Establishment of a collagen-induced arthritis model in human FcαRI/human IgA mice

Esil Aleyd¹, Marieke H. Heineke¹, Louis Boon², Marjolein van Egmond¹,³

¹Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands
²Bioceros BV, Utrecht, The Netherlands
³Department of Surgery, VU University Medical Center, Amsterdam, The Netherlands

Manuscript in preparation
Abstract
Rheumatoid arthritis (RA) is a chronic autoimmune disease that is characterized by inflammation of the joints and the presence of autoantibodies, including rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA). Interestingly, several studies showed a correlation between the presence of immunoglobulin A (IgA) autoantibodies and extra-articular manifestations as well as worse disease prognosis in RA patients. We recently showed that IgA immune complexes, present in plasma and synovial fluid (SF) of RA patients, induced prominent neutrophil activation and the formation of neutrophils extracellular traps via FcαRI in vitro. Since neutrophils are found in high numbers in the SF of RA patients, we hypothesize that IgA autoantibodies may be involved in inducing joint damage and in this way worsen disease in RA patients. To explore the in vivo role of neutrophil FcαRI/IgA interactions in RA, we investigated the susceptibility of human FcαRI transgenic/human IgA knock-in (FcαRI/hIgA) mice to collagen induced arthritis. Immunization of FcαRI/hIgA mice with bovine collagen II induced IgA autoantibody production against mouse collagen II in pilot experiments. Neutrophil numbers in blood and bone marrow of mice increased over time, and a small number of neutrophils migrated to the knees of immunized FcαRI/hIgA mice. This was not sufficient to induce clinical signs of arthritis, such as inflamed paws. However, we observed loss of joint function in FcαRI/hIgA mice, a feature also observed in RA patients. Thus, active immunization with collagen II induces inflammatory responses in FcαRI/hIgA mice, but more research is needed to further establish the role of FcαRI/IgA interactions in the pathogenesis of RA.
**Introduction**

Immunoglobulin A (IgA) represents the most prominent antibody class at mucosal surfaces and has an important role in mucosal immunity. Whereas secretory IgA is considered as a non-inflammatory antibody, since it is unable to bind the IgA Fc receptor FcαRI, complexed monomeric or dimeric IgA effectively activate immune cells that express FcαRI. Neutrophils induce robust proinflammatory responses after binding IgA immune complexes. Crosslinking of FcαRI by IgA immune complexes induces production of reactive oxygen species (ROS), release of cytokines, phagocytosis and release of neutrophil extracellular traps (NETs). Moreover, crosslinking of FcαRI by IgA immune complexes induces the release of the potent neutrophil chemoattractant leukotriene B4 (LTB4) and concomitant neutrophil recruitment.

Abnormal accumulation of IgA-antigen complexes in chronic inflammation may lead to continuous neutrophil activation, which can result in serious tissue damage due to persistent release of harmful inflammatory cytokines, ROS and proteases. For instance, patients with Linear IgA Bullous Diseases (LABD) have deposits of IgA autoantibodies directed against collagen XVII and neutrophil accumulation in the skin. LABD patients develop blisters, and we previously showed that neutrophil activation and concomitant tissue damage is a direct consequence of neutrophil FcαRI triggering through IgA autoantibodies using an ex-vivo model of this skin blistering disease. Moreover, we recently established a novel LABD mouse model and showed a critical in vivo role of neutrophil FcαRI and IgA autoantibody interactions in activating neutrophils resulting in accumulation of cells and tissue damage (chapter 5).

Neutrophil activation through IgA autoantibodies might also play a role in other autoimmune diseases, such as rheumatoid arthritis (RA). This is a chronic inflammatory disease affecting the joints, causing pain, stiffness and inflammation of the synovial membrane ultimately leading to articular destruction. Interestingly, several studies reported a correlation between the presence of IgA autoantibodies and extra-articular manifestations and worse disease prognosis of patients with RA, which has been suggested as diagnostic tool.

We recently demonstrated that IgA immune complexes present in plasma and synovial fluid (SF) of RA patients induce prominent neutrophil activation and release of NETs via FcαRI in vitro. As such, we hypothesize that IgA autoantibodies may be involved in the pathogenesis of RA. The contribution of IgA in autoimmune diseases is poorly understood as in vivo evidence for a pathogenic role of FcαRI/IgA autoantibody interactions is limited. This is largely due to the absence of suitable in vivo models, as mice do not express FcαRI. Previously, human FcαRI transgenic mice were generated that express human FcαRI on cells of the myeloid lineage, mostly neutrophils, closely corresponding to the human situation. Additionally, human IgA knock-in mice were generated. These models now allow the investigation of the role of FcαRI/IgA interactions in RA.

The contribution of autoantibodies in pathogenesis of arthritis can be investigated with different models. The presence of anti-collagen type II (COL2) antibodies and collagen II-specific T cells indicates that COL2 is one of the candidate autoantigens in human RA. Since COL2 is exclusively expressed in the articular cartilage of joints, the
presence of autoantibodies against COL2 induces inflammation and ultimately joint destruction in mice. Arthritis can be induced by passive transfer of a cocktail of monoclonal antibodies that target various regions of collagen type II, referred to as collagen antibody induced arthritis (CAIA). An advantage of this model is that autoantibodies can initiate disease independently of B and T cells during the effector phase of arthritis and that it is characterized by macrophage and polymorphonuclear inflammatory cell infiltrates. Therefore the CAIA model can provide insight into the separate roles of innate and adaptive immune responses in the development of arthritis. Furthermore, disease is induced within a few days rather than the several weeks that are required in other models. The clinical signs of disease are polyarthritis, which is most prominent in the limbs, and the presence of inflammatory cells in the synovium, as well as cartilage and bone erosions, and synovial hyperplasia, similar to human RA.

Another frequently used animal model is collagen induced arthritis (CIA). Two characteristics of the CIA model, namely breaking of tolerance and generation of autoantibodies directed against self-collagen, makes it a ‘golden standard’ in preclinical rheumatology research. CIA can be induced in genetically susceptible mouse strains by active immunization with heterologous COL2 with adjuvant. The development of CIA is associated with the production of anti-COL2 autoantibodies by B cells and COL2-specific T cells. The clinical development of arthritis in CIA is similar to CAIA, but is associated with T-and B-cell responses.

We now used CAIA and CIA models in FcαRI/hIgA mice to investigate whether IgA-induced neutrophil activation via FcαRI contributes to the pathology of arthritis.

Materials and Methods

Mice

Transgenic mice that express FcαRI on neutrophils were backcrossed onto BALB/c genetic background. FcαRI mice were crossed with mice that were human IgA (hIgA) knock-in on a BALB/c background. FcαRI/hIgA BALB/c mice were 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 a hybridoma cell line producing anti-bovine collagen II hIgA antibodies

hIgA knock-in mice were immunized subcutaneously with 100 μg bovine collagen II (Chondrex, Redmond, WA) in Complete Freund’s Adjuvant (Sigma-Aldrich, St. Louis, MO), followed by three booster injections (day 14, 28 and 42) of 100 μg bovine collagen II (bCOL2) in Incomplete Freund’s Adjuvant (Sigma-Aldrich). Mice were sacrificed and mouse spleens were harvested. SP2/0 mouse myeloma cells were fused with splenocytes from immunized mice according to standard protocols. Positive hybridoma clones that produced anti-bCOL2 hIgA antibodies were selected by a bCOL2 hIgA ELISA and were also tested for their ability to bind to mouse collagen II (mCOL2, cross-reactivity; see
also ELISA M&M). To obtain monoclonal lines, cells from positive wells were subcloned by limiting dilution. Subcloning of monoclonal lines was repeated twice to make sure that the lines that were generated were derived from single clones and then frozen in liquid nitrogen. Anti-bCOL2hIgA 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).

**Isolation of human neutrophils from healthy control donors**
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) supplemented with 10% FCS, glutamine and antibiotics. 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). Neutrophils were labeled with calcein-acetoxyxymethylester (1 μmol/L; Molecular Probes Inc, Eugene, OR) for binding assays according to the manufacturer’s instructions.

**Ligand binding assay**
Flat well microtitre ELISA plates (Nunc-Immuno MaxiSorp, Roskilde, Denmark) were coated with 100 µl anti-bCOL2 hIgA (10 µg/ml) or bovine serum albumin (10µg/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 to remove non-bound cells and used for lactoferrin (degranulation marker for neutrophils) ELISA to investigate activation of neutrophils. 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**
*Bovine/mouse collagen type II ELISA*
Flat well microtitre ELISA plates (Nunc-Immuno MaxiSorp) were coated with bovine collagen II or mouse collagen II (500 ng/well; Chondrex) 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-bCOL2 hIgA as positive control (1 hour, 37 °C). Platers were washed with PBST, followed by incubation with biotin-labelled mouse anti-human IgA mAbs or anti-mouse IgG 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-Immuo 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.

**ROS production**
The wells of flat well microtitre ELISA plates (Nunc-Immuo MaxiSorp) were coated with anti-bCOL2 hIgA or BSA (negative control). PMNs were preloaded for 20 minutes at 37°C with the fluorescent probe CM-H$_2$DCFDA (10 µM; Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s guidelines. After incubation, cells were washed twice and resuspended in RPMI 1640 (Gibco BRL) supplemented with 10% FCS, glutamine and antibiotics and transferred to the pre-coated black 96 wells plate. Optical density was measured every 5 minutes for 1 hour in a preheated fluorimeter at 37°C (FLUOstar/ POLARstar) at 480 nm excitation, 520 nm emission.

**Injection of anti-bovine collagen hIgA antibodies (CAIA)**
One group of FcaRI/hIgA mice (n=3) was injected with 4 mg anti-bCOL2 hIgA antibodies on day 0, whereas a second group received 4 mg on day 0 and 4 mg on day 1 (8 mg in total) (n=3). Blood samples were collected on day 0, 1, 2, 3, 4. Blood was centrifuged and serum was stored at -20 °C until further use.

**Induction of CIA and evaluation of arthritis**
FcaRI/hIgA mice (Sacrificed week 3, 5, 7 and 10, n=5 each group) were immunized intradermally at the base of the tail with 100 µg bovine collagen II (Chondrex) in Complete Freund’s Adjuvant (Sigma-Aldrich), followed by a booster injection on day 14 with 100 µg bovine collagen II in Incomplete Freund’s Adjuvant (Sigma-Aldrich). Development of clinical arthritis was followed based on scoring the number of inflamed joints in each paw using an scoring protocol. In this scoring system, each inflamed toe or knuckle gets 1 point, whereas an inflamed wrist of ankle gets 5 points, resulting in a score of 0-15 (5 toes + 5 knuckles + 1 wrist/ankle) for each paw and 0-60 points for each mouse. To assess articular function, mice were placed on a grid. The grid was then slowly flipped, and the length of time that the mice held on to the grid was recorded (maximal 60
This test was repeated 3 times.

**Flow cytometry**
Mice were sacrificed, and blood was collected by cardiac puncture. Bone marrow cells were isolated from femur and tibia. After removing hind legs, muscles were cleaned from the bones with cotton tissue. Cleaned bones were cut open on both sides with scissors and bone marrow was flushed with a 1 ml syringe and 24G needle. Single-cell suspensions were obtained by resuspending cell clumps through a 26G needle a few times and isolated cells were centrifuged.
Blood and single cell suspensions of bone marrow were stained for 30 minutes at 4 °C with anti-GR-1 PE antibody to identify neutrophils (Clone RB6-8C5, BD Biosciences), followed by lysis of red blood cells by BD FACS lysing solution (BD Bioscience). Cells were washed with FACS buffer (0.5% BSA in PBS) and samples were analyzed by flow cytometry (FACSCalibur).

**Immunofluorescence**
Mouse knee or feet cryosections (12-14 μm) of FcαRI/hIgA transgenic mice and hIgA knock-in mice were fixed in acetone and air-dried. Sections were stained with anti-mouse GR-1 FITC mAb (Clone RB6-8C5, eBioscience, San Diego, CA; 1 hour, RT), anti-hIgA FITC mAb (DAKO, Heverlee, Belgium; 1 hour, RT) or anti-mIgG 488 mAb (Life Technologies; 1 hour, RT). For nuclear staining diamidino-2-phenylindole (DAPI) was used. Cryosections were analyzed with a Leica DM6000 microscope (Leica Microsystems B.V., Rijswijk, The Netherlands).

**Statistical analysis**
All statistical analyses were performed using GraphPad Prism version 4.03 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 tests (comparing 2 groups) or ANOVA (comparing >2 groups) or Chi-square test. Significance was accepted when p < 0.05.
## Results

### Generation and characterization of bovine collagen II specific antibodies

*In vivo* studies investigating the role of IgA and FcαRI have been limited, since mice do not express a FcαRI homologue and the murine IgA system differs from the human situation. We used a human FcαRI transgenic mouse model to overcome the lack of expression of a FcαRI homologue in mice. However, mouse IgA does not bind to human FcαRI. To overcome this limitation, hIgA knock-in mice were immunized with bovine collagen II to obtain a hybridoma cell line producing anti-bCOL2 hIgA. After primary immunization and in between booster injections (data not shown), the production of antibodies was tested by obtaining serum samples of immunized mice. Antibody titers were determined with a binding ELISA specific for bovine collagen II (bCOL2). On day 58 after immunization, hIgA specific antibodies directed against bCOL2 were detected in the serum of immunized mice (Figure 1A). Additionally, cross-reactivity with mouse collagen II (mCOL2) was checked, to assure their ability to bind to mouse collagen II expressed at the articular cartilage of joints of mice (Figure 1A). Purified anti-bCOL2 hIgA antibodies were first characterized for binding capacity. No binding of PBS (as control) but strong binding of anti-bCOL2 hIgA to bCOL2 and mCOL2 was demonstrated (Figure 1A). This validated that purified anti-bCOL2 hIgA antibodies were directed against bCOL2 and cross-react with mCOL2, which enabled us to study the role of neutrophil FcαRI and IgA interactions in experimental arthritis.

A ligand binding assay was performed to investigate whether human neutrophils that express FcαRI were able to bind to purified anti-bCOL2 hIgA antibodies. Wells of 96 wells plate were coated with anti-bCOL2 hIgA or BSA as control, after which calcein labeled neutrophils were added. Unbound cells were washed away and the number of bound cells was quantified by measuring the fluorescence after cell lysis. Minimal neutrophil binding was observed to BSA, whereas a high number of neutrophils was able to bind to anti-bCOL2 hIgA antibodies (Figure 1B). Next we determined whether anti-bCOL2 hIgA antibodies were functional and induced cell activation. Neutrophil activation initiates degranulation, with release of granules and production of ROS. Lactoferrin, a secondary granule component, was used as a measure of degranulation. We analyzed the concentration of lactoferrin in supernatants of ligand binding assays. Addition of neutrophils to BSA coated wells did not induce neutrophil degranulation, which is in line with the inability of neutrophils to bind to BSA. In contrast, lactoferrin release by neutrophils was observed when neutrophils were added to anti-bCOL2 hIgA coated wells (Figure 1C). Furthermore, we demonstrated ROS production over time after addition of neutrophils to anti-bCOL2 hIgA coated wells (Figure 1D). Taken together, these data demonstrate the successful generation of an anti-bCOL2 hIgA antibody that crossreacts with mCOL2, is able to bind human FcαRI and induces neutrophil activation, measured by the release of lactoferrin and the production of ROS. Thus these anti-bCOL2 hIgA antibodies can be used to investigate the *in vivo* role of IgA-induced neutrophil activation in CAIA using human FcαRI transgenic mice.

### The presence of human IgA antibodies in mice after injection

CAIA is the preferable experimental arthritis model to investigate the role of neutrophil
CIA in FcαRI transgenic mice

Figure 1. Anti-bCOL2 hIgA antibodies bind and activate neutrophils. (A) Bovine and mouse collagen type II ELISA. Anti-bCOL2 hIgA antibodies were measured in serum of immunized mouse (left panel) and purified anti-bCOL2 hIgA antibody (right panel). The presence of hIgA antibodies directed against bCOL2 was tested and cross-reactivity with mCOL2 was checked, as the produced autoantibodies against bCOL2 need to cross-react with mCOL2 to assure their ability to bind to mouse collagen II expressed at the articular cartilage of joints of mice. (B) A ligand binding assay was performed in which fluorescence labeled neutrophils were added to BSA or anti-bCOL2 hIgA coated wells. Non bound cells were washed away and the number of bound cells was quantified by measuring the fluorescence after cell lysis. (C) Concentration of lactoferrin in supernatants of ligand binding assay, as a measure of neutrophil degranulation. (D) ROS production after addition of neutrophils to wells that were coated with BSA or anti-bCOL2 hIgA antibodies. Data are presented as mean ± SD. ****p<0.0001.

FcαRI/IgA interactions, as it is characterized by macrophage and polymorphonuclear inflammatory cell infiltrates. Additionally, it bypasses the initial phase of immune responses involving T and B cells allowing investigation of the effector phase of arthritis. We first tested the period of time that antibodies were still present in mice blood after injection of anti-bCOL2 hIgA antibodies in FcαRI/hIgA mice (see injection scheme in Figure 2A). One group of mice received one intraperitoneal injection with 4 mg of antibodies (day 0), whereas the second group received two intraperitoneal injections over two days (day 0 and 1) and thus received a total of 8 mg antibodies (Figure 2A). Blood samples were taken during 5 consecutive days and serum was tested for the presence of injected antibodies with a hIgA bCOL2 ELISA. Anti-bCOL2 hIgA antibodies were detectable in the serum of injected mice one day after injection (Figure 2B). However, antibodies (4 mg) were cleared from the circulation of mice within 48 hour. The injection of twice the amount of antibodies (8 mg) resulted in the presence of specific anti-bCOL2 hIgA antibodies in the blood of mice up to 48 hours (Figure 2B). Nonetheless, IgA had been cleared at day 3.
Chapter 4

**Figure 2. Concentration of anti-bCOL2 hIgA antibodies in mice** (A) Experimental setup. One group of mice (n=3) received one intraperitoneal injection with 4 mg of anti-bCOL2 hIgA antibodies (day 0). The second group (n=3) received two intraperitoneal injections over two days (day 0 and 1) and thus received a total of 8 mg antibodies. Blood samples were taken on day 0, 1, 2, 3 and 4. (B) Presence of anti-bCOL2 hIgA antibodies in serum of mice (group 1 and 2) at different time points.

**Immunization against bovine collagen II induces antibody production**

The short half-life of hIgA antibodies in mice precluded the use of CAIA as a model, as this would require daily injections of antibodies for an extended period. We therefore switched to CIA, in which we immunized mice with bCOL2 and sacrificed mice on different time points (week 3, 5, 7 and 10) after immunization. We first verified whether immunized mice produced antibodies against bCOL2. As the presence of antibodies that are cross-reactive with mCOL2 is required to induce arthritis, we tested antibody production in a bCOL2 and mCOL2 binding ELISA. Mice generated anti-bCOL2 hIgA antibodies (x-as), which were cross-reactive with mCOL2 (y-as) (Figure 3A). Anti-bCOL2 hIgA antibodies were detected in the serum of all immunized mice after 3, 5, 7 and 10 weeks (Figure 3A). Antibody production increased over time. Most mice also produced anti-bCOL2 mIgG antibodies, although antibody production decreased over time compared to hIgA (Figure 3B). Immunofluorescence staining of knee cryosections of immunized mice showed binding of hIgA antibodies (Figure 3C) and mIgG antibodies (Figure 3D) to the cartilage, as collagen II is expressed in the cartilage of joints. Although in low numbers, neutrophil influx was detected in the knees of immunized mice, compared to their absence in non-immunized mice as shown in the red circles that represent part of the synovium (Figure 3E).

**Collagen II-induced inflammation in FcαRI transgenic mice**

In addition to specific antibody production, a significant increase in blood neutrophil numbers was observed 3, 5 and 7 weeks after immunization and a massive increase was seen after 10 weeks (Figure 4A). Additionally, bone marrow neutrophil numbers were also
CIA in FcαRI transgenic mice

Figure 3. The production of autoantibodies and the presence of neutrophils after immunization with bovine collagen II in FcαRI/hIgA mice. CIA was used as a model in which mice were immunized with bCOL2 in CFA on day 0. A booster injection with bCOL2 in IFA was given on day 14. Mice were sacrificed 3, 5, 7 and 10 weeks after immunization. (A-B) The presence of antibodies directed against bCOL2 was tested, and cross-reactivity with mCOL2 was checked, which is necessary to assure binding of antibodies to articular cartilage. Bovine (x-as) and mouse (y-as) collagen II binding ELISA for (A) hIgA and (B) mIgG in mice serum 3, 5, 7 and 10 weeks after immunization. (C-E) Cryssections of knees of non-immunized (upper row) or immunized (sacrificed at week 10; lower row) FcαRI/hIgA mice stained with (C) anti-hIgA (green, left panels), (D) mIgG (green, middle panels) and (E) the neutrophil marker GR-1 (green, right panels). DNA was visualized with DAPI (blue). Arrows represent binding of hIgA or mIgG antibodies to the articular cartilage. (E) Red circles represent the presence of neutrophils in part of the synovium. Arrows represent the presence of neutrophils in the bone marrow. AC: articular cartilage; BM: bone marrow; M: meniscus; S: synovium.

increased (Figure 4B), which supports the presence of an inflammatory response in these mice. However, a decrease in bone marrow neutrophils was observed on week 10 when compared to week 7, which might be explained by the increased neutrophil number in the blood and knees of mice on week 10. We monitored the mice for inflamed joints in the paws and they were sacrificed in groups on week 3, 5, 7 and 10. Mice did not develop clinical signs of arthritis 10 weeks after immunization, as we did not observe paw and ankle swelling. However, as functionality/strength of the joints could be already affected, we tested the ability of the mice to hold on to a grid as a measure of joint function 24.
Control mice were able to hold on to the grid for 60 seconds (maximum duration of assay) while in contrast, 4 out of 5 of the immunized mice did not succeed in holding on to the grid (Figure 4C). This suggests the initiation of loss of joint function, a feature also observed in RA patients.

Figure 4. Induction of inflammatory responses after immunization with bovine collagen II in FcαRI/hIgA mice. (A-B) Neutrophil numbers determined with a GR-1 staining at 3, 5, 7 and 10 weeks after immunization of FcαRI/hIgA mice in (A) blood and (B) bone marrow. (C) Immunized mice were tested for their joint functionality with an articular function test. Their ability to hang on a grid for maximal 60 seconds was tested 10 weeks after immunization. Bars represent mean ± SD. *p<0.05, ***p<0.001, ****p<0.0001.
Discussion

Several studies reported a correlation between the presence of IgA autoantibodies and worse disease course in RA patients. Recently we have shown that IgA RF present in plasma and synovial fluid of RA patients induce neutrophil activation and the release of NETs. The activation of neutrophils by IgA immune complexes suggests that neutrophils can play an important role in the pathogenesis of RA. Excessive neutrophil activation through IgA autoantibodies and FcαRI interactions can induce tissue damage, but knowledge about their in vivo role in autoimmune diseases is lacking. In this study we set out to develop an in vivo model to study the interaction of IgA and FcαRI during the pathogenesis of arthritis.

To investigate the role of neutrophil FcαRI/IgA interactions in vivo, CAIA is the most suitable model to use, as it is a T cell and B cell independent model. Although we succeeded to obtain hIgA antibodies against mouse collagen II, anti-bCOL2 hIgA antibodies were not detectable in serum two days after passive transfer. Due to the short half-life of hIgA antibodies in mice, daily injections for an extended period would be required to reach an effect comparable to IgG antibodies (commercial available cocktail of anti-COL2 IgG antibodies) in the passive transfer CAIA model. The short half-life of human IgA in mice was previously shown to be problematic to use for in vivo antibody therapy of cancer. Recently it has been shown that this limitation can be successfully addressed by antibody engineering strategies to prolong the in vivo half-life of IgA. Implementation of these strategies may result in prolonged in vivo half-life of our anti-bCOL2 antibodies. As these modified IgA antibodies are currently not available, we switched to the CIA-model, in which we immunized FcαRI/hIgA mice with bovine collagen II. Immunization resulted in anti-bovine collagen II antibody production and increased neutrophil numbers in blood and bone marrow. This is an advantage compared to the CAIA model as there is a persistent anti-bCOL2 hIgA antibody production by plasma cells, which assures the continuous presence of these antibodies in the circulation of mice. Although clinical signs of disease did not develop in FcαRI/hIgA mice 10 weeks after immunization, joint functionality was decreased since immunized mice could not hold to the grid as long as control mice, supporting the induction of joint inflammation.

In other mice strains, mice develop clinical signs of arthritis over a wide range of time, starting around day 21, with peak incidence reaching from 40 to 50 days after immunization. IgG antibodies directed against collagen II, inflammatory infiltrates and joint destruction are detectable at early (up to 2 weeks after onset) and late (6 to 8 weeks after onset) stage of disease. The phenotype of arthritis was shown to be milder in C57BL/6 mice than in DBA/1 mice, with less swelling and a more gradual increase in clinical score. Our mice were of the BALB/c background, which are less susceptible for the induction of arthritis, which may explain the absence of clinical signs. In our FcαRI/ hIgA mouse model, modest neutrophil influx to the knees was observed at 10 weeks, suggesting that the inflammation was just starting. Reduced neutrophil numbers were observed in bone marrow 10 weeks after immunization, which might be explained by recruitment to blood and joint cartilage. The lack of massive neutrophil migration and accumulation in the joints can be the reason of the lack of clinical signs of arthritis in these.
mice, although the starting inflammation was sufficient to diminish joint functionality. Immunized FcαRI/hIgA mice produced both hIgA and mouse IgG anti-bCOL2 antibodies. As binding of IgG antibodies to mouse cartilage was detected, this implies that these complexes might also activate neutrophils via Fcγ receptors. It has been shown that DBA/1 mice lacking FcγR chain were protected from CIA in contrast to wild-type mice, even though both groups produced similar levels of IgG anti-collagen antibodies. This suggests a crucial role of Fcγ receptors in triggering autoimmune arthritis. However, previously we demonstrated that crosslinking of Fcγ receptors by IgG did not induce LTB4 release and consequently did not mediate neutrophil migration as this process seems to be specific for FcαRI triggering by IgA.

We recently established a novel LABD mouse model and showed a critical role of neutrophil FcαRI and IgA autoantibody interactions in vivo in activating neutrophils, which resulted in accumulation of neutrophils and severe tissue damage (chapter 5). In our pilot arthritis model, the inflammatory process of the joints just started, and did not lead to overt clinical symptoms. Nonetheless, it is likely that neutrophil FcαRI and IgA autoantibody interactions play a role in RA in vivo comparable to LABD, as neutrophils are found in high numbers in the synovial fluid of RA patients.

We hypothesize that immune complexes of collagen II and IgA induce neutrophil activation via FcαRI. This leads to secretion of chemoattractants, resulting in amplification of neutrophil migration to the joints of RA patients. Subsequently, these newly recruited neutrophils will also get activated and this results in a positive feedback loop. The current conducted study was a pilot experiment in which we only used FcαRI/hIgA mice and control hIgA mice were not included. As such, formally evidence is lacking that the observed effects in immunized mice are FcαRI specific. In future experiments, control hIgA mice should be included in the studies and mice should be followed for a longer period than 10 weeks to see whether they will develop signs of disease on a later time point. Moreover, susceptibility to develop arthritis has been associated with the expression of certain major histocompatibility complex (MHC) class II molecules. FcαRI/hIgA mice have a BALB/c background, which is considered to be a more resistant strain. Backcrossing of this mouse model onto C57BL/6 or DBA/1 genetic background would be preferable, as those mice are more susceptible to develop CIA.

Taken together, based on the results with our novel in vivo LABD model (chapter 5), the in vitro study with RA patients’ material (chapter 3) and our current study, we propose that FcαRI/hIgA interactions might play a role in the pathogenesis of RA. However, this need to be further investigated to fully establish the in vivo role of neutrophil FcαRI/IgA interactions in the pathogenesis of RA.
References


CIA in FcαRI transgenic mice