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Abstract

Interleukin 22 (IL-22) expression is associated with increased joint destruction and disease progression in rheumatoid arthritis (RA). Although IL-22 is considered a pro-inflammatory cytokine, its mechanism of action in RA remains incompletely understood. Here, we used the collagen-induced arthritis model in IL-22 deficient (IL-22$^{-/-}$) mice to study the role of IL-22 in RA. In spite of normal disease incidence, disease severity is significantly diminished in IL-22$^{-/-}$ mice. Moreover, pathogenicity of Th17 cells and development and function of B cells are unaffected. In contrast, splenic plasma cells, as well as serum autoantibody titers, are reduced in the absence of IL-22. At the peak of disease, germinal centers (GCs) are severely reduced in the spleens of IL-22$^{-/-}$ mice, correlating with a decline in GC B cell numbers. Within the GC, we identified IL-22R1 expressing follicular dendritic cell-like stromal cells. Human lymphoid stromal cells respond to IL-22 *ex vivo* by inducing transcription of CXCL12 and CXCL13. We therefore postulate IL-22 as an important enhancer of the GC reaction, maintaining chemokine levels for the persistence of GC reactions,

essential for the production of autoantibody-secreting plasma cells. Blocking IL-22 might therefore prevent immune-complex deposition and destruction of joints in RA patients.

Introduction

Interleukin-22 (IL-22) is an IL-10 family member that signals through a heterodimer of the IL-10 receptor 2 (IL-10R2) and the IL-22 specific IL-22R1 subunits, which is expressed by non-hematopoietic cells only [1, 2]. IL-22 can be produced by CD4+ and CD8+ T cells, NK T cells and innate lymphoid cells [3-5]. It plays a major role in the defense against microbes at mucosal barriers by preventing systemic inflammation with (commensal) gut flora, and is involved in tissue repair [5-10].

Interestingly, IL-22 has also been implicated in the pathogenesis of rheumatoid arthritis (RA), a systemic autoimmune disease affecting the joints [2, 11-13]. Higher proportions of IL-22 expressing T cells were found in the circulation and in the inflamed synovium of RA patients [14-17]. Furthermore, IL-22 induces osteoclastogenesis through fibroblast activation, and serum IL-22 levels in RA patients correlate with radiographic progression of the disease. It has been demonstrated that IL-22 deficient (IL-22-/-) mice are partially protected against pannus formation and joint destruction upon collagen-induced arthritis (CIA), a mouse model for RA. Despite a reduced clinical arthritis score, an unexpected increase in pathological anti-collagen type II immunoglobulins was observed [12]. This is counterintuitive, as deposition of immune-complexes of autoantibodies directed against collagen type II (CII) in the joints is crucial for disease development. In contrast, IL-22 is not involved in experimental autoimmune encephalomyelitis (EAE) [18], and has a protective role in mouse

models for inflammatory bowel disease [19-22]. Finally, in bleomycin-induced airway inflammation, IL-22 can have both a protective and a pathological role, depending on the co-expression of IL-17A [23]. Clearly, this versatile role of IL-22 in diverse immune-related diseases justifies further investigation of IL-22, which could be regulated at the level of B cell selection and antibody affinity maturation that has been largely neglected to date.

The formation of high-affinity antibody producing plasma cells is T-cell dependent and initiates in the germinal center (GC) of B cell follicles. GCs can be divided into a dark zone, where B cells proliferate, and a light zone, in which selection of high affinity B cells is regulated by antigen presenting follicular dendritic cells (FDC) and follicular helper T (Tfh) cells. B cells migrate between these zones to assure the selection of high-affinity B cell clones only. The chemokines CXCL12 and CXCL13 are crucial for the guided migration of GC B cells within the GC, and disruption of either signal perturbs the development of high-affinity antibody producing plasma cells [24-26]. As ectopic GCs can also be formed in synovial tissues, and synovial fibroblasts are shown to express IL-22R1 [13, 27, 28], we were prompted to more closely investigate the IL-22/IL-22R1 axis in the regulation of the GC reaction.

Here, we demonstrate that IL-22-/- mice have reduced disease severity in a CIA model displaying lower levels of chicken and mouse CII-specific autoantibodies in the serum and fewer antibody producing plasma cells in the spleen. Supporting the decline in plasma cell numbers, we found that most GCs in the spleen were lost at 30 days post immunization. Analysis of secondary lymphoid organs at the peak of the immune reaction revealed IL-22R1 expression, specifically on stromal cells of the GC. Most interestingly, stimulation of cultured lymphoid-like stromal cells or

purified primary human FDC with IL-22 induced the expression of CXCL12 and CXCL13, respectively, suggesting that IL-22 is required to prolong the GC reaction through modulation of essential chemokines that are critical for the generation of high affinity (auto)antibodies.

Results

Severity of collagen-induced arthritis is reduced in IL-22-/- mice

Wild type and IL-22-/- mice were immunized with chicken collagen type II (CII) in complete Freund's adjuvant (CFA) and boosted 21 days after initial challenge to induce arthritis in the joints. Lack of IL-22 did not significantly change the incidence of arthritis (Figure 1A), but clearly diminished the severity of the disease (Figure 1B). In the absence of IL-22, mice developed a significantly less severe joint pathology and this was maintained for two weeks. These data confirm and extend the notion that IL-22 exacerbates autoimmune arthritis [12]. Notably, the incidence and onset of arthritis were similar in wild type and IL-22-/- mice, suggesting that IL-22 is not required for the early events leading to arthritis development. On the other hand, while boost challenge clearly worsened disease progression in wild type mice, this was not seen in IL-22-/- mice (Figure 1B). Taken together, the data demonstrate that IL-22 increases the severity of collagen-induced arthritis (CIA), which is predominantly evident after boost challenge.

Th17 cells from IL-22-/- mice retain pathogenic capacity

Previous studies have shown that IL-17A and Th17 cells play a critical role in the incidence and progression of arthritis [27, 29, 30]. In addition to IL-17A, Th17 cells also produce IL-22 [5, 14]. Therefore, we analyzed whether the absence of IL-22 had a negative effect on the development of IL-17A producing splenic CD4+ T cells in wild type and IL-22-/- mice at different time points after CII-immunization. Flow cytometry analysis revealed that the lack of IL-22 production did not hamper development of splenic CD4+ T cells producing IL-17A (Figure 2A). Moreover, the percentage of splenic Th17 cells within the CD4+ population was even higher in these mice ten days after immunization (Figure 2A). However, this difference in Th17 percentage was lost after 3 weeks, and was not observed after boost immunization. Since initially more IL-17A producing T cells were detected while the severity of arthritis was significantly lower, we investigated whether IL-22-/- and wild type Th17 cells were equally pathogenic. For this purpose, CD4+CCR6+ effector T cells, enriched for Th17 effector cells [31], were FACS sorted from spleens of mice 10 days after immunization with CII/CFA. The sorted cells were co-cultured with synovial fibroblasts to determine their capacity to induce a pro-inflammatory response in IL-22R1+ synovial fibroblasts, as measured by the expression of IL-6 [13, 27]. After three days, IL-6 production by synovial fibroblasts in the culture supernatant was measured by ELISA. As expected based on the increased frequency of Th17 cells in IL-22-/- mice ten days after immunization, IL-6 production by the synovial fibroblasts was increased after co-culture with IL-22-/- T cells as compared to co-culture with wild type T cells (Figure 2B). Hence, despite reduced arthritis severity, effector T cells isolated from challenged IL-22-/- mice retained the ability to activate synovial fibroblasts. In line with this, the proportion of IL-17A producing T cells after culture remained significantly elevated in IL-22-/- T cells compared to wild type T cells (Figure

2C). These findings show that IL-17A producing T cells are formed in the absence of IL-22, and that these cells retain pathogenic potential as demonstrated by this *in vitro* assay.

As the *in vitro* activity of the Th17 cells is unaffected by the absence of IL-22, we set out to test whether in vivo Th1 and Th17 cell responses were altered IL-22-/- mice. For this reason, we induced a T cell-dependent delayed-type hypersensitivity (DTH) response in these mice [32]. Wild type and IL-22-/- mice were immunized with mBSA/CFA followed by a secondary injection with mBSA in the footpads, seven days later. Supporting the *in vitro* data, mice showed comparable DTH responses measured by swelling of the footpad (Figure 2D). Together, these data imply that Th1/Th17-mediated cell responses are unaffected in IL-22-/- mice. Therefore the reduced severity of CIA in IL-22-/- mice is not likely caused by hampered T-cell immunity.

Plasma cell formation and CII-specific antibody production are impaired in IL-22-/- mice

In addition to Th17 cells, CIA development depends highly on the humoral immune response through the production of pathogenic antibodies, formation of immune complexes and activation of complement [33]. We therefore analyzed B cell responses to collagen in wild type and IL-22-/- mice during CIA development. Just before boost immunization (day 20), FACS analysis for IgM or IgG2 positive plasma cells in the spleen revealed no differences between the two groups (Figure 3A). Nine days after boost immunization with CII/CFA (day 30), the number of IgM and IgG2 expressing plasma cells were elevated in wild type mice but not in IL-22-/- mice. Importantly, the lower number of class-switched plasma cells in the spleen was associated with a marked decrease in the concentration of chicken CII-specific IgG2c antibodies in the serum of IL-22-/- mice during CIA (Figure

3B). Moreover, the serum levels of pathogenic self-reactive anti-mouse CII-specific IgG2c antibodies were also significantly lower when compared to wild type controls. The large variation in antibody levels reflects the variability in disease scores found in CIA, as both diseased animals and animals that remained disease free were included in the analysis. This impaired humoral autoimmune response suggests that IL-22 is required for the differentiation or maintenance of autoantibody producing plasma cells.

Development and activation of IL-22-/- B cells is normal

Neither naïve B cells nor plasma cells express IL-22 or the IL-22 receptor [1, 3-5, 13]. However, to exclude indirect developmental defects caused by the genetic deletion of IL-22, homeostatic B cell differentiation was analyzed in wild type and IL-22-/- mice. Upon leaving the bone marrow as immature B cells, they arrive in the spleen as IgM+IgD(low) transitional B cells. Upon maturation into naïve B cells, they reduce the expression of the IgM B cell receptor (BCR) and highly express IgD at their surface. The ratio of transitional versus mature follicular B cells in the spleen was comparable between wild type and IL-22-/- mice (Figure 4A), as were total numbers of splenic CD19+B220+ B cells (data not shown). Importantly, immunization with CII/CFA did not alter total splenic B cell numbers (Figure 4B). To further demonstrate that B cells from IL-22-/- mice were functional, we MACS purified naïve splenic B cells and cultured them with anti-IgM $F(ab)_2$ fragments, LPS or LPS + IL-4. Proliferation was comparable between IL-22-/- and wild type B cells after 2 days of BCR or LPS stimulation (Figure 4C). There were no differences between wild type and IL-22-/- B cells in expression of activation markers, including CD25, CD69 and CD86, when comparing similar time

points (data not shown). Furthermore, we found no intrinsic defects in class-switch recombination potential in IL-22-/- B cells after four days of LPS or LPS + IL-4 stimulation (Figure 4D), as the cells readily switched towards IgG1 or IgG3 expressing plasmablasts. Importantly, this was independent of disease progression, as MACS-purified B cells at day 30 after immunization of both genotypes responded equally well to the same stimuli (data not shown). Together, these data reveal that *in vivo* B cell development is normal in IL-22-/- mice and that these B cells respond equally well *in vitro*, compared to their wild type counterparts.

Fewer and smaller germinal centers during CIA in the absence of IL-22

Since the development and *in vitro* activation of B cells was normal in IL-22-/- mice, we hypothesized that development or maintenance of germinal centers (GCs), the anatomical structures needed for immunoglobulin generation *in vivo*, might be altered in the absence of IL-22. This would fit with the notion that the IL-22R is expressed on non-hematopoietic rather than on hematopoietic cells. The largest difference in CII-specific autoantibodies in the CIA model was observed after boost immunization (Figure 3B). Therefore we analyzed spleens from wild type and IL-22-/- mice for the presence and phenotype of GCs ten days after the first immunization and at the peak of the secondary immune response (day 30). At the early time-point (d10) we found no difference in GC formation in the spleen by immunohistochemistry (data not shown). However, at day 30, we found not only fewer (Figure 5A), but also much smaller GCs in the absence of IL-22 (Figure 5B). These findings suggest that IL-22 is a limiting factor for the maintenance of GCs during CIA. Crucially,

plasma cells and autoantibodies mostly arise after boost immunization in wild type mice (Figure3A-B), at which time-point GCs are almost gone in IL-22-/- mice.

IL-22 induces GC chemokines in IL-22R1 expressing lymphoid stromal cells

IL-22R is expressed on non-hematopoietic epithelial and stromal cells. However, little is known about the expression of the IL-22R on stromal cells within lymphoid organs. To gain insight into the cells that might link IL-22 to GCs we immunized mice with the model antigen TNP-KLH and analyzed reactive lymph nodes for expression and localization of IL-22R. In naïve lymph nodes, we could not detect any IL-22R expression. However, 10 days after immunization, at the peak of the GC reaction, we were able to detect IL-22R protein exclusively within the GCs (Figure 6A). IL-22R1 was expressed in both the dark and light zone of the GC. Since the staining pattern resembled FDC, we labeled sections with FDC-specific antibodies and indeed observed a partial overlap with IL-22R staining. In addition, dark zone stromal cells not expressing classic FDC markers were also IL-22R positive. These data provide the cellular link between IL-22 and GC stromal cells. Both FDC and dark zone stromal cells are functionally involved in GC reactions by the production of the B cell chemokines CXCL13 and CXCL12. To link IL-22 to chemokine production we isolated FDC from human pediatric tonsils. By using human lymphoid tissues we were able to isolate sufficient FDC for overnight culture. Upon culture of FDC from 3 different donors in the presence or absence of IL-22 an increase in *CXCL13* transcription was detected (Figure 6B). In addition, when we stimulated human IL-22R1+ adipocyte-derived stromal cells (ADSCs) with IL-22, we observed a significant

induction of *CXCL12* expression. Notably, *IL-22R1* expression could be further increased on these ADSCs upon activation with the pro-inflammatory cytokine TNFα (Figure 6C).

Taken together, our data imply that IL-22R1 is expressed on GC stromal cells, and that IL-22 may be involved in regulating B cell chemokines within the GC.

Discussion

Rheumatoid arthritis (RA) is a complex autoimmune disease of which the etiology is still largely unknown. Despite the identification of multiple genetic and risk factors, it is still unclear how the autoimmune reaction is transferred to the joints, and which cells are the most important inducers [34]. However, it has become apparent that multiple cell types are involved in the inflammation of the joint, and the subsequent joint destruction. Synovial fibroblasts and endothelial cells are activated, which attract immune cells, including B and T cells, by the production of chemokines and upregulation of adhesion molecules [35]. Of note, Th17 cells have been identified to be important drivers of RA, which was mainly ascribed to their production of IL-17A. Th17 cells also produce IL-22 and higher proportions of IL-22 expressing T cells were found in the circulation and in the inflamed synovium of RA patients, in combination with elevated serum IL-22 levels that correlated with radiographic progression of the disease. We therefore set out to identify the role of IL-22 in the onset and progression of arthritis. For this purpose we made use of IL-22 deficient mice (IL-22-/-) and subjected them to collagen-induced arthritis (CIA).

IL-22-/- mice developed less severe arthritis upon induction of CIA. Importantly, this was only observed when a homozygous allele for IL-22 deficiency was present, as IL-22+/- developed an equally severe collagen-induced arthritis compared to wild type littermates (not shown), ruling out a role for the abnormal microbiota in IL-22-/- mice in the development of autoimmune diseases [36]. Remarkably, however, the incidence was rather similar or even slightly higher compared to the wild type counterparts. The increase in incidence may be explained by the observed increase in pathogenic IL-17A producing T cells in the spleens of IL-22-/- mice ten days after immunization. In support, these Th17 cells were more potent at inducing pro-inflammatory IL-6 when co-cultured with synovial fibroblasts, and we found a significant increase in IL-17A producing cells after co-culture. A delayed-type hypersensitivity response, however, showed no functional differences in Th1/Th17 function *in vivo*. Therefore, we speculate that IL-17A producing CD4+ T cells are important for the initial trigger to develop arthritis upon CIA, but their pathogenicity in synovium could not explain the difference observed in severity of the arthritis.

Since arthritis development also highly depends on B cells, as μMT mice that lack all B cells were completely protected against CIA [33], we more closely examined the B cell response. Nine days after boost immunization, we found fewer plasma cells in the spleens of IL-22-/- mice. This impaired plasma cell formation resulted in an evident block in the production of CII-specific autoantibodies measured at almost 3 weeks after re-challenge. While in general we observed similar results as previously demonstrated [12], some important discrepancies have to be mentioned. First, in the earlier study, the initial onset of the disease was observed 25 days after immunization, which is 10 days later compared to our experiments. Secondly, the maximum severity score at 37 days

after immunization was approximately 35% (5.5 out of 16) for both genotypes, while we measured a maximum severity score of ~50% (4 out of 8) in wild type and 25% (2 out of 8) in IL-22-/- mice. Thirdly, we included more than twice the number of mice per group to avoid unwanted low incidence, which is a precedent of this RA mouse model and which could affect data interpretation. Indeed, they found a lower severity in IL-22-/- mice, but due to a low number of animals no significant difference was measured. Finally, the authors observed that with equal severity and shortly after onset of the disease (3 days) CII-specific antibody titers were higher in IL-22-/- mice. Although unexpected, it is important to note that in general the total IgG titers in the IL-22-/- mice were higher compared to wild type controls, while still 4-5 times lower compared to our anti-mouse CII-specific IgG2c autoantibody titers (data not shown). This suggests that the time point of measurement by Geboes and colleagues might be too early to detect the differences we observed. As such, in line with lower disease severity, we found lower autoantibody titers after an additional 3 weeks of disease development (at the peak of disease severity) in IL-22-/- mice.

It is important to note that the numbers of B cells in IL-22-/- spleens before and after immunization were comparable to wild type, and that the ratio of transitional/follicular mature B cells was similar. In addition, *in vitro* stimulations resulted in equal proliferation and class-switch recombination. Hence, IL-22-/- B cells are fully functional and the lack of IL-22 should be an indirect effect. As we found a significantly lower amount of plasma cells and autoantibodies in IL-22-/- mice, we shifted our focus towards GCs. GCs are essential for the generation of class-switched plasma cells, which are selected for high affinity towards presented antigens [25, 37]. At the peak of disease development, IL-22-/- mice had significantly fewer GCs and overall GC B cells in the spleen. Most

interestingly, we could not only identify a decrease in numbers of GCs, but the GCs that were still present were also much smaller. To date it has been appreciated that the chemokines CXCL12 and CXCL13 play a key role in the regulation, endurance and maintenance of the GC reaction [24, 37-39]. More specifically, reticular cells in the dark zone express CXCL12, while FDCs in the light zone of the GC express CXCL13 [38, 39]. Both recruitment and retention of CXCR5+ B cells and follicular helper T cells (Tfh) to the GC is essential since the interaction of all the aforementioned cells has to take place at this specific location for a proper reaction [25, 37, 40]. Interference with chemokine (CXCL12 or CXCL13) or chemokine receptor (CXCR4 or CXCR5) expression could change GC stability and endurance. Interestingly, in BXD2 mice, Th17-derived IL-17 was shown to be important in the development of autoimmune disease by orchestrating the formation of spontaneous GCs through direct modulation of the chemotactic response to CXCL12 by B cells [41]. Here, we show that IL-22 could add another level of complexity to the GC reaction, by differentially regulating the expression levels of CXCL12 and CXCL13 by specific stromal cells of the GC, as was also shown for tertiary lymphoid structures [42]. Importantly, we show that IL-22R is expressed on stromal cells in the GC. The experiments described in this study were not designed to identify the cellular source of IL-22 during experimental RA, however a similar mechanism was shown for tertiary lymphoid structures, with the major source of IL-22 being (γδ)T cells [42].

Th17 cells, through their production of IL-17A have been shown to play an important role in GC formation in other models of autoimmunity and in the formation of tertiary lymphoid structures in the lung (iBALT) [41, 43]. Our data suggests that in CIA, Th17 cells may be important for GC development but might also be involved in GC maintenance through local IL-22 production. Of note,

we found that iBALT formation upon influenza infection was unaffected in IL-22-/- mice (data not shown). In addition, we show that TNFα, which is also produced by Th17 cells, can induce the expression of the IL-22R on stromal cells. Th17 cells are therefore likely candidates to facilitate several aspects of the GC reaction. In addition, stromal cells have also been shown to produce IL-22 and might potentially be involved.

In summary, our findings and those of others suggest that the Th17 cytokines IL-17A and IL-22 are partners in crime in the development of severe CIA [12, 13, 18, 41, 44, 45]. Where IL-17A most likely has a prominent role in the early onset of arthritis, IL-22 is mainly required in the later and more chronic phase of disease development. We therefore postulate that, unlike IL17A, IL-22 is not a major pro-inflammatory cytokine in the inflammatory phase of CIA, but it has a vital role in the regulation of effective secondary follicular structures. As IL-22 has a pivotal role in the chronic phase of the disease, interfering with IL-22 rather than IL-17A could be valuable for RA patients. Beneficial effects of IL-22 blocking antibodies have been found in several experimental arthritis models, including CIA [46, 47]. Administration of blocking antibodies after onset of arthritis in CIA significantly reduced anti-CII IgG2a antibodies [46]. Blockage of IL-22 expression might therefore abrogate ongoing GC reactions, thereby preventing the development of autoantibody producing plasma cells, ultimately leading to a decline in immune complex deposition and destruction of the joints.

Materials and methods

Mice

IL-22-/- mice on a C57BL/6 background [8] were kindly provided by Dr. Wenjun Ouyang, Genentech Inc., USA. Wild type C57BL/6 control mice were bred at the Erasmus Medical Center animal facility (EDC) or purchased from Harlan Laboratories B.V. (Horst, The Netherlands). Mice were kept under SPF conditions and were provided with food and water *ad libitum*. All experiments were approved by the Erasmus Medical Center Animal Ethical Committee. All mice were 8-12 weeks of age at the start of the experiments.

Immunizations

For collagen-induced arthritis (CIA), 2 mg/ml chicken collagen type II (CII) (Chondrex, USA) was emulsified in an equal Freund's adjuvant (CFA) volume containing 1 mg/ml heat-killed *Mycobacterium tuberculosis* (strain H37Ra; Difco Laboratories, Inc., Detroit, Mi). Mice were immunized by intra-dermal (i.d.) injection with 100µg CII/CFA emulsion and 21 days later mice were boosted subcutaneously (s.c.) with 100µg CII/CFA. Arthritis severity was scored macroscopically by assessing redness and swelling of all four paws. The maximum score per mouse was 8 (2 per paw), however mice were sacrificed for ethical reasons at a score ≥6.

For delayed-type hypersensitivity (DTH) reaction, 8 mg/ml methylated bovine serum albumin (mBSA, Sigma-Aldrich, St. Louis, MO) was emulsified in an equal CFA volume containing 1 mg/ml heat-killed *Mycobacterium tuberculosis* (strain H37Ra; Difco Laboratories). Mice were immunized with 100µg mBSA/CFA emulsion i.d. and were injected with 200µg mBSA in 10 µl 0.9% NaCl in the footpads 7 days later. Swelling of the footpads was measured 24 and 48 hours after challenge.

For TNP-KLH, immunizations were performed exactly as previously described [48].

Flow cytometry

For intracellular cytokine staining, $2*10^6$ splenocytes were stimulated with PMA (0.05 µg/ml) and Ionomycin (0.5µg/ml) in the presence of Golgi stop (BD Biosciences) for 4 hours. Cells were fixed with 2% PFA and permeabilized in 0.5% saponin. Anti-CD4 and anti-IL-17A antibodies were obtained from BD BioSciences.

For B cell stainings, $2*10^6$ splenocytes were incubated with antibody mix for 20 minutes at room temperature. For intracellular staining, cells were subsequently fixed and permeabilized using BD Cytofix/Cytoperm (BD BioSciences) and stained for intracellular markers for 20 minutes at room temperature. Anti-CD19, anti-B220 and anti-IgM were obtained from eBioscience (San Diego, CA, USA), anti-IgD, anti-IgG1, anti-IgG2ab and anti-CD95 from BD BioSciences and PNA from Sigma-Aldrich (St Louis, USA).
Samples were acquired on a FACS Canto II HTS or a FACS LSR II flow cytometer (BD BioSciences) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Lymph node stromal cells were isolated and FACS-sorted essentially as described [49, 50]. After single cell suspensions were obtained, cells were stained on ice for CD45 (clone MP-33, eBioscience, Halle-Zoersel, Belgium) PE.Cy7, unlabeled hamster anti-mouse gp38 (clone 8.1.1), developed with goat anti-hamster AlexaFluor 647, and CD31 labeled with AlexaFluor 488. Cells were sorted on a FACS ARIA (BD Bioscience) flow cytometer and files were analyzed with FlowJo software (TreeStar Inc.).

Co-culture experiments

Th17 cells were sorted from spleens obtained ten days after CII/CFA immunization by FACS sorting CD4-high, CD62L-low, CD25-low, CCR6-high expressing cells. Antibodies were obtained from BD BioSciences (San Diego, CA, USA) or Biolegend (San Diego, CA, USA) (anti-CCR6). Purity of obtained fraction was >98%. $15*10^3$ sorted cells were co-cultured with 10^4 synovial fibroblasts in IMDM medium (Lonza, Verviers, Belgium), supplemented with 10% FCS (Invitrogen, Carlsbad, CA), 100U/ml Penicillin/Streptomycin, 2mM L-Glutamin (Lonza) and 50μM β-mercapto-ethanol (Merck, Darmstadt, Germany). Th17 cells were stimulated with anti-CD3 and anti-CD28 (both 4μg/ml, BD Biosciences) for 96 hours. After 96 hours, IL-6 levels in supernatant were measured by ELISA and IL17A expressing cells were measured by flow cytometry.

B cell isolation and culture

Naïve B cells were isolated by depleting non-B cells, B1 cells, activated B cells and (pre)plasma cells by MACS sorting using anti-CD5, -CD43, -CD138, -CD11b, -GR-1 and -TER-119 antibodies (BD Biosciences). Purity of obtained cells was >95%. 10^5 purified cells were cultured for four days in IMDM medium (Lonza), supplemented with 10% FCS (Invitrogen), 100 U/ml Penicillin/Streptomycin, 2 mM L-Glutamin (Lonza) and 50 μM β-mercapto-ethanol (Merck) with LPS (5μg/mL, own production) in the presence or absence of recombinant IL-4 (50ng/mL, R&D systems).

Stromal cell culture, RNA extraction and quantitative PCR

An adipocyte-derived stromal cells kit was purchased from Life Technologies (Bleiswijk, the Netherlands) and maintained according to manufacturer's protocol. For stimulation, cells were plated in 24-wells plates at $5*10^4$ cells per well. After stimulation with TNFα or IL-22 (both from R&D Systems, Minneapolis, MN, USA) for indicated time duration, RNA was extracted using the RNA-XS kit (Machery Nagel) followed by reverse-transcription with random hexamer primers. For quantitative PCR, a Neviti Thermal Cycler (Applied Biosystems) and DyNAmo Flash SYBR Green qPCR kit (Finnzymes) were used, with the addition of $MgCl_2$ to a final concentration of 4mM. All reactions were done in duplicate and are normalized to the expression of GAPDH (glyceraldehyde phosphate dehydrogenase).

Human tonsils were obtained from children undergoing routine tonsillectomy after recruitment under institutional review board approval and informed consent process according to the Declaration of Helsinki. After enzymatic dissociation using dispase and collagenase (Worthington Biomedical Corp. Lakewood, NJ), FDC were cell-sorted on a FACSAria from $DAPI^{neg}CD45^{neg}CD31^{neg}CD34^{neg}CD11b^{neg}$ viable stromal cells (Abs were purchased from Miltenyi Biotech) based on the co-expression of gp38/podoplanin (clone eBio8.1.1, eBioscience) and CD21 long isoform (clone DRC1, Dako, Les Ulis, France). Cells were maintained overnight at $15*10^3$ cells /mL in MEM-α/10% FCS (Hyclone) in the presence or absence of IL-22 (20ng/mL, R&D systems) before RNA extraction and amplification (Nugen). For quantitative RQ-PCR, we used assay-on-demand primers and probes, and Taqman Universal Master Mix (Life Technologies). Gene expression was measured using the StepOnePlus (Life Technologies) based on the ΔC_t calculation method. *RPS17* was determined as the appropriate internal standard gene using TaqMan Endogenous Control

Assays (Life Technologies). Cell purity was checked by RQ-PCR for *CD19*, and *CD3*, before quantification of *CXCL13* expression.

ELISA

For CII-specific antibody measurement in serum, wells of microtiter plates were coated with 1µg/mL chicken or mouse CII (Chondrex, USA) in PBS overnight. Serum was added in serial dilution and incubated for three hours, followed by biotinylated anti-IgG2a antibodies (Southern Biotechnology) for one hour at room temperature. Optical density at 450nm was measured.

Measurements of IL-6 levels in culture supernatant were performed using a mouse IL-6 ELISA Duoset (R&D Systems, Minneapolis MN). ELISA was performed according to the manufacturer's instructions.

Immunohistochemistry

Spleen tissue was frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) and stored at -80^{o}C. 6µm thick samples were cut. Slides (Superfrost, Gerhard Menzel GmbH, Braunschweig, Germany) were fixed in acetone and endogenous peroxidase was blocked using 30% H_2O_2 (Sigma-Aldrich). Gal-β(1-3)-GalNAc expression on GC B cells was stained with biotinylated peanut agglutinin (PNA) (Sigma-Aldrich) and streptavidin (peroxidase labeled, Jackson ImmunoResearch, Westgrove, PA, USA) or rat-anti-IgD (eBioscience) and anti-rat (alkaline phosphatase labeled, Sigma-Aldrich). GCs were counted by screening a complete cross-section of

each spleen in low magnification. Pictures were made using a NanoZoomer (Hamamatsu Photonics Deutchland GmbH, Herrsching am Ammersee, Germany).

For fluorescent microscopy, 6μm sections were fixed in acetone for 10 minutes, blocked with 5% normal mouse serum, and subsequently incubated with rat anti-IgD, -CD35, -GL7 and -IL-22R1, as indicated. Secondary goat anti-rat antibodies labeled with AlexaFluor 488, 555, or 647 (all purchased from Life Technologies, Bleiswijk, the Netherlands) were used to visualize specific staining. Sections were analyzed on a Leica microscope and images were captured using FW4000 software (Both from Leica Microsystems, Rijswijk, The Netherlands). All images were processed in Adobe Photoshop CS3 (Adobe Systems, San Jose, CA).

Statistical analysis

Data was analyzed using Prism software v5.04 (GraphPad Software Inc. La Jollla, CA). A two-sided student's T-test was performed, unless indicated otherwise. P-values <0.05 were considered significant.

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Conflict of interest

The authors declare no financial or commercial conflict of interest

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Figure Legends

Figure 1. Scoring of collagen-induced arthritis in wild type and IL-22-/- mice. (A and B) WT and IL-22$^{-/-}$ mice were immunized at day 0 and boosted at day 21 with chicken CII/CFA. [A] Incidence of collagen-induced arthritis in wild type and IL-22-/- mice is shown. [B] Arthritis score of animals with disease development. Maximum score per mouse is 8. Mice with a score >6 were sacrificed for ethical reasons. Data are shown as mean + SEM (n = 20 - 21/mice group). (A and B) Data shown are from 1 experiment representative of 5 independent experiments. *p<0.05; Mann Whitney U test.

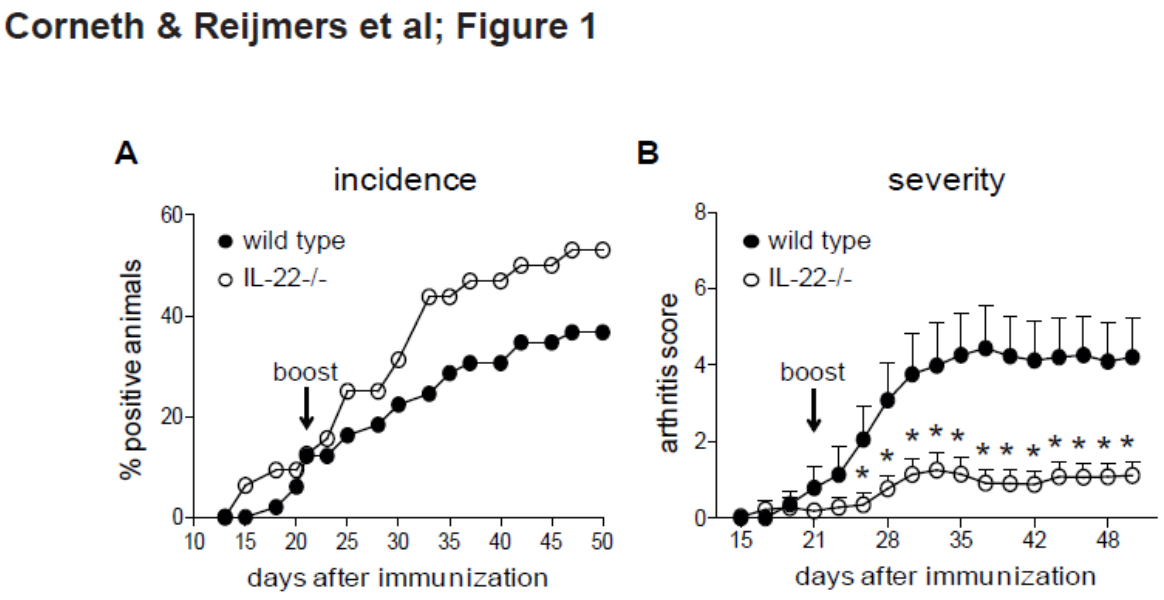


Figure 2. Th17 cells are functional both *in vitro* and *in vivo*. (A) Percentage of IL-17A-producing $CD4^+$ T cells in wild type and IL-$22^{-/-}$ mice (n=6/10 mice/group) at several time points during collagen-induced arthritis (CIA). Data are shown as mean + SEM and are from 1 experiment representative of 4 independent experiments. (B and C) Wild type and IL-22-/- mice (n=6 mice/group) were immunized with CII/CFA and sacrificed ten days later. $15*10^3$ FACS sorted $CD4^+CCR6^+$ T cells from the spleens were co-cultured with mouse synovial fibroblasts for three days. (B) Levels of IL-6 in co-culture supernatant were measured by ELISA. (C) Percentage of IL-17A-producing T cells was measured by flow cytometry. (D) Wild type and IL-22-/- mice (n=5 mice/group) were immunized with mBSA/CFA. After seven days an mBSA trigger was given in the footpads of these mice. Swelling of the footpad was measured to determine the inflammatory T-cell response. (B-D) Data are shown as mean + SEM and are from 1 experiment representative of 2 independent experiments. *$p<0.05$; Mann Whitney U test.

Corneth & Reijmers et al; Figure 2

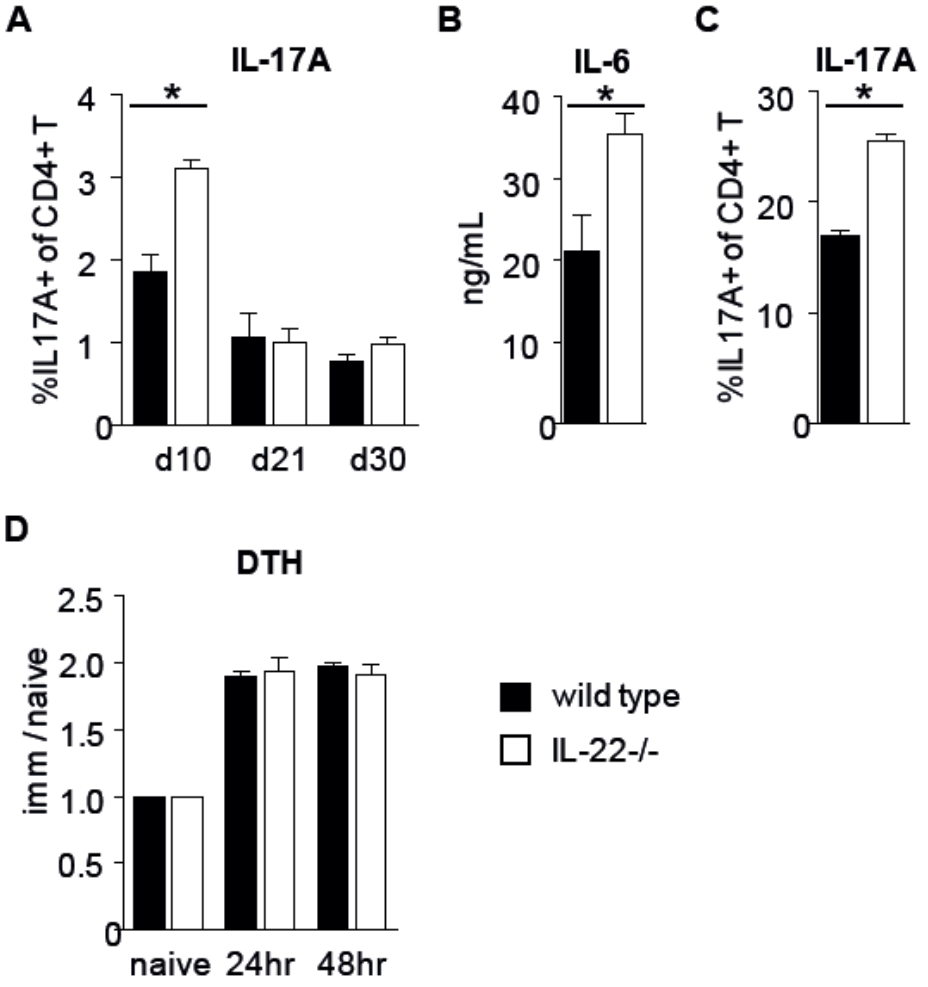


Figure 3. Plasma cell formation and autoantibody formation upon collagen-induced arthritis. (A) IgM and IgG2ab plasma cells numbers in CIA wild type and IL-22-/- mice were measured 20 and 30 days after immunization by flow cytometry. (Left) Total numbers of splenic IgM and IgG2ab plasma cells. (Right) Flow cytometry graphs show IgM^+ and $IgG2ab^+$ plasma cells in live CD11b-gated lymphocytes. (B) Chicken (left panel) and mouse (right panel) CII-specific IgG2c antibody levels in serum of wild type (n = 20) and IL-22-/- (n = 21) CIA mice, as measured by ELISA. (A and B) Each circle represents one mouse. Data shown are of from 1 experiment representative of 2 independent experiments. **p<0.01; *** p<0.001; Mann Whitney U test.

Corneth & Reijmers et al; Figure 3

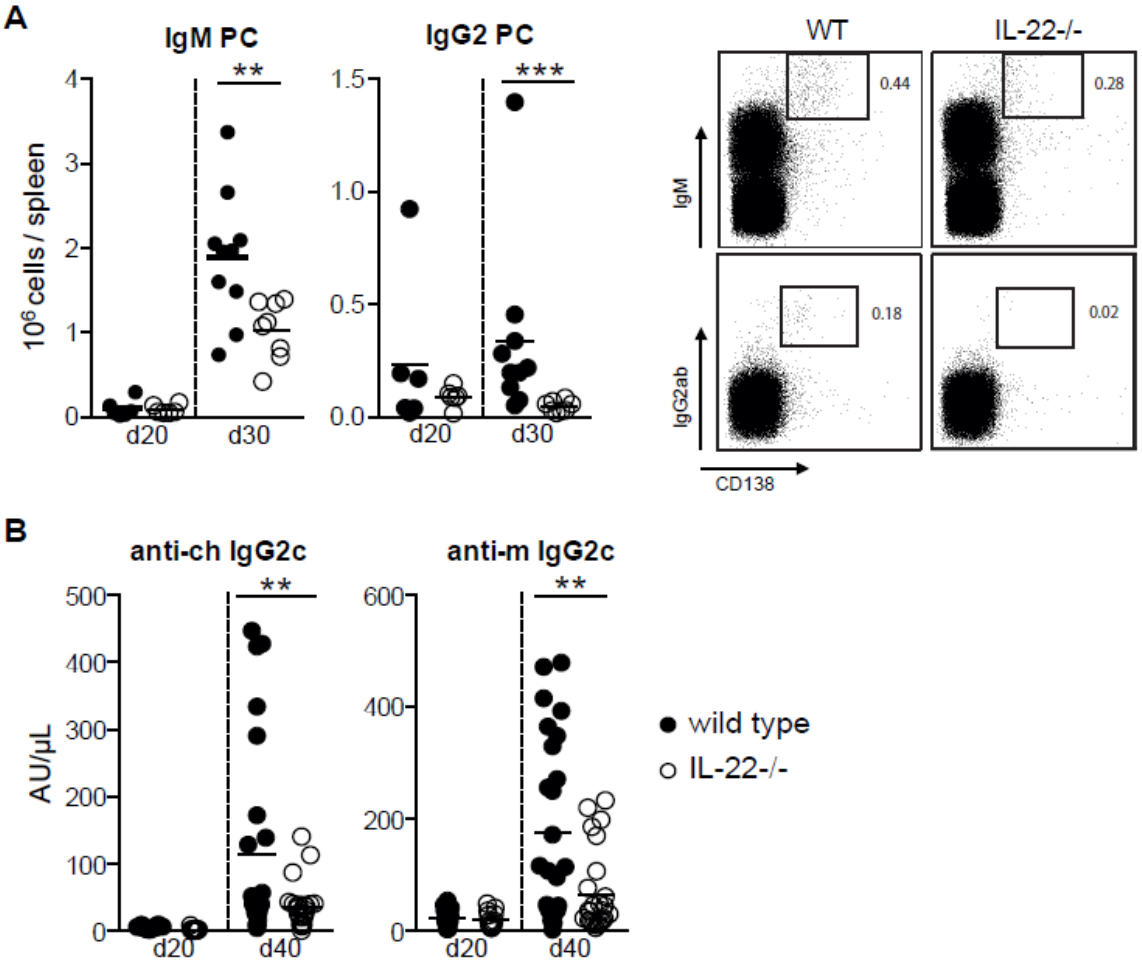


Figure 4. Normal B-cell development, activation and differentiation in IL-22$^{-/-}$ mice. (A) B-cell distribution in spleens obtained from naïve wild type and IL-22$^{-/-}$ mice (n=6 per group) analyzed for mature follicular B cells (IgD+IgMlow) and immature transitional B cells (IgM+IgDlow) by flow cytometry. Two representative FACS plots (left) and average ratio of mature/immature B cells (right) are shown. (B) Total splenic B cell numbers in wild type and IL-22$^{-/-}$ mice after CIA. (C) Proliferation of naive splenic B cells (n=4 mice per group) after two day cultures with anti-IgM and LPS measured by H^3 incorporation. (D) IgG3 and IgG1 plasma cell formation after four day cultures with LPS or LPS + IL-4 respectively (n=4 mice per group). Data shown are from a single experiment.

Corneth & Reijmers et al; Figure 4

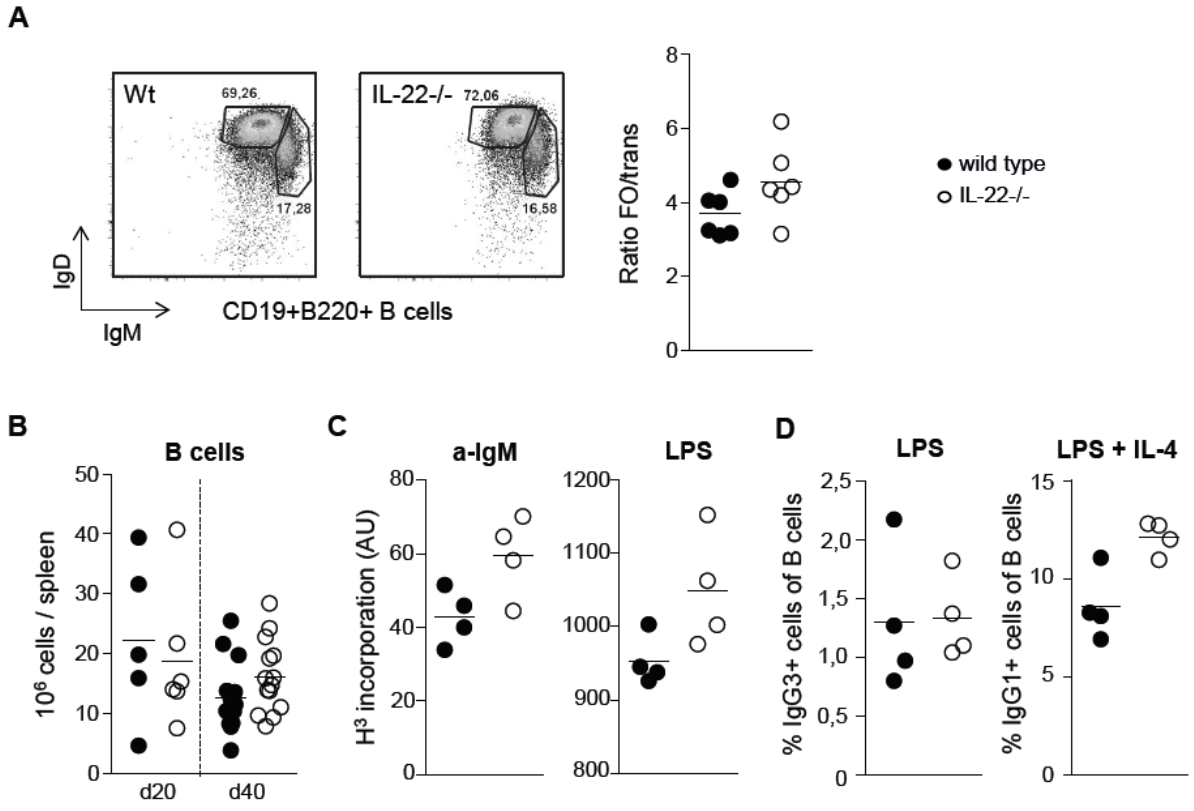


Figure 5. Rapid decay of germinal centers in IL-22-/- mice after boost challenge. (A) Number of PNA+ germinal centers (GCs) in the spleen per field of vision at the peak of disease severity (d30), analyzed by immunohistochemistry. Mean and SEM are shown for wild type (n = 10) and IL-22$^{-/-}$ (n = 8) mice; *p<0.05; Mann Whitney U test. (B) Immunohistochemistry on spleen sections of wild type and IL-22$^{-/-}$ mice at the peak of disease severity, B cell follicles are identified by IgD (blue) and GCs by PNA (brown). Two representative samples of wild type (n = 10) and IL-22$^{-/-}$ (n = 8) mice are shown. Data shown are from 1 experiment representative of 2 independent experiments.

Corneth & Reijmers et al; Figure 5

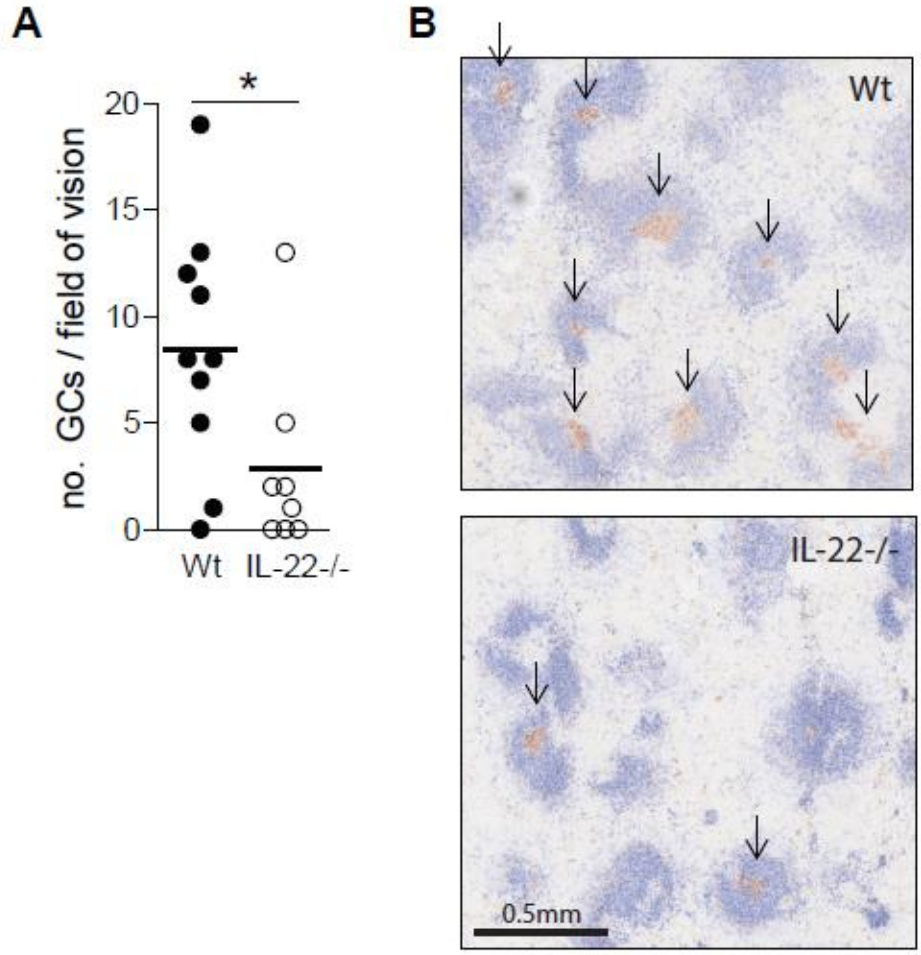
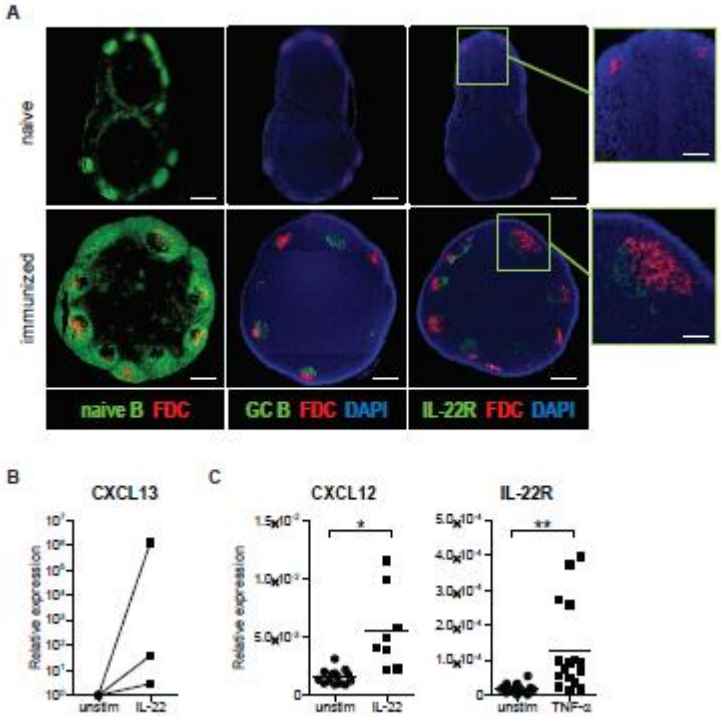
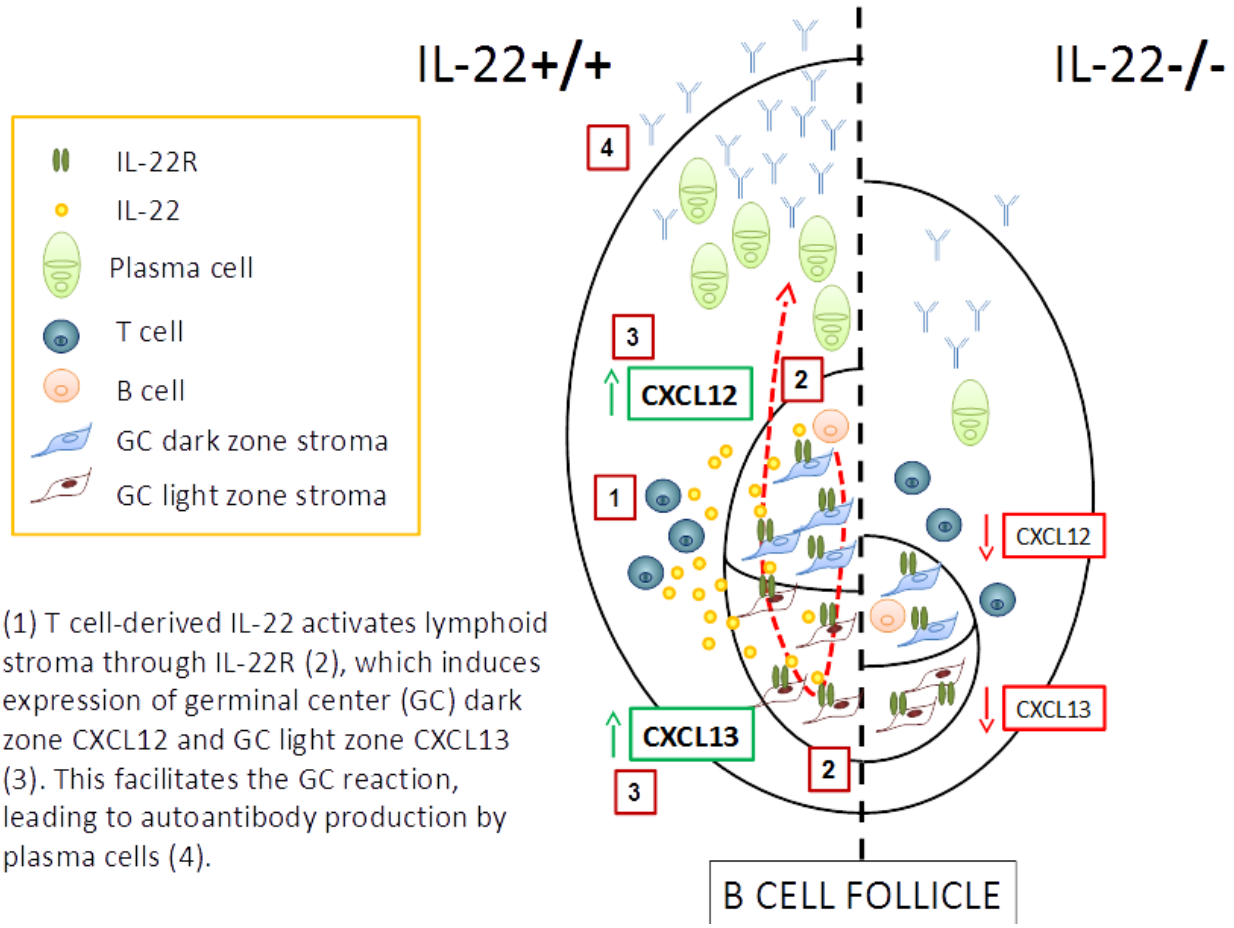


Figure 6. IL-22R1-expressing stromal cells induce CXCL12 and CXCL13 upon IL-22 stimulation. (A) Expression of IL-22R1 was found on stromal cells in the germinal center (GC) of the B-cell follicle. IgD, staining naïve B cells (left panel, green) was lost in the GC, and GL7 (middle panel, green) was expressed by GC B cells. Follicular dendritic cells (FDCs) were identified by the expression of CD35 (red). IL-22R expression can be seen in the GC (right panel, green). Scale bars represent 200 µm (full picture) or 100 µm (inserts). (B) Incubation of purified human FDCs with recombinant IL-22 induced the expression of the GC chemokine CXCL13 (n = 3). (C) Human adipocyte-derived stromal cells expressed IL-22R1, which could be induced after TNF-α incubation for 48 hours. Basal expression of IL-22R1 was sufficient to increase the expression of CXCL12 after 48 hour stimulation with IL-22. N = 8-16 per group. Data shown are pooled data points of replicate experiments performed 3-4 times per condition as indicated. *$p<0.05$; **$p<0.01$; Mann Whitney U test.

Corneth & Reijmers et al; Figure 6





(1) T cell-derived IL-22 activates lymphoid stroma through IL-22R (2), which induces expression of germinal center (GC) dark zone CXCL12 and GC light zone CXCL13 (3). This facilitates the GC reaction, leading to autoantibody production by plasma cells (4).