animal Facility of the VU University (Amsterdam, 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.

**Generation of hybridoma cell line producing anti-mCOL17 hIgA antibodies**

Three recombinant GST fusion fragments of the extracellular domain (GST-mCXVII-EC1, GST-mCXVII-EC3, GST-mCXVII-EC7) and one of the intracellular domain (GST-mCXVII-IC2) of mCOL17 were cloned and purified as previously published \(^\text{21}\). hIgA knock-in mice \(^\text{20}\) were immunized subcutaneously with 40 \(\mu\)g of a mixture of these four different constructs of mCOL17 in Complete Freund’s Adjuvant (Sigma-Aldrich, St. Louis, MO), followed by four booster injections of 40 \(\mu\)g of the mixture of recombinant mCOL17 in Incomplete Freund’s Adjuvant (Sigma-Aldrich). Mice were sacrificed at day 83 and mouse spleens were harvested. SP2/0 mouse myeloma cells were fused with splenocytes from immunized mice according to standard protocols \(^\text{22}\). Positive hybridoma clones that produced anti-mCOL17 hIgA were selected by a mCOL17 hIgA ELISA (see below). To obtain monoclonal cell lines, cells from positive wells were subcloned by limiting dilution. Subcloning of monoclonal lines was repeated twice to make sure generated hybridoma lines were derived from single clones. Hybridomas were frozen in liquid nitrogen till further use. Anti-mCOL17 hIgA mAb were isolated using a *Staphylococcus aureus* superantigen-like protein 7 agarose (SSL7/Agarose) affinity column according to the manufacturers’ instructions (InvivoGen, San Diego, CA).
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SDS-PAGE and Western Blot
Anti-mCOL17 hIgA and controls were prepared in reducing and non-reducing Laemmli sample buffer and analyzed by SDS-PAGE for detection of human IgA. Gels were stained with Silver Staining according to the manufacturers’ instructions (Thermo Scientific, Waltham, Massachusetts, USA). Polyacrylamide gels were transferred on polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore corporation, Bedford, MA), and blocked with 5% BSA in Tris (hydroxymethyl)aminomethane (TBS) for 1 hour at room temperature (RT). Membranes were probed with mouse anti-human IgA mAb (BD Pharmingen™, Erembodegem, Belgium) for 1 hour at RT followed by incubation for 45 minutes with a secondary anti-mouse 800 Odyssey labelled antibody (Li-Cor Biosciences, Lincoln, NE). After extensive washing in TBST (TBS, 0.05% Tween-20), bound antibody was detected with an Odyssey Infrared Imaging System (Li-Cor Biosciences).

Isolation of human neutrophils from healthy controls
Polymorphonuclear cells (PMNs) were isolated from human peripheral blood that was obtained from healthy donors using Lymphoprep (Axis-Shield, Oslo, Norway) density gradient centrifugation, after which erythrocytes were lysed in ammonium chloride buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.11 mM EDTA, 10 minutes, RT). After lysis, PMNs were washed with phosphate-buffered saline (PBS; B.Braun, Melsungen, Germany). Cells were resuspended in RPMI 1640 (Gibco BRL, Paisley, UK) that was supplemented with 10% FCS, glutamine and antibiotics. Neutrophils were labeled with calcein-acetoxymethylster (1 μmol/L; Molecular Probes Inc, Eugene, OR) for binding assays according to the manufacturer’s instructions. Blood samples were collected after donors gave informed consent, in accordance with the guidelines of the Medical Ethical Committee of the VU University Medical Center (The Netherlands).

Ligand binding assay
Flat well microtitre ELISA plates (Nunc-Immuno MaxiSorp, Roskilde, Denmark) were coated with 100 μl anti-mCOL17 hIgA (10 μg/ml) or bovine serum albumin (10 μg/ml; BSA, negative control). After washing, wells were incubated with calcein labeled neutrophils (2.5 x 10⁵ cells/well) for 20 minutes at 37 °C. Subsequently, supernatant was harvested and used for lactoferrin (degranulation marker for neutrophils) ELISA to investigate activation of neutrophils (see below). Plates were then washed and bound cells were lysed and fluorescence of supernatant (reflecting number of calcein-labeled neutrophils) was measured using a fluorimeter. A standard curve generated from 0-600.000 of calcein-labeled neutrophils was used to quantify neutrophil binding (485 nm excitation/520 nm emission filters; Fluostar Galaxy, BMG Labtechnologies, Offenburg, Germany).

ELISA
Mouse collagen type XVII ELISA
Flat well microtitre ELISA plates (Nunc-Immuno MaxiSorp) were coated with mouse collagen XVII EC7 construct (500 ng/well), followed by a blocking step for non-specific
binding sites with PBS with Tween-20 (PBST, 200 µl) containing 0.5% BSA for 1 hour at 37 °C. Coated plates were incubated with diluted sera from mice or purified anti-mCOL17 hIgA (1 hour, 37 °C). Plates were washed with PBST, followed by incubation with biotin-labelled mouse anti-human IgA mAbs (1 hour, 37 °C, BD Biosciences, FranklinLakes, NJ). Plates were washed and further incubated with streptavidin horseradish peroxidase (HRP) (30 minutes, RT). As substrate (3,3', 5,5')-tetramethylbenzidine (TMB) was used. Plates were read with a microplate reader (Bio-Rad) at 450 nm.

**Lactoferrin ELISA**
Lactoferrin was measured in the supernatants of ligand binding assays. Flat well microtitre ELISA plates (Nunc-Immuno MaxiSorp) were coated with 100 µl rabbit anti-human lactoferrin mAb (50 µg/ml, Sigma) followed by a blocking step for non-specific binding sites with PBST containing 0.5% BSA for 1 hour at 37 °C. Plates were then incubated with 2x diluted supernatant for 1 hour at 37 °C, followed by incubation with alkaline phosphatase–labeled rabbit anti-human lactoferrin antibodies (1:2500; MP Biomedicals, LLC, Solon, OH) for 1 hour at 37 °C. Plates were washed between incubation steps with PBST. After addition of the chromogenic substrate P-nitrophenyl phosphate (Sigma), plates were read with a microplate reader (Bio-Rad) at 405 nm. Purified human lactoferrin (Sigma) was used as a standard to calculate the amount of lactoferrin in the measured samples.

**Amplex Red assay**
Wells of a 96-wells plate (Nunc-Immuno MaxiSorp) were coated with anti-mCOL17 hIgA or BSA (negative control). Neutrophils were incubated with 100 µl Hepes+ buffer (132 mM NaCl, 20 mM hepes, 6 mM KCl, 1 mM MgSO$_4$·7H$_2$O, 1.2 mM K$_2$HPO$_4$·3H$_2$O, 1 mM CaCl$_2$, 0.5% BSA, 1 mg/ml glucose) supplemented with 50 µl Amplex red reaction mix (200 µM Amplex red reagent (Molecular Probes Inc) and 4 U/ml horse radish peroxidase in 1× Hepes+ buffer). Fluorescence of the produced resorufin (a red fluorescent compound) was measured every minute for 1 hour at 37°C in a fluorimeter (Galaxy Fluorstar) with an excitation of 550 nm and an emission of 590 nm. A standard curve of H$_2$O$_2$ in Hepes+ buffer was used as standard measure.

**Animal experiments**
LysEGFP versus FcαRI/LysEGFP or hIgA versus FcαRI/hIgA BALB/c mice were used for experiments. All ear injections were performed after mice were narcotized by administration of a mixture of Ketamine/Xylazine anesthesia.

**Intravital imaging**
Intravital imaging of blood vessels was used to examine the potential of anti-mCOL17 hIgA to activate neutrophils *in vivo*. Mouse ears are optimal imaging sites as they are thin and relatively transparent allowing for non-invasive imaging imaging of the blood circulation to analyze neutrophil activation and migration. For intravital imaging, anti-mCOL17 hIgA mAb (35 µg; right ear) or PBS (left ear) was injected intracutaneously in
ears of LysEGFP or FcαRI/LysEGFP transgenic mice. After 48 hours, mice were narcotized by administration of a mixture of Hypnorm/Dormicum and intravital imaging recordings were performed with a ZEISS Axiovert 200 Marianas inverted microscope (Marianas, I.I.I., Denver, CO). A cooled EM-CCD camera (Photometrics, Tucson, AZ; 512 x 512 pixel) recorded images with 16-bit capability. Imaging was performed with a 10X air lens (ZEISS) and standard GFP filter set. In total 2400 frames were taken with an interval of 0.5 second (20 minutes). After the recordings, injected ears were harvested and snap frozen for immunofluorescence staining.

**In vivo inflammation experiments**

For *in vivo* inflammation experiments, FcαRI/hIgA or control hIgA mice received anti-mCOL17 hIgA (70 µg) subcutaneously every other day for 1 week (4 injections in total) or for 14 days (7 injections in total) (See Supplementary Figure 2 for injection scheme). Additionally, in some experiments mice were injected intraperitoneally with 100 µg anti-FcαRI mAb (MIP8a; Serotec, Uden, the Netherlands) on day 0 or day 3 (prevention model) with 150 µg on day 7 or day 11 (treatment model). Control mice received an isotype control (Ultra-LEAF™ Purified Mouse IgG1, κ Isotype Ctrl Antibody, Biolegend) at the same days. Mice were monitored daily for discomfort and at the end of the experiments, ears were harvested and snap frozen to use for immunofluorescence staining.

**Immunofluorescence**

Mouse ear cryosections (6-8 µm) of FcαRI/hIgA transgenic mice were fixed in acetone and air-dried, after which they were incubated with purified anti-mCOL17 hIgA (1 hour, RT), followed by washing and incubation with anti-hIgA FITC mAb (DAKO, Heverlee, Belgium). Alternatively, ear cryosections of LysEGFP, FcαRI/LysEGFP, hIgA and FcαRI/hIgA transgenic mice were stained with anti-mouse GR-1 FITC mAb (eBioscience, San Diego, CA; 1 hour, RT) or with anti-hIgA FITC mAb (DAKO; 1 hour, RT). Diamidino-2-phenylindole (DAPI) was used for nuclear staining. Cryosections were analyzed with a Leica DM6000 microscope (Leica Microsystems B.V.). Tile scanning scan was performed to obtain a composite image of the whole ear. Additional analyses were performed using ImageJ software measuring ear thickness and RawIntDen/Area of GR-1 staining.

**Quantification images**

GR-1 staining of cryosections was analyzed using ImageJ. Tile scans of ears were used for analysis. Region of interest was determined based on DAPI staining. Raw integral density (RawIntDen, the sum of pixels in image) was measured for GR-1 staining. Quantification was calculated as the RawIntDen of GR-1 staining divided by area of DAPI staining.

**Statistical analysis**

Data analysis was performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA). Data are expressed as mean ± SD. Statistical differences were determined using two-tailed unpaired Student’s t tests (comparing 2 groups) or ANOVA (> 2 groups). Differences were considered statistically significant if p < 0.05.
Results and Discussion

Generation of specific and functional human IgA anti-mouse collagen XVII antibodies (anti-mCOL17 hIgA)

Because mice do not express FcαRI, we made use of a human FcαRI transgenic mouse model, which was generated under its own promotor, resulting in expression on neutrophils \(18\). However, murine IgA binds poorly to human FcαRI. We therefore immunized hIgA knock-in mice \(20\) with mouse collagen XVII proteins \(21\), which yielded hybridomas producing anti-mCOL17 hIgA (Supplementary Figure 1A-C). Neutrophils bound firmly to anti-mCOL17 hIgA antibodies, resulting in lactoferrin release (as measure of degranulation), and ROS production (Figure 1A-C), indicating that anti-mCOL17 hIgA antibodies were functional. Additionally, cryosections of mouse ears were incubated with anti-mCOL17 hIgA, leading to binding to keratinocytes and hair follicles (Figure 1D), demonstrating that antibodies recognized mouse collagen XVII in situ. Thus, obtained anti-mCOL17 hIgA antibodies were specific for mouse collagen XVII and able to activate FcαRI-expressing neutrophils in vitro.

Anti-mCOL17 hIgA induces neutrophil migration in mice expressing human FcαRI

To study IgA-induced inflammation in vivo, we examined neutrophil migration 48 hours after injection of anti-mCOL17 hIgA antibodies using intravital imaging of blood vessels in ears of LysEGFP mice \(19\) crossed with human FcαRI transgenic mice \(18\), referred to as FcαRI/LysEGFP mice. Minimal neutrophil activation and recruitment was observed in ears of LysEGFP mice that had been injected with either PBS or anti-mCOL17 IgA (Figure 2A-B...
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and Video 1). Similarly, minimal neutrophil activation was observed in ears of FcαRI/LysEGFP mice that had been injected with PBS (Figure 2A-B and Video 2). By contrast, injection of anti-mCOL17 hIgA antibodies in ears of FcαRI/LysEGFP mice resulted in neutrophil accumulation at the blood vessel wall, as well as migration and extravasation into ear tissue (Figure 2A-B and Video 2).

Importantly, IgA-induced neutrophil activation was completely prevented in FcαRI/LysEGFP mice that received an FcαRI blocking antibody (MIP8a) (Figure 2A-B and Video 2). Neutrophil influx in ears of LysEGFP mice 48 hours after anti-mCOL17 hIgA injection was minor (Figure 2C). However, massive neutrophil accumulation was observed in ears of FcαRI/LysEGFP mice that had been injected with anti-mCOL17 hIgA (Figure 2D), which was prevented when mice had additionally been treated with MIP8a (Figure 2E).

![Figure 2](image-url)

**Figure 2. Anti-mCOL17 hIgA antibodies induce FcαRI-dependent neutrophil recruitment.**

(A) Still frames from intravital imaging movies, 48 hours after injection of PBS (first panel) or anti-mCOL17 hIgA (second panel) in ears of LysEGFP mice. Ears of FcαRI/LysEGFP mice were injected with PBS (third panel), anti-mCOL17 hIgA (fourth panel) or anti-mCOL17 hIgA in combination with systemic treatment with an FcαRI blocking antibody MIP8a (fifth panel). Red arrowhead indicates neutrophil extravasation (see also Videos 1&2). (B) Quantification of neutrophil numbers in contact with blood vessels in intravital imaging movies. n = 3 per group. (C-E) Cryosections of ears of LysEGFP mice injected with anti-mCOL17 hIgA (upper panels), FcαRI/LysEGFP mice injected with anti-mCOL17 hIgA without (middle panels) or with the treatment anti-FcαRI mAb MIP8a (lower panels). Three examples per group are shown. Cryosections were stained with the neutrophil marker GR-1 (green) and DAPI (DNA; blue). Data are presented as mean ± SD. * p < 0.05.
Induction of tissue damage by aberrant accumulation of neutrophils initiated by IgA autoantibody binding to FcαRI

A single injection of anti-mCOL17 hIgA did not result in visible tissue damage (data not shown), and long-term exposure to IgA-antigen complexes is required to induce chronic inflammation. However, human IgA has a short half-life in mice. To potentially increase half-life of injected human IgA, we first crossed FcαRI transgenic mice with hIgA knock-in mice, and we investigated the presence of anti-mCOL17 hIgA in tissue over time. Antibodies were observed up to 48 hours after injection, after which IgA presence rapidly declined, and was absent after 72 and 96 hours (Figure 3A). Therefore, to induce chronic inflammation, ears of FcαRI/hIgA or control littermates hIgA mice were injected with anti-mCOL17 hIgA or PBS for 4 times every other day (for injection scheme, see Supplementary Figure 2). Mice were sacrificed at day 7, after which IgA-induced neutrophil migration and tissue damage was investigated.

Low neutrophil numbers were observed in ears of hIgA or FcαRI/IgA mice that had been injected with PBS (Figure 3B-C, left panels). Frequent injection of anti-mCOL17 hIgA in ears of hIgA mice induced some neutrophil influx, but massive recruitment and accumulation of neutrophils was observed in ears of FcαRI/hIgA mice (Figure 3B-D). Importantly, in addition to augmented accumulation of neutrophils, severe tissue damage and blisters were observed in ears of FcαRI/hIgA mice in response to multiple injections of anti-mCOL17 hIgA (Figure 3C). Additionally, ear thickness was measured to assess the degree of inflammation. Ears of FcαRI/hIgA mice that had been injected with anti-mCOL17 hIgA showed increased thickness ~ 800 µm when compared to ears of hIgA mice ~ 400 µm (Figure 3E-F). Thus, continuous presence of anti-mCOL17 hIgA in ears of FcαRI/hIgA transgenic mice induced chronic inflammation and tissue damage.

FcαRI blocking antibodies prevent detrimental IgA autoantibody-induced neutrophil accumulation

To investigate whether blocking FcαRI prevents accumulation of neutrophils and subsequent induction of chronic inflammation, mice were injected with anti-mCOL17 hIgA or PBS every two days in the ears together with systemic injection of the FcαRI blocking antibody MIP8a or an isotype control (Figure 4). Some neutrophil influx was found in ears of hIgA mice that had been injected with anti-mCOL17 hIgA, but no difference in neutrophil recruitment was observed between mice that had been treated with MIP8a or an isotype control (Figure 4A-C). In contrast, massive neutrophil accumulation was observed in ears of FcαRI/hIgA mice that had been injected with anti-mCOL17 hIgA and an isotype control antibody (Figure 4D+F). Importantly, injection with the FcαRI blocking antibody MIP8a prevented neutrophil recruitment and accumulation (Figure 4E-F). Similarly, systemic injections of MIP8a prevented ear thickening in FcαRI/hIgA mice, but not in hIgA mice (Figure 4G-J).
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Figure 3. hIgA anti-mCOL17-induced neutrophil migration and chronic inflammation in FcαRI/hIgA mice. (A) Cryosections of ears of FcαRI/hIgA mice were stained with anti-human IgA (red) and DAPI (DNA; blue) after injection of anti-mCOL17 hIgA antibodies. Mice were sacrificed at indicated time points. (B-C) hIgA (B) and FcαRI/hIgA (C) mice were injected with PBS (left panel) or anti-mCOL17 hIgA (right panels) every other day till sacrifice on day 7 (in total 4 injections). Cryosections were stained with the neutrophil marker GR-1 (green) and DAPI (DNA; blue). Representative examples of ears that had been injected with anti-mCOL17 hIgA are shown. Red arrowheads indicate blister formation and tissue damage. (D) Quantification of GR-1 staining. Each dot represents one mouse. N = 8 per group (E-F) Analysis of ear thickness. (E) A representative cryosection example of a hIgA mouse ear (left panel) or FcαRI/hIgA mouse ear (right panel) are shown. (F) Quantification of ear thickness. N = 8; each dot represents one mouse. Data are presented as mean ± SD. *p < 0.05, **p < 0.001.
Figure 4. Blocking FcαRI prevets IgA-induced neutrophil accumulation and thickening of mice ears. (A + B) hIgA and (D + E) FcαRI/hIgA mice were injected with PBS (left panels) or anti-mCOL17 hIgA (right panels, 3 examples) every other day till sacrifice on day 7 (in total 4 injections) in combination with an (A + D) isotype control antibody or (B + E) anti-FcαRI mAb MIP8a. (C + F) Quantification of GR-1 staining. (G + I) Analysis of ear thickness of (G) hIgA or (I) FcαRI/hIgA mice after treatment with an isotype control (left panel) or MIP8a (right panel). A representative example of a mouse ear cryosection is shown. (H + J) Quantification of ear thickness. Each dot represents one mouse. hIgA mice (isotype control or MIP8a) n = 4; FcαRI/hIgA mice (isotype control) n = 3; FcαRI/hIgA mice (MIP8a) n = 8. Ears cryosections stained with neutrophil marker GR-1 (green) and DAPI (DNA; blue). Data are presented as mean ± SD. *p < 0.05, **p < 0.001, n.s., non significant.

Anti-FcαRI monoclonal antibodies as novel therapeutic target for IgA-induced chronic inflammation

Thus, blocking neutrophil FcαRI prevented IgA-induced inflammation in vivo. However,
patients with IgA-mediated autoimmune diseases mostly benefit from a therapy that is able to diminish already existing active disease. Therefore, FcαRI/hIgA mice received 4 injections of anti-mCOL17 hIgA antibodies in the ears every other day, which induced neutrophil accumulation and concomitant chronic inflammation (as seen in previous experiments; see Figure 3). Treatment with MIP8a or an isotype control commenced on day 8 (see Supplementary Figure 2 for injection schedule). Treatment with an isotype control antibody was ineffective as massive neutrophil accumulation was observed in ears that had been injected with anti-mCOL17 hIgA (Figure 5A+C). Moreover, ear thickness increased to ~1500 µm, after two weeks of exposure to anti-mCOL17 hIgA and significant tissue damage and blister formation was observed (Figure 5D+F). Treatment with MIP8a for 7 days, however, reduced neutrophil influx to background levels and ear thickness was reduced to ~ 700 µm (Figure 5E-F).

Figure 5. Anti-FcαRI monoclonal antibodies as treatment for IgA-induced neutrophil recruitment, chronic inflammation and tissue damage. FcαRI/hIgA mice were injected with PBS (left ear) or anti-mCOL17 hIgA (right ear) every other day till sacrifice on day 14 (in total 7 injections). Mice were treated with an isotype antibody or anti-FcαRI mAb MIP8a at day 8 and 11. (A - B) FcαRI/hIgA mice were treated with an isotype control (A) or with MIP8a (B). Left panels; PBS injection. Three representative examples of ears that had been injected with anti-mCOL17 hIgA are shown. (C) Quantification of GR-1 staining. (D - F) Analysis of ear thickness. A representative example of mouse ear cryosection is shown after treatment with an isotype control (D) or MIP8a (E). Inserts are magnifications. (F) Quantification of ear thickness. Each dot represents one mouse. (Isotype control n = 3; MIP8a n = 8. Cryosections of ears stained for the neutrophil marker GR-1 (green) and DAPI (DNA; blue). Data are presented as mean ± SD. **p < 0.05, ***p < 0.001.
We propose that in LABD the presence of IgA autoantibodies in the skin directly contributes to activation and recruitment of neutrophils into the skin through interaction with Fc\(\alpha\)RI. Newly recruited neutrophils will also encounter IgA complexes and become activated, which will lead to secretion of more LTB4 \(^9\), resulting in amplification of neutrophil migration. Perpetuating neutrophil accumulation will ultimately result in severe tissue damage and blister formation (Figure 6A). An Fc\(\alpha\)RI blocking antibody resolved pre-existing IgA autoantibody-induced inflammation in mice, which emphasizes the therapeutic potential of anti-Fc\(\alpha\)RI antibodies for IgA-mediated diseases (Figure 6B).

The current treatment of neutrophil-mediated chronic inflammatory skin diseases is amongst others systemic treatment with the antibiotic dapsone, although the mechanisms of action are poorly understood \(^{24,25}\). In most cases, long-term treatment is necessary, and dapsone is often poorly tolerated with considerable side-effects \(^{26}\). Removing pathogenic autoantibodies by immunoabsorption or immunoapheresis has been proposed as another strategy \(^{27,28}\), but this is an intensive treatment because patients’ plasma needs to be filtered multiple times. A more specific treatment for IgA-mediated diseases is therefore essential.

![Figure 6. Schematic model of the role of neutrophils and Fc\(\alpha\)RI/IgA interactions in Linear IgA Bullous Disease. (A) IgA autoantibodies bind collagen XVII at the basement membrane of the skin. Crosslinking of Fc\(\alpha\)RI on neutrophils by IgA immune complexes induces release of the potent chemoattractant LTB4, resulting in a continuing neutrophil migration loop, inducing tissue damage and ultimately blister formation. (B) Treatment with an anti-Fc\(\alpha\)RI antibody blocks Fc\(\alpha\)RI/IgA interactions hereby inhibiting neutrophil accumulation, inflammation and tissue damage in tissues.](image)

We now demonstrate for the first time that anti-Fc\(\alpha\)RI monoclonal antibody therapy resolves already existing inflammation in vivo, which represents a promising novel therapeutic strategy for patients with LABD, and possible dermatitis herpetiformis. However, this may also constitute an attractive approach for other autoimmune diseases in which IgA autoantibodies are likely to play a harmful role through over-activation of neutrophils. For instance, we showed that IgA rheumatoid factors induced neutrophil activation and release of NETs \(^{15}\). Interestingly, patients with high IgA RF levels poorly respond to TNF-\(\alpha\) inhibitors, suggesting that IgA-activated neutrophils may be less responsive to this treatment \(^{29,30}\). Similarly, ulcerative colitis, which is characterized by massive neutrophil influx in the colon, can be difficult to treat with current immunosuppressive and/or
biological therapies, which sometimes requires surgical removal of the colon. Anti-FcαRI monoclonal antibody therapy may reduce neutrophil activation and migration in these diseases as well.

Collectively, IgA is not a harmless anti-inflammatory molecule, but has a critical role in activation of neutrophils. When IgA autoantibodies are present, interactions with neutrophil FcαRI induces continues neutrophil activation and accumulation, resulting in severe tissue damage \textit{in vivo}. Consequently, anti-FcαRI monoclonal antibodies represent promising novel therapeutic tools to specifically treat IgA-induced inflammation in autoimmune diseases.

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Supplementary Figure 1. Characterization of anti-mCOL17 hIgA antibodies. (A) Presence of specific anti-mCOL17 hIgA in serum of immunized mouse and after purification as detected by a mCOL17 binding ELISA. (B) SDS-PAGE and silver staining of anti-mCOL17 hIgA antibody under non-reducing (left panel) and reducing conditions (right panel). Lane 1: pooled human serum IgA; lane 2: FCS; lane 3: anti-mCOL17 hIgA. H: heavy chain; L: light chain. (C) Anti-human IgA western blot. A second band was observed in the anti-mCOL17 hIgA sample that reflects dimeric IgA, as serum IgA is mainly dimeric in mice. Of note, we and others previously showed that dimeric IgA is equally capable of inducing inflammatory responses via FcαRI compared to monomeric IgA.

Supplementary Figure 2. Experimental setup in vivo experiments. (A) Induction of inflammation (Figure 3): FcαRI/hIgA or hIgA mice received intracutaneous injections of anti-mCOL17 hIgA or PBS in the ears every other day for 7 days (4 injections in total). (B) Prevention of inflammation (Figure 4): hIgA or FcαRI/hIgA mice received intracutaneous injections of anti-mCOL17 hIgA or PBS in the ears every other day for 7 days (4 injections in total). In addition, mice received intraperitoneal injections of anti-FcαRI mAb or an isotype control on day 0 and 3. (C) Treatment of inflammation (Figure 5): FcαRI/hIgA mice received subcutaneous injections of anti-mCOL17 hIgA or PBS in the ears every other day for 14 days (7 injections in total). In addition, mice received intraperitoneal injections of anti-FcαRI mAb or an isotype control on day 8 and 11.
Supplementary Video 1
Intravital imaging of blood vessels of mouse ears and LysEGFP neutrophils are displayed in green. Video of blood vessels were taken 48 hours after injection of anti-mCOL17 hIgA antibodies or PBS in the ears of mice. Video 1 demonstrates ears of LysEGFP mice after injection with PBS or anti-mCOL17 hIgA.

https://www.youtube.com/watch?v=sQx506lMyRs

Supplementary Video 2
Intravital imaging of blood vessels of mouse ears and LysEGFP neutrophils are displayed in green. Video of blood vessels were taken 48 hours after injection of anti-mCOL17 hIgA antibodies or PBS in the ears of mice. Video 2 demonstrates ears of FcαRI/LysEGFP mice after injection with PBS, anti-mCOL17 hIgA or with anti-mCOL17 hIgA in combination with an FcαRI blocking antibody.

https://www.youtube.com/watch?v=X8CL1AXH9f0
Chapter 5

References


