CHAPTER 3

Blocking FcαRI on granulocytes prevents tissue damage induced by immunoglobulin A autoantibodies

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**ABSTRACT**

Immunoglobulin A (IgA) represents the most prominent antibody class at mucosal surfaces and the second most prevalent antibody in human blood after IgG. We recently demonstrated that cross-linking of the granulocyte IgA Fc receptor (FcαRI) by IgA induces a chemotactic driven positive-feedback migration loop, hereby amplifying recruitment of granulocytes to IgA deposits. Therefore, we postulated that aberrant IgA-antigen complexes, which can be found in tissues in IgA-mediated diseases, are responsible for tissue damage by inducing continuous granulocyte migration and activation. Using an IgA-dependent skin blistering disease as a model system, we demonstrated co-localization of FcαRI-positive granulocyte infiltrates with IgA in cryosections of lesional skin of patients suffering from this disease. Furthermore, we showed granulocyte migration to IgA deposits injected in human skin explants and in murine skin of FcαRI transgenic mice *in vivo*. Importantly, *ex vivo* migration and tissue damage was inhibited by blocking FcαRI indicating that these events are dependent on the interaction of IgA autoantibodies with FcαRI. Thus, interrupting the granulocyte migration loop by blocking FcαRI reduces tissue damage in diseases with aberrant IgA-immune complexes. As such, our results may lead to development of new therapies for IgA-mediated chronic inflammatory diseases, hereby decreasing severe morbidity and improving quality of life for these patients.
INTRODUCTION

Immunoglobulin A (IgA) represents the most prominent antibody class at mucosal surfaces. It is produced by plasma cells in the lamina propria in dimeric form (dIgA), which is transported through mucosal epithelial cells and excreted at the apical membrane as secretory IgA (SIgA). Here it mainly serves as an antiseptic coating of the mucosal wall by neutralizing bacterial toxins and preventing adherence and invasion of microorganisms.\(^1,2\) Thus, SIgA provides a first line of defense against invading bacteria, but is generally considered as a non-inflammatory antibody, since it is a poor opsonin.\(^3\) In addition to mucosal SIgA and dIgA, a monomeric form of IgA (mIgA) is present in human serum, which has long been considered as a non-inflammatory antibody as well.\(^4,5\) Compared with IgM and IgG, IgA is a poor activator of complement.\(^1,6,9\) It was furthermore demonstrated that targeting of the myeloid Fc receptor for IgA (Fc\(\alpha\)RI, CD89) with soluble mIgA in the absence of antigen led to inhibitory signals or apoptosis in monocytes,\(^10-12\) which is due to weak phosphorylation of the FcR\(\gamma\)-chain ITAM, resulting in recruitment of SHP-1. By contrast, complexed mIgA has been shown to trigger a plethora of inflammatory functions through cross-linking of Fc\(\alpha\)RI, as this leads to heavy phosphorylation of FcR\(\gamma\)-chain ITAM, hereby engaging Syk and resulting in activation. Inflammatory functions comprise amongst others endocytosis, phagocytosis, release of cytokines, superoxide release or inflammatory mediators, antibody-dependent cellular cytotoxicity (ADCC), and antigen presentation.\(^13\) Interestingly, we recently demonstrated that cross-linking of Fc\(\alpha\)RI by complexed mIgA or dIgA induced granulocyte migration.\(^14\) This initiated a self-controlled positive-feedback loop, which resulted in enhanced recruitment of granulocytes to IgA deposits. Thus, it is now clear that cross-linking of Fc\(\alpha\)RI by complexed IgA induces robust inflammatory responses as protective mechanisms against invading pathogens. Aberrant deposits of IgA-antigen complexes are found in several chronic inflammatory diseases, including celiac disease, IgA nephropathy, Henoch-Schönlein purpura, IgA pemphigus, dermatitis herpetiformis, and linear IgA bullous disease (LABD).\(^6,15-17\) The latter two diseases are chronic skin diseases associated with IgA autoantibodies, which are characterized by subepidermal blisters with dense inflammatory infiltrates that are dominated by granulocytes. Several molecular targets to which IgA autoantibodies are directed have been identified. For instance, anti-epidermal transglutaminase IgA autoantibodies are found in dermatitis herpetiformis,\(^18\) whereas IgA autoantibodies in LABD are directed against collagen XVII, also referred to as the bullous pemphigoid antigen of 180 kDa (BP180), which is a transmembraneous hemidesmosomal protein involved in maintaining cell-matrix adhesion in the skin.\(^17,19\) The potential of IgA autoantibodies to induce tissue damage and the pathogenic relevance of their interaction with granulocytes have, however, not yet been investigated in great detail. In the present study, we used an IgA-dependent skin blistering diseases as a model system to investigate the role of IgA autoantibodies in recruitment of granulocytes and the involvement of Fc\(\alpha\)RI in inducing tissue destruction in diseases with aberrant IgA complexes.
**MATERIAL AND METHODS**

**Isolation of human neutrophils and eosinophils**

Standard Lymphoprep (Axis-shield, Dundee, Scotland) density gradient centrifugation (800 x g, 25 minutes) was used to isolate granulocytes from heparinized peripheral blood samples, which were obtained from healthy donors. Erythrocytes were removed by hypotonic lysis (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) for 10 minutes. Granulocytes were resuspended in RPMI 1640 (Gibco BRL, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum, L-glutamine, penicillin and streptomycin. For the cryosection assay, human granulocytes were isolated from the peripheral blood of healthy donors. After 3% dextran sedimentation, remaining erythrocytes were lysed using a hypotonic solution of 0.2% NaCl. Human granulocytes were washed and resuspended in DMEM medium w/o supplements. Eosinophils were isolated from granulocyte suspension according to the manufacturer’s instructions using a MACS-Eosinophil Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of isolated neutrophils (> 95%) or eosinophils (> 95%) was confirmed by cytospin preparation. Studies were approved by the Medical Ethical Committee of VU University Medical Center (The Netherlands) and by the Ethics Committee of the Medical Faculty of the University of Freiburg (Germany), in accordance with the Declaration of Helsinki. All donors gave informed consent.

**Preparation of immunoglobulin-coated beads**

N-hydroxysuccinimide (NHS)-activated sepharose beads (GE Healthcare, Uppsala, Sweden) were coated under sterile conditions with either IgA or bovine serum albumin (BSA) according to the manufacturer’s instructions. Briefly, beads were washed in 1 mM HCl, and resuspended in 1 ml 0.2 M NaHCO₃/0.5 M NaCl pH 8.3 containing 300 µg/ml serum IgA (Sigma-Aldrich, St. Louis, MO) or BSA and incubated overnight (4°C, head over head). Beads were washed with 0.5 M ethanolamine/0.5 M NaCl pH 8.3 and 0.1 M sodiumacetate/0.5 M NaCl pH 4.

**Granulocyte migration and activation assays**

Two dimensional (2D) migration assay

The potential of eosinophils to migrate towards IgA-coated beads was assessed with a previously described assay. Briefly, isolated human eosinophils were labeled with PKH-67 (green fluorescence), according to the manufacturer’s instructions (Sigma-Aldrich). 2.5 x 10⁵ eosinophils/well were seeded in 96 wells flat bottom plates (Greiner Bio-one North America Inc., Monroe, NC), after which IgA-coated beads or BSA-coated beads as control were added. Eosinophils were incubated for 20 minutes at 37°C, after which supernatants were removed and beads were washed 2 times with PBS to remove unbound eosinophils. Beads were incubated with a buffer containing 2.0 g/l hexadecyltrimethyl ammonium bromide, 1.0 g/l tween 20, 2.0 g/l BSA and 7.44 g/l EDTA in PBS to lyse bound eosinophils. Fluorescence was measured using 485 nm excitation and 520 nm emission filters (Fluostar Galaxy, BMG Labtechnologies, Offenburg, Germany).

Three dimensional (3D) migration assay

Collagen (isolated from rat tail tendons and dissolved in 0.1% acetic acid) was mixed with
NaOH and DMEM (10x, Sigma-Aldrich) as described.\textsuperscript{20} IgA- or BSA-coated beads (100\mu l/ml) were added and 1 ml of this mixture was plated in a 24-well plate and allowed to coagulate, after which 2x10\textsuperscript{6} granulocytes were added. After 4 hours incubation (37°C) collagen gels were fixed and embedded in paraffin. Sections were stained with Mayer’s hematoxyline & eosine (Klinipath, Duiven, The Netherlands).

**Real-Time video recordings of neutrophil/eosinophil migration**

Isolated human neutrophils or eosinophils were labeled with PKH-67 (green fluorescence) or PKH-26 (red fluorescence) respectively, according to the manufacturer’s instructions (Sigma-Aldrich). Mixtures of neutrophils and eosinophils (ratio 5:1) per well were seeded in 8 wells plates (Greiner Bio-One). Real time video recordings were performed with an inverted phase-contrast microscope (Olympus, IX81-ZDC, Suffolk, UK) housed in a humidified, 5% CO\textsubscript{2} gassed, temperature-controlled (37°C) chamber. Randomly selected fields were recorded for 50 minutes. Pictures were taken every 15 seconds with an Olympus ColorView II camera (Olympus Nederland BV, The Netherlands). For tracking experiments an interval of 7 seconds was used. Recordings were analyzed using CELL F trackIT software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

**Human skin migration assay**

Full thickness mammary skin grafts (epidermis and dermis) were placed in an ex vivo tissue incubation chamber (method adapted from Oosterling,\textsuperscript{21} with the dermis face up. IgA- or BSA-coated beads were injected intracutaneously via the dermis, followed by addition of PKH-67 labeled granulocytes (4x10\textsuperscript{6} cells/well) on the dermis in the absence or presence of 10 \mu g/ml MIP8a. To allow detection of the injection spot, Indian ink was added to the beads. In alternative experiments serum of patients with LABD was injected in the skin instead of beads. Of note, due to the short half-life of granulocytes after isolation, cells were supplemented with IFN\gamma to prevent early apoptosis (300 units/ml; Boehringer Ingelheim, Ingelheim am Rhein, Germany). Skin was incubated overnight at 37°C, after which biopsies of the injected skin were taken and snap frozen. Cryosections were analysed with a Leica DM6000 microscope (Leica Microsystems B.V., Rijswijk, The Netherlands).

**Autoantibody-induced granulocyte-dependent dermal-epidermal separation assay**

The potentiality of IgA autoantibodies to activate granulocytes and induce dermal-epidermal separation was assessed, with a previously described assay.\textsuperscript{22,23} Briefly, 5x10\textsuperscript{6} granulocytes from healthy donors were added to cryosections of normal human skin, which had been incubated with serum of patients with LABD, bullous pemphigoid (an IgG and complement mediated autoimmune bullous disease, used as control) or healthy donors. Incubation with granulocytes was performed in the absence or presence of 10 \mu g/ml MIP8a, 100 \mu M Apocynin (Sigma-Aldrich), or 100 \mu M N-(methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone (Sigma-Aldrich). Cryosections were then incubated for 2 hours at 37°C, followed by a 20 minutes wash in PBS and a standard hematoxyline and eosine staining (Klinipath).

**ELISA**

The concentration of eosinophil cationic protein (ECP) in the supernatants of neutrophil/
eosinophil migration assays was analysed by ELISA (USCN, Wuhan, China) according to the manufacturer’s instructions.

**Animal experiments**

Mice were bred and maintained at the Central Animal Facility of the VU University, Amsterdam, The Netherlands under standard conditions. IgA- or BSA-coated beads were injected intracutaneously in non transgenic or FcαRI transgenic mice under isoflurane anesthesia. To allow detection of the injection spot, Indian ink was added to the beads. After 48 hours mice were sacrificed, and biopsies of injected skin were taken and snap frozen. All experiments were performed according to institutional and national guidelines. The animal ethical committee of the VU University Medical Center approved all experiments.

**Immunofluorescence and immunohistochemistry**

Human normal- or LABD skin cryosections (6 μm) were fixed in anhydrous acetone, air-dried and non-specific binding was blocked by incubation (15 minutes, RT) with 10% normal rabbit serum diluted in 0.5% BSA in PBS. Skin sections were stained as indicated in the figure with FITC or PE conjugated anti-human CD66b, IgA and FcαRI mAbs (BD, Franklin Lakes, NJ) (1 hour, RT). Detection of serum autoantibodies was performed as described by Sitaru et al. Briefly, cryosections of normal human skin were incubated with serum of patients with LABD, bullous pemphigoid or healthy donors (1 hour, RT), after which they were washed and subsequently incubated with FITC anti-human IgA (Dako Netherlands bv, Heverlee, Belgium), anti-IgG or an irrelevant isotype control (BD). Granulocyte presence in mouse skin cryosections was determined by staining sections with rat anti-mouse GR-1 mAb (BD), and horseradish peroxidase (HRP)-labeled goat anti-rat antibodies. 3-Amino-9-ethylcarbazole (AEC, Zymed, San Francisco, CA) was used as a peroxidase substrate. Cryosections were analysed with a Leica DM6000 microscope (Leica Microsystems B.V.).

**Statistical analysis**

Data are shown as mean ± standard deviation (SD). Statistical differences were determined using two–tailed unpaired Students t-tests (comparing 2 groups) or ANOVA (> 2 groups). Significance was accepted when p < 0.05.
RESULTS

Co-localization of IgA deposits and FcαRI-expressing granulocytes in skin of LABD patients

Cryosections of skin lesions of LABD patients, or of normal healthy skin as control, were analyzed by immunofluorescence microscopy to evaluate co-localization of IgA deposits and FcαRI-expressing cells. Neither IgA deposits nor FcαRI expression was observed in normal skin (Figure 1A, upper panels). In contrast, in cryosections of LABD patients IgA (shown in green) was deposited along the basement membrane in a linear pattern as also described in Sitaru et al.26 (Figure 1A, lower panels). Interestingly, an infiltrate of FcαRI-expressing cells (shown in red) was observed in the dermis adjacent to the basement membrane of the skin (Figure 1A, lower panels). Importantly, the overlay panel shows co-localization of IgA and FcαRI (Figure 1A insert: yellow staining). Furthermore, double staining for the granulocyte marker CD66b (green) and FcαRI (red) (Figure 1B, co-localization; yellow staining), or IgA (green) and CD66b (red) (Figure 1C), demonstrated that FcαRI positive cells consisted of granulocytes, which were situated adjacent to IgA deposits. Thus, FcαRI-expressing granulocytes were located in contact with IgA-immune complexes at the epidermal basement membrane of lesional skin of LABD patients.

IgA induces both neutrophil and eosinophil migration

Granulocyte infiltrates of lesional skin of LABD patients consisted mostly of neutrophils and to a lesser extent of eosinophils (Figure 2A, eosinophils; arrowheads) and as earlier described.27,28 When collagen gels containing IgA-coated beads or BSA-coated beads as control were incubated with granulocytes and stained with hematoxyline and eosine to distinguish neutrophils from eosinophils, neither neutrophils nor eosinophils migrated towards BSA-coated beads. However, granulocytes that were recruited towards IgA-coated beads consisted of both neutrophils and eosinophils (Figure 2B). We previously demonstrated rapid neutrophil migration to IgA-coated beads with a 2D migration assay.14 Since eosinophils also express a functional FcαRI,29,30 we now investigated whether eosinophils were equally capable of migrating to IgA-coated beads. Therefore, we first performed a 2D migration assay in which IgA- or control (BSA) beads were added to a monolayer of fluorescently labeled eosinophils. The amount of bound cells was quantified by measuring fluorescence after the cells had been lysed, which confirmed that only few eosinophils had bound to control beads, whereas a large number of eosinophils had migrated towards and adhered to IgA-coated beads (Figure 2C). Next, cell tracking experiments were performed with a granulocyte suspension to compare neutrophil versus eosinophil migration in response to IgA-coated beads (Figure 2D, and supplementary video). Neutrophils (green fluorescent cells) responded within two minutes after addition of IgA-coated beads (Figure 2D, upper panels; arrows), whereas eosinophils (red fluorescent cells) were still inactive at this time point. After 4 minutes, eosinophils became active (Figure 2D, lower panels; arrows). Both neutrophils and eosinophils specifically travelled in the direction of IgA-coated beads, indicating induction of chemotaxis instead of random chemokinesis (Figure 2E and F). Travelled distances were similar between neutrophils and eosinophils (Figure 2G, left panel). However, neutrophils migrated with
Figure 1. Co-localization of IgA deposits and FcαRI-expressing granulocytes in skin of LABD patients. (A) Cryosections of normal human skin (upper panels) and of skin lesions of LABD patients (lower panels) were stained with anti-human IgA-FITC (left panels, green) and anti-human FcαRI-PE (middle panels, red). Right panels: overlay pictures. Insert: magnification of the basement membrane. (B) Skin cryosections of lesions of LABD patients were stained with the neutrophil marker anti-CD66b-FITC (left panel, green) and anti-human FcαRI-PE (middle panel, red). Right panel: overlay picture. (C) Skin cryosections of lesions of LABD patients were stained with anti-human IgA-FITC (left panel, green) and anti-CD66b-PE (middle panel, red). Right panel: overlay picture. Dotted lines: top of epidermis. n = 3.
blocking FCαRI prevents tissue damage

higher speed, compared to eosinophils (Figure 2G, right panel). To assess whether eosinophils may contribute to the pathogenesis of IgA-mediated tissue damage, we analyzed the concentration of eosinophil cationic protein (ECP) as a marker of eosinophil degranulation. The concentration of ECP was significantly higher in supernatants of the granulocyte suspension in response to IgA-coated beads compared to BSA-coated beads (Figure 2H), which support that eosinophils had degranulated.

**FCαRI mediates IgA induced granulocyte migration in ex vivo human skin and in vivo**

To mimic granulocyte migration towards aberrant IgA-antigen complexes in skin, we next established an *ex vivo* migration assay. Full thickness human skin grafts were injected with BSA- or IgA-coated beads and incubated for 24 hours with fluorescently labeled granulocytes. Bright field analyses of skin sections showed localization of the injected BSA- or IgA-coated beads (Figure 3A, left panels). Random migration of a small number of granulocytes (green fluorescence) at the top of the dermis was observed in skin sections in which BSA-coated beads had been injected. In contrast, massive granulocyte infiltration was observed towards IgA-coated beads. Overlay images showed no binding of granulocytes to BSA-coated beads, but extensive binding of cells to IgA-coated beads (Figure 3A, middle and right panels). When an anti-FCαRI mAb was added to the culture that blocks the binding site for IgA on FCαRI (MIP8a) migration of granulocytes was strongly reduced (Figure 3B, lower panels). Thus, the presence of IgA-beads induced granulocyte migration, which was depended on FCαRI. To confirm that IgA autoantibodies of LABD patients induce granulocyte migration, *ex vivo* skin experiments were repeated after injection of serum of LABD patients into skin samples. Similarly, without blocking FCαRI, granulocytes migrated towards injected serum of LABD patients (Figure 3C, left panel). Blocking FCαRI with MIP8a, however, inhibited migration of granulocytes almost completely (Figure 3C, right panel).

In *vivo* studies investigating the role of IgA have been restricted because mice do not express an FCαRI homologue. To overcome this limitation human FCαRI transgenic mice were generated, in which FCαRI expression, regulation, interaction with human IgA, and function mimic the human situation. Human IgA-coated beads (or BSA-coated beads as control) were injected in the skin of human FCαRI transgenic mice or non transgenic littermates to study the *in vivo* role of FCαRI in IgA-induced granulocyte migration in skin. Mice were sacrificed after 48 hours. Only minimal influx of granulocytes was observed in skin of non-transgenic mice injected with either BSA- or IgA-coated beads (Figure 3D, panels I and III), or in the skin of FCαRI transgenic mice injected with BSA-coated beads (Figure 3D, panel II). In contrast, massive granulocyte infiltration was observed in skin of human FCαRI transgenic mice that had been injected with IgA-coated beads (Figure 3D, panel IV). Thus, only aberrant IgA deposits in the skin of human FCαRI transgenic mice induced massive granulocyte migration and infiltration.
Figure 2. IgA induces both neutrophil and eosinophil migration. (A) Paraffin sections of lesional skin of LABD patients were stained with hematoxyline and eosine to distinguish neutrophils from eosinophils. Insert: higher magnification of neutrophils and eosinophils, arrows: eosinophils. (B) Paraffin sections of collagen gels, in which IgA-coated beads (*) had been embedded and had been incubated with granulocytes, were stained with hematoxyline and eosine. Insert: higher magnification of neutrophils and eosinophils, arrows: eosinophils. (C) Control (BSA) or IgA-coated beads (IgA) were added to monolayers of resting fluorescently labeled eosinophils for 20 minutes. Cells were lysed, after which fluorescence was determined as measure of the number of cells that had adhered to the beads. (D) Cell tracking experiments were performed with a mixture of PKH-67 labeled neutrophils (upper panels, green) and PKH-26 labeled eosinophils (lower panels, red). White spheres indicate location of IgA-coated beads, arrows: activated neutrophils (green) or eosinophils (red). (E) Migratory direction of neutrophils is indicated by green arrows, eosinophils by red arrows. White spheres indicate location of IgA-coated beads. (F) IgA-coated beads after 20 minutes. Both neutrophils (green) and eosinophils (red) have attached. (G) Distance traveled in 20 minutes (left panel) and speed (right panel) of neutrophils (white bars) and eosinophils (black bars) to control beads (BSA) and to IgA-coated beads (IgA). (H) Eosinophil cationic protein (ECP) was measured as indication of eosinophil degranulation. Data are represented as mean ± SD. * P < 0.05. A representative experiment out-of-three is shown.
Figure 3. FcαRI mediates IgA induced granulocyte migration in ex vivo human skin and in vivo. (A) Cryosections of ex vivo skin explants injected with control beads (BSA) or IgA-coated beads (IgA) and incubated with PKH-67 labeled granulocytes (green). Bright field (left panels), FITC channel (middle panels) or overlay (right panels) are shown. Black staining: Indian ink to mark injection spot. (B) Cryosections of ex vivo skin explants injected with IgA-coated beads in the absence or presence (+ MIP8a) of FcαRI blocking mAb, and incubated with PKH-67 labeled granulocytes (green) in absence (left panel) or presence (right panel) of MIP8a. Insert: higher magnification of migrated neutrophils. Dotted line: top of the skin where neutrophils had been added. (C) Cryosections of ex vivo skin explants injected with serum of LABD patients and incubated with PKH-67 labeled granulocytes (green) in absence (left panel) or presence (right panel) of MIP8a. Insert: higher magnification of migrated neutrophils. (D) Cryosections of skin of non transgenic (non Tg; left panels) or human FcαRI transgenic (Tg; right panels) mice that had been injected with control beads (BSA; upper panels) or IgA-coated beads (IgA; lower panels) were stained for granulocytes (shown in red). Insert: higher magnification of beads. Black staining: Indian ink to mark injection spot. n = 3.
Blocking FcαRI prevents IgA induced tissue damage

To analyze whether blocking FcαRI would prevent tissue damage, normal human skin was incubated with serum from healthy donors, patients with LABD, or patients with bullous pemphigoid as control. The latter is an autoimmune blistering disorder of the skin similar to LABD, but mediated by IgG autoantibodies against collagen XVII. First, we analyzed the presence of auto-reactive antibodies to the epidermal basement membrane in human sera with immunofluorescence microscopy. No specific staining was detected in skin cryosections incubated with normal human serum (NHS) of healthy donors (Figure 4A, left panel). In contrast, both IgG or IgA autoantibodies from patients with bullous pemphigoid (BP) or LABD, respectively, stained the basement membrane of human skin cryosections (Figure 4A, middle and right panel). Subsequently, cryosections were incubated with granulocytes. No blister formation was observed in cryosections incubated with NHS (Figure 4B, left panel). However, separation of the dermis and epidermis (DES) was observed when skin cryosections were incubated with serum of either BP or LABD patients in the presence of granulocytes (Figure 4B, middle and right panel). Next, we investigated the therapeutical potential of blocking FcαRI. When cryosections of human skin were incubated with patients’ sera, and granulocytes in the presence of an irrelevant isotype control (ISO) (Figure 4C, upper panels), dermal-epidermal separation was not inhibited. In addition, blister formation was not abolished when skin cryosections were incubated with sera from patients with BP and granulocytes in the presence of MIP8a (Figure 4C, lower, middle panel). Importantly, IgA autoantibodies against the basement membrane did not induce granulocyte-dependent dermal-epidermal separation in cryosections of human skin, when granulocytes were incubated with MIP8a (Figure 4C, lower, right panel and 4D). Thus, blocking FcαRI prevented *ex vivo* tissue damage in skin induced by LABD IgA-autoantibodies and granulocytes.

To successfully kill microbes after their phagocytosis and in the pericellular tissue environment, granulocytes use efficient weapons, such as reactive oxygen species (ROS), and serine proteases like neutrophil elastase. Although intracellular killing of pathogens by ROS and serine proteases is a beneficial function for the host, extracellular spillage can results in significant collateral damages of tissues. To assess whether ROS or neutrophil elastase is responsible for the tissue damage observed in patients, granulocytes were incubated with inhibitors for NADPH-oxidase (Apocynin), which is involved in ROS production, or neutrophil elastase (chloromethyl ketone), and added to normal human skin, incubated with serum from healthy donors or patients with LABD. Inhibition of ROS production resulted in ~50% reduction of DES (Figure 4E). Additionally, blocking elastase activity (Figure 4F) resulted in decreased DES as well, albeit to a lesser extend. Thus, both pathways contributed to blister formation in the skin.
Figure 4. Blocking FcαRI prevents tissue damage in ex vivo human skin, induced by IgA autoantibodies of LABD-patients and granulocytes. (A) Cryosections of normal human skin were incubated with normal human serum (NHS), serum of patients with bullous pemphigoid (BP) or serum of patients with Linear IgA Bullous Disease (LABD), and stained with anti-human IgG-FITC (BP) or anti-human IgA-FITC (LABD). Arrows: basement membrane. (B) Cryosections were incubated with NHS (left panel), BP serum (middle panel) or LABD serum (right panel) and with granulocytes. Sections are stained with hematoxyline and eosine. Arrows: dermal-epidermal separation. (C) Cryosections were incubated with NHS (left panels), BP serum (middle panels) or LABD serum (right panels) and with granulocytes in the presence of an isotype mAb (ISO; upper panels) or anti-FcαRI blocking mAb MIP8a (lower panels). Sections are stained with hematoxyline and eosine. Arrows: dermal-epidermal separation. (D) Percentage of inhibition of dermal-epidermal separation (DES) in cryosections that had been incubated with granulocytes pretreated with MIP8a and serum of LABD patients (IgA-LABD), bullous pemphigoid patients (IgG-BP) or normal human serum (NHS) in relation to incubation with the vehicle alone. (E, F) Percentage of inhibition of DES in cryosections that had been incubated with serum of LABD patients and granulocytes in presence of Apocynin (E) or Chloromethyl-Ketone (ClMeKetone, F) in relation to incubation with the vehicle alone. Data are represented as mean ± SD. *: p< 0.05
DISCUSSION
Granulocytes are the first cells that migrate into tissues to engulf and kill microorganisms at sites where the skin or mucosal barriers are damaged. They arrive within an hour of tissue damage and their number increases significantly over time, particularly when the lesion is infected. Release of anti-microbial products by granulocytes efficiently kills pathogens, but can also lead to serious collateral damage to normal host cells. It is therefore imperative that activation is tightly regulated and that granulocytes are removed from the tissue as soon as the infection is cleared.

We recently demonstrated that cross-linking of granulocyte FcαRI by IgA induces the release of leukotriene B4, which is a strong chemoattractant for granulocytes. This induces a positive migration loop which can be very efficient for killing invading bacteria at mucosal sites where IgA is the predominant antibody. Moreover, we also showed that targeting FcαRI very effectively induced LTB4-dependent neutrophil recruitment into tumour colonies, which led to destruction. Thus, we postulated that FcαRI may promote tissue damage in diseases mediated by IgA autoantibodies. We now show that granulocyte migration and activation is deranged in diseases in which aberrant IgA-antigen complexes are formed. Because granulocytes are not able to clear such complexes, newly recruited granulocytes are continuously activated, leading to perpetuating inflammation and tissue damage. For instance, anti-collagen type XVII IgA antibodies that are deposited at the basement membrane of the skin induce continued granulocyte activation resulting in separation of the dermis and epidermis, which constitutes a pathological hallmark of LABD. Importantly, blocking FcαRI with a monoclonal antibody prevented ex vivo skin cleavage. Furthermore, both the respiratory burst and elastase were responsible for tissue damage, as separation of the dermis and epidermis was (partly) blocked when these pathways were inhibited. The infiltrates of LABD patients often contain also eosinophils, but the role of these cells in the pathology of LABD is not completely understood. We demonstrated that eosinophils had degranulated after migration towards IgA-coated beads, as indicated by increased ECP concentration, which supports the hypothesis that eosinophils likely contribute to the pathology of LABD. Eosinophils were previously shown to express FcαRI. It was furthermore shown that cross-linking of FcαRI on eosinophils induced respiratory burst activity, albeit smaller compared to the response of neutrophils. This may be due to the fact that FcαRI is inactive on resting eosinophils, and has to be primed by cytokines to become fully active.

FcαRI induced tissue damage may also apply to other diseases characterized by aberrant IgA-antigen complexes and granulocyte infiltrates, such as Henoch-Schönlein purpura, IgA pemphigus and dermatitis herpetiformis. However, this mechanism may also play an important role in diseases, which are generally not considered as strictly mediated by granulocytes. Rheumatoid arthritis for instance is characterized by chronic joint inflammation, in which mononuclear cells like T and B lymphocytes, and macrophages play a prominent role. Infiltration of granulocytes into the synovial membrane and fluid compartment is, however, also observed. Moreover, it was shown that patients with elevated levels of serum IgA immune complexes or IgA rheumatoid factor have worse prognosis as this is associated with development of rheumatoid vasculitis and predicts...
development of active, erosive disease of the joints.\textsuperscript{42-45} It was, furthermore, suggested that granulocyte recruitment to inflamed joints was mediated by leukotriene B\textsubscript{4}.\textsuperscript{46} Thus, IgA-Fc\alpha RI interactions may induce granulocyte recruitment to the joints of rheumatoid arthritis patients, leading to increased destruction of joint tissues. Furthermore, also in mucosal diseases in which aberrant IgA complexes have not yet been recognized as potentially damaging, IgA-induced migration of neutrophils may contribute to the pathogenesis of disease. Ulcerative colitis is a chronically remitting and relapsing disorder, characterized by severe inflammation of the intestinal tract.\textsuperscript{47} One of the hallmark features of ulcerative colitis are large granulocytic infiltrates. Because the mucosal barrier is impaired in these patients, leading to translocation of luminal contents, it is likely that excessive IgA-antigen complexes are formed. Because we previously demonstrated that granulocytes in the colon of ulcerative colitis patients had taken up IgA complexes\textsuperscript{14} and we now demonstrate that excessive IgA deposits lead to an expanding inflammation loop of infiltrating granulocytes and tissue damage, we hypothesize that cross-linking of Fc\alpha RI by aberrant IgA-antigen complexes might be a key process in causing severe tissue damage in this disease.

In conclusion, although the origins of aberrant IgA-antigen complexes in most diseases are currently unknown and our data as such do not clarify the initiation of disease, this study clearly demonstrates that abnormal depositions of IgA-antigen complexes in tissues induce sustained granulocyte recruitment through cross-linking of Fc\alpha RI. As a consequence, our results provide an explanation for the constant activation and infiltration of granulocytes, which causes severe tissue damage and aggravation of the symptoms of these diseases. Importantly, we now demonstrate that blocking IgA-Fc\alpha RI interactions abolishes the perpetuating inflammatory loop of granulocytes, hereby significantly reducing damage and morbidity. As such, these results hold promise for development of new therapeutics for diseases associated with aberrant tissue deposits of IgA-antigen complexes.

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REFERENCES


SUPPLEMENTARY INFORMATION
Supplementary video 1. IgA induces both neutrophil and eosinophil migration. IgA coated beads were added to a mixture of neutrophils (green fluorescent cells) and eosinophils (red fluorescent cells) at a ratio of 5:1 and recorded with real time video for 50 minutes. Neutrophils responded within 2 minutes after addition of the IgA-coated beads by binding to and spreading on these beads, whereas eosinophils were still inactive at this time point. After 4 minutes eosinophils became active and also bound to and spread on the beads. Bar represents 20 μm.

http://youtu.be/bike0JTIKVk