Analysis of the role of the IL-23/Th17 axis on B cell-mediated autoimmunity

Analyse der Rolle der IL-23/Th17 Achse während B-Zell vermittelter Autoimmunität

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Great Are The Works Of The LORD,

Studied By All Who Delight In Them

Psalm 111:2 (ESV)

Table of Content

SUMMARY

Rheumatoid arthritis (RA) is a common autoimmune disease. Early RA is characterized by an initial break in immunological tolerance, the appearance of specific autoantibodies and a phase of "asymptomatic autoimmunity". Finally, RA shifts into a state of active disease and manifests itself in autoantibody-mediated chronic synovitis which leads to cartilage and bone destruction. Apart from autoantibodies, the IL-23/Th17 axis has been identified as a major driving force during RA pathogenesis. The exact contribution of the IL-23/Th17 axis, however, has remained incompletely understood. Multiple "hits" through infectious agents and other environmental factors are suggested to be responsible for the transition from asymptomatic autoimmunity into active RA. However, checkpoints and mechanisms regulating this transition still remain obscure.

In this work, we aimed to elucidate the role of the IL-23/Th17 axis during the pathogenesis of autoimmune arthritis. Therefore, we dissected the role of IL-23 during the initial break of tolerance and during the autoantibody mediated joint inflammation.

Here we report, that the IL-23/Th17 axis does not directly contribute to antibody induced joint inflammation. Instead, IL-23 was found to be decisively involved in orchestrating the transition from asymptomatic autoimmunity into chronic autoimmune disease. Prior to the onset of experimental arthritis Th17 cells accumulated inside germinal centers of secondary lymphatic organs. There, IL-23 activated Th17 cells increased the pro-inflammatory activity of autoreactive antibodies by suppressing the expression of β-galactoside α2,6 sialyltransferase (*st6gal1*) in developing plasma cells in a IL-21 and IL-22 dependent manner. Subsequently, autoantigen specific IgG glycosylation shifted towards a pro-inflammatory status which triggers the onset of arthritis. Similarly, plasmablasts from RA patients show a decreased *st6gal1* activity compared to healthy control. IgG from these individuals showed corresponding changes in its glycosylation profile and inflammatory activity. The work presented in this thesis identifies a so far unrecognized mechanism by which Th17 cells unmask a preexisting breach in humoral tolerance and initiate the transition from a stage of asymptomatic autoimmunity into inflammatory autoimmune disease. The key findings of the work presented here have previously been published in Nature Immunology (Pfeifle et al. 2017[1]).

ZUSAMMENFASSUNG

Rheumatoide Arthritis (RA) ist eine der häufigsten Autoimmunerkrankungen. Sie äußert sich typischerweise in der Form einer symmetrischen Polyarthritis und befällt zumeist die kleinen diarthrodialen Gelenke an Hand und Fuß. Dort verursachen Antikörper gegen körpereigene Proteine chronische Entzündungen des Synoviums und führen letztendlich zu irreversibler Schädigung der betroffenen Gelenke.

Diese Autoantikörper markieren den Beginn einer „asymptomatischen Phase", welche bis zu 10 Jahre andauern kann. Aus bisher ungeklärten Gründen kommt es im Verlauf der Erkrankung zum Ausbruch einer chronischen Synovitis.

Bemerkenswerterweise lassen sich vor dem Auftreten erster klinischer Symptome Veränderungen in der Glycanstruktur der funktionsvermittelnden Fc Teile der Autoantikörper feststellen. Die endständige Sialylierung dieser Fc Glycane nimmt ab, wodurch den betreffenden Antikörpern die Fähigkeit abhandenkommt an anti-inflammatorische Rezeptoren zu binden. Stattdessen werden verstärkt pro-inflammatorische Rezeptoren aktiviert und das Krankheitsbild verschiebt sich von befundloser Autoimmunität zur rheumatoiden Arthritis. Warum und aufgrund welcher Mechanismen sich diese Glycanstrukturen auf den Autoantikörpern ändern, war jedoch bisher unverstanden.

In dieser Arbeit zeigen wir, dass die IL-23/Th17 Achse, während der entzündlichen Phase der Arthritis nicht direkt involviert ist. Stattdessen war IL-23 unverzichtbar für die Entwicklung pathogener Antikörper. Während der prodromalen Phase der Arthritis wurde beobachtet, dass Th17 Zellen in den Keimzentren sekundär lymphatischer Organe akkumulieren. Diese durch das Zytokin IL-23 aktivierten Th17 Zellen waren in der Lage mittels IL-21 und IL-22 die Expression der β-galactoside α2,6 sialyltransferase (*st6gal1*) in sich entwickelnden Plasmazellen zu hemmen. Dies führte zu einer verminderten Sialylierung an den Fc Teilen von Autoantikörpern. Diese „desialylierten" Autoantikörper hatten *in vitro* eine höhere inflammatorische Aktivität und lösten *in vivo* eine Arthritis aus. Durch die Neutralisierung von IL-23 während der prodromalen Phase der Arthritis, oder durch enzymatische Sialylierung der betreffenden Autoantikörper konnte dies verhindert werden.

Im Rahmen dieser Arbeit konnte eine bisher unbekannte Funktion der IL-23/Th17 Achse aufgezeigt werden, welche Autoantikörper eine pro-inflammatorische, arthritogene Funktion verleiht. Hierdurch nun kann ein bereits existierender Bruch der immunologischen Toleranz zu Tage treten und den Übergang von asymptomatischer Autoimmunität zur inflammatorischen Autoimmun-erkrankung bewirken.

Ähnlich der Situation im Tiermodell, konnte in dieser Arbeit auch bei RA Patienten eine verminderte Aktivität des Enzyms St6gal1 nachgewiesen werden. Gleichsam wurden bei RA Patienten eine verminderte Fc-Sialylierung und eine erhöhte inflammatorische Aktivität festgestellt. Darüber hinaus konnte bei Personen unmittelbar vor dem Auftreten erster Symptome eine Desialierung spezifischer Autoantikörper beobachtet werden. Dies legt nahe, dass es im humanen System einen ähnlichen Mechanismus gibt, welcher die Aktivität der Autoantikörper kurz vor dem Ausbruch einer aktiven RA reguliert.

Dieser Arbeit bietet eine molekulare Erklärung für den Übergang von asymptomatischer Autoimmunität zur aktiven RA. Darüber hinaus liefert sie Hinweise auf neue therapeutische Möglichkeiten bei der Behandlung dieser Erkrankung.

Ausgehend von den hier vorliegenden Daten ist eine Neutralisation von IL-23 während einer aktiven Arthritis wenig erfolgversprechend, da hiermit der Antikörper vermittelten Entzündung nicht mehr Einhalt geboten werden kann. Da die IL-23/Th17 Achse jedoch den Übergang von beschwerdefreier Autoimmunität zu aktiven Arthritis vermittelt, könnte die Neutralisation präventiv eingesetzt werden. Darüber könnte der Erhalt der Remission nach Rituximab Behandlung durch IL-23 Depletion unterstützt werden.

Teile dieser Arbeit wurden 2016 in Nature Immunology veröffentlicht [1].

I. INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system is a sophisticated network. It consists of various interacting cells, cell products and organs and is designed to protect living organisms against invading pathogens like viruses, bacteria, fungi and parasites, but also against malignant cells.

In order to fulfill this task, the immune system needs to mount powerful defense mechanisms against dangerous invading "non-self" structures, while tolerating healthy "self" antigens. This finely balanced discrimination between "self" and "non-self" is crucial for the effective maintenance of an organism's integrity, while preventing chronic inflammation or autoimmune disease.

In general, one can distinguish two different branches of the immune system, shared within all gnathostome species: the innate and the adaptive immune system [2]. These two branches combine both, an immediate defense against broadly distributed foreign structures and as well the capacity to recognize almost any possible molecular structure. Furthermore, the adaptive immune system enables the organism to develop an immunological memory leading to a more specific and faster defense against reoccurring infections with the same pathogen.

Both components of the immune system are, however, not independent, but cooperate with each other and partially use the same effector cells [3, 4].

1.1.1 INNATE IMMUNITY

The innate immune system is genetically programmed to provide a basic recognition capability for foreign structures. Therefore, cells of the innate immune-system are equipped with receptors detecting pathogen-associated molecular patterns (PAMPs). PAMPs are invariant structures exclusively found on microbial pathogens and often are essential for the survival or pathogenicity of microbes. Although the variety of pathogens is immense, PAMPS are shared by entire classes of prokaryotes. Well known examples are bacterial lipopolysaccharide (LPS), lipoteichonic acid, mannans, peptidoglycans, bacterial DNA and double stranded RNA [5, 6, 7, 8].

Recognition of PAMPS by pathogen recognition receptors (PRR) rapidly triggers a series of anti-microbial immune responses involving antimicrobial peptides, the complement system, various inflammatory cytokines, chemokines, and type I interferons. Furthermore several cells are

empowered to phagocytose and destroy microorganisms via lysosomes [7, 8], thus building a first line of defense against invading pathogens. Furthermore, triggering several PRRs simultaneously can induce very different innate immune responses, enabling the innate immune system to organize a precise answer for the particular threat [9].

However, the innate immune system is not only responsible for controlling the replication of microbes. Specially equipped representatives of the innate immune system, namely the dendritic cells (DC), macrophages and fully differentiated mature B cells serve as so called "antigen-presenting cells" (APCs) for cells of the adaptive immune system. These APCs are not only capable to engulf and digest pathogens and dying cells, but also are empowered to present immunogen particles to T cells and thereby able to activate the adaptive immune system [3, 10].

1.1.2 ADAPTIVE IMMUNITY

While the innate immune recognition is focused on the detection of conserved and broadly expressed PAMPs, the adaptive immune system has remarkable capacity to recognize almost any molecular structure via specific receptors. Moreover, it grants the organism the benefit of establishing an immunological memory, conferring an often lifelong protection against pre-encountered pathogens to the individual [5].

Adaptive immune responses are organized around its effector cells, called lymphocytes. Almost 50 years ago it had become clear, that lymphocytes can be divided in two distinct lineages: thymus-matured T cells and bone marrow-derived B cells. Both lymphocyte lineages together enable the immune system to establish its "immunological memory" and orchestrate effective, highly specific cellular and humoral responses against reoccurring pathogens [11, 12].

For this purpose, the adaptive immune system employs antigen receptors, which are not germline encoded, but stochastically formed through somatic gene segment rearrangement, which creates a tremendous quantity of antigen receptors. Due to the fact that these gene recombination events occur independently in every single lymphocyte, all T and B cells bear a unique, clonal expressed antigen receptor. Thus, the overall population of lymphocytes ($2x\ 10^{12}$ in humans [13]) provides a wide range of receptor specificities.

However, due to the fact that the lymphocyte receptors are created randomly, lymphocytes end up with recognizing antigens regardless of their origin: microbial, environmental, and even self. Hence, self-reactivity is an inevitable by-product of creating the vast diversity of antigen

receptors and effective mechanisms must be in place to prevent massive collateral damage through autoimmune activation. Even though this is almost perfectly accomplished through control mechanisms of the innate immune system, errors may happen, leading to allergy or autoimmune diseases [3, 5].

1.1.3   T-LYMPHOCYTES

T cells develop out of a common lymphoid progenitor cell which is generated inside the bone marrow, but many of the key differentiation steps of T cells occur in the thymus, where they mature.

During maturation in the thymus T cells begin to express T cell receptors (TCR) on their surface which allows them to recognize their cognate antigen. The TCR is not designed to bind its antigen independently, rather it detects short peptide sequences processed and presented to T cells via "major histocompatibility complexes" (MHC) which are expressed on the surface of APCs.

To form an immunocompetent repertoire immature thymocytes initially generate highly diverse T cell receptors through recombination of germline encoded V, (D) and J receptor gene segments. To prevent autoreactivity and useless TCR clones the processes of T cell differentiation and T cell activation are strictly controlled [14].

a.   T cell development

When lymphoid progenitor cells arrive in the thymus, they lose their ability to develop into B- or natural killer cells [15, 16, 17] and are instructed to develop into T cells.

Differentiating "mainstream" $\alpha\beta$-T cells pass through a series of developmental phases. During the first phase they are characterized by the absence of the co-receptors CD4 and CD8 and are therefore called double-negative (DN) cells. During the DN phase somatic gene segment rearrangement begins in the TCRβ locus and the newly formed protein forms a heterodimer with the surrogate pre-TCRα chain, the pTα protein [18]. The pre-TCR combines with the CD3/ξ complex to form a functional receptor on the cell surface [19]. Active signaling represents the first selection step and is required for further T cell maturation.

The pre-TCR guides T cell differentiation through the double negative stage into the double positive stage (CD4$^+$CD8$^+$). Double positive (DP) thymocytes rearrange the TCRα gene locus, which will, if rearrangement succeeds, together with the TCRβ-chain replace the tentatively pre-TCR. After that the T cells enter an important selection process to ensure the TCR specificity and to prevent strong autoreactive TCRs. For this process "promiscuous" tissue-restricted antigens

(TRAs) are ectopically expressed by a special antigen-presenting cell (APC), the cortical thymic epithelial cells (cTECs) [20]. During the selection process called "positive selection" all T cells which are unable to bind self-peptide:MHC-complexes are sentenced to die in the thymus. Thus all useless TCRs are eliminated [21, 22]. The remaining T cells enter the selection process called "negative selection" where strongly self-reactive TCR-bearing lymphocytes are determined to undergo apoptosis. This "intra-thymic" negative selection also applies to proteins which are otherwise restrictively expressed in other organs but the thymus [23, 24].

Ideally, this somatic "education" known as "central tolerance" or "clonal selection", as it was named by Burnet and Talmage in 1959[25, 26, 27], would remove self-reactivity and select reactivity to foreign "non-self", including "near-self" antigens[28]. If self-antigens are only expressed in very low levels inside the thymus [29], or post-translational modifications of autoantigens affect T cell epitopes only in the periphery but not inside the thymus[30], autoreactive T cells may escape clonal deletion. Escaping autoreactive T cells are known to correlate with the development of several autoimmune diseases [21].

Mature T cells are single-positive for CD4 or CD8 and leave the thymus through the blood stream or via the lymphatic system [21, 31]. Arriving inside the secondary lymphatic organs, T cells are not independent from the innate immune system. Indeed, the recognition of the peptide-MHC ligand presented by APCs is not sufficient to activate T cells. To be activated, T cells require at least one additional signal [32].

While the first activation signal comes from the TCR:peptide-MHC interaction, it is the interaction with costimulatory receptors, that determines a T cells fate. Costimulatory molecules (e.g. CD80/86) are only expressed on the cell surface of APCs if these cells sense the presence of pathogens or endogenous danger signals via their PRRs [3, 10].

Self-antigens however, are tolerated by PRRs and therefore do not induce the expression of costimulatory molecules. This mechanism, known as 'peripheral tolerance', is meant to ensure that only pathogen-specific T cells will be activated [3].

b. Effector CD4^{+} T cells

Once a T cell is activated by its antigen:MHC complex and receives costimulatory signals it starts to proliferate (clonal expansion) and differentiates into an effector cell. This effector cell differentiation is directed through cytokines, also produced by APCs in response to PRR stimulation. CD4^{+} T cells, along with CD8^{+} T cells, make up the majority of T lymphocytes outside the thymus. After antigen encounter and subsequent activation, CD4^{+} T cells are committed to differentiate into one out of a variety of effector subsets. These lineage specific

decisions are predominantly tailored by the cytokine microenvironment, but also by APC types, costimulatory molecules and TCR:antigen interactions involved during T cell activation.

Differentiated CD4^{+} T helper cells may produce various cytokines and perform a variety of different functions. Therefore they are categorized into distinct subsets including Th1, Th2, Th17, Tregs (regulatory T cells), Th22 cells[33] and T follicular helper cells (Tfh) [34].
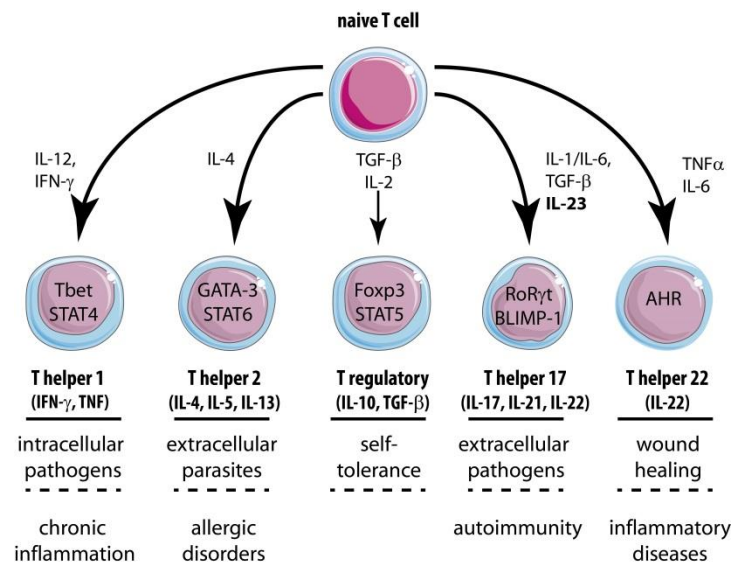
Figure 1: Differentiation of T cell subsets
The different CD4^{+} subsets are generated from the naive T cells by the different cytokines. For each T helper subset a distinct transcription factor has been identified. Furthermore, each Th subset produces a different set of key cytokines and is important during the defense against certain pathogens or immune disorders. Figure was adapted from Golubovskaya & Lijun [35]

Th1 cells differentiate in the presence of IL-12 and IFNγ depending on the T-box transcription factor (Tbet) and on STAT4 [36, 37]. Th1 cells produce IFN-γ, lymphotoxin α (Lfα) and IL-2. They are especially important in the IFN-γ–driven activation of macrophages. This results in an increased phagocytic activity and an enhanced microbial killing ability which is necessary to eliminate intracellular pathogens.

Th2 cells are important in the humoral immunity to control helminths and other extracellular pathogens but are also involved in allergic reactions. They differentiate under the impact of IL-4 which upregulates the transcription factor GATA-3 in a STAT6 dependent manner. Effector cytokines of Th2 are IL-4, IL-5, IL-9, IL-10 and IL-13 [37].

Tregs develop in dependence of TGF-β and under the control of the transcription factors Foxp3 and STAT5. Tregs are critical for maintaining self-tolerance and modulate immune responses to infections [37, 38].

c.   Th17 cells

Th17 were described as distinct CD4^{+} T helper subset in 2005 [39, 40]. The breakthrough for the discovery of Th17 cells was obtained during animal studies of autoimmune diseases, like experimental autoimmune encephalomyelitis (EAE) [41] or Collagen-induced arthritis (CIA)[42] Th17 cells were shown to be critical for host defense against extracellular bacteria such as *Citrobacter rodentium* [43] and *Mycobacterium tuberculosis* [44]. Furthermore, the clearance of *Propionibacterium acnes*, *Klebsilla pneumonia* and *Candida albicans* requires inflammation of the type engendered by Th17 cells[45]. However, Th17 cells are also considered as involved in the pathogenesis of several autoimmune diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), juvenile idiopathic arthritis, type 1 diabetes, multiple sclerosis, psoriasis and inflammatory bowel disease[46] .

Th17 cell differentiation

Th17 differentiation is controlled by the transcription factors 'RAR-related orphan nuclear receptor (RORγt) and retinoid-related orphan receptor alpha (RORα) [47, 48]. However, which cytokines are needed for the initial regulation of the Th17 differentiation was unclear for a long time.

In 2006, it was suggested, that TGF-β in the presence of IL-6 was sufficient to induce Th17 cells, since the blockade of both cytokines resulted in the abrogation of Th17 cell differentiation and IL-6 together with TGF-β also enhanced RORγt mRNA expression [47, 49, 50]. Nevertheless, the role of TGF-β in Th17 generation was heavily discussed [39, 51, 52]. Among others, this was because of the dualistic function of TGF-β during T helper cell differentiation. While the presence of high concentrations of TGF-β instructs naïve T cells to develop into Tregs, low concentrations of TGF-β, especially in the presence of inflammatory cytokines like IL-6 and IL-23, tip the balance for T cells to differentiate into the more aggressive Th17 subset [50, 51, 52].

Interleukin 23 (IL-23) is another inflammatory cytokine that contributes to Th17 generation. While TGF-β together with IL-6/IL-21 are inducing the IL-23R expression on the cell surface, CD4^{+} T cells get responsive to IL-23 produced from myeloid cells. Although IL-23 is not

critically required for the differentiation of IL-17 producing T helper cells, it is required for their terminal differentiation and responsible for their pathogenicity[53]. This is known as the "Th17 plasticity" resulting in "classical" activated and thereby more regulated Th17 cells (TGF-β and IL-6) and "alternative" activated Th17 cells (IL-6, IL-1β, IL-23) which are more pathogenic and importantly characterized by the expression of IL-22 [54, 55, 56, 57]. Thus, IL-23p19 knockout mice (*Il23a*^-/-) infected with *Citrobacter rodentium,* although they induce the proper Th17 response, fail to clear the infection [43]. *Il23a*^-/- mice are, on the other hand, completely protected against Th17 cell driven autoimmune diseases [41, 42].

The role of Th17 cells in immunity

Like all other T helper cells Th17 cells exert their effector functions via the defined expression of certain effector cytokines. For Th17 cells, the prototypic cytokine is IL-17A. But apart from IL-17A, Th17 cells also express IL-17F and depending on the signaling of IL-1β and IL-23 during their differentiation also IL-21, IL-22, or GM-CSF [58].

Interleukin 17A (IL-17A, initially described in 1993[59]) is a pro-inflammatory cytokine that is predominantly expressed by Th17 cells, but also by different innate immune cells, such as macrophages, mast cells and neutrophils. IL-17A belongs to the IL-17 cytokine family, consisting of six identified proteins (IL17A-F) and is able to form disulfide-linked homo- and heterodimers with IL-17F, leading to three different isoforms (IL-17A/A, IL-17F/F, IL17A/F). These isoforms potentially exert three different biological activities[59, 60, 61]. IL-17A and IL-17F are both major contributors to host defence against fungal pathogens and bacteria. They induce the secretion of proinflammatory cytokines (e.g. IL-6, IL-1, prostaglandin E2 and TNFα), and antimicrobial peptides (β-defensins 1, 3, 4 and lipocalin 2 [62, 63, 64, 65, 66]). IL-17 is also able to induce neutrophil recruitment (via cytokine-induced neutrophil chemoattractant (KC) or human CXCL8 respectively [63, 67]) , is thought to recruit B cells into the germinal centres through IL-17A-induced CXCL12. Furthermore, a potential role during B cell-mediated antibody production is discussed [68, 69, 70].

IL-21 (described in 2000) is a pro-inflammatory cytokine, which belongs to the type I cytokine four-α-helical bundle cytokines and is predominantly produced by activated T cells. IL-21 was shown to have pleiotropic functions and potently regulates both adaptive and innate immune responses. IL-21 is able to activate macrophages and NK cells, increases the cytotoxicity of CD8^{+} T cells and is important for the differentiation of Th17 cells [71, 72]. Apart from being an important amplifying cytokine for Th17 differentiation, the IL-21 receptor is predominantly expressed on B

cells, and IL-21 is implicated in germinal center (GC) function, B cells survival and apoptosis, plasma cell differentiation and immunoglobulin production [72, 73, 74, 75].

Interleukin-22 was discovered in 2000 as an IL-9-inducible cytokine sharing 22% of its amino acids with IL-10 and was therefore initially described as IL-10-related T cell-derived inducible factor (IL-TIF) [76]. Not unlike other cytokines IL-22 has anti-inflammatory and pro-inflammatory effects. IL-22 was shown to be an important factor in the host defense against bacterial inflammation through orchestrating the mucosal immune defense (antimicrobial peptides and inflammatory mediators) [77, 78] and contributing to tissue regeneration [79]. On the other hand, IL-22 is pathogenic in certain inflammatory diseases, such as psoriasis and rheumatoid arthritis [80, 81, 82, 83]. IL-22 was shown to act through a transmembrane receptor consisting of two different subunits: IL-10R2 and IL-22R1.While IL-10R2 is ubiquitously expressed the expression of IL-22R1 is more restricted. IL-22R1 was shown to be hardly detectable on resting B cells and completely absent on resting and activated T cells, while being mainly expressed in epithelial tissues including keratinocytes, hepatocytes and intestinal and respiratory tissues [82]. Nevertheless, IL-22R1 expression was detected in lymphoid tissues in mice during chronic inflammation like autoimmune collagen-induced arthritis [80].

GM-CSF was first purified from LPS treated lung-conditioned medium in 1977 by Burgess *et al.* [84]. As "colony-stimulating factor" GM-CSF is mostly known for its capability to generate both granulocytes and macrophage colonies from bone marrow precursor cells [85]. But aside from its well established function as "colony-stimulating factor", GM-CSF exhibits pleiotropic effects on diverse cell types like epithelial cells, fibroblasts, neutrophils, dendritic cells, eosinophils and maybe B cells (an object of ongoing discussion) [86, 87].While playing a merely maintaining, and possibly even redundant role under physiological conditions, during inflammation GM-CSF is becoming a critical pro-inflammatory factor affecting microglia[88], neutrophils[89], monocytes[90, 91, 92] and dendritic cells[93] by stimulating the release of inflammatory mediators and promoting cytotoxic functions. In line with this, TH17-derived GM-CSF has been suggested to be a key cytokine during a variety of auto-immune diseases, such as rheumatoid arthritis [94, 95, 96, 97] and autoimmune neuroinflammation [88, 90], while on the other hand being protective in Crohn's disease[98].

1.1.4　B LYMPHOCYTES

Both, T and B cells derive from a multipotent lymphoid progenitor cell (CLP) [99, 100]. But unlike T cells B lymphocytes are continuously generated and the bone marrow is also the place of major maturation steps. During maturation, B cells begin to express the immunoglobulin B-Cell Receptor (BCR). It is this highly diverse receptor which empowers B cells to bind their antigen. To achieve its necessary diversity the BCR is not germline encoded but, quite like the TCR, is generated through somatic gene segment rearrangement.[101]

a.　B cell differentiation

Shortly, the differentiation process of B cells involves the "pro-B cell" stage in which the V(D)J rearrangement of the immunoglobulin heavy chain (IgH) is conducted and the "pre-B cell" stage where V-J segments of the light chain are rearranged [101]. During the subsequent "immature B cell" stage the mechanisms of the central tolerance are performed while a "transitional B cell" is able to leave the bone marrow but is further subject to negative selection inside the spleen. Finally, the term "mature B cell" describes a fully matured B cell "in waiting" for activation through its specific antigen.

At the end of their differentiation program, each mature B cell is ought to express a unique, self-tolerant and mono-reactive B cell receptor (BCR) [26, 102, 103]. The entire B cell pool (10^9 B cells in mice and nearly 10^{12} B cells in humans) [104], on the other hand, is able to detect an enormous array of structures and therefore almost any pathogen which possibly could invade the body.

While correctly tailored effector functions promote a lifelong immunity, inadequate effector choices may lead to autoimmunity or even autoimmune disease. Therefore, the B cell development process is supervised on various checkpoints to assure the mature B cells are committed to self-tolerance.

The first of those major checkpoints, which differentiating B cells have to pass is described as "central tolerance" because it takes place when the B cells are still in the bone marrow, directly after the first productive V-J recombination of the IgL-chain (VL-JL). For this purpose the newly formed BCR is expressed and tested for reactivity against autoantigens supposedly present in the developmental environment. B cells expressing high-affinity autoreactive BCRs are deleted, either by apoptosis or by a reoccurring process named "receptor editing" where secondary VL-JL rearrangements take place until the autoreactivity of the BCR is lost or the developing B cell dies in failing to do so [105]. In the line of this process, many but not all, polyreactive and autoreactive B cells are deleted. However, B cells expressing only a low-affinity autoreactive BCR survive and leave the bone marrow via the blood and travel to secondary lymphoid organs in the periphery [25,

[106]. Only if they past this negative selection step, they are empowered to react upon antigen-recognition with proliferation and differentiation into memory B cells or plasma cells [107, 108]. However, despite an extensive negative selection of B cells (central checkpoint and peripheral checkpoint) a substantial portion of autoreactive specificities remains present in the peripheral B cell pool.

b. B cell maturation

Once these "immature" transitional B cells arrived in the spleen they have to pass a secondary checkpoint to become "mature" long-lived peripheral B cells. Immature B cells are, in contrast to mature B cells short-lived and particularly susceptible to BCR-induced apoptosis. This condition is used by the immune systm to establish the secondary selection process called "peripheral tolerance" where autoreactive immature B cells were actively deleted or functionally inactivated [104, 108, 109] Mature B cells can be divided into three major subsets: follicular B cells, marginal zone (MZ) B cells and B1 cells. While MZ B cells and B1 cells are able to mount a quick response to T cell independent antigens (TI antigens), follicular B cells seem to be more specialized for responding to protein antigens, which require T cell help (TD-response) [110].

Upon T cell-dependent activation of follicular B cells, the development of antibody producing B cells is organized into two steps. During the first step, known as "extra-follicular response", B cells differentiate into dividing B lymphoblasts. B lymphoblasts may undergo class-switch recombination and differentiate into short-lived plasmablasts which secrete early protective antibodies. But due to their limited capability to adjust their BCR unto the actual antigen, the BCR binding affinity tends to be moderate. Therefore, a second step is crucial. When follicular B cells are activated and receive T cell help, they re-enter the B cell follicle and organize themselves in germinal centers which are specialized micro-anatomical compartments inside secondary lymphoid organs. Germinal centers are a site of vigorous proliferation the so called "affinity maturation", resulting in the development of highly antigen-specific plasma cells [110].

Plasma cells are terminally differentiated post-mitotic B cells, specialized to secrete vast amounts of the soluble form of their former B cell receptor [111]. Furthermore PCs differ from their precursors in their loss of migratory capabilities, the downregulation of membrane-bound BCR and other characteristic B cell markers, such as CD19, B220, CD20, CD22 and HLA-DR/MHC class II, the prominent expression of Taci (CD267) and the upregulation of Syndecan-1 (CD138) and the PAX-5 repressor protein BLIMP-1 (B-lymphocyte-induced maturation protein) [111, 112].

Even though plasma cells exist in multiple lymphoid organs and during active inflammation even in non-lymphoid organs, the majority is located in plasma cell niches in the bone marrow. Inside these bone marrow niches plasma cells receive necessary survival factors (e.g. APRIL, BAFF, IL-6 and CXCL12 [113, 114]) to grant them the environment to become long-lived plasma cells. Inside the bone marrow, plasma cells are able to survive irradiation and B cell depletion and are refractory to signals from antigen or antibody complexes [113, 115, 116], providing a humoral memory with the extraordinary half-life reaching from 11 (tetanus) to >200 years (measles, mumps) [117]. But since the number of bone marrow niches for long-lived plasma cells seems to be limited to encompass only 0.1-1% of all bone marrow cells [118] a settled plasma cell is not guaranteed to stay there once and for all, but instead is competing against freshly immigrating plasmablasts to remain inside the plasma cell niches. This model was supported by recent data which demonstrates that each infection is accompanied by a loss of existing long-lived plasma cells [119]. Nevertheless, after protective humoral memory specific for an antigen has been formed, it could last a lifetime [120].
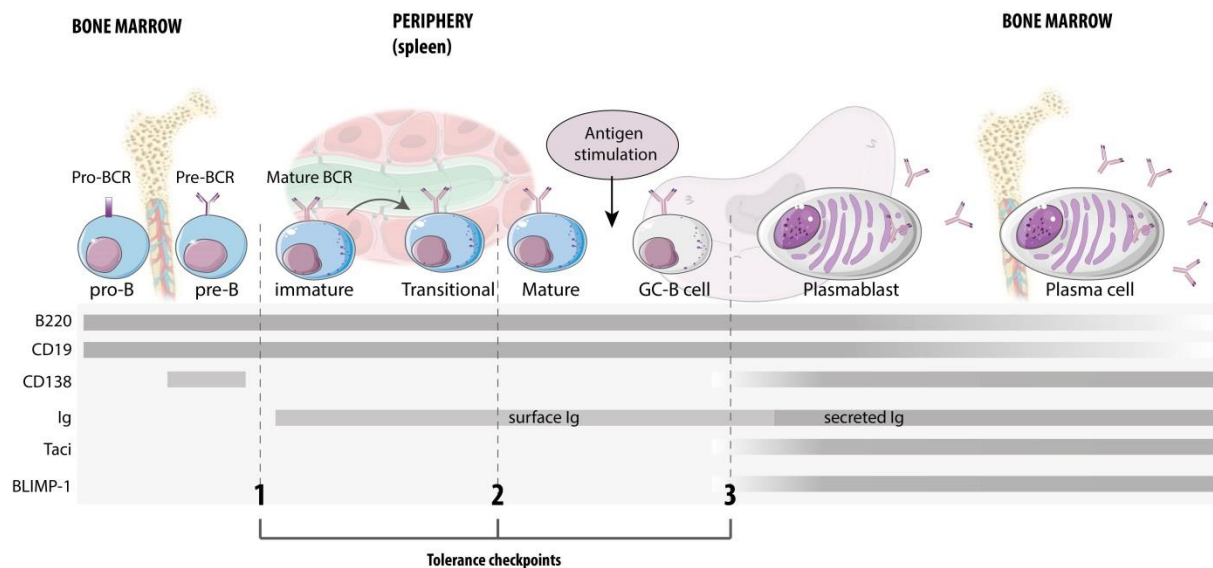
Figure 2: Antigen expression and tolerance checkpoints of normal B cell development
Stages of the development of antibody-secreting plasma cells are characterized by the differential expression of surface markers, presence of surface or secreted forms of Ig, and the increasing expression of the transcription factor BLIMP-1 [110, 121]. Tolerance checkpoints during B cell development are organized in the bone marrow (1), followed by several tolerance checkpoints in the periphery (2-3) [104, 122]. These checkpoints ensure the removal of the vast majority of autoreactive or polyreactive B cells which come into being during the BCR generation and/or during somatic hypermutation during the germinal center reaction. Figure was adapted from Kallies et al. 2004 [121] and Mouquet and Nussenzweig 2015 [123].

c. BCR affinity maturation

Ideally, the repertoire of naïve recirculating B cells has to be able to recognize any possible threat to the host's health at least with a low-affinity interaction unto its BCR for initial B cell activation. Nevertheless, for an efficient neutralization of harmful invaders, it requires antibodies with high affinity.

To accomplish this, activated B cells are empowered to increase the BCR affinity for their cognate antigens through the phenomenon called "affinity maturation", occurring in germinal centers. It is inside these anatomical structures, where their antibody encoding genes are modified by the highly regulated process called "somatic hypermutation" (SHM) and the Ig isotype is created through "class-switch recombination". SHM is describing a process (localized in the so called "dark-zone" of germinal centers) which diversifies the original BCR of antigen-activated B cells by randomly introducing point mutations at a high rate (one mutation for each 1000 bases) into the variable regions of the immunoglobulin genes [124, 125, 126, 127]. By this means diversified BCRs, are afterwards selected for their capability to bind their antigen with a high affinity, while less affine and self-reactive BCR clones have to be deleted. This selection processes take place in the "light zone" of germinal centers and are governed by a specialized T helper cell subset, the T follicular helper cells (T_{FH}) [128]. High BCR affinity for the antigen presented by follicular dendritic cells, correlates with higher antigen uptake and thus higher antigen-peptide presentation to surrounding antigen specific T_{FH} cells, which provide survival signals to the developing B cells [128, 129, 130]. In this way, more affine BCR clones get more T_{FH} help and the most successful BCR clone is enabled to out-compete the others, leading to its positive selection.

According to the current model, this fraction of positively selected B cells returns into the DZ for further proliferation and mutation, a process repeated for numerous times. During the whole process, GC B cells compete with each other for the greatest share of T cell help, which will result in higher proliferation rates of the more affine BCR clones in the DZ and hereafter accounts for efficient affinity maturation [128, 131, 132]. Prevailing B cell clones differentiate into either memory B cells or into class-switched highly specific antibody producing plasma cells.

Apart from T_{FH} cells, there are several other T cells like Foxp3^{+} Tregs or TH17 cells described inside the germinal center [133, 134]. Together, these T cells regulate the outcome of a germinal center reaction, but their contribution remains poorly defined.

While SHM is beneficial for the host through promoting affinity maturation, the largely random somatic mutation inevitably leads to a second wave of self-reactive B cell clones, which unless

they are getting inactivated, bear the danger of developing into autoantibody-producing plasma cells. The low prevalence of autoimmune diseases, and research done by Notidis *et al*. has given evidence for an efficient deletion of self-reactive B cell clones while or after the germinal center reaction [135]. While the precise mechanism is still subject for discussion, it has become clear, that B cell intrinsic checkpoints include surface proteins like FcγRIIb, CD22 as well as intracellular components like *bcl-2*, *fas* and the signaling molecules *lyn* and *syk* [135, 136, 137, 138, 139]. A recent study argues for an efficient depletion of self-reactive B cell clones governed by ubiquitous or GC-close-by expressed self-proteins. By contrast, GC B cells which recognized rare self-antigens were found to be susceptible to escape the negative selection if they cross-react to foreign antigen [140].

The fact that many autoantibodies bear evidence of somatic point mutations strongly suggests that failure to sustain self-tolerance during the GC-reaction may contribute to autoimmune diseases. Therefore, self-tolerance archived within the germinal center is not absolute, but can be broken in certain circumstances [141, 142, 143, 144].

1.1.5   IMMUNOGLOBULINS

Immunoglobulins are glycoproteins which function as antibodies. Antibodies, initially discovered in 1890[145], play a crucial role in the humoral adaptive immune system by linking highly specific antigen recognition to the powerful effector functions of the innate immune system.

Immunoglobulins are composed of two identical heavy chains and two identical light chains (L) that form the Fab (fragment of antigen binding) and the Fc (fragment crystallible) region of the Y shaped antibody. The Fab-region, located in the upper part of the Y structure contains the variable "paratop" that binds the cognate epitope. The defining characteristic of this paratop is its capacity to target a seemingly limitless number of antigens, a diversification accomplished by somatic recombination and mutation discussed before. The lower part of the Y-shaped antibody structure, the Fc part, is formed by the carboxy-terminal constant domains of the two heavy chains. This Fc part serves as a multi-functional interaction platform [146] which orchestrates the multiple effector functions of immunoglobulins. In mammals, five different heavy chains are encoded inside the genome, leading to five different immunoglobulin isotypes: IgA, IgD, IgE, IgG and IgM [147]. IgM and IgA are known to establish a first line of defense against invading pathogens, with IgA being enriched in mucosal surfaces and secretions mounting. IgE, as the

least abundant Ig isotype is associated with hypersensitivity and allergic reactions, as well as in the defense against parasites [147].

The immunoglobulin G is the most abundant isotype in the serum. It is generated after B cell class-switch recombination following antigen encounter and CD4$^+$ T cell help. In humans, IgG exists in four subclasses, numbered in reference to their natural abundance inside the serum: IgG1, IgG2, IgG3 and IgG4. In the murine system the IgG subtypes are classified as IgG1, IgG2a, IgG2b and IgG3. In several mouse inbred strains (e.g. C57BL6 or NOD) the IgG2b subtype is replaced by IgG2c [148]. These four IgG subclasses are highly diverse in the amino acid sequence of the antibody hinge region and the Fc part, resulting in different structural and functional properties [149]

IgG molecules are glycoproteins composed out of four polypeptide chains. The constant region of the heavy chain consists of a variable domain (VH) and three constant domains: CH_1, CH_2 and CH_3. In the cleft between the two CH_2 domains, attached on Asn297, lies a carbohydrate structure that shields the hydrophobic core of the Fc part from the solvent and, by its sequence variations, ads more structural determinants to the Fc fragment.

a. IgG interactions with classical "type I" Fc receptors

IgG mediate a variety of effector functions. On one hand, antigen-IgG immune complexes promote pathogen clearing through neutralization, opsonization for antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). Paradoxically, IgG is also involved in anti-inflammatory immunomodulation by preventing inappropriate maturation in dendritic cells, controlling the antibody-dependent inflammation caused by neutrophils or macrophages and down regulate the BCR-mediated activation of B cells [150].

To organize all this contrary effector functions, a variety of different type I and type II Fc receptors, expressed on diverse cells of the immune system, interact with the structural diverse Fc parts of IgG.

The type I Fc receptors (FcR), belong to the immunoglobulin receptor superfamily and recognize the hinge-proximal region of the Fc part[151, 152, 153]. Broadly, the type I FcR family is composed out of five subfamilies (FrαR, FcγR, FcμR, FcεR and FcδR); according to their binding-specificity to distinct immunoglobulin isotypes. Furthermore, each FcR subfamily further contains several FcR classes [147]. Accordingly, the IgG binding family FcγR is further subdivided

into FcγRI, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa and FcγRIIIb in humans and FcγRI, FcγRIIa, FcγRIIb, FcγRIII, FcγRIV in mice, respectively [154]. Thereby, each FcγR class differs from others in its unique IgG subtype binding capacity, cellular distribution and signaling pathways [147, 155].

Generally, the FcγR family members are widely expressed on many hematopoietic cells, including B cells (only FcγRIIb) [156], NK cells (only FcγRIII), monocytes, macrophages, granulocytes, osteoclasts and mast cells, but also in non-hematopoietic cells such as endothelial cells [147, 157, 158]. Thus, FcγR provide myeloid cells with the whole antigen-recognizing repertoire of B cells.

Importantly, even so most human and mouse FcγRs bear the same name and CD as the murine receptors, they have quite distinct binding abilities unto various IgG subclasses and are also dissimilar in their expression patterns, making it difficult to translate murine findings into humans [154].

Nevertheless, activation of pro-inflammatory responses through IgG is largely dependent on the interaction of the IgG-Fcs with one out of four activating FcγR family members (human: FcγRIA, IIA, IIC, IIIA / mouse: FcγRI, IIA, III, and IV) [157, 159].

Broadly, FcγR crosslinking initiates the phosphorylation of "immuno-receptor tyrosine-based activation motif" (ITAM) at the intra cytoplasmatic domain of the FcγR (for human FcγRIIA) or the associated signal-transducing adaptor molecule the common γ-chain, by kinases of the *src* family. Tyrosine phosphorylation of ITAM leads to the recruitment signaling proteins which contain a Src homology 2 (SH_2) domains, particularly members of the *syk* kinase family. Depending on the particular activated cell type different kinases may be involved. An important downstream target is known to be phosphoinositide 3-kinase (PI3K) and subsequently phospholipase Cγ (PCγ) which leads to the influx of the second messenger ion Ca^{2+} into the cytoplasm. Cellular phenotypes of successful signaling through FcγR include degranulation, phagocytosis, ADCC, cytokine expression and release of inflammatory mediators [151, 158, 160, 161].

Suppression of pro-inflammatory responses through IgG is achieved through inhibitory receptors. The most famous inhibitory FcR is the type I Fc receptor FcγRIIB. This receptor contains an immune-receptor tyrosine-based inhibition motif (ITIM) instead of an ITAM in its cytoplasmic domain, which can be phosphorylated by Lyn [162, 163]. Therefore FcγRIIB crosslinking is not leading to the recruitment of activating kinases, but instead creating a SH2 recognition domain

for the phosphatase SHIP, which results in dephosphorylation of downstream targets and inhibition of activation [164]. Therefore, to activate an immune cell the activating signals have to surpass the threshold established by FcγRIIB, enabling a fine tuning of antibody response [158, 165]. The lack of FcγRIIb, on the other hand, was shown to cause autoimmune disease [136].

b. IgG interactions with type II Fc receptors

Apart from FcγR, IgGs can also bind type II Fc receptors, expressed on dendritic cells, macrophage subsets and monocytes. Type II FcRs like CD209 (DC-SIGN and its murine orthologue SIGN-R1) and CD23, recruit themselves from the C-type lectin receptor family [9]. Unlike type I Fc receptors, these receptors recognize the CH3-CH3 region and exclusively bind IgG with a "closed" Fc conformation [166, 167].

One important mechanism mediating the anti-inflammatory activity of IgG through type II FcRs was characterized by Ravetch *et al.* elucidating the SIGN-R1 dependent upregulation of FcγRIIb on inflammatory monocytes during K/BxN serum transfer arthritis. Subsequently the threshold necessary for an inflammatory antibody response increased and symptoms of arthritis declined [166]. Nevertheless, further studies suggest that there are also other receptors and signaling pathways involved in the immunosuppressive response to IVIG. Because in other disease models, this therapy offers protection even in SIGN-R1$^{-/-}$ mice [168, 169], highlighting the need for further studies.

c. Glycosylation of the Fc domain

Immunoglobulins, like many other glycoproteins, are post-translationally modified through covalent, asparagine-linked sugar moieties (N-glycans) attached on highly conserved "glycosylation sites" (N-X-S/T) [170]. Basically, N-linked glycosylation, modification and degradation are the most common co-valent protein modifications in eukaryotic cells and control many diverse functions involving intermolecular recognition events as well as protein structure [166, 170, 171].
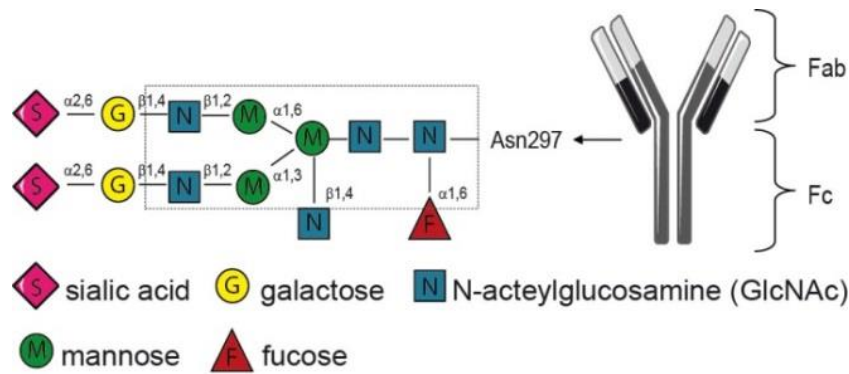
Figure 3: IgG glycan structure
IgG molecules are organized into a "fragment of antigen binding" (Fab) and a "fragment crystallible" (Fc) part which resembles an interaction platform for many Fc receptors and organizes the effector functions of IgG. The IgG glycan is attached at asparagine 297 on both the CH_2 domains located inside the Fc fragment. The Fc glycan is composed out of a heptameric core glycan extended by various possible branching residues. These branching residues include sialic acid (S), galactose (G), N-acetylglycosamine (N) and fucose (F) [172, 173]. These branching glycan- moieties shape the structure of the Fc part and thereby regulate its function. Picture was published in Pfeifle *et al.* 2017 [1]

Unlike the Fc fragments of other immunoglobulins, which can be modified on multiple glycosylation sites, IgG has only one highly conserved N-X-S/T motif located at asparagine 297 (N297) in the CH2 domain. In addition, in roughly 15-20% of IgG-Fab regions, a glycosylation-site is generated by chance during somatic hypermutation [174]. Concerning Fab glycosylation impacts on antigen-recognition, protein-protein interactions, IgG half-life and immune-complex formation are discussed. But in contrast to Fc glycan, which has been extensively studied in the last decade, the effect of Fab-glycosylation is still poorly understood [175].

The carbohydrates which are attached on the Fc part are complex-type biantennary structures N-linked glycans are not exposed on the surface of IgG but rather intercalate between the two heavy chains, and fine tune Fc structure [166, 176, 177]. The core glycan is formed by a highly conserved heptamer composed of mannose and N-acetylglucosamine (GlcNAc). In addition, branching residues of fucose, galactose, sialic acid and bisecting GlcNAc can be attached to the glycan creating a multitude of different Fc glycans [172]. Some glycan modifications are quite common: e.g. 85% of IgG are fucosylated, while 40 %, of IgG are mono- and 20-40 % digalactosylated. Terminal sialic acid, on the other hand, is a more modestly used modification: Only around 10 % of IgG-glycans bear sialic acid on one arm and disialylated glycans are sparsely found [178].

The core glycan is critical for prober IgG-FcR interaction [179, 180], and alterations in the branching residue composition strongly influences the binding-affinity between the Fc and the pro-inflammatory FcRs. Defucosylated IgG1 was shown to have a 50-fold higher FcγRIIIA binding affinity and therefore is much more competent in inducing ADCC, a capacity recently taken

advantage of to design the more efficient CD20-depleting antibody *Obinutuzumab*.[181, 182, 183]. The galactose content in IgG glycans has shown to be involved in the regulation of CDC activity: galactose-rich IgGs (G2) show two to five fold decreased C1q binding efficiency than the G0 glycoform [184].

Reduced degrees of galactose in IgG core glycan have been associated with autoimmune diseases like rheumatoid arthritis. During pregnancy RA often goes into remission [185, 186]. This is a phenomenon, which correlates with increasing degrees of galactose in Fc glycans, suggesting an anti-inflammatory potential of Fc galactosylation. Nevertheless, it has to be taken into account, that galactosylated IgG is the necessary for terminal sialic acid. Indeed, the degree of terminal $\alpha2,6$-linked sialic acid, which represents the only negatively charged carbohydrate inside the Fc glycan, was especially linked to anti-inflammatory IgG activity [168, 184].

This anti-inflammatory IgG activity was first recognized in 1981 during the treatment of idiopathic thrombocytopenic purpura, where intravenously applied high doses of polyclonal IgG (IVIG) therapy resulted in an immediate increase in platelet counts [187]. Since then, IVIG therapy has proven to be an effective treatment for a wide range of autoimmune diseases including multiple sclerosis, rheumatoid arthritis, chronic inflammatory demyelinating polyneuropathies, immune thrombocytopenia (ITP) and Kawasaki syndrome [188, 189]. Nevertheless, the mechanisms responsible for this phenomenon remained elusive for a long time.

Finally, it was shown that the immune suppression by IVIG depends on three key components: the inhibitory receptor FcγRIIb, the type II FcR DC-SIGN/SIGN-R1 and the small fraction of sialic acid-rich Fc glycans present inside the IVIG infusion [168, 190]. In the meantime, the anti-inflammatory effect of terminal sialic acid was proven several times, in mouse and man [168, 169, 178, 191]. Recently, sialylation of IgG was also shown to be able to convert arthritogenic IgG into inhibitors of arthritis[192].

In structural studies it was shown that terminal sialic acid is forcing the Fc part in a more closed conformation. Thereby, binding to pro-inflammatory FcγRs is getting significantly impaired, but an otherwise cryptic binding site is revealed. These conformational alterations enabling the recognition by type II Fc receptors leading to an active anti-inflammatory activity [166].

1.2 AUTOIMMUNE DISEASES

Immunity and inflammation are crucial for a body's defense against invading pathogens. However, it may lead to substantial damage and disease if the cautiously balanced immune responses are directed against the organisms own proteins or when inflammation is insufficiently resolved. The resulting "autoimmune" diseases group together in a heterogeneous group of more than 80 illnesses which develop when the immune system attacks its own cells, tissues or organs.

1.2.1 RHEUMATOID ARTHRITIS

The first description of rheumatoid arthritis (RA) which is acknowledged by modern scientists was provided in the dissertation of Landré-Beauvais in 1800 [193]. But possibly, RA itself is a very old disease, since descriptions of erosive arthritis resembling RA were found as well in ancient descriptions from the Greek philosopher Hippocrates [193] and the Byzantine physician Soranus [194] as well as in ancient skeletons [195, 196] and paintings [197, 198, 199].

Today, RA is one of the most common autoimmune diseases affecting around 0.5-1% of the world's population [199, 200]. RA is a symmetric poly-articular inflammatory disease, mainly affecting the small diarthrodial joints of hands and feet. It is characterized by autoantibody production, chronic synovial inflammation, hyperplasia of the normally relatively acellular synovial lining and ultimately through the destruction of cartilage and bone.

If inflammation is not sufficiently suppressed, these processes lead to irreversible joint damage and to a progressive loss of functional capacity. Furthermore, RA is associated with several comorbid risks that include vasculitis, interstitial lung disease, secondary amyloidosis, lymphoma and cardiovascular disease resulting in increased mortality [201]. Therefore, traditional medications of RA target the inflammatory response and reduce inflammatory signs, irreparable joint destruction and systemic symptoms as quickly as possible. Although there has been a remarkable progress in the available therapeutic possibilities, curing RA is still out of reach. Deciphering the pathogenesis of RA remains an essential challenge to address unmet clinical needs.
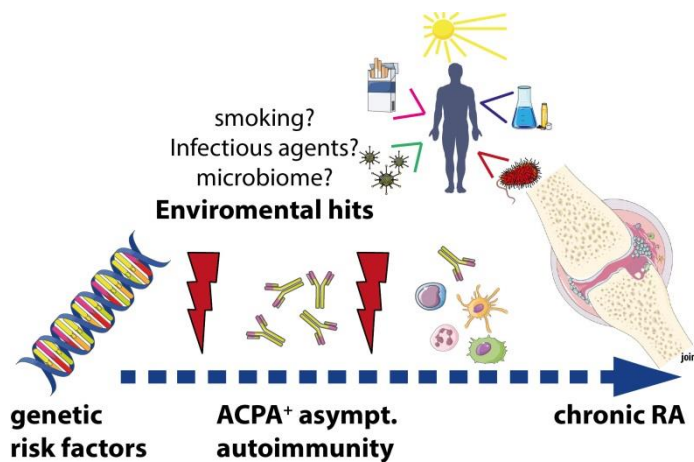
a. The pathophysiology of RA

Figure 4: "Multistep" progression during RA pathogenesis

Genetically predisposed individuals, epigenetic modifications, environmental stochastic insults lead to breach of immunological tolerance and to the development of asymptomatic autoimmunity characterized by the appearance of autoantibodies such as ACPA, a condition that can precede RA for up to 10 years. An additional "hit" through yet unknown factors (presumable environmental stimuli or infectious agents) triggers the expansion of T cell mediated autoimmunity and subsequent transition to chronic synovitis and non-resolving clinical RA. Figure was adapted from McInnes and Schett [202, 203].

In contrast to other inflammatory diseases, RA requires the collaboration of a variety of different cell types of the immune system involving both innate and adaptive immunity. The inflamed synovium is characterized by massive infiltration of neutrophils and monocytes as well as T cells, B cells, plasma cells, natural killer cells, dendritic cells and mast cells, and additionally shows extensive proliferation of pro-inflammatory synovial fibroblasts and differentiation of osteoclasts. During activation and recruitment of these cells several cytokines become important (e.g. TNFα and IL-6) thereby further fueling chronic inflammation and leading to the destruction of cartilage and bone through metalloproteinases, nitric oxide and products of cyclooxygenases [204]. While several pathways involved in arthritic inflammation have been already identified, the basic mechanisms causing arthritis remain still elusive [205]. Several gene mutations are associated with RA development. Risk genes include the *HLA-DRB1* gene which plays an important role in T cell activation through APCs. But also genes of pro-inflammatory signaling pathways such as *TRAF1-C5* and *c-REL* are involved. Apart from genetic prepositions, several stochastic events that can occur years before arthritis becomes clinically evident and that include environmental exposure to infectious agents and changes in the microbiome seem to play an important role in onset of arthritis. These different stimuli are assumed to activate the innate immune system and eventually lead to the break of tolerance during the asymptomatic, "pre-articular" phase of RA [202].

b. Autoantibodies in RA

During the "pre-articular" phase of RA no sign of inflammation can be detected inside the joints, but autoreactive plasma cells and autoantibodies appear [175, 202, 204, 206, 207].

Historically, the first autoantibody associated with RA, was the rheumatoid factor which recognizes IgG (RF, described 1940 by Waaler) [199, 208]. RF positive RA was subsequently described as "sero-positive" RA [209]. However, is not disease specific and has also been described in patients with other autoimmune or infectious diseases [210]. Today, also anti-citrullinated protein antibodies (ACPA) are used as biomarkers for arthritis. A large portion of RA patients (70-80% of all seropositive RF$^+$ RA patients [206]) have been shown to produce also anti-citrullinated protein antibodies (ACPA) [211]. In contrast to RF, ACPAs have been shown to be highly specific (specificity >95%) for RA. Furthermore, ACPA which are mostly composed of the IgG isotype [212] have also been shown to predict a more erosive disease with greater bone and cartilage damage [213, 214, 215]. Studies in animal models for RA have indicated, that ACPA are able to enhance arthritis [216], activate macrophages and the complement system [217, 218], but also contribute to bone erosion through the activation of osteoclasts [219].

Intriguingly, the mere presence of ACPA is not sufficient to cause autoimmune disease, since presence of ACPA may precede the clinical appearance of RA for more than ten years [206, 220, 221]. These "pre-rheumatoid arthritis" associated ACPA have been shown to display highly sialylated Fc parts, giving them an anti-inflammatory rather than an inflammatory capacity [179, 222]. However, at a certain moment prior to the onset of clinical arthritis, ACPA undergo significant transformations. ACPA titer levels increase [223], epitope spreading takes place [224] and isotype usage changes [225]. In line with these events also the effector functions, regulated by Fc glycosylation are modified and ACPA acquire their pathologic capabilities [226]. In recent studies desialylated and degalactosylated ACPA G0-IgG forms correlated with disease activity and significantly differed from non-related serum IgG[185]. Furthermore, autoreactive IgG in synovial fluid of RA patients displayed a further decrease in sialylation than serum IgG in the same patient [227].

Also in murine models of RA, the role of autoantibodies has been highlighted. B cell deficient mice have demonstrated to be resistant to Collagen-induced arthritis [228]. Collagen-type II (CII)-specific antibodies precede arthritis in the collagen-induced arthritis model and anti-CII-IgG against four different epitopes transferred into wildtype mice was able to induce arthritis [229]. The

same was true for glucose-6-phosphate isomerase G6PI-autoantibodies, which cause arthritis in K/BxN serum-transfer arthritis [230].

Taken together, ACPA have been shown to be deeply involved in the development of clinical RA. Nevertheless, ACPA per se have been proven to be not pathogenic, suggesting that pathogenic ACPA arise from multiple hits driving the development of non-sialylated, pro-inflammatory ACPA and culminating in the transition from the "pre-rheumatoid" phase into chronic synovitis [203]. Despite major advances in understanding the factors that predispose for development of RA, the mechanisms regulating this transition from systemic autoimmunity into clinical arthritis are largely unknown.

c. T cells in RA

There are at least two lines of evidence that implicate T cells in initiating and promoting several aspects of RA. The first is supported by the fact, that ACPA bear evidence of T cell help, since they are often class-switched to IgG and their "complementarity determining region" (CDR) show evidence of somatic hyper mutation (SHM) [175]. Secondly, the HLA locus (especially HLA-DRB1) was found to be the most important risk factor and accounts for 30-50% of overall genetic susceptibility to RA, emphasizing that peptide presentation and deregulated T cell repertoire selection is important for RA development [231, 232]. Furthermore, *abatacept,* a fusion protein containing the extracellular CTLA-4 domain fused to the Fc part of IgG1, disrupts antigen-presentation by blocking T cell costimulation and is an efficient therapeutic treatment of RA[233].

Both, $CD8^+$ and $CD4^+$ T cells have been found inside joints of RA patients. While $CD8^+$ T cells are crucial in the onset of several autoimmune disease models, there is much data arguing for the superior role of $CD4^+$ T helper cells in RA onset. In the murine disease model of collagen-induced arthritis, it was established, that anti-CD4 treatment prevented disease [234], and that transfer of $CD4^+$ T cell from arthritic mice into T cell and B cell deficient SCID mice was able to induce arthritis [235].

The recently identified Th17 cells have been shown to express a wide range of pro-inflammatory cytokines (IL-17A-F, IL-21, IL-22)[58]. It is therefore not surprising that they have been shown to be dominant pathogenic players in many autoimmune diseases. In RA, an increased proportion of $CCR6^+$ Th17 cells were detected in the peripheral blood of patients suffering from early arthritis [236] and recent research has provided more evidence that Th17 cells are a key players during the pathogenesis of RA.

In 2003, it was shown, that mice deficient for the cytokine IL-23, a cytokine crucial for the development of pathogenic Th17 cells, were completely protected against collagen-induced arthritis [42, 237]. Also systemic overexpression of IL-23 leads to arthritis [238, 239].

More evidence suggests that Th17 cells themselves may take part in two separate parts of RA pathogenesis. The most discussed involvement is their part in promoting chronic inflammation and joint destruction in established RA. IL-17A in particular is considered to be osteoclastogenic and blockade or deletion of IL-17 results in reduced severity of arthritis, while local or systemic IL-17 overexpression exacerbated symptoms of CIA [240, 241]. The pro-inflammatory capacity of IL-17 is also underlined by the fact, that $Il17^{-/-}$ mice develop a less severe arthritis during the K/BxN serum transfer model, where arthritogenic serum initiates a transient and effector cell dependent arthritic reaction [242]. In human RA patients, IL-17 has been detected inside the inflamed synovium [240, 243].

However, the contribution of Th17 cell-derived IL-17 to this disease still remains incompletely understood, since IL-17 depletion was also effective in SCID mice lacking functional B and T cells during K/BxN serum transfer [242]. IL-17$^+$ CD4$^+$ T cells are reported to be found in close proximity to osteoclasts in the joints of RA patients, yet these reports are inconsistent and in other studies it was demonstrated that even though Th17 cells were more abundant in peripheral blood of RA patients, there was no difference in the affected joints [243, 244].

Thus, even though the role of IL-17 in RA patients is established inside the scientific community, anti-IL-17A treatment (secukinumab) was not highly effective [245, 246] questioning its role [247] during joint inflammation.

The same holds true for the Th17-cytokine IL-22. IL-22 was significantly elevated in the serum during RA in comparison to healthy controls and osteoarthritis patients. Elevated IL-22 levels were also found in inside the synovial fluid [248, 249]. These serum levels of IL-22 correlated with erosive disease [83]. In contrast less severe arthritis was observed in $Il22^{-/-}$ mice (CIA model) [250]. Furthermore, IL-22 showed the ability to induce synovial fibroblast proliferation and chemokine production [81].

Nevertheless, IL-22 had no direct role in an IL-17/Th17-mediated model of human synovial inflammation (antigen-induced arthritis), favoring the speculation that IL-22 contributes to arthritis in an indirect way and influences systemic immunity rather than because of its direct pro-inflammatory actions in the joint[249].

Although the differences between these various studies can be partially attributed to the heterogeneity of RA, the exact role of Th17 cells in contributing to chronic inflammatory processes inside the joint remains largely elusive.

Th17 cells can also contribute to RA pathogenesis during the initiation phase and early arthritis. While dissecting the IL-23-dependent and -independent phases of arthritis, it was shown, that IL-23 blockade before clinical onset of collagen-induced-arthritis lead to suppressed severity of arthritis. Administration of an αIL-23A antibody after the first clinical signs of arthritis (CIA) however, had no significant effect on the severity of the disease [251]. These findings are in line with the experience with IL-23 blockade in RA patients, which showed no clinical improvement in a phase IIA randomized controlled trial [252].
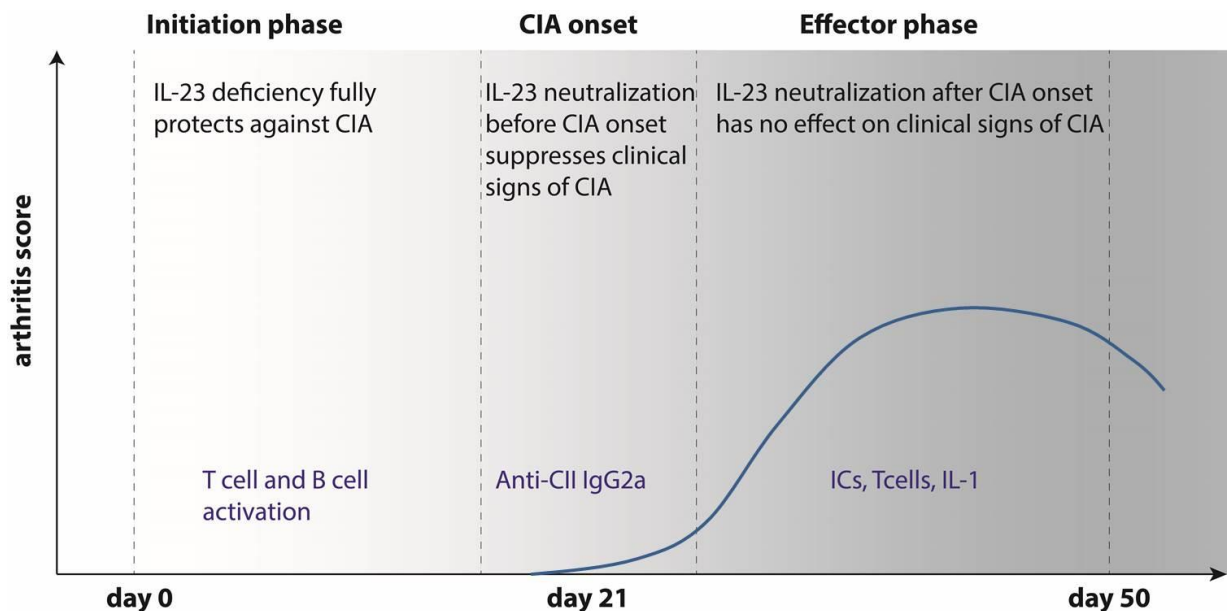
Figure 5: IL-23 dependent and independent stages of arthritis
Overview of the role of the IL-23/Th17 axis during the pathogenesis of experimental arthritis. Therapeutic neutralization during different stages of CIA leads either to a complete protection (initiation phase), had suppressive effects (onset of CIA) or showed no beneficial effects on the progression of the disease (effector phase): Figure was adapted from Lubberts et al. 2016 [253].

II. MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 MEDIA

Medium name	Composition
Ag8653 myeloma cells medium	Roswell Park Memorial Institute 1640 Medium; 10% FCS (heat-inactivated), 2mM L-Glutamine, 1% Penicillin/Streptomycin and 50 μM β-mercaptoehtanol [254].
B cell medium	Roswell Park Memorial Institute 1640 Medium; 10% FCS (heat-inactivated); 1% Penicillin/Streptomycin; 1% Sodiumpyruvat; 10 mM HEPES; 55 μm β-mercaptoethanol
Bone marrow derived dendritic cells medium	Roswell Park Memorial Institute 1640 Medium; 10% FCS (heat-inactivated); 2mM L-Glutamine; 1% Penicillin/Streptomycin (10 000 U/ml); 55 μM β-mercaptoethanol; 10% GM-CSF containing conditioned medium from Ag8653 myeloma cells[10]
Monocyte-derived dendritic cells medium	Roswell Park Memorial Institute 1640 Medium; 5% FCS (heat-inactivated); 2 mM L-Glutamine; 1% Penicillin/Streptomycin; 1 mM HEPES; 1 mM Sodium Pyruvat; 800 U/ml (40 ng/ml) GM-CSF; 500 U/ml (10 ng/ml) IL4.
T cell skewing medium	Iscove's Modifieds Dulbeccos's Medium; 10% FCS (heat-inactivated); 2mM L-Glutamine; 1% Penicillin/Streptomycin (10 000 U/ml); 50 μm β-mercaptoehtanol.

2.1.2 ENZYME & PROTEINS

Enzyme	Supplier
Albumin from bovine serum	Sigma-Aldrich (#A7030-100G)
Aleuria aurantia lectin (AAL), biotinylated	Biozol (#VEC-B-1395)
ArthritoMAB^{TM} monoclonal antibody cocktail for C57BL/6 incl. LPS	Mdbiosciences. (#CIA-MAB-2C/)
Bovine type II collagen	Sigma-Aldrich (#C7806-10MG)
Chicken type II collagen	Sigma-Aldrich (#C9301-100MG)
Collagenase from Clostridium histolyticum (CAS-9001-12-1)	Sigma-Aldrich (#C5138)
Erythrina cristagalli lectin (ECL), biotinylated	Biozol (#VEC-B-1145)
Lens culinaris agglutinin (LCA), biotinylated	Biozol (#VEC-B-1045)
Maackia amurensis Lectin II (MAL II)	Biomol (#M1061-15B.1)
Mycobacterium tuberculosis (H37RA)	BD (#231141)
Neuraminidase	New England Biolabs GmBH (#P0720L)
Ovalbumin	Sigma-Aldrich
Ovalbumin, Alexa Fluor 488 conjugate	Thermo-Fisher (O34781)
Peanut Agglutinin (PNA), biotinylated	Biozol (#VEC-BA-0074)
Peroxidase anti-biotin	Biozol (#VEC-SP-3010)
Sambucus nigra lectin (SNA), fluorescein	(Biozol (#VEC-FL-1301)
Sambucus nigra lectin (SNA), biotinylated	Biozol (#VEC-B-1305)
α-2,6-sialyltransferase from *photobacterium damsela*	Sigma-Aldrich (#2076-UN)
β-1,4-galactosyltransferase	Sigma-Aldrich (#48279-5mg-F)

2.1.3 CYTOKINES

Cytokine	Supplier
Recombinant h-IL-4	Peprotech (#200-04)
Recombinant h-GM-CSF	R&D Systems®, Minneapolis (#215-GM)
Recombinant h-TGFβ	BioLegend® (#580702)
Recombinant m-GM-CSF	PeproTech (#315-5)
Recombinant m-IL-17A	R&D Systems®, Minneapolis (#421-ML)
Recombinant m-IL-17F	R&D Systems®, Minneapolis (#2057-IL)
Recombinant m-IL-21	R&D Systems®, Minneapolis (#594-ML-010/CF)
Recombinant m-IL-22	R&D Systems®, Minneapolis (#582-ML-010/CF)
Recombinant m-IL-23	R&D Systems®, Minneapolis (#1887-ML/CF)
Recombinant m-IL-6	BioLegend®, San Diego (#575702)

2.1.4 BUFFER

Buffer	Composition	
10x Perm buffer		ebioscience
CII-IgG binding buffer	0.05 M Tris 0.15 M NaCl pH 7.5	sterile filtrated (0,4 µM)
CII-IgG elution buffer	0.1 Glycin-HCl pH 9.0	sterile filtrated (0,4 µM)
Decalcification buffer	14 % EDTA 2,25 % NH^3 pH 7.2 with NH$_4$OH	
ELISA blocking buffer	1% BSA in PBS	
ELISA stop solution	-	eBioscience
ELISA substrate solution I	(tetramethylbenzidine)	eBioscience
ELISA substrate solution II	(H$_2$O$_2$)	eBioscience
ELISA wash buffer	0,05% Tween 20 in PBS	-
FACS buffer	4% FCS in PBS	sterile filtrated (0,4 µM)
Galactosylation buffer	50 mM MOPS 20 mM MnCl$_2$ pH 7,2	sterile filtrated (0,4 µM)
Lectin-binding buffer	1 mM MnCl$_2$ 1 mM CaCl$_2$ 1 mM MgCl$_2$ in TBS	-
Lectin-blocking buffer	3% acid-treated Gelatine dialysed in TBS pH 7,6	-
Protein G - binding buffer	20 mM Na$_2$HPO$_4$ pH 7.0	sterile filtrated (0,4 µM)
Protein G - elution buffer	0,1 M Glycin pH 2,7	sterile filtrated (0,4 µM)

Protein G - neutralization buffer	1 M Tris HCL pH 9,0	sterile filtrated (0,4 μM)
RBC lysis buffer	4g NH$_4$Cl 2,35g HEPES 100 μL 0.5 M EDTA pH 8 add 500 ml H$_2$O	sterile filtrated (0,4 μM)
Rotiphorese® 50x TAE buffer	-	Carl Roth (#CL86.1)
Sepharose-acetate washing buffer	0,1 M C$_2$H$_3$NaO$_2$ 0,5 M NaCl pH 4,0	sterile filtrated (0,4 μM)
Sepharose-blocking buffer	0,2 M Glycin pH 8,0	sterile filtrated (0,4 μM)
Sepharose-cleaning buffer	1mM HCl	sterile filtrated (0,4 μM)
Sepharose-coupling buffer	0,1 M NaCO$_3$ 0,5 M NaCl pH 8,0	steril filtrated (0,4 μM)
Sepharose-Tris washing buffer	0,1 M Tris 0,5 M NaCl pH 8,5	sterile filtrated (0,4 μM)
Sialylation Puffer	50 mM MES 10 mM MnCl$_2$ pH 6,0	sterile filtrated (0,4 μM)
Tail lysis buffer	100 mM Tris-HCLpH8.5	-
TE buffer	10 mM TRIS-HCL 1 mM EDTA in H$_2$O ~ pH 8	-
Tris-buffered saline (TBS)	50mM Tris 150 mM NaCl; pH 7,6	

2.1.5 CELL CULTURE

Reagent	Supplier
DMEM	GIBCO® by life technologies (#41965-039)
FCS	GIBCO® by life technologies (#10499-044)
IMDM with HEPES	Lonza (#12-726F)
L-Glutamine	GIBCO® by life technologies (#25030-081)
PBS	GIBCO® by life technologies (#14200-067)
Penicillin/Streptomycin	GIBCO® by life technologies (#15140-122)
Plates	Greiner bio-one CELLSTAR®
RPMI 1640	GIBCO® by life technologies (#11875-093)
β-mercapto-ethanol	GIBCO® by life technologies (#31350010)

2.1.6 CHEMICALS

Chemical	CAS- number	supplier
2-(N-Morpholino)ethanesulfonic acid (MES)	71119-23-8	Sigma-Aldrich(#M3885)
2-Propanol	67-63-0	Merck Millipore (#109634)
3-(N-Morpholino)propanesulfonic acid (MOPS)	1132-61-2	Sigma-Aldrich (#M1254)
4-nitro blue tetrazolium chloride / 5-bromo-4-chloro-3-indolyl phosphate	-	Roche
Acetic acid	64-19-7	Sigma-Aldrich (#735020)
Ammonia solution	1336-21-6	Carl Roth (#A990.1)
Ammonium chloride	12125-02-9	Carl Roth (#5050.1)
Ammonium persulfate (APS)	7727-54-0	Sigma-Aldrich (#A3687)
Brefeldin A sol.	20350-15-6	BioLegend (#420901)
BSA	-	Sigma-Aldrich (#A7030)
Calcium chloride	10043-52-4	Carl Roth (#A119.1)
Chloroform	67-66-3	Merck Millipore (#102445)
CMP-Neu5Ac, disodium salt	3063-71-6	Merck Millipore (#233264)
Cyanogen bromide-activated-Sepharose® 4B	68987-32-6	Sigma-Aldrich (#C9142)
Dimethyl sulfoxide (DMSO)	67-68-5	AppliChem (#A3608,001)
di-Sodium-hydrogenphosphate-dihydrate	10028-24-7	Merck Millipore (#106476)
ECL Western blotting substrate	-	ThermoScientific (#32106)
ELISA stop solution	-	eBioscience (#BMS409)
ELISA substrate II and II	-	eBioscience (#BMS402/3)
Eosin	15086-94-9	Sigma-Aldrich (#230251)

Ethanol p.a.	64-17-5	Merck Millipore (100983)
Ethanol spoiled	-	Carl Roth (#T913.3)
Ethylenediaminetetraacetic acid (EDTA)	62-33-9	Sigma-Aldrich (#E9884)
Gelatine	9000-70-8	Carl Roth (#4308.3)
Glycine	56-40-6	Sigma-Aldrich (#G8898)
Guandidin HCL	50-01-1	Carl Roth (#0035.1)
HEPES	7364-49-9	Carl Roth (#9105.4)
Hydrochloric acid	7647-01-0	VWR 310701.1000
Imperial^{TM} Protein Stain	-	Thermo scientific (#24615)
Incomplete Freund's Adjuvant	-	Sigma (#F5506)
Ionomycin calcium salt	56092-82-1	Sigma (#10634)
Isopropanol	67-63-0	Sigma-Aldrich (#190764)
Laemmli buffer Biorad	-	Biorad (#161-0737)
LPS from E.coli O111:B4	-	Sigma-Aldrich (#L2630)
Lymphoflot	-	Bio-Rad (#824012)
Magnesium chloride	7786-30-3	Sigma-Aldrich (# 208337)
Manganese chloride	64333-01-3	Sigma-Aldrich (# 13220)
Methanol	67-56-1	Sigma-Aldrich (#322415)
Monensin	22373-78-0	ebioscience (#00-4505-51)
Mounting medium	-	Dako (#S3025)
peqGOLD Trifast	-	Peqlab (#30-2020)
Peroidic acid	10540-60-9	Carl Roth (#3257.1)
Phorbol 12-myristate 13-acetate (PMA)	16561-29-8	Sigma-Aldrich (# P8139)

Potassiumthiocyanat	333-20-0	Carl Roth GmBH&Co KG (#P753.1)
Powdered milk	-	Carl Roth (#T145.2)
RNAse free water	-	Fermentas (#R0581)
Roti Histofix®	-	Carl Roth (#P087-3)
Sodium acetate	127-09-3	Sigma-Aldrich (# S2889)
Sodium chloride	7647-14-5	Carl Roth (#3957.3)
Sodium dihydrogene carbonate	497-19-8	Carl Roth (#HNO1.1)
Sodium dodecyl sulfate (SDS)	151-21-3	Sigma-Aldrich (# 74255)
Sodium Pyruvat	113-24-6	Sigma-Aldrich (#58636)
Sodiumhydrogencarbonat	144-55-8	Carl Roth GmbH&Co KG (#6885.2)
Tissue Tek® O.C.T. ™ Compount	-	SAKURA (#4583)
Tris	77-86-1	Carl Roth (#48855.2)
Tween 20	9005-64-5	Sigma-Aldrich (#P2287)
UDP-α-D –galactose, disodium salt	137868-52-1	Merck Millipore (#670111)
β-mercapto-ethanol	60-24-2	Sigma-Aldrich (#6250)
glucose-6-phosphate	54010-71-8	Sigma-Aldrich (#7879)

2.1.7 ANTIBODIES

Antigen	Clone	Supplier
goat α-hamster biotin	Poly 4055	BioLegend ® (#405501)
goat α-rabbit IgG HRP	Polyclonal	Dako (#2016-03)
goat-α-rabbit-HRP		Promega
muIgG1 Ctrl (α-TNP)	muIgG1 SYNG-PRB000935	Pfizer
murine CD138	281-2	BioLegend ® (#142507) BioLegend ® (#142504)
murine CD16/32, TrueStain fcK^{TM}	93	BioLegend ® (#101319)
murine CD19	6D5 (rat IgG2a,κ)	BioLegend ® (#115537)
murine CD19 biotin	eBio1D3 (rat IgG2a,κ)	eBioscience (#13-0193)
murine CD21/35	7E9	BioLegend ® (#123411) BioLegend ® (#123407) BioLegend ® (#123413)
murine CD25	PC61	BioLegend ® (#101907)
murine CD254 (RANKL),LEAF	IK22/5 (rat IgG2a,k)	BioLegend ® (#510007)
murine CD267	281-2	BioLegend ® (#133403)
murine CD267 (TACI)	8F10 (rat IgG2a, κ)	BioLegend ® (#133404)
murine CD28, LEAF	37.51 (Syrian hamster IgG)	BioLegend ® (#102111)
murine CD3	17A2	BioLegend ® (#100210) BioLegend ® (#122126) BioLegend ® (#100214)
murine CD3ε, LEAF	145-2C11 (armenian hamster IgG)	BioLegend ® (#100331)
murine CD4	GK1.5 (rat IgG2b,κ)	BioLegend ® (#-00406)

murine CD45R (B220)	RA3-6B2 (rat IgG2a,κ)	BioLegend ® (#103227)
		BioLegend ® (#103206)
		eBioscience (#17-0452)
murine CD8a	53.6-7	BioLegend ® (#100711)
murine GL-7	GL7	BioLegend ® (#144605
		BioLegend ® (#144613)
murine GM-CSF biotin	MP1.31G6 (rat IgG1)	BioLegend ® (#505501)
		BioLegend ® (#576308)
murine GM-CSF, LEAF	MPA-22E9 (rat IgG2a,k)	BioLegend ® (#505407)
murine GR1	RB6-8C5	BioLegend ® (#108405)
murine IFNγ, LEAF	AN-18 (rat IgG1,k)	BioLegend ® (#517903)
murine IgD	11-26c2a	BioLegend ® (#405711)
		BioLegend ® (#405717)
murine IL17A biotin	TC11.18H1C1 (rat IgG1,k)	BioLegend ® (#507001)
murine IL17A, LEAF	TC11.18H1C1 (rat IgG1,k)	BioLegend ® (#506905)
murine IL17F biotin	8F5.1A9 (mouse IgG1,k)	BioLegend ® (#517105)
murine IL21 biotin	7H20-I19-M3 (mouse IgG1,k)	BioLegend ® (#514303)
murine IL-22	Polyclonal Goat IgG	BioLegend ® (#516407)
		BioLegend ® (#516404)
murine IL22, purified	Poly5164 (polyclonal goat)	BioLegend ® (#516401)
murine IL-22Rα1	Clone #496514 (rat IgG2a)	R&D Systems®, Minneapolis #FAB42941
murine IL-23A	BiP1027B-5B5-1 Khaverty 2015.01.1	Pfizer
murine IL-4	11B11	BioLegend ® (#504104)
murine RoRγt	B2D	BioLegend ® (#126981-80)
murine/human IL-22	IL22JOP	eBioscience 17-7222-80

rabbit IgG Ctrl	SC2027	Santa Cruz Biotechnology (#F0313)
rat IgG1 biotin, Ctrl	RTK2071	BioLegend ® (#400403)
rat IgG1,κ Ctrl PE	eBRG1	eBioscience (#12-4301-83)
human CD38	HIT2 (RUO)	BD (#551400)
human CD27	M-T271 (RUO)	BD (#555441)
human CD19	LT19	Miltenyi Biotech

2.1.8 KITS

Kit	Supplier
Avidin/Biotin Blocking kit	VectorLabs (#SP-2001)
CD43 (Ly48) MicroBeads, mouse	MACS Miltenyi Biotech (# 130-049-801)
EasySep Mouse CD4+ TCell Isolation Kit	STEMCELL Technologies (#19852)
Foxp3/Transcription factor staining buffer	ebioscience (#005223-56)
Human IL-17A Duoset® ELISA	R&D Systems®, Minneapolis (#DY317)
Human IL-21 Duoset® ELISA	R&D Systems®, Minneapolis (#DY782)
Human IL-6 Duoset® ELISA	R&D Systems®, Minneapolis (#DY206)
Human IL-8 Duoset® ELISA	R&D Systems®, Minneapolis (#DY208)
Human TNFα Duoset® ELISA	R&D Systems®, Minneapolis (#DY210)
Mouse CXCL1/KC Duoset® ELISA	R&D Systems®, Minneapolis (#DY453)
Mouse Ig2b –HRP (ELISA)	Bethyl Laboratories, inc (#A90-109P)
Mouse IgG1 –HRP (ELISA)	Bethyl Laboratories, inc (#A90-105P)
Mouse IgG2c ELISA Quantification set	Bethyl Laboratories, inc (#E90-136)
Mouse IgG3 –HRP (ELISA)	Bethyl Laboratories, inc (#A90-111P)
Mouse IgM –HRP (ELISA)	Bethyl Laboratories, inc (#A90-101P)
Mouse IL-6 Duoset® ELISA	R&D Systems®, Minneapolis (#DY406)
Mouse TNFα Duoset® ELISA	R&D Systems®, Minneapolis (#DY410)
Mouse total IgG ELISA	
Protein-G Gravi-Trap	GE-Healthcare (28-9852-55)
Protein-G HP-SpinTrap	GE-Healthcare (28-9031-34)

2.1.9 PRIMERS

Gene name	Sequence
β-actin	Forward: 5'-TGTCCACCTTCCAGCAGATGT-3' Reverse: 5'-AGCTCAGTAACAGTCCGCCTAGA-3'
blimp1	Forward: 5'-ACACACAGGAGAGAAGCCACATGA-3' Reverse: 5'-TCGAAGGTGGGTCTTGAGATTGCT-3'
b4galt1	Forward: 5'-GCCATCAATGGATTCCCTAA-3' Reverse: 5'-CATTTGGACGTGATATAGACATGC-3'
fut8	Forward: 5'-CTGGTCCAGCGGAGAATAAC-3' Reverse: 5'-CATAGCCACAGCCTTTATTGATG-3'
st6gal1	Forward: 5'-TGAGCCTTCCCCAAATACCT-3' Reverse:5'-TTCACAGGATGATCAAAAACCA -3'

2.1.10 PLASTIC-WARE

Plastic ware	Company
Amicon Ultra-0.5, Ultracell, 30 kDa	Millipore (#UFC503096)
Amicon Ultra-0.5, Ultracell, 50 kDa	Millipore (#UFC505096)
Amicon Ultra-15 Ultracell, 50 kDa	Millipore (#UFC905024)
Amicon Ultra-4 Ultracell, 50 kDa	Millipore (#UFC805024)
Bio-spin chromatography columns empty, 100	BioRad (#732-6008)
Cell culture plates	Greiner bio-one CELLSTAR®
Cell strainer 40,70,100 µm	Falcon
CellTrics 50,100 µm	Sysmek
Colstar® EIA/RIA 1*8 Stripwell^{TM} Plate	Corning
Cover glasses (Ø12mm)	Thermo-Scientific
ImmunoBlot® PDVF membrane	Biorad
Mausinjektionskafig Typ A	G&P Kunststofftechnik
Serological pipettes	Sarstedt
Micro Hämakrit-Kapillaren, 75mm, heparinisiert	Schubert&Weiss OMNILAB (#5411215)

2.1.11 MACHINES

Machine	Company
7300 Real Time PCR System	Applied Biosystems
BBD 6220 CO$_2$ Incubator	Thermo Scientific
BD FACSCanto^{TM} II	BD Biosciences
DELFIA^{TM} system	Perkin Elmer
EpiShear^{TM} Probe Sonicator & EpiShear^{TM} Cooled Sonication Platform	Active Motif
FACSCalibur^{TM}	BD Biosciences
Gallios^{TM} Flow Cytometer	Beckman Coulter
Heraeus Megafuge 1.0R	Thermo Scientific
Microcentrifuge 5417R	Eppendorf
Mini-PROTEAN® Tetra Cell	Bio-Rad (#1658000)
Olympus DP72 digital camera	Olympus
PowerPac^{TM} HC power supply	Bio-Rad
QuantStudio^{TM} 7 Flex Real-Time PCR System	Applied Biosystem
Sonopuls HD 2070	Bandelin Electronic
Spectra MAX 190 spectrophotometer	Molecular Devices
Thermomixer® comfort	Eppendorf
TProfessional Thermocycler	Biometra
Trans-Blot® SD Semi-Dry Transfer Cell	Bio-Rad
Zeiss Axioskop (LabA1) microscope	Zeiss

2.1.12 SOFTWARE

Software	Company
Prism 5	GraphPad Software
Abdobe Illustrator CS5	Abdobe
Abdobe Photoshop CS6	Abdobe
7300 System Software	Applied Biosystems
QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Base v1.x	Applied Biosystems
OsteoMeasure™	OsteoMetrics
Bio-Rad CFX Manager 3.0	Bio-Rad
FlowJo software Version 7.6.5	Tree Star Inc.
Kaluza® 1.2	Beckman Coulter Inc. Brea, CA(#B16406)

2.2 ANIMALS

Mice used in this dissertation are listed in table 2.1.

$Il23a^{-/-}$, $Il12a^{-/-}$, $st6gal1^{-/-}$ mice were backcrossed for m 12 generations to the C57BL/6 background. Most mice were used at 6-12 weeks of age unless otherwise stated. The mice were housed in the animal facility of the University Erlangen-Nuremberg and the animal experiments were approved by the government of Mittelfranken (Franconia, Bavaria, Germany).

Mouse strain	General remarks and references
C57BL/6j	Purchased from Charles River Laboratories (San Diego, CA, USA)
DBA/1	Purchased from Charles River Laboratories (San Diego, CA, USA)
$Il23a^{-/-}$	Kindly provided by Prof. Dr. Christoph Becker [255]
$Il12a^{-/-}$	Kindly provided by Prof. Dr. Bogdan [256]
NOD	Purchased from Jackson Laboratories (Stock No: 001976)
KRN	Generated from D. Mathis and C. Benoist (Harvard Medical School, Boston, MA) [257]
K/BxN	Male KRN T-cell receptor transgenic mice were bred to NOD mice to generate K/BxN mice.

2.3 HUMAN SUBJECTS

Informed consent from all healthy controls and all RA patients was obtained. Analysis of human material was approved by the ethics committee of the University Hospital Erlangen.

2.4 METHODS

2.4.1 GENOTYPING

Genomic DNA was isolated by digestion of a piece of tail of *Il23a*$^{-/-}$ the mice in so called tail lysis buffer overnight by 55°C, and the PCR was performed with the following primers:

primer 1: 5'- GGA CTA CAG AGT TAG ACT CAG -3'
primer 2: 5'- GTC ACA ACC ATC TTC ACA CTG -3'
primer 3: 5'- GAA ACG CCG AGT TAA CGC CAT -3'

With the following program:

94°C 3 min   1 cycle

94°C 0:45 min   32 cycle
56°C 0:30 min
72°C 1:30 min
72°C 7 min   1 cycle
15°C   ∞

2.4.2 ARTHRITIS MODELS

a. Induction of Collagen-induced arthritis (CIA)

Collagen-inducedarthritis (CIA) was induced as described elsewhere [258]. Briefly, collagen type II (CII, Sigma C-930) was emulsified in the Incomplete Freund's Adjuvance (IFA) using a pulse sonificator. Mice were immunized intradermal with 100 µL of the emulsion.

CIA in high responder strains (H-2^q)
DBA/1 mice were immunized with 100µg type II collagen and 100µg heat-inactivated *M.tubercolosis* emulsified in IFA on day 0 and boosted with 50 µg in IFA on day 21.

CIA in low responder strains (H-2^b)
C57BL6 mice where immunized with 200µg type II collagen together with 250µg heat-inactivated *M.tubercolosis* emulsified in IFA on day 0 and day 21.

b. Collagen-antibody-induced arthritis (CAIA)

Collagen antibody-induced arthritis (CAIA) was induced by using a cocktail containing M2139 (γ2b), CIIC1 (γ2a), CIIC2 (γ2b), and UL1 (γ2b) monoclonal antibodies binding to triple helical J1 [MP*GERGAAGIAGPK; P* indicates hydroxyproline], C1 (GARGLTGRO), D3 (RGAQGPOGATGF), and U1 (GLVGPRGERGF) CII epitopes was used to induce arthritis. The cocktail was kindly provided by MD bioscience. 4 mg of total IgG was injected on two consecutive days and mice additionally received LPS (25 µg per mouse i.p.) at day 5 as a secondary stimulant [229, 259]. Arthritis development was monitored following the protocol described above for collagen-inducedarthritis.

c. K/BxN arthritis

K/BxN arthritis, which spontaneously develops in the F1 generation of KRN mice crossed to NOD mice, has been described before [260, 261]. Shortly, male KRN T vcell receptor transgenic mice were bred to NOD mice to generate K/BxN mice. Mice were scored from week 2 until week 6 of age. During anti IL-23 blockade mice received 5 mg/kg αIL-23A antibody (5B5; fully murine IgG1) or a similar amount of an isotype twice a week starting treatment on day 14 after birth. 5B5 was provided by Boehringer Ingelheim and displayed a IC50/90 during the inhibition of IL-17A secretion from mouse IL-23 stimulated splenocytes of 5+/- 0.2/66+/-43nM.

d. Induction of K/BxN serum transfer arthritis

Serum transfer arthritis was induced by an intraperitoneal injection of K/BxN serum (150µL/mouse) and was monitored until day 14. Paw thickness was measured with a calliper (Peacock) and the results were expressed as the change of the joint thickness (in mm) relative to that value measured on day 0.

Clinical arthritis severity was graded by scoring each limb on a scale from 0 to 4, where 0 = no erythema of swelling; 1 = mild swelling or erythema of ankle, wrist or individual digits; 2 = moderate, but defined erythema and swelling of ankle or wrist; 3 = severe erythema and swelling extending from the entire paw including digits; 4 = maximal inflamed limb with involvement of multiple joints. The total clinical score was determined by adding the individual scores for each limb.

2.4.3 IMMUNIZATION AGAINST OVALBUMIN

8 to 12 week old C57BL/6 mice were immunized intradermal with 200 µg of chicken derived ovalbumin (Sigma) together with 250 µg of heat-inactivated *M. tuberculosis* H37RA (BD, 231141) emulsified in Incomplete Freund's Adjuvant (IFA, Sigma) on day 0 and day 21. Splenic ovalbumin specific plasmablasts were analysed for St6gal-1 on day 0 and day 26.

2.4.4 HISTOLOGY

Bones were fixed for 6 h in 4% paraformalin and decalcified in an EDTA containing (table 2.1.4). Serial paraffin sections (2 µm) were stained with TRAP for osteoclast detection or with hematoxylin-eosin for morphological analysis of joints. All analyses were performed using a microscope (Nikon, table 2.1.11) equipped with a digital camera and an image analysis system for performing histomorphometry (Osteomeasure; OsteoMetrics, table 2.1.11).

2.4.5 IMMUNOFLUORESCENCE

The spleens were snap-frozen in O.C.T Tissue-Tek compound (table 3.1.6) and kept for at least 2 days at -80°C. Sections of 4 µm were performed and fixed for 2,5 min in a cold acetone bath (-20°C) and stored in -20°C till staining.

The slides were rehydrated with PBS for 5 min and unspecific IgG binding sites were blocked with TruStain fcX^{TM} blocking antibody (1:100; table 3.1.7) in PBS containing 10% rat serum for 30 min at room-temperature. Possible endogenous biotin-binding sites were blocked with the Avidin/Biotin Blocking kit (Vector, table 3.1.8) according to the manufacturer's instructions.

Sections were stained in 5% rat serum/PBS with Pacific-Blue, FITC, PE and APC-labelled antibodies at 4°C overnight. Afterwards, sections were washed three times for 5 min with PBS and the slides were covered using mounting medium. Micoscropy was performed with an Eclipse-80i microscope and a monochromatic camera (DS-Qi1MC; both from Nikon). Photomicrographs are shown with indicated pseudocolors produced by NIS elemts software BR3.0 (Nikon, table 3.1.12).

2.4.6 ISOLATION AND DIFFERENTIATION OF CELLS

a. Tissue Preparation

Human mononuclear cells were isolated from full blood. PBMCs were separated from healthy donors by Ficoll-Diatrizoate-density gradient centrifugation (Biorad). After centrifugation the PBMC containing interphase was isolated and washed two times with 1mM EDTA in PBS.

Mice were killed in a CO_2 chamber before spleen; bone marrow and inguinal lymph nodes were harvested. For cell extraction from spleen, lymph nodes or bone marrow, tissues were passed through a 100 μm nylon cell strainer (FALCON) to obtain single-cell suspensions. Red-blood cells were lysed in 5 ml of RBC-lysis buffer (table 3.1.4) for 3 min. For calculating cell concentrations, cells were counted using the "Neubauer-counting-chamber". For single cell isolation from murine paws the paws were incubated together with collagenase (2mg/ml, table 3.1.2) at 37°C for 1 hour while shaking with 350 rpm. Digested pieces were forced through a 100 μm cell strainer and erythrocytes were lysed as described above.

b. Generation of CD4+ Th17 cells

Splenocytes from C57BL/6 mice were isolated as described above and murine splenic $CD4^+$ T cells were isolated by immune-magnetic separation using EasySep (STEMCELL Technologies, table 3.1.8) according to the manufacturer's instructions.

For cytokine-mediated Th17 cell differentiation, $1x10^6$ $CD4^+$ cells (purity > 90%) were cultured in 1 ml "T cell skewing medium" (table 3.1.1) in a 48-well plate which have been coated for 3 hours with 5 μg/ml αCD3 antibody. Furthermore α-CD28 (3 μg/ml), IL-6 (10 ng/ml) and TGFβ (5 ng/ml) were added to the medium. To obtain pathogenic Th17 cells isolated $CD4^+$ cells were differentiated in the presence of IL6 (10 ng/ml) and IL-23 (20 ng/ml). After 4 days of culture 40% of the T cells were positive for IL-17A.

c. Isolation of B cells

CD43-negative splenic B cells were isolated from C57Bl/6 mice by CD43 depletion using MACS technology (table 3.1.8) to a purity of approximately 95-99%. Purified splenic B cells were stimulated with 5 µg/ml LPS (Sigma) for 72 h with or without following cytokines: IL-17A, IL-17F, IL-21, IL-22, IL-23, GM-CSF (R&D Systems®, table 3.1.3).

CD138$^+$ CD19$^+$ B220$^-$ CD3$^-$ GR1$^-$ plasma cells were sorted out using the spleens of *Il23a$^{-/-}$* and C57BL/6j mice before and during the induction of the collagen-induced arthritis.

d. Generation of bone marrow-derived dendritic cells

For measuring the intrinsic inflammatory capacity of murine total and collagen type II specific IgGs bone marrow cells were isolated from femora and tibiae of 6- to 8 week old mice. Erythrocyte depleted cells were cultured for 8 days in R10 medium at a concentration of 2×10^6 cells per 10-cm dish (Falcon, no. 1029, bacterial quality) and granulocyte macrophage colony-stimulating factor (GM-CSF). R10 culture medium is composed of RPMI 1640 (Lonza) supplemented with penicillin (100 U/ml, Lonza), streptomycin (100 mg/ml, Lonza), L-glutamine (2 mM, Lonza), 2-mercaptoethanol (50 mM, Sigma-Aldrich), and 10% heat-inactivated FCS (PAA Laboratories). GM-CSF supernatant (1:10) from a cell line transfected with the murine GM-CSF gene was used [262]. On day 3 a volume of 9 ml R10 medium containing 1 ml GM-CSF supernatant was added to the cultures. 50% of the culture supernatant was removed on day 6, and cells were fed again with fresh 10 ml of 1:10 diluted R10 medium containing GM-CSF supernatant. On day 8, non-adherent cells (>95% CD11c$^+$) were harvested and used for the different experiments [263].

Total IgG immune complexes (HAG) were obtained by heat aggregation of the IgG at 63°C for 30min. Collagen type II specific complexes were obtained by coating chicken collagen on sterile cell culture plates (10 µg/ml) followed by incubation with pooled IgG from collagen-induced arthritis mice. Efficiency of CII-IgG complex generation was tested via ELISA.

e. Generation of monocyte-derived dendritic cells (MoDC)

Monocytes were isolated from PBMCs (via their capability to bind on plastic surfaces) and cultivated in RPMI supplemented with 5% FCS (Biochrom), 1% penicillin/streptomycin (Gibco), 1% L-Glutamin (Gibco) 1mM HEPES, 1mM Sodium Pyruvate (Sigma) in the presence of 800 U/ml GM-CSF (Peprotech) and 500 U/ml IL4 (Peprotech). Cells were fed on day 3 and day 5. Experiments were performed on day 6 after pre-conditioning MoDCs with 0.5ng/ml LPS.

2.4.7 FLOW CYTOMETRY CELL SORTING (FACS) ANALYSES

a. Surface staining

Single cell suspensions were done as described before. After isolating and counting of the cells, Fcγ receptors were blocked by incubating the cells in 100 µl "FACS buffer" (table 3.1.4) containing TruStain fcX^{TM} blocking antibody (1:100, table 3.1.7). After 10 min incubation at room temperature, 100 µl of staining solution containing the diluted antibodies in FACS buffer at a saturating concentration was added, and the cells were incubated in the dark, at 4°C for 30 minutes. Afterwards, the cells were washed with 1ml FACS buffer and finally resuspended in 200 µl FACS buffer.

b. Intracellular staining

Intracellular staining was performed after surface staining, done as described above. Surface stained cells were fixated and permeabilized with the "Foxp3/Transcription factor staining buffer set" from ebioscience (table 3.1.8) according to the manufacturer's instructions. Permeabilized cells were stained intracellularly overnight at 4°C. Afterwards cells were washed 2 times with 1ml 1xPERM buffer (table 3.1.8) and resuspended in 200 µL FACS-Puffer. Intracellularly cytokines were stained after *in vitro* activation of single cell suspensions with 50 ng/ml PMA and 1 µg/ml ionomycin for 4-6 hours (table 3.1.6). Brefeldin A (1:1000) and monensin (1:1000) were also added for at least 4 hours. After that cells were harvested and used for following surface and intracellular cytokine staining. OVA-specific plasma cells were stained intracellularly using OVA-A488 (5 µg/ml) from Thermo-Fisher for 20 min at 4°C. The flow-cytometric analysis was performed with a GALLIOS flow cytometer (Beckmann Coulter) and analysed with FlowJo and Kaluza$^{®}$ software (table 3.1.12).

c. Flow-cytometry based sialic acid analysis on human plasma cells

Human PBMCs were isolated from the blood of RA patients and healthy donors, respectively. Human PBMCs were stained with anti-CD19 (Miltenyi Biotech), anti-CD27 and anti-CD38 antibodies all purchased by BD. Afterwards surface sialic acid was stained using Sambuccus nigra agglutinin (SNA)-FITC from Vector Laboratory (1:800). For the purpose of a negative control *st6gal1*$^{-/-}$ mice derived B cells were used, and human B cells were desialylated using Neuraminidase (100 mU/mio cells at 37°C for 1h) or sialic acid was oxidized using mild iodate treatment (2mM iodate in PBS for 30 min at 4°C).

2.4.8   IGG TREATMENT

a. IgG Isolation

Human IgG was obtained from healthy controls, ACPA-positive asymptomatic individuals that had not experienced arthritis yet or active ACPA-positive RA patients (DAS28 > 3.2). Murine serum was obtained from arthritic C57BL/6 (Charles River), DBA/1 or *Il23a*$^{-/-}$ mice on day 50. IgG were isolated by purification using a protein G column (GE Healthcare) according to the manufacturer's instructions.

b. Autoantibody isolation

For coating with chicken type II collagen, CNBr-activated Sepharose 4B beads were suspended on ice cold "Sepharose cleaning buffer" (2.1.3) and incubated for 1 h on ice. Every 20 minutes "Sepharose cleaning buffer" was exchanged. Then the beads were washed briefly with ddH$_2$O , followed by coupling with collagen (2 mg/g beads) in "Sepharose-coupling buffer" (table 2.1.3) over night at 4°C on a rolling device. Afterwards unreacted groups were blocked with "Sepharose-blocking buffer" (table 2.1.3) for 2 h, followed by five washing steps with "Sepharose-acetate" and "Sepharose-Tris" washing buffer (table 2.1.3). Collagen-coupled beads were stored at 4°C in 1 M NaCl. Protein-G isolated total IgG was dialysed in "CII-IgG binding buffer" (table 2.1.3) and enriched over collagen type II-coupled Sepharose 4B beads (chicken or bovine, respectively), washed with 1 M NaCl for four times and eluated with "CII-IgG elution buffer" (table 2.1.3). Enrichment of CII-specific IgG was confirmed by ELISA.

c. IgG modification

For desialylation, IgG was incubated with 100 U/mg (human) or 12500 U/mg (mouse) neuraminidase (NEB). Human IgG was desialylated within 24 hours, while desialylation of murine was done within 48 hours at 37°C. The digested IgG was purified using a protein G column (GE Healthcare) according to the manufacturer's instructions. Protein concentration was determined with nanodrop. The efficiency of the enzymatic digestion was tested via lectin blot.

For galactosylation 1 mg of murine IgG was incubated with 0.8 mM UDP-galactose (Calbiochem) and 50 mU of b-1-4 galactosyl transferase (Sigma) in 50 mM MOPS, pH 7.2 with 20 mM $MnCl_2$ for 48 h at 37°C. For subsequent sialylation 1mg of IgG was incubated with 0.5 mM CMP-sialic acid (Calbiochem) and 25 mU of α2-6 sialyl transferase (Sigma) in 50 mM MES, pH 6,0 with 20 mM $MnCl_2$ for 48h at 37°C. The reactions were confirmed with a lectin blot.

2.4.9   IgG GLYCOSYLATION PROFILING

Total-IgG, CII-IgG specific and CII-depleted IgG were purified from mice on day 50 after CII immunization from arthritic mice.

a. Lectin blotting

IgG was resolved on a 10% sodium dodecyl sulfate-PAGE (SDS-PAGE) gel under reducing conditions, transferred to PVDF membranes and blocked with 3% deglycosylated gelatin (Sigma). Gelatin was deglycosylated by incubating with 20 mM periodic acid for 8h at room temperature. Deglycosylated gelatin was dialysed in 1x TBS overnight.

Blots were incubated with biotinylated sumbuccus nigra lectin (2µg/ml) for sialic acid, erythrina cristagalli lectin (5µg/ml) for galactose, aleuria aurantia lectin for fucose, or lens culinaris agglutinin (5µg/ml, all vector laboratories) for the detection of the core glycan, followed by incubation with streptavidin-HRP and detection via Pierce ECL (Thermo-Scientific). Loading was checked by anti-mouse IgG-HRP Western Blot antibody.

b. Mass-spectrometry

For the analysis of Fc glycans, the IgG eluates were dried in a vacuum centrifuge and subjected to tryptic digest by adding 200 ng trypsin (sequencing grade, Promega) in 40 µL ammonium bicarbonate buffer followed by overnight incubation at 37°C. Digested IgG from *Il23a*^{-/-} and wild type mice were separated and analyzed on an Ultimate 3000 UPLC system (Dionex Corporation, USA) coupled to a maXis™ HD Ultra-High Resolution Q-TOF mass spectrometer (MS) (Bruker Daltonics, Germany) as described previously with minor modifications[264, 265]. Following extraction of tryptic glycopeptides by a C18 solid phase extraction trap column (Dionex Acclaim PepMap100), separation was achieved on an Ascentis Express C18 nano-liquid chromatography (LC) column (Supelco, USA) conditioned at 900nl/min with 0.1% TFA (mobile phase A) after which the following gradient of mobile phase A and 95% acetonitrile (mobile phase B) was applied: 0min 3% B, 2 min 6% B, 4.5 min 18% B, 5 min 30% B, 7 min 30% B, 8 min 0% B and 11min 0% B. The UPLC was interfaced to the MS with a sheath-flow ESI sprayer. Mass spectra were recorded from m/z 600 to 2000 with 2 averages at a frequency of 0.5 Hz. Quadrupole ion energy and collision energy of the MS were set at 2 and 4 eV, respectively. The total analysis time per sample was 13min. Quality of mass spectra was evaluated based on intensities of total IgG1 glycoforms. Data processing and calculations of the level of galactosylation, sialylation and fucosylation residues of IgG1and IgG2 were performed as described earlier [222, 265, 266].

2.4.10   WESTERN BLOT ANALYSIS

Cells and tissue were homogenized and lysed in 1x Laemmli buffer. Protein concentration were determined using a RC/DC Protein Quantification Kit (Bio-Rad). IgG concentration was determined using the Nanodrop technique. Proteins were separated by electrophoresis in 10% SDS poly acrylamide gels. Proteins were blotted on polyninylidene difluoride membranes and, after blocking with 5% dry mikl/0.1% Tween 20, incubated with rabbit anti-mouse *st6gal1*(C) antibody (1µg/ml; IBL) or anti-Hsp90 antibody (1:1000) over night at 4°C. A secondary antibody was applied the next day for 1 h at room temperature and the proteins were visualized by enhanced chemiluminescence (ECL) procedure, according to the manufacturer's instructions. Polyclonal anti-rabbit HRP secondary antibody was used for detection of *st6gal1*, while anti-mouse Hsp90 was detected with anti-mouse-HRP secondary antibody (all 1:2000).

2.4.11   RT-PCR ANALYSIS

Total RNA was isolated from cells using peqGOLD TriFastTM (Peqlab). 500 ng was used for the first strand complementary DNA synthesis (Amersham Biosciences), which was then used for SYBR Green-based quantitative RT-PCR. Relative quantification was performed by calculating the difference in cross-threshold values (Δct) of the gene of interest and a housekeeping gene according to the formula 2-ΔCt. Normalized gene expression values were calculated as the ratio of expression of messenger RNA (mRNA) for the gene of interest to the expression of mRNA for *β-actin*.

2.4.12   ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

a. Cytokine quantification

Cell culture supernatants were analysed for murine TNFα, CXCL1 and IL-6 as well as human IL-17A, IL-22, TNFα, IL-6 and IL-8 were performed with ELISA kits (R&D-systems). The protocols of the company were strictly followed.

b. Autoantibody quantification

For analysis of collagen type II autoantibodies, microtiter plates were coated overnight with 5 µg/ml chicken collagen type II. The coated plates were washed 3 times with ELISA-washing buffer and unspecific binding sites were blocked with 3% BSA in PBS for 90 minutes. Sera where added at 1:1000 dilution in 3% BSA/PBS for 90 min at room temperature. Bound IgG where detected with horseradish peroxidase-conjugated anti-mouse IgG, IgG1, IgG2b, IgG2c, IgG3 or IgM antibody (1:1000 dilution at room temperature for 90 minutes). The detection of the IgG-HRP was performed with ELISA substrate for no longer than 10 min and the reaction was stopped by acid (ELISA stop solution).

2.4.13   DETECTION OF HIGH AFFINITY IgG ANTIBODIES

Mice were immunized i. p. with 100 µg Alum-precipitated (Thermo Scientific) 4-hydroxy-3-nitrophenylacetyl (NP) coupled to chicken γ-globulin (NP-CGG), with a ratio of NP to CGG of 21 (BioSearch Technologies). NP-specific IgG in the sera were measured by ELISA, using two different coupling ratios of NP–BSA (8 and 26) as the coating antigens. To estimate the affinity of NP-binding antibody the ratio of NP8-binding antibody to NP26-binding antibody was

calculated. Briefly, Maxisorp plates (Nunc) were coated with 10 µg/ml NP-BSA in PBS at 4°C overnight and blocked with 3% BSA in PBS. Serially diluted sera were then added and incubated at 4°C overnight. Subsequently, HRP-conjugated goat anti–mouse IgG (Southern Biotech) was added and incubated at room temperature for 1 h. Between incubation steps, the plates were washed with PBS containing 0.05% Tween 20. HRP activity was detected using TMB peroxidase substrate and stop solution (eBioscience). Optical densities were determined at 450 nm. As standard we used pool of sera of immunized mice, which was applied to every plate.

2.4.14 QUANTIFICATION OF MOUSE CII-SPECIFIC IgG

a. DELFIA™ system

The detection of mouse specific αCII-IgGs were carried out by europium (EU+) -labeled α–mouse IgG antibody and the DELFIA™ system (Perkin Elmer) according to the manufacturer's recommendations.

b. Luminex Assay

NeutrAvidin (Thermo Fischer) was coupled with carboxylated beads (COOH Microspheres, Luminex-Corp.) in accordance to previously published antigen coupling protocols with minor modifications [267]. In brief, 10^6 beads per bead identity were distributed across 96-well plates (Greiner BioOne), washed and resuspended in phosphate buffer (0.1 M NaH2PO4, pH 6.2) using a plate magnet and a plate washer (EL406, Biotek). The carboxyl groups on the surface of the beads were activated by 0.5 mg of 1-ethyl-3(3-dimethylamino-propyl) carbodiimide (Pierce) and 0.5 mg of N-hydroxysuccinimide (Pierce) in 100 µl phosphate buffer. After 20 min incubation on a shaker (Grant Bio) beads were washed in MES buffer (0.05 M 2-(N-morpholino)ethanesulfonic acid, pH 5.0). 250 µg/ml of NeutraAvidin was prepared in MES buffer and added to the beads. The coupling reaction was allowed to take place for 2 h at room temperature (RT), the beads were then washed 3x in PBS-T (0.05% Tween20 in PBS), resuspended in 100 µl storage buffer (1% BSA, 0.05% Tween20, ProClin300 in PBS) and stored in plates at 4°C overnight. Following day 100 µl of each different biotinylated peptide at 50 µM concentration was added to NeutrAvidin-coated beads. Following an overnight incubation at 4°C beads were washed 3x in PBS-T and resuspended in 100 µl storage buffer. The final antigen suspension bead array was prepared by

combining equal volumes of each bead identity. Triple helical CII peptides containing defined epitopes have previously been described [268]. Immobilization of the peptides was confirmed by the use of monoclonal CII-epitope specific antibodies used in collagen antibody induced arthritis.

Serum samples were diluted 1:100 (v/v) in assay buffer (3% BSA, 5% milk powder, 0,1% ProClin300, 0,05% Tween 20, 100 ug/mL Neutravidin in PBS) and incubated for 60 min at RT on a shaker for preadsorption of unspecific antibodies. Using a liquid handler (CyBi-SELMA, CyBio), 45 µl of 1:100 diluted serum samples were transferred to a 384 well plate containing 5 µl bead array per well. Following incubation at RT on a shaker (Grant Bio) for 75 min, beads were washed 6x with 60 µl PBS-T on a plate washer (EL406, Biotek) and resuspended in 50 µl of each secondary antibody solution. Anti-mouse IgG Fcγ–PE diluted 1:500 in a buffer consisting of 5% BSA, 0.05% Tween20 in PBS and used for detection of antibodies. After incubation with the secondary antibodies for 40 min, the beads were washed 3x with 60 µl PBS-T and resuspended in 60 µl PBS-T for measurement by a FlexMap3D instrument (Luminex Corp.). The median fluorescence intensity (MFI) was chosen to display serum antibody-peptide interactions.

2.4.15 STATISTICAL ANALYSES

All data are shown as means. Group values are analyzed by two tailed Student's t test. If three different groups are compared the Two-way ANOVA test was used. Outliers were identified using the ESD-method. Significant differences are indicated as: *$p<0.05$; **$p<0.01$; *** $p<0.001$.

III.   RESULTS

3.1   IL-23 DURING PRIMING PHASE AND EFFECTOR PHASE OF AUTOIMMUNE ARTHRITIS

RA has been demonstrated to be a T cell dependent disease. For decades Th1 cells were considered to be critical during the pathogenesis of RA. In 2003 the contribution Th1 cells was questioned through the discovery of Th17 cells. Extensive research during the last decade has identified the IL-23/Th17 axis as critical for developing experimental arthritis[42, 53, 239].

However, studies trying to describe the exact role of the IL-23/Th17 axis remained contradictory. On the one hand, Th17-derived cytokines have been shown to mediate pro-inflammatory effects and can be associated with destructive RA[65, 67, 83, 240]. On the other hand, disappointing results from efforts of treating RA through targeting IL-23 have fueled questions concerning the specific role of Th17 cells during RA[245].

To dissect the contribution of Th1 cells and the IL-23/Th17 axis to RA pathogenesis, we used *Il12a*$^{-/-}$ and *Il23a*$^{-/-}$ mice or αIL-23 blocking antibody in several animal models of RA. Since the *Il12a*$^{-/-}$ mice are unable to develop Th1 cells, and *Il23a*$^{-/-}$ mice cannot differentiate pathogenic Th17 cells [269, 270] these knockout strains were used to study the influence of Th1 and Th17 cells.

In general, there is no "universal" model for RA. Due to the complexity of this disease, numerous animal models for RA exist, each of them suitable to study a certain aspect of RA pathogenesis. For analyzing the priming phase of arthritis, we used the collagen-induced arthritis model (CIA)[271]. In this model, arthritis is induced through immunization, leading to a break of tolerance, the generation of autoreactive T cells and subsequently the development of pathogenic autoantibodies. A second model we used to study the immunological priming phase of RA was the K/BxN model. In the K/BxN model the antigen is defined by a transgenic TCR (KRN, apparently bispecific for bovine RNase and glycose6-phosphate isomerase). Mice harboring this TCR are crossed into the autoimmune-prone non-obese diabetic (NOD, harboring the MHC class II molecule I-Ag7) mice. T cells of the F1 generation recognize a self-peptide inside the ubiquitously expressed glycose-6-phosphate isomerase (G6PI) and interact with B cells, leading to production of autoantibodies and severe arthritis by the age of 4-5 weeks [257].
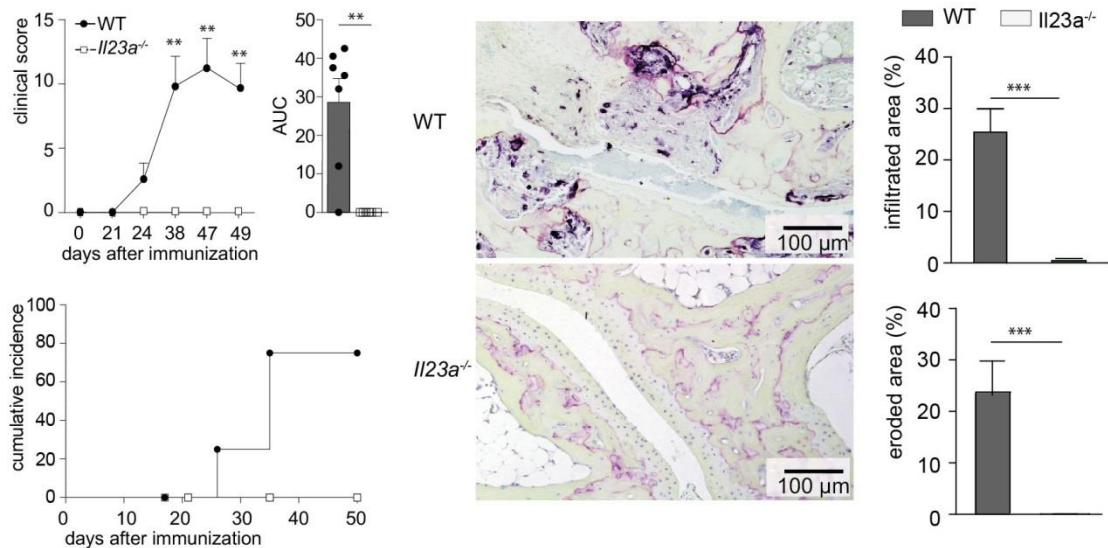
Figure 6: *Il23a$^{-/-}$* **mice are inert against collagen-induced arthritis**
Clinical score, area under the curve (AUC), incidence and histology of C57BL/6 (WT) and *Il23a$^{-/-}$* mice during the collagen-induced arthritis model. After secondary booster immunization on day 21 mice were scored periodically. At climax of arthritis mice were sacrificed and ankle joints were analyzed histologically with TRAP staining. Histology shows representative images stained for TRAP. Data is representative of at least 3 independent experiments (n=7). Error bars represent SEM * $P \le 0.05$, ** $P \le 0.005$, *** $P \le 0.001$; Student's *t* test. Figure 6 was adapted from Pfeifle *et al.* 2017 [1]

For analyzing the antibody-driven effector phase, we used the collagen antibody-induced arthritis model (CAIA)[272] and the K/BxN serum transfer model (STA)[230]. Both models depend on the transfer of arthritogenic IgGs into healthy recipient mice which subsequently develop a transient antibody-driven joint inflammation. While during the STA model pooled serum from K/BxN mice is used, CAIA is induced by the intravenous injection of four monoclonal IgGs recognizing four different epitopes of collagen type II.

When comparing wildtype and *Il23a$^{-/-}$* mice during CIA, we observed that *Il23a$^{-/-}$* mice were fully protected against arthritis (Figure 6). Around 80% of all wildtype mice developed severe inflammatory arthritis and histology showed hyperplasia, cartilage and bone erosions. *Il23a$^{-/-}$* mice, however, showed no clinical signs of arthritis and no synovial hyperplasia or bone erosion, when analyzed histologically via staining for TRAP positive cells. These data was in line with previously published data [42](Figure 6). Similar results were achieved when we treated K/BxN mice with αIL-23 neutralizing antibody (5 mg/kg from day 14 after birth, twice a week) (Figure 7). Isotype-treated K/BxN mice developed a severe arthritis starting at day 28 of age. Furthermore, massive joint infiltration and bone destruction could be detected through histological analysis of the ankle joints. In contrast, anti-IL-23 treatment led to a delay of joint

inflammation and less severe arthritis. Histologically, less inflammation inside the ankle joints was observed in αIL-23 treated K/BxN mice and less cartilage destruction and bone erosion was noted.
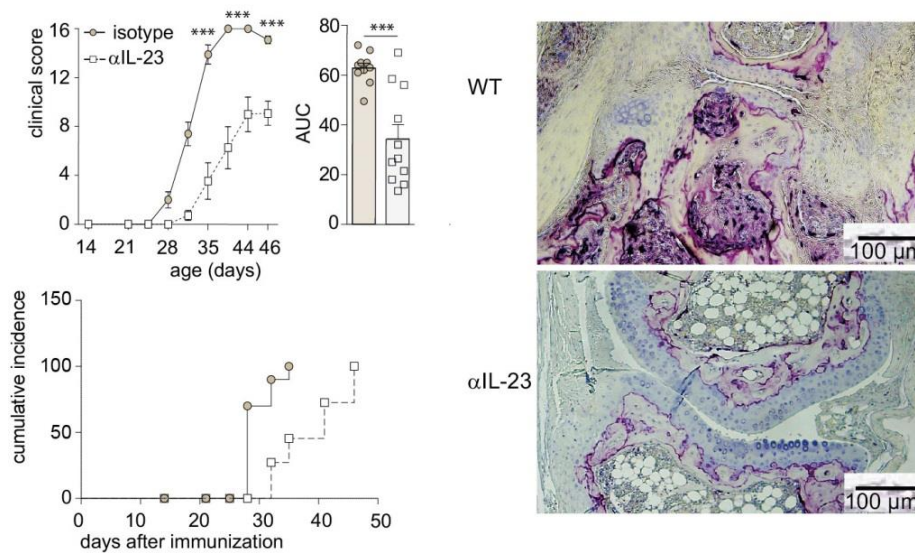

Figure 7: αIL-23 antibody protects during K/BxN arthritis
Clinical score, incidence and histology and area under the curve (AUC) of either isotype, or αIL-23 treated (5mg/kg from day 14 after birth, twice a week) K/BxN mice (n=11). Histology shows representative images stained for TRAP. Data is representative of at least 3 independent experiments ($n= 5$) Error bars represent SEM * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.001$; Student's t test. Figure 7 was adapted from Pfeifle *et al.* 2017[1].

IL-23 has been shown to be crucial in arthritis models, depending on functional priming and effector mechanisms. To further dissect the role of IL-23 during arthritis, we used *Il23a*^{-/-} mice and αIL-23 treated mice in the CAIA and the K/BxN model. Both models depend on the effector mechanisms initiated by the respective arthritogenic antibodies.

In contrast to the crucial participation of IL-23 in the priming phase of arthritis, IL-23 was completely dispensable in models depending only on the effector mechanisms of joint inflammation. Like WT control, *Il23a*^{-/-} mice did develop a full-blown joint inflammation during CAIA (Figure 8A) and STA (Figure 8C). Similar infiltration and bone erosions were detected during histological TRAP-staining of ankle joints. There was also no detectable difference between isotype and αIL-23 treated mice during K/BxN serum transfer arthritis (Figure 8B).

This data demonstrates that IL-23 is critically involved in the priming phase of experimental arthritis. However, during the IgG-mediated effector phase, IL-23 presence was fully dispensable.
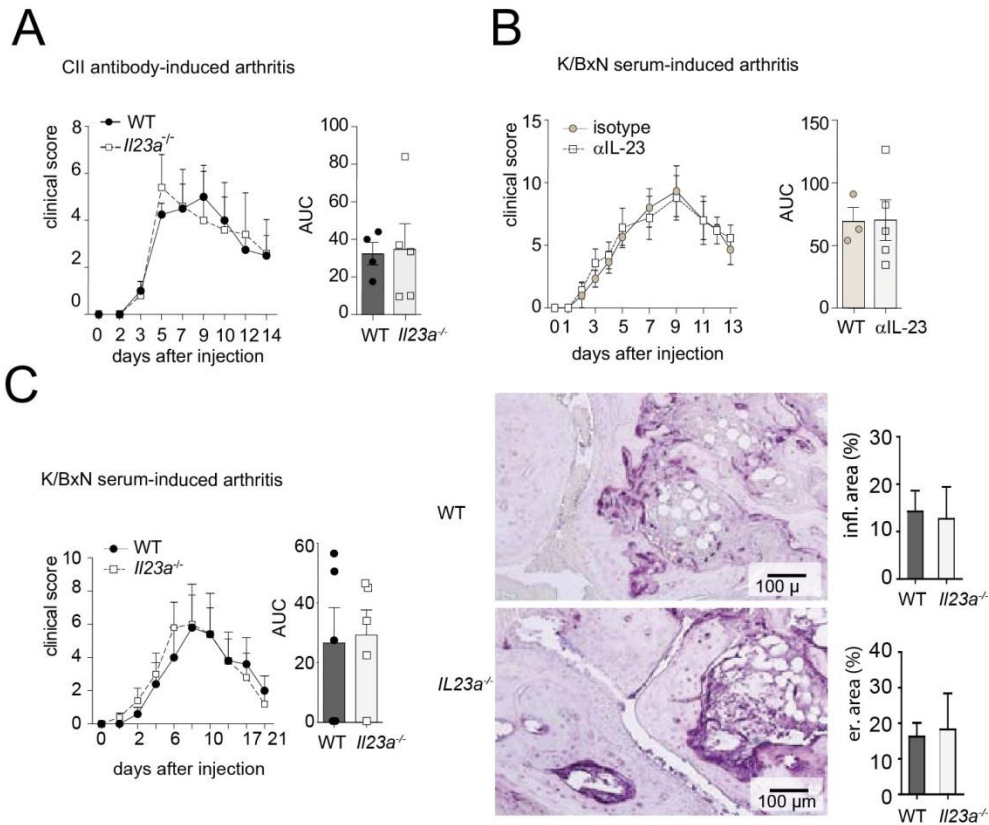
Figure 8: IL-23 neutralization is not protective during CAIA and STA
Clinical score and area under the curve (AUC) of *Il23a*[-/] (n=4) and αIL-23 treated mice (n=3/5) during (A) CAIA and (B) K/BxN serum-induced arthritis. (C) K/BxN serum-induced arthritis in *Il23a*^{-/-} mice. At climax of STA, mice were sacrificed and ankle joints were analyzed histologically with TRAP staining. Histology shows representative images stained for TRAP. Data is representative of at least 3 independent experiments (*n*= 5) Error bars represent SEM * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.001$; Student's *t* test. Figure 8 was adapted from Pfeifle *et al.* 2017[1]

3.2 T HELPER CELLS DURING EXPERIMENTAL ARTHRITIS

Recent research implicated autoreactive Th17 cells mostly as direct contributors to inflammation in the affected organs. For many autoimmune diseases, this suggestion seems to hold true[273]. However, in RA and its animal models, the role of Th17 cells remains unclear. Our data showed that the IL-23/Th17 axis was dispensable in passive arthritis models. To address the role of Th17 cells in arthritis, we induced CIA in wildtype mice and studied the overall T cell response in arthritic hind paws, and in secondary lymphatic organs like spleen and draining lymph nodes.
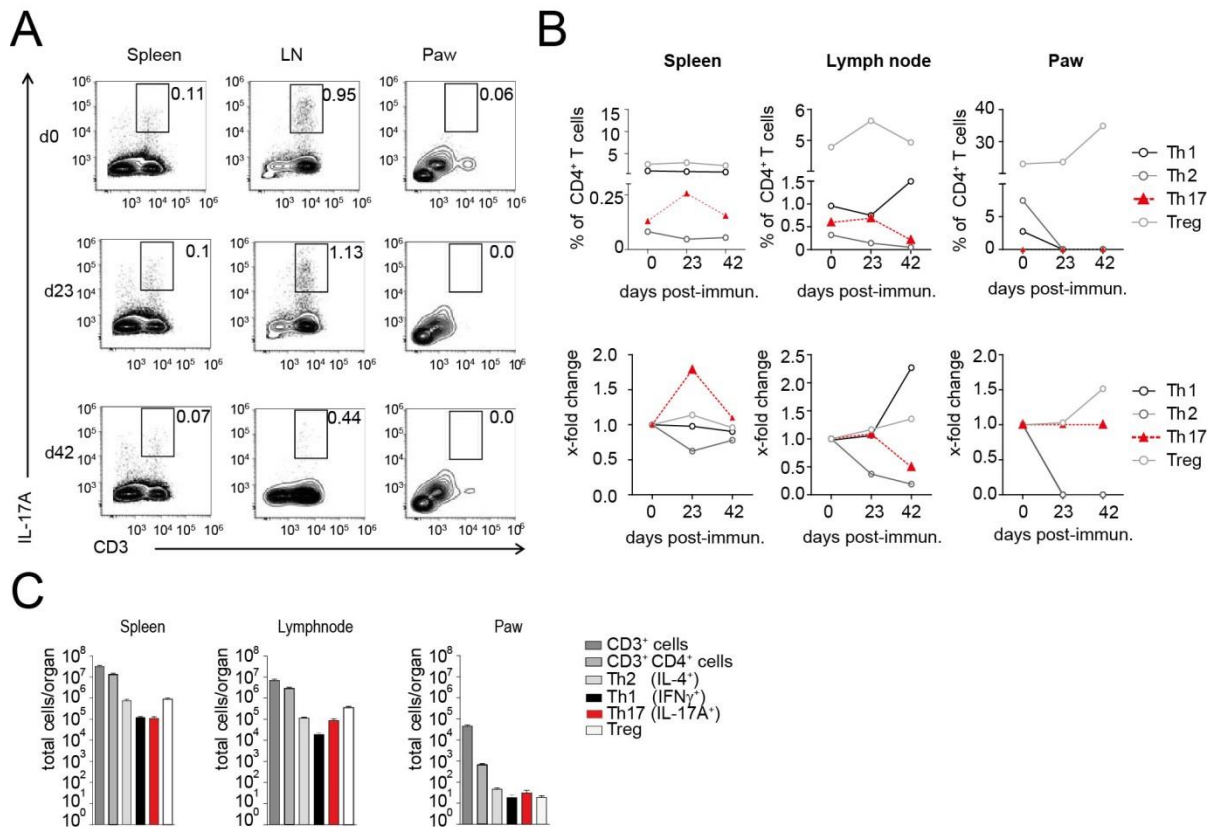
Figure 9: Th cell frequencies during collagen-induced arthritis
(A) Flow cytometric analysis of the frequency of IL-17 expressing CD3$^+$ T cells during collagen-induced arthritis (CIA) in the hind paws of wildtype C57BL/6 mice at indicated time points. Shown are representative FACS plots. (B) Mean percentages and fold change of T helper cell subsets DBA/1 mice during CIA. CD3$^+$CD4$^+$ T cells were subcategorized into IFNγ$^+$ Th1, IL-4$^+$ Th2, IL-17A$^+$ Th17 and Foxp3$^+$ Tregs. (C) Total cell counts per organ of CD3$^+$CD4$^+$ T helper cell subsets in C57BL/6 mice at day 26 of CIA. Error bars represent SEM. Figure 9 was adapted from Pfeifle *et al.* 2017[1]

When analyzing T helper cell (Th) subsets, we found, that Th17 cells were generally present in spleen and draining lymph nodes at all time points investigated. However, in arthritic paws Th17 cells were hardly detectable. During inflammation this phenotype was even more pronounced. IL-17$^+$ T cells were present in spleens and lymph nodes at all time points, while in paws no IL-17$^+$ CD4$^+$T cell expression was detected (Figure 9A).

When comparing CD3$^+$CD4$^+$ T helper cell subsets, we found Th17 frequencies increasing during CIA in spleens and draining lymph nodes, especially two days after booster injection (day 21). An increase of Th1 cells was detected in the draining lymph nodes, while inside arthritic paws only increasing frequencies of Tregs but no Th17 cells were detected (Figure 9B). When calculating the total amount of T cells and Th subsets on day 26 of CIA, we again found Th17 cells to be present inside spleen and lymph nodes, but we were hardly able to detect IL-17$^+$ T

helper cells inside the paws. Furthermore, the majority of T cells inside the joints were found to be CD3⁺CD4⁻ T cells, suggesting cytotoxic T cells being more abundant effector cells than T helper cells in arthritic joints (Figure 9C).
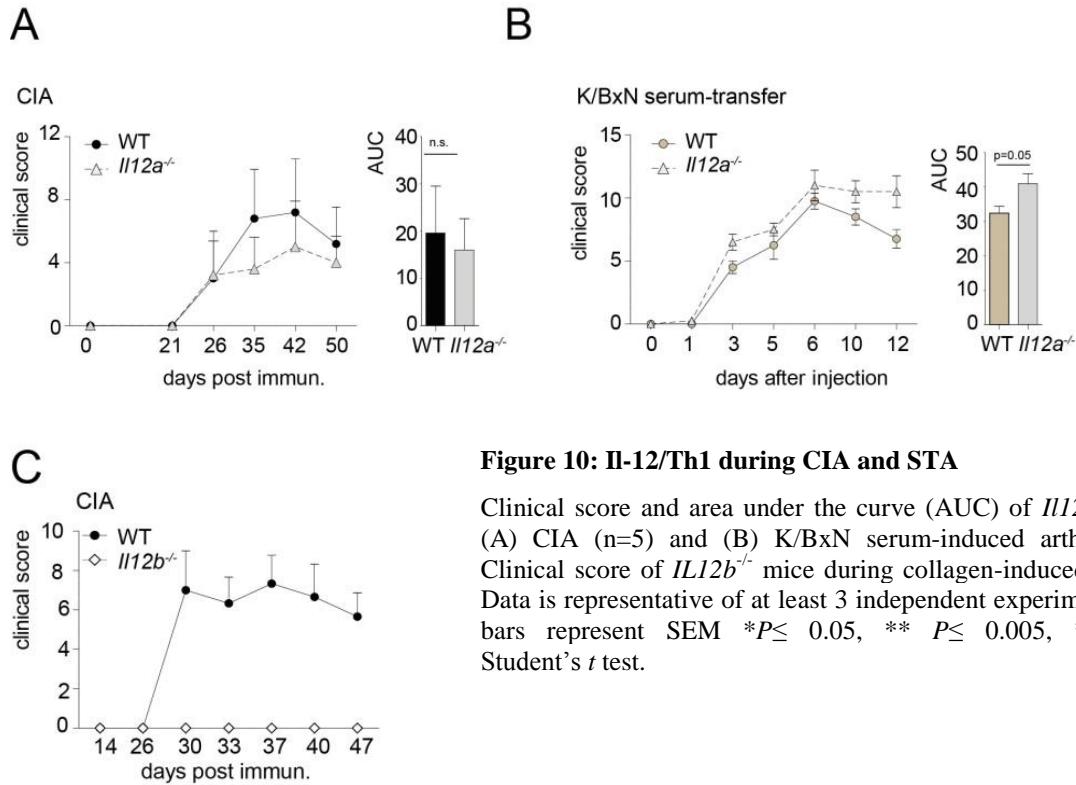

Figure 10: Il-12/Th1 during CIA and STA

Clinical score and area under the curve (AUC) of $Il12a^{-/-}$ mice during (A) CIA (n=5) and (B) K/BxN serum-induced arthritis (n=4). (C) Clinical score of $IL12b^{-/-}$ mice during collagen-induced arthritis. (n=4) Data is representative of at least 3 independent experiments (*n*=5) Error bars represent SEM *$P \leq$ 0.05, ** $P \leq$ 0.005, *** $P \leq$ 0.001; Student's *t* test.

Since Th1 cells were increased in lymph nodes, we studied the impact of the lack of Th1 cells during collagen-induced arthritis and K/BxN serum transfer arthritis. We used $Il12a^{-/-}$ mice, which lack the subunit IL12p35 and therefore lack IL-12-dependent Th1 cells. $Il12b^{-/-}$ were used as control, since they lack the IL-12p40 subunit which is shared between both IL-23 and IL-12, leading to a lack of Th1 and to impaired Th17 cells[42, 269, 270].

Evaluation of clinical score during CIA showed that in contrast to $Il23a^{-/-}$ mice (Figure 7) and $Il12b^{-/-}$ mice (Figure 10C) which were completely protected against CIA, $Il12a^{-/-}$ mice were not protected against arthritis (Figure 10A).

During K/BxN serum transfer we even identified a tendency for an even more severe phenotype. However this tendency was not significant (Figure 10B). This data argues against an involvement of the IL-12/Th1 axis during the pathogenesis of arthritis and even point towards a potentially suppressive role of Th1 cells during antibody-mediated inflammation.

3.3 ROLE OF IL-23 IN REGULATING HUMORAL RESPONSE

Since our data suggested that Th17 cells regulated the onset of humoral (auto)immunity, we analyzed serum-IgG levels in wildtype and IL-23-deficient mice during CIA and K/BxN arthritis. When analyzing total collagen type II specific IgG (CII-IgG), we found that CII-IgG precede the appearance of clinical signs of arthritis. After the booster injection on day 21, the IgG concentration further increased and subsequently joint inflammation could be detected. These results indicate that the regulation of autoantibody formation is an important factor during development of autoimmune arthritis (Figure 11).
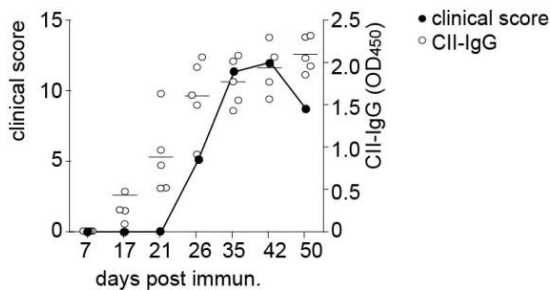
Figure 5: Collagen-type II specific IgG precede arthritis

Development of the levels of collagen-type II specific IgG (CII-IgG) and clinical score at indicated time points during collagen-induced arthritis. Figure 11 was adapted from Pfeifle *et al.* 2017[1].

To elucidate the role of the IL-23/Th17 axis in regulating the humoral immune response, we analyzed the germinal center reaction in *Il23a*^{-/-} in comparison to WT mice during collagen-induced arthritis by flow cytometry and by immunofluorescence. First we analyzed the general capacity for germinal center (GC) formation by immunofluorescence staining for PNA, IgD and CD21/35 and we found that GCs form normally inside spleens of *Il23a*^{-/-} mice (Figure 12A). Furthermore, we analyzed the expansion of germinal center B cells by flow cytometry and found IgD[-] PNA[+] GL7^{+} Fas^{+} B cells to expand normally during experimental arthritis (Figure 12B).
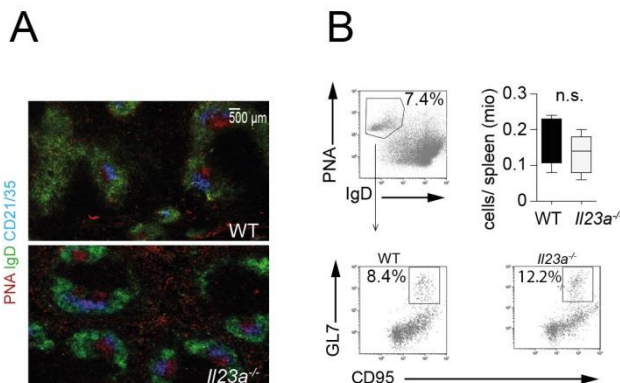

Figure 12: IL-23 is dispensable for germinal center reaction

(A) Immunofluorescence analysis of germinal center formation at day 26 after induction of CIA in WT and *Il23a*^{-/-} Pictures are representative for analysis in at least 5 mice per group. (B) Flow cytometric analysis of germinal center B cell (CD19^{+} pre-gated) frequencies in wildtype and *Il23*^{-/-} mice at day 26 after induction of CIA (n=5). Error bars represent SEM *$P \leq$ 0.05, ** $P \leq$ 0.005, *** $P \leq$ 0.001; Student's *t* test. Figure 12 was adapted from Pfeifle *et al.* 2017 [1].

Additionally, we performed ELISA analysis from sera derived from wildtype and $Il23a^{-/-}$ mice during arthritis and compared CII -specific IgG levels.

However, during CIA we found no differences in CII-IgG levels between wildtype and $Il23a^{-/-}$ mice (murine and chicken CII-IgG), neither in total CII-IgG levels nor in CII- specific IgG subtypes. The comparison of the binding of IgG to several murine CII epitopes between wildtype and $Il23a^{-/-}$ showed no differences (Figure 13 A-C). Similarly to CII-specific IgG during CIA, there was no difference in αG6PI antibody titers between αIL-23 treated K/BxN mice and K/BxN mice who received isotype (Figure 13D-E). Thus, this data show IgG subtype production during autoimmune arthritis is independent of the IL-23/Th17 axis.
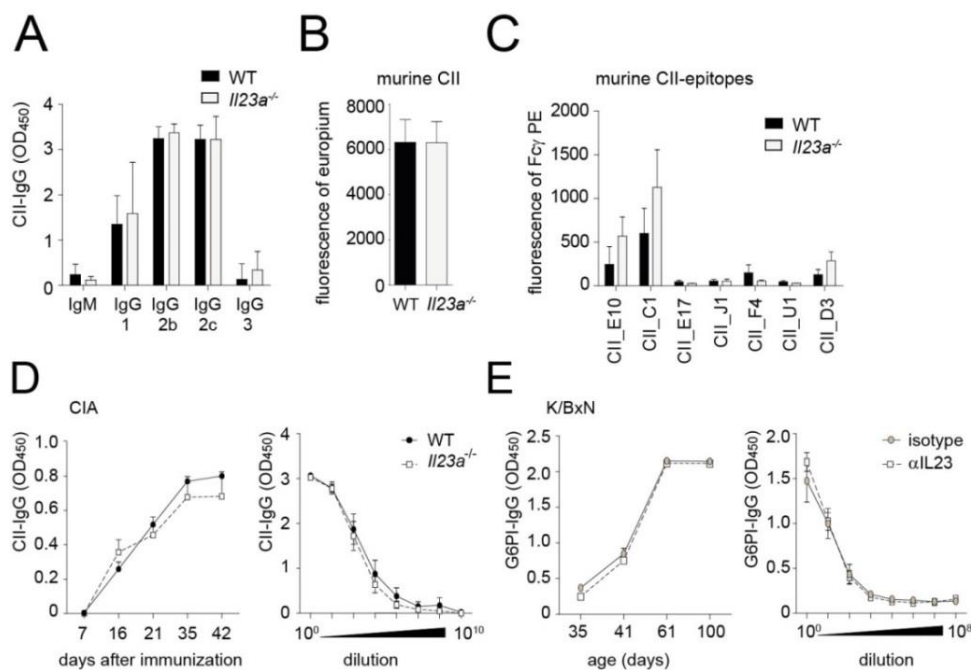
Figure 13: IgG subtype regulation is independent of IL-23 during autoimmune arthritis
(A-C) Analysis of levels of antibody subsets against (A) chicken type II collagen (CII-IgG), (B) against murine CII and IgG against (C) several murine CII-epitopes in wildtype (WT) and $Il23a^{-/-}$ mice at day 50 after CII-immunization. (D,E) Analysis of the levels and titers of (D) CII-IgG during collagen-induced arthritis and in WT and $Il23a^{-/-}$ (n=5) and (E) in αIL-23 and isotype treated mice K/BxN mice (n=3). Error bars represent SEM * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.001$; Student's t test. Figure 13 was adapted from Pfeifle *et al.* 2017 [1].

Apart from IgG subtypes, we also analyzed CII-IgG affinity. Therefore, we isolated total IgG and studied the IgG affinity to CII via a titration assay with the chaotropic salt potassium thiocyanate (Figure 14A). Furthermore we analyzed the generation of high affinity antibodies in response to the T cell-dependent immunization against 4-hydroxy-3-nitrophenylacetyl (NP) coupled to chicken gamma globulin (NP-CGG). For this purpose we immunized wildtype and $Il23a^{-/-}$ mice NP-CGG in Alum. During B cell maturation antibodies against NP-8 and NP-26 are generated. While NP-8 is only detected by high-affinity antibodies, NP-26 is detected by less affine IgG. The ratio gives information about the efficiency by which highly affine IgG is being generated (Figure 14B). In both approaches we found that there was no difference between $Il23a^{-/-}$ and wildtype mice.
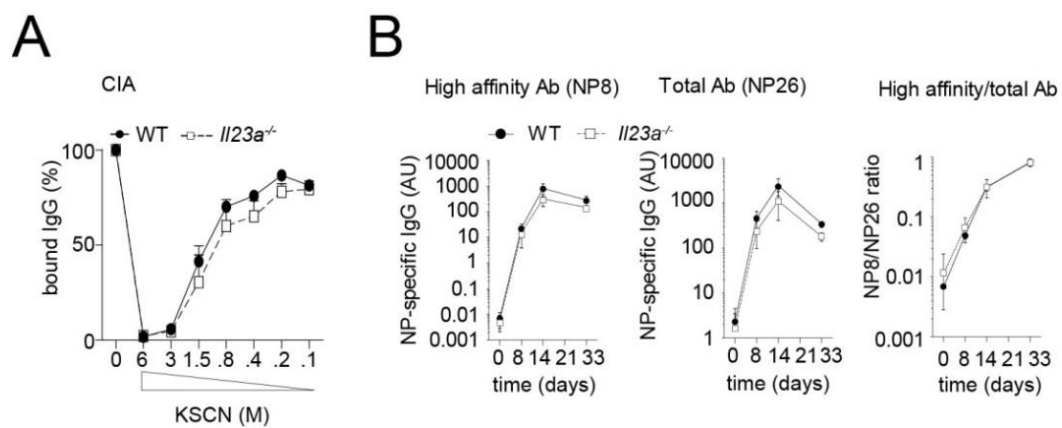

Figure 14:IL-23 is dispensable for affinity maturation of arthritogenic autoantibodies
(A) Potassium thiocyanate (KSCN) titration assay as a way to determine the binding affinity of CII-IgG isolated from $Il23a^{-/-}$ mice at climax of collagen-induced arthritis. (B) Measurement of the levels of regular and high affinity antibodies in response to a T cell dependent immunization with 4-hydroxy-3-nitrophenylacetyl (NP) coupled to chicken γ-globulin (NP-CGG) in WT and $IL23a^{-/-}$ mice. Error bars represent SEM. Figure 14 was adapted from Pfeifle *et al.* 2017.

Humoral immunity can be regulated by total IgG generation, IgG affinity and IgG subclass distribution [159]. But furthermore, IgG can be regulated through post-transcriptional modifications [166, 179, 184]. Therefore we compared protein-G-isolated IgG from wildtype and $Il23a^{-/-}$ mice after onset of arthritis for their capacity to evoke inflammatory responses from members of the innate immune system. To this end we stimulated wildtype bone marrow derived dendritic cells (BM-DCs) with monomeric IgG, heat-aggregated non-specific IgG or CII-specific IgG complexes (IC) from wildtype and $Il23a^{-/-}$ mice. Subsequently, we performed an ELISA-based measurement of IC-induced pro-inflammatory cytokines TNFα, Il-6 and CXCL-1 in the supernatant.
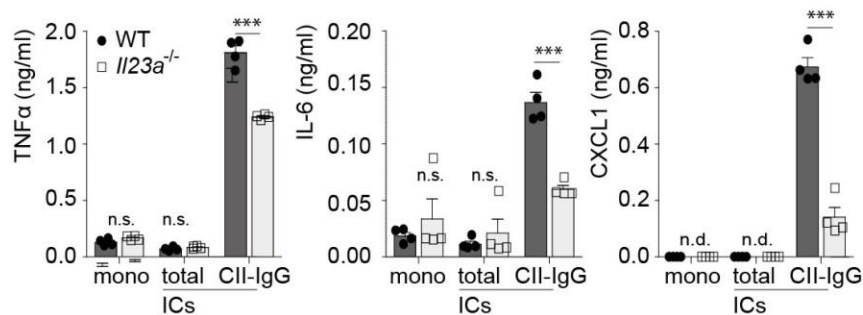
Figure 15: *Il23a-/-* mice generate CII-IgG with lower inflammatory activity
Inflammatory potential of IgG isolated from wildtype and *Il23a-/-* mice at the climax of experimental arthritis (day 50). WT bone marrow-derived dendritic cells were incubated with monomeric (mono), heat-aggregated (total) IgG-immune complexes (ICs) and CII-specific ICs for 24h before cytokine levels were analyzed inside the supernatant by ELISA (n=4) Error bars represent SEM *P≤ 0.05, ** P≤ 0.005, *** P≤ 0.001; Student's t test. Figure 15 was adapted from Pfeifle et al. 2017[1].

Incubation of BM-DCs with monomeric or non-specific IgGs did not lead to a significant induction of TNFa, IL-6 or CXCL-1. CII-specific ICs (CII-ICs) strongly induced TNFα and IL-6 production. CXCL-1 secretion was moderately induced. Notably, CII-ICs generated by *Il23a-/-* displayed a significant reduction in their inflammatory capacity. TNFα production was one third lower, while IL-6 and CXCL-1 production induced by *Il23a-/-* isolated was more than one half reduced (Figure 15).

These data showed that the inflammatory property of IgGs during CIA is regulated not at the level of total IgGs, but inside the fraction of CII-specific IgG. Furthermore, the data demonstrated that part of the inflammatory properties of CII-ICs depend on the presence of IL-23.
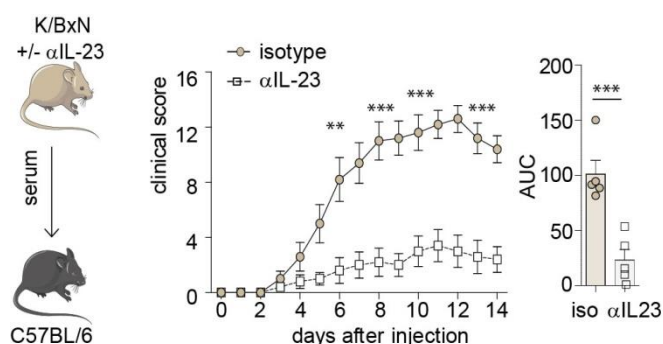

Figure 16: IL-23 neutralization in K/BxN mice reduces IgG activity in K/BxN serum transfer model
Arthritis score in C57BL/6 wildtype mice, receiving serum from K/BxN mice which have been treated with either isotype of αIL-23 antibody from day 14 after birth (5mg/kg, twice a week). Serum of K/BxN was taken from week 6-12, pooled and injected in recipient mice. Error bars represent SEM * P≤ 0.05, ** P≤ 0.005, *** P≤ 0.001; Student's *t* test. Figure 16 was adapted from Pfeifle *et al.* 2017[1].

To evaluate the relevance to this finding *in vivo*, we analyzed the effect of a αIL-23 treatment of K/BxN mice on the arthritogenicity of IgG. Therefore, we treated K/BxN mice with either isotype or αIL-23 (5 mg/kg from d14 after birth, twice a week) before we collected serum (weekly at day 6-12). Although the neutralization of IL-23 had no impact on the serum levels of G6PI-specific

autoantibodies (Figure 13E), the capability to cause arthritis was conspicuously reduced (Figure 16). Thus, these data strongly link IL-23 mediated signaling with the regulation of IgG activity *in vitro* and *in vivo* during CIA and K/BxN arthritis.

3.4   IL-23 UNLEASHES AUTOIMMUNE INFLAMMATION BY MODIFYING Fc GLYCOSYLATION

IgG is known for mediating a variety of effector functions. These are, in part, regulated by post-transcriptional modifications such as sugar residues attached to highly conserved "glycosylation sites" (N-X-S/T) inside the Fc region. Paradoxically, these modifications are able to turn IgG from a promoter of pro-inflammatory functions into a potent suppressor of inflammation [179, 192].

To determine Fc glycosylation, we isolated total IgG, collagen-specific IgG and a fraction of IgGs in which all CII-specific IgGs were depleted. These fractions were isolated from wildtype and *Il23a*^{-/-} mice at the climax of collagen-induced arthritis (day 50) and subsequently analyzed via mass spectrometry.
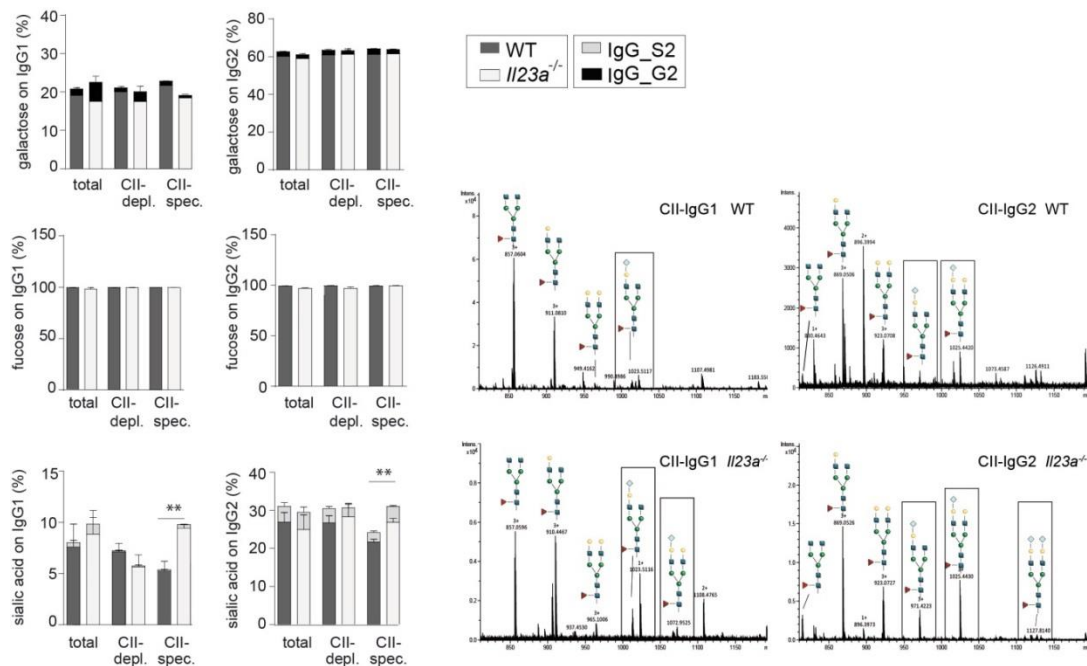

Figure 6: IL-23/Th17 axis downregulates sialylation of CII-IgG during arthritis
Mass-spectrometry based analysis of galactose, fucose and sialic acid content at the asparagine 297 residue of total, CII-IgG depleted and CII-specific IgGs isolated from wildtype and *Il23a*^{-/-} mice (n=4) at climax of arthritis (day 50). Error bars represent SEM * P≤ 0.05, ** P≤ 0.005, *** P≤ 0.001; Student's *t* test. Pictures show representative mass spectra illustrating IL-23 dependent changes of glycosylation. Framed peaks indicate sialylated core glycans. Figure 17 was adapted from Pfeifle *et al.* 2017 [1].

No differences could be detected inside the total IgG and the collagen-IgG depleted (CII-IgG depleted) fraction in terms of fucosylation, galactosylation or sialylation between WT and $Il23a^{-/-}$ mice. However, we detected a significant decrease in sialylation of both CII-specific IgG1 and IgG2 in arthritic wildtype mice in comparison to the total and CII-IgG depleted fraction. In contrast to CII-IgG from wildtype mice, this downregulation was absent in $Il23a^{-/-}$ mice (Figure 17), arguing for a IL-23 dependent mechanism involved in reducing sialic acid content during the onset of arthritis.

Especially Asn297-associated terminal sialic acid residues have been implicated to be a master regulator of IgG activity. It has been shown, that terminal sialic acid critically controls changes in IgG-Fc conformation leading to decreased binding affinity for activating FcRs and increased binding unto inhibitory Fc receptors [178, 184].

To address the question, if the observed IL-23 dependent pro-inflammatory capacity of hypo-sialylation of IgG is in fact causative for the observed differences in IgG activity (0 and 0), we isolated IgG from $Il23a^{-/-}$ and wildtype mice and added terminal sialic acid to the IgG by using a commercial α2,6 sialyltransferase *in vitro*. Subsequently, we analyzed the IgG activity by incubating IgG with BM-DCs and determining the concentration of pro-inflammatory cytokines inside the supernatant (Figure 18).
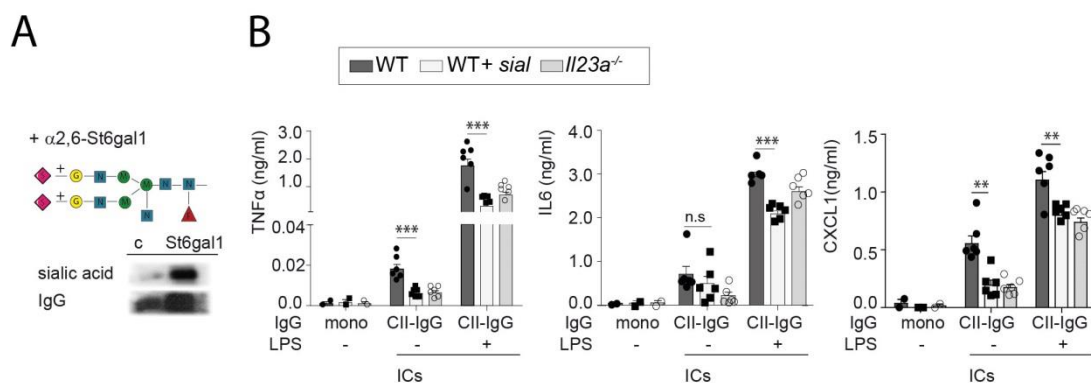

Figure 18: IgG sialylation decreases activity of wildtype CII-ICs
(A) *St6gal1*-mediated sialylation of wildtype IgG. Schematic representation and quality check of sialylated IgG by Lectin Blot with SNA directed against α2,6-linked sialic acid. Loading control is α-mouse IgG-Hrp. (B) Analysis of IgG activity analyzed by the incubation of wildtype BM-DCs with monomeric or collagen specific ICs (n=6) for 24h in absence or presence of 1ng/ml LPS before cytokine levels were analyzed in the supernatant by ELISA. Error bars represent SEM * P≤ 0.05, ** P≤ 0.005, *** P≤ 0.001; Student's *t* test. Figure 18 was adapted from Pfeifle *et al.* 2017[1].

When comparing wildtype and *Il23a*^{-/-} mice we found that sialyation of wildtype IgG reduced their capability to induce a pro-inflammatory cytokine production in BM-DCs. Concentrations of TNFα, IL-6 and CXCL-1 induced through sialylated wildtype ICs were significantly reduced in comparison to untreated wildtype ICs, and were even quenched again to cytokine levels produced after incubation with *Il23a*^{-/-} ICs (Figure 18).
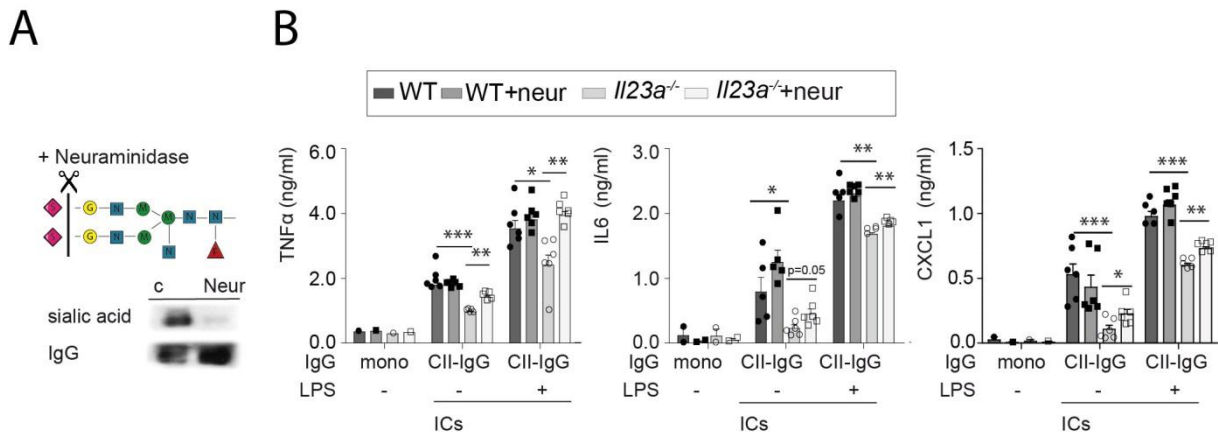

Figure 19: IgG desialylation increases activity of *Il23a*^{-/-} CII-ICs
(A) Neuraminidase digestion of wildtype and *Il23a*^{-/-} IgG. Schematic representation and quality check of desialylation of IgG by Lectin Blot with SNA directed against α2,6 linked sialic acid. Loading control is α-mouse IgG-HRP. (B) Analysis of IgG activity assed by the incubation of wildtype BM-DCs with monomeric, or Collagen-specific-ICs (n=6) for 24h in absence or presence of 1ng/ml LPS before cytokine levels were analyzed in the supernatant by ELISA. Error bars represent SEM *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$; Student's *t* test. Figure 19 was adapted from Pfeifle *et al.* 2017[1].

Contrary, if we removed terminal sialic acid from IgG by neuraminidase digestion, ICs isolated from *Il23a*^{-/-} mice induced higher TNFα, IL-6 and CXCL-1 production in wildtype BM-DCs (Figure 19). Wildtype IC-induced cytokine production was not affected by neuraminidase treatment, suggesting a regulatory sialic acid-dependent threshold present in IgG that regulated its activity (Figure 19). Finally, we wanted to test these findings in an *in vivo* situation. Therefore, we isolated total IgG from K/Bxn mice, which have been treated from day 14 after birth with either isotype or αIL-23 neutralization antibody. In the next step we incubated the IgGs either with or without neuraminidase and performed a K/BxN IgG transfer into healthy recipient mice and subsequently scored arthritis. Mice injected with isotype-treated K/BxN IgG developed a normal transient arthritis; reaching the peak of disease at day 5. IgG isolated from αIL-23-treated K/BxN mice again developed a less severe arthritis. Strikingly, this effect was abolished, if the IgGs were treated with neuraminidase prior to injection. Mice receiving neuraminidase-treated IgGs developed a normal arthritis as observed in the isotype group. These data clearly demonstrated a role of IL-23 in unlocking antibody activity through regulating IgG sialylation (Figure 20).
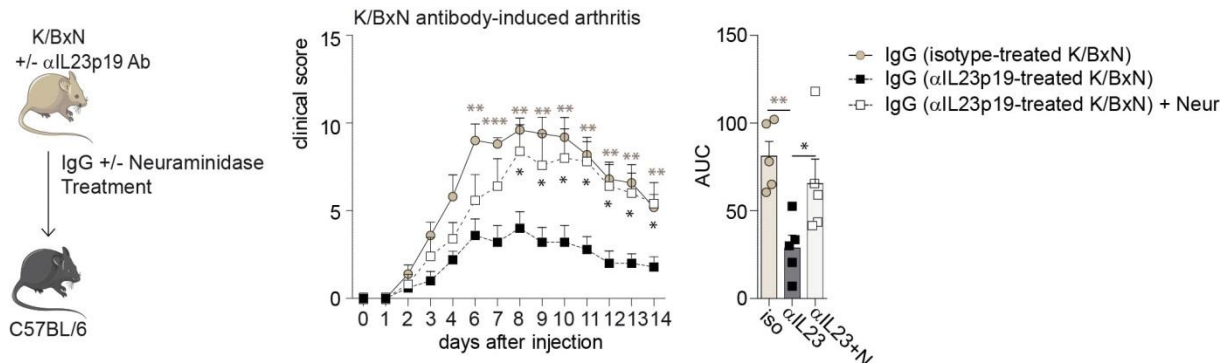
Figure 20: Neuraminidase treatment abolishes anti-inflammatory potential of aIL-23-treated K/BxN derived IgG

Arthritis after transfer of purified IgG from K/BxN mice treated with isotype or neutralizing αIL-23 antibody and neuraminidase-treated IgG (+Neur) from K/BxN + αIL-23. Error bars represent SEM * P≤ 0.05, ** P≤ 0.005, *** P≤ 0.001; Student's *t* test. Figure 20 was adapted from Pfeifle *et al.* 2017 [1]

3.5   IL-23 REGULATES ST6GAL1 IN DEVELOPING PLASMA CELLS

In mice, the typical attachment of sialic acid is carried out by the enzyme beta-galactoside-alpha-2,6-sialyltransferase 1 (*st6gal1*). Consequently, as IL-23 controls IgG glycosylation, this enzyme has to be regulated in developing B cells by the IL-23 axis. To investigate this, we sorted plasma cells and plasmablasts from wildtype and *Il23a*^{-/-} mice (pooled from 6 mice) at day 0 and shortly after booster immunization (day 26) of collagen-induced arthritis and performed quantitative RT-PCR analysis of *st6gal1* and enzymes responsible for other glycosylation events (including *b4galt1*).
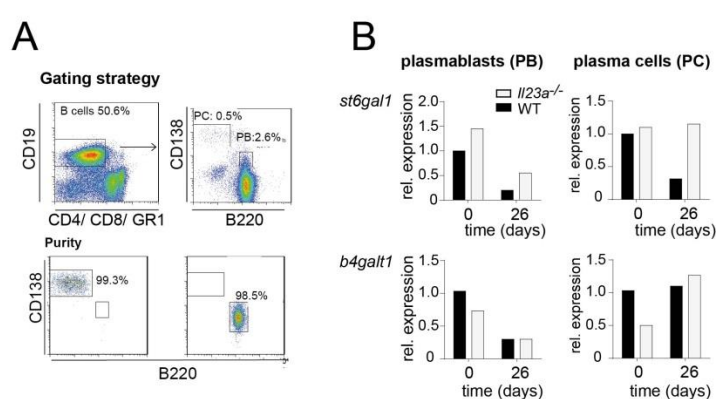

Figure 21: *St6gal1* downregulation during arthritis is Il-23 dependent
(A) Sorting strategy and sorting purity analysis of splenic plasma cells (PC; CD19⁺CD138⁺B220⁻) and plasmablasts (PB; CD19⁺CD138⁺B220⁺). (B) RT-PCR analysis of splenic plasma cells and plasmablasts from wildtype and *Il23a*^{-/-} mice (pooled from 6 mice per group) at indicated time points. Bar graphs represent SEM of triplicates. Figure 21 was adapted from Pfeifle *et al.* 2017 [1]

Sorting of plasma cells and plasmablasts achieved a purity of 99.3% and 98.5%, respectively (Figure 21A). While analyzing mRNA expression of glycosylation enzymes we detected similar levels of *st6gal1* in day 0 controls, but increased *st6gal1* expression in plasma cells (PC) isolated on day 26 from *Il23a*^{-/-} mice. B220⁺CD138^{low} plasmablasts (PB) isolated from *Il23a*^{-/-} displayed a

higher *st6gal1* expression on both time points when compared to wildtype cells. *B4galt1* expression on the other hand, showed no such regulation and remained largely similar between *Il23a*^{−/−} and wildtype mice (Figure 21B).

To verify these mRNA data on the protein level, we preformed intracellular flow cytometry analysis of *st6gal1* expression in developing plasma cells in spleens of wildtype and *Il23a*^{−/−} mice before (day 0) immunization and shortly before outbreak of full-blown arthritis (day 26). During flow-cytometric analysis plasma cells were defined as B220^{low} CD267^{+} CD138^{+}.
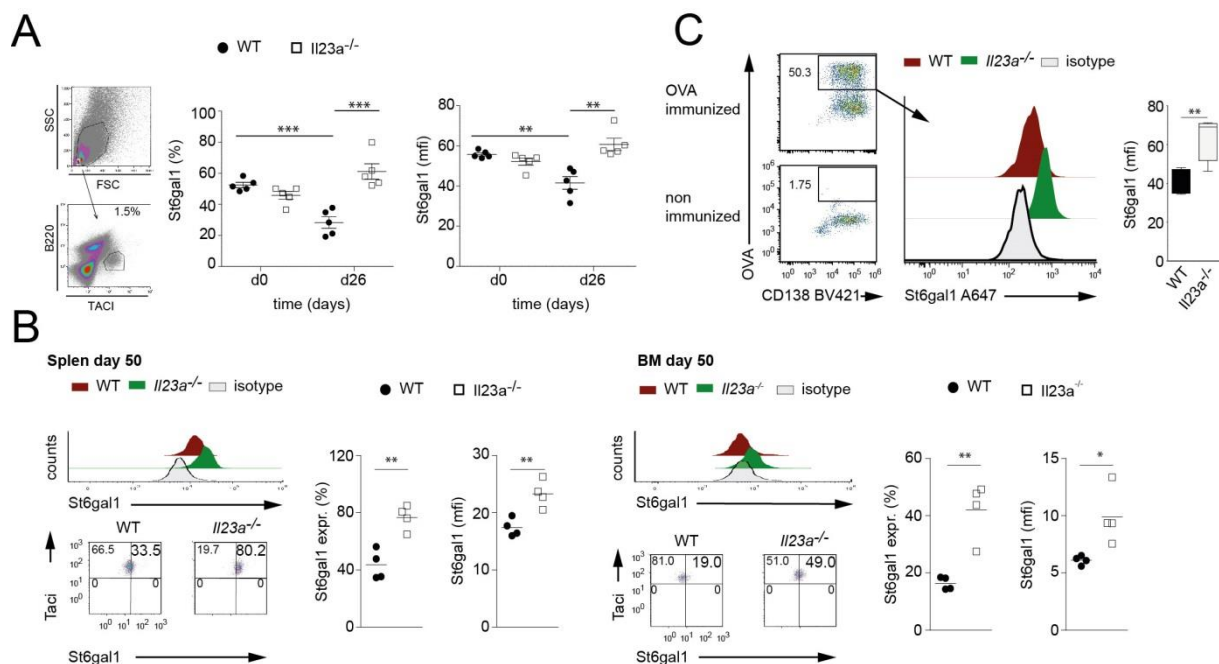

Figure: 7 *St6gal1* is regulated by IL-23 in an antigen-specific manner
(A) Gating strategy for flow cytometry-based quantification analysis of st6gal1 in splenic and bone marrow derived plasma cells. Plasma cells were defined to be B220^{low}Taci^{+}and CD138^{+}. St6gal1 regulation was analyzed on day 0 and day 26 during collagen-induced arthritis in wildtype and Il23a-/- mice (n=5). (B) Flow cytometry-based quantification of α2,6-sialyltransferase 1 (st6gal1) protein expression in plasmablasts and plasma cells in spleen and bone marrow (BM) of WT and *Il23a*^{−/−} mice (n=4) on day 50 after induction of CIA. (C) c) Flow cytometry-based analysis of st6gal1 expression in ovalbumin (OVA)-specific plasma cells (CD19^{dim}Taci^{+}) from WT and *Il23a*^{−/−} mice 5 days after secondary immunization with OVA. Error bars represent SEM * P≤ 0.05, ** P≤ 0.005, ***P≤ 0.001; Student's t-test. Figure 22 was adapted from Pfeifle et al. 2017 [1].

The results supported the mRNA data (Figure 22A). While *st6gal1* was found to be down regulated in wildtype plasma cells, *Il23a*^{-/-} mice were unable to do so. Similarly, when analyzing plasma cells in mice suffering from active arthritis on day 50 in spleen and bone marrow, protection against arthritis in *Il23a*^{-/-} mice was associated with significantly higher *st6gal1* levels in B220^{low} CD267^{+} CD138^{+} plasma cells (Figure 22B).To investigate IL-23-regulated *st6gal1* inside antigen-specific plasma cells, we immunized wildtype and *Il23a*^{-/-} mice against chicken ovalbumin (OVA) in the presence of complete freund's adjuvant. Subsequently on day 26 (5 days after booster immunization), we tracked freshly formed splenic plasma cells intracellularly with A488-labeled OVA. When comparing *st6gal1* expression in OVA-specific plasma cells, again *st6gal1* was found to be down regulated in wildtype mice, but in *Il23a*^{-/-} mice *st6gal1* expression remained significantly higher (Figure 22C). Together, these results suggested a direct or indirect regulation of *st6gal1* through Il-23 in an antigen-specific manner. Precisely, IL-23 was found to be crucial for the down-regulation of *st6gal1* during the prodromal phase of arthritis.

3.6 IL-23 SUPRESSES ST6GAL1 EXPRESSION VIA TH17 CELLS

IL-23 is known to be crucial for the differentiation of pathogenic Th17 cells [270]. Therefore, we addressed the question, if IL-23 acts itself on developing plasma cells or via licensing Th17 cells to favor pro-inflammatory antibody responses.

To address this question, we isolated B cells from murine spleens and stimulated them with 5 µg/ml LPS to differentiate into antibody-producing plasma cells. Meanwhile, we co cultivated these developing plasma cells in presence or absence of IL-23 and Th17 cells (Figure 23). Differentiated plasma cells were isolated via fluorescence-activated cell sorting and analyzed for *st6gal1* mRNA levels. To analyze protein levels, we again stained intracellularly for *st6gal1* in B220^{low}CD138^{+} plasma cells.

After analyzing *st6gal1* expression we found that IL-23 itself was unable to suppress *st6gal1* in developing B cells. Similarly, Th17 cells in absence of IL-23 stimulation were insufficient to downregulate *st6gal1*. However, co-cultivation with IL-23 stimulated Th17 cells suppressed *st6gal1* leading to a significant suppression of *st6gal1* on mRNA and protein level, indicating that IL-23-activated Th17 cells are able to suppress sialylation enzymes and thereby regulate IgG activity (Figure 23 A/B).
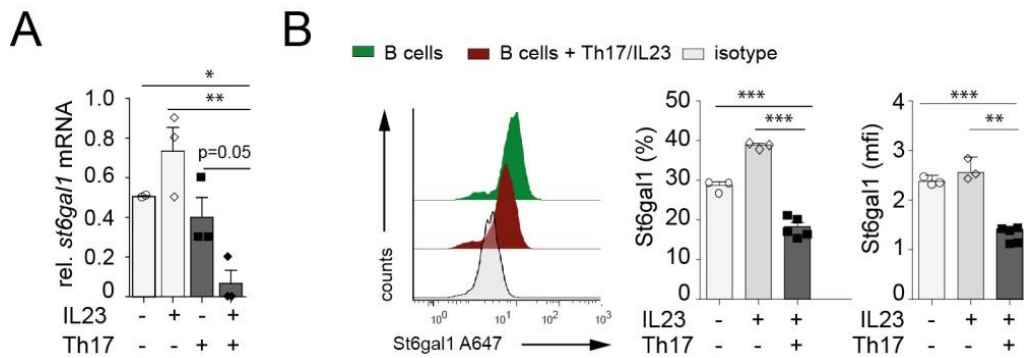
**Figure 23: Il-23 activated Th17 cells downregulate *st6gal1* in developing plasma cells**

(A) Quantification of st6gal1 mRNA expression in in vitro-differentiating plasma cells after co-incubation with IL-23 (25ng/ml) and/or in vitro-differentiated Th17 cells (n=3;p≤0.0081). (B) Flow cytometry-based quantification of st6gal1 protein expression in in vitro-differentiating plasma cells after incubation with IL-23 (25ng/ml) alone or after co-incubation with in vitro-differentiated and IL-23-activated Th17 cells (n=3-5; p≤0.009). Error bars represent SEM * P≤ 0.05, ** P≤ 0.005, *** P≤ 0.001; Student's t test. Figure 23 was adapted from Pfeifle et al. 2017 [1].

Th17 cells were found to increase during experimental arthritis inside secondary lymphatic tissues (Figure 9). Yet, to directly instruct developing plasma cells, one would expect to find them in close proximity to germinal centers. To address the open question, if Th17 cells are found in proximity to developing plasma cells, we preformed immunofluorescence analysis and stained for Th17 cells and germinal centers during the prodromal phase of experimental arthritis (day 26).

When we analyzed murine spleens during the prodromal phase of collagen-induced arthritis, we found RORγt⁺ and IL-17⁺ cells located inside the germinal center and in close contact to follicular B cells (Figure 24A). Furthermore, we wanted to quantify these germinal center associated Th17 cells during arthritis. Therefore, CIA was induced in wildtype and *Il23a*⁻/⁻ mice and splenic T cells were stained for T follicular helper cell markers together with RORγt, the classical transcription factor of Th17 cells.

We found RORγt⁺ Th17 cells to express markers of T follicular helper cells like PD-1. Strikingly, these PD-1⁺ Th17 cells were drastically reduced in *Il23a*⁻/⁻ mice (Figure 24B), suggesting an IL-23 dependent expansion of germinal center associated Th17 cells.
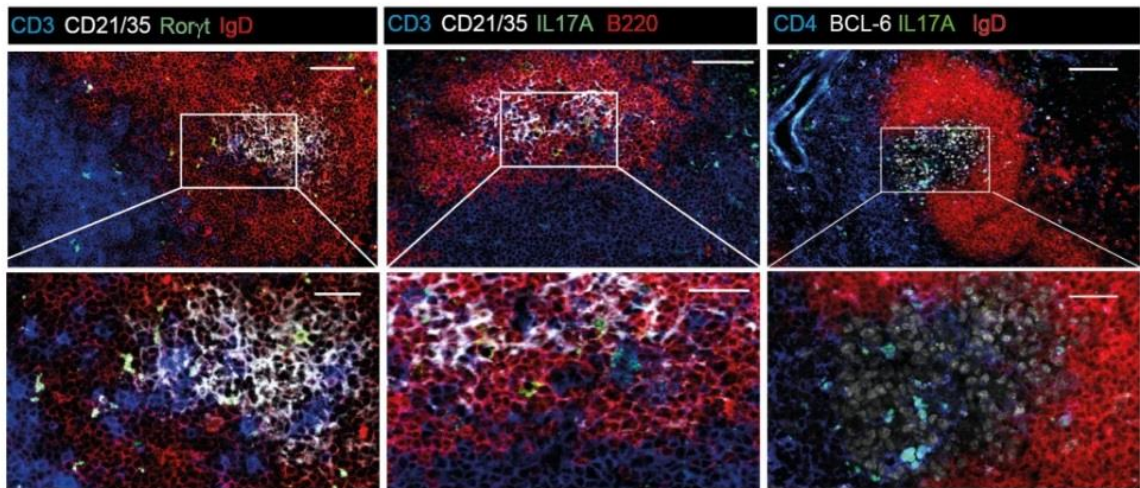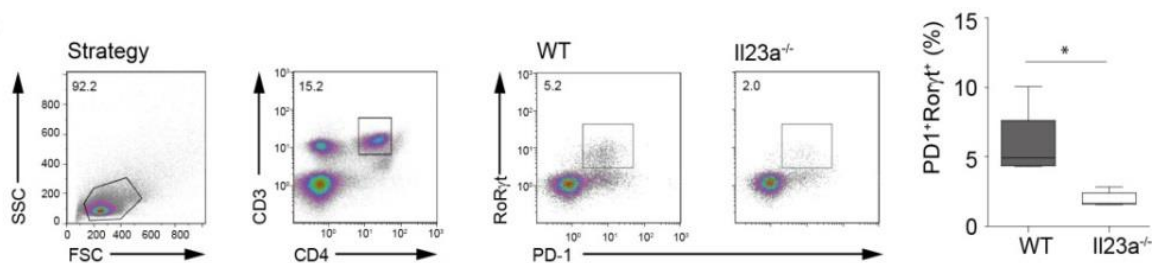
Figure 24: Presence of Th17 cells inside germinal centers.
(A) Immunofluorescence-based analysis of the location of RoRγt⁺IL-17⁺Th17 cells in the spleen of WT mice on day 26 after induction of CIA. B cell follicles and germinal centers were identified via staining with CD12/35 or BCL (both white), while T cells were stained for CD3 or CD4 (blue) and B cells with B220 or IgD (red). IL17A and RORγt are shown in green. Scale bars represent 200 μm (upper lane) and 100 μm (lower lane), respectively. Experiments were repeated for at least 3 times. (B) Flow cytometry based analysis of Rorγt⁺PD-1⁺ Th17 cells in the spleen of WT and *Il23a*⁻/⁻ mice at day 26 (n=5); *P≤ 0.0081; Student's t test. . Figure 24 was adapted from Pfeifle et al. 2017 [1]

3.7 TH17 CELLS SUPRESS ST6GAL1 THROUGH IL-21 AND IL-22

Although, Th17 cells were found in proximity of developing plasma cells, the exact mechanism that mediated *st6gal1* suppression remained elusive. Therefore, we wanted to identify the cytokines by which Th17 cells act on developing plasma cells. Therefore, we performed a B cell/Th17 co-culture experiment and neutralized the Th17-derived cytokines IL-17A/F, IL-21, IL-22 or GM-CSF [54, 57] trough blocking antibodies, while controls were incubated with IgG isotypes (Figure 25).
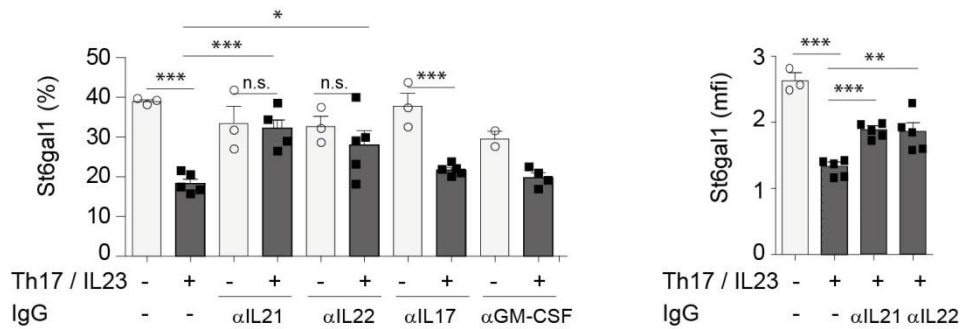
Figure 8: Il-23/Th17 axis regulate *st6gal1* by IL-21/22
Effect of the antibody-mediated neutralization of IL-21 (αIL21), IL-22 (αIL22), IL-17A/F (αIL17A/F) or GM-CSF (αGM-CSF) on st6gal1 protein expression in differentiating plasma cells that were co-cultivated within presence or absence of Th17 cells (n=3-5). Error bars represent SEM * P≤ 0.05, ** P≤ 0.005, *** P≤ 0.001; Student's t test. Figure 25 was adapted from Pfeifle et al. 2017 [1]

While determining protein levels by flow cytometric analyzes in developing plasma cells we found that IL-17A/F and GM-CSF neutralization was unable to interfere with the Th17-mediated suppression of *st6gal1*. However, neutralization of IL-21 and IL-22 drastically interfered with the downregulation of *st6gal1*, suggesting those two Th17-derived cytokines to be critically involved in the Th17 controlled inhibition of IgG sialylation and the resulting switch of anti-inflammatory to pro-inflammatory activity (Figure 25).

To test the relevance of this finding *in vivo*, we performed flow cytometry and analyzed the frequency of IL-17, IL-22 and IL-17/IL-22 producing CD3$^+$CD4$^+$ T cells in the spleen and lymph nodes during the course of arthritis. While IL-17$^+$ T helper cells increased from 0.5 to 1.0 percent, we found that IL-22$^+$ and IL-17$^+$IL-22$^+$ T helper cells increased significantly inside spleen and lymph nodes of wildtype mice (Figure 26).
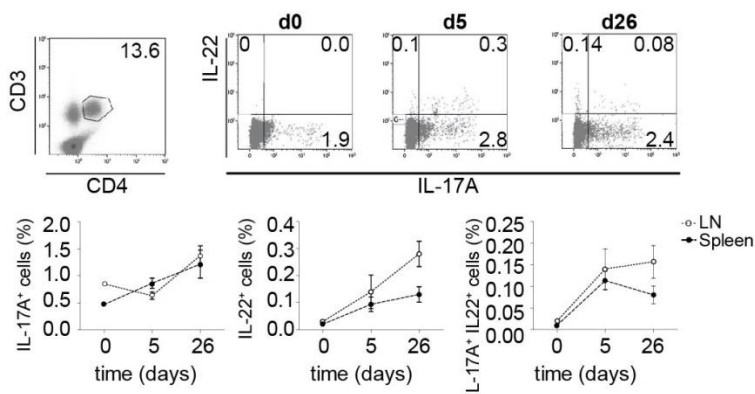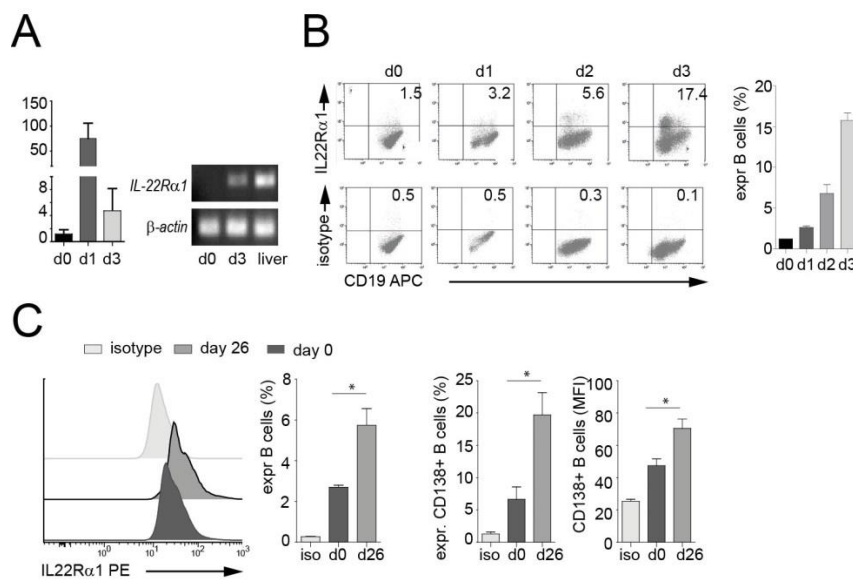

Figure 9: T helper cell frequencies in spleen and lymph node during arthritis
Flow cytometry based quantification of the frequency of IL-17 positive, IL-22 positive and IL-17/IL-22 double positive T cells in spleen and lymph nodes (LN) at indicated time points during collagen-induced arthritis. Error bars represent SEM. Figure 26 was adapted from Pfeifle et al. 2017 [1].

While IL-21 has been recognized to be involved in plasma cell differentiation as well as in the development of autoimmune diseases [274], the potential impact of IL-22 on developing plasmablasts has not been studied so far, since it has been published that the IL-22R subunit IL-22rα1 is not expressed in lymphoid cells [82].

To address this question we carefully analyzed IL-22Rα1 expression in naïve B cells, but furthermore studied the expression of IL-22Rα1 in developing plasma cells *in vitro* and during autoimmune arthritis *in vivo*. Therefore, we stimulated naïve B cells with LPS (5µg/ml) and analyzed IL-22Rα1 expression on mRNA and protein level.

Figure 10: IL-22Ra1 expression during plasma cell differentiation (A) mRNA- and (B) flow-cytometry based quantification of the expression of the IL-22 receptor (IL-22Rα1) on CD19⁺B220⁺ B cells during differentiation to plasma cells after LPS stimulation (5µg/ml). (C) Quantification of IL-22Rα1 surface expression on splenic CD19⁺B220⁺ B cells and B220⁺CD138⁺ plasmablasts after induction of CIA. Error bars represent SEM * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.001$; Student's *t* test. Figure 27 was adapted from Pfeifle *et al.* 2017[1]

Indeed, we found *Il22ra1* to be absent in naïve B cells. However, we detected *Il22ra1* being upregulated during plasma cell differentiation (Figure 27 A/B). Furthermore, we were able to confirm the expression of IL-22Rα1 on splenic B cells as well as on plasmablasts (B220⁺CD138⁺) during the prodromal phase of collagen-induced arthritis (Figure 27 0C), a phenomenon that coincided with the arrival of IL-22 producing T cells in the spleen (Figure 26).

3.8 PLASMABLASTS SIALYL-TRANSFERASE ACTIVITY PARALLELS CLINICAL ONSET OF RA

To analyze if, these changes in *st6gal1* activity also reflect human disease, we established a method, by which we were able to measure the overall content of sialic acid on the surface of CD19$^+$CD27$^+$CD38$^+$ plasmablasts by flow cytometry staining with the lectin *sambuccus nigra* agglutinin (SNA). This lectin exclusively binds to sialic acid α2,6 linked to galactose. Sialic acid served as surrogate marker for sialyltransferase activity.

The specificity was confirmed by flow cytometric and immunofluorescence analysis with *st6gal1*$^{-/-}$ mice. Furthermore we stained for human plasmablasts and used cells as negative controls that underwent specific oxidation or digestion of sialic acid by iodate or neuraminidase treatment (Figure 28 A-C).
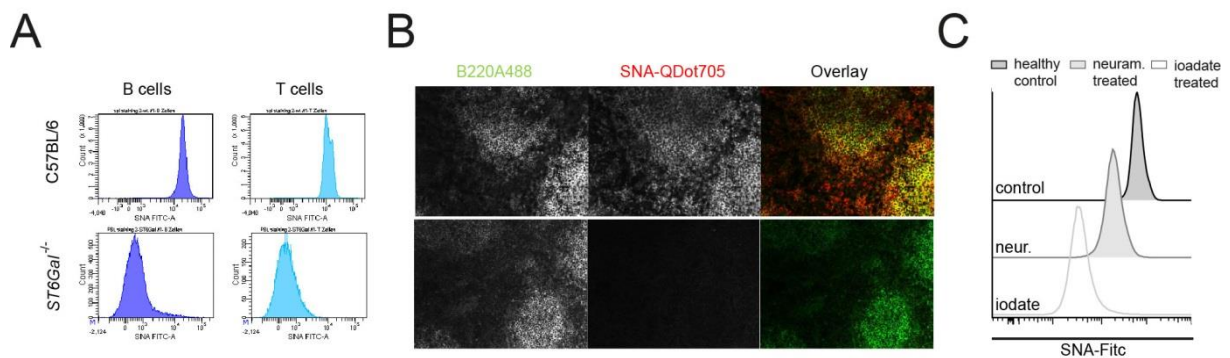

Figure 11: SNA specifically recognizes α2,6 linked sialic acid
(A) B and T cells of wild-type (WT) and *st6gal1*$^{-/-}$ mice were stained with a FITC-coupled SNA-lectin to determine the levels of surface sialic acid. (B) Immunofluorescence microscopy of spleens of WT and ST6gal1$^{-/-}$ mice determining the levels of sialic acid on B220$^+$ B cells by co-staining with an antibody against B220 (A488, green) and a SNA lectin (QDot705, red). (C) Human CD19$^+$ B cells were stained with FITC-labeled SNA-lectin to determine the levels of surface sialic acid. B cells were pretreated with neuraminidase (100 mU , 1h at 37°C) or iodate (2mM, 1h at 4°C) to remove sialic acid where indicated. Error bars represent SEM * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; Student's t test. Figure 28was adapted from Pfeifle *et al.* 2017 [1]

With this method we analysed peripheral plasma cells of healthy controls and RA patients for their overall content of surface sialic acid. Plasmablasts in the peripheral blood of healthy individuals showed a considerable heterogeneity, but plasmablasts of RA patients showed a significantly lower sialic acid content on the cell surface, illustrating that a reduction in sialyltransferase activity paralleled onset of disease (Figure 29).
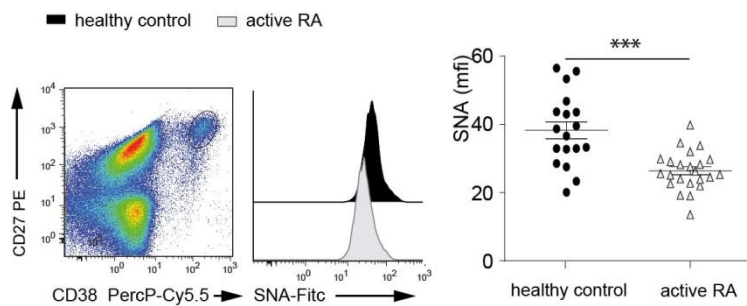
Figure 12: Overall content of sialic acid on plasmablasts parallels arthritis
SNA-lectin based quantification of the levels of surface sialic acid on CD27^{hi}CD38^{hi}CD19$^+$ plasmablasts from healthy controls and ACPA$^+$ RA patients. Each symbol represents the data from an individual donor (n=18 healthy controls and 23 RA patients; $P \leq$ 0.0001). Data is presented as mean values. Each data point reflects one individual. Student's t test. Figure 29 was adapted from Pfeifle *et al.* 2017[1]

Recent research has revealed that asymptomatic autoimmunity can be present a long time before clinical manifestations in RA patients occur [220, 221]. To determine if our findings were also reflected by changes in IgG glycosylation and to test the predictive value of this analysis, we isolated total IgG1 and ACPA$^+$ IgG from a cohort of ACPA$^+$ asymptomatic pre-RA [206, 223] individuals and analysed the content of sialic acid, galactose and fucose of the Asn297 glycans by mass spectrometry. Furthermore, we monitored the clinical progression towards RA.

Strikingly, we detected a significant decrease of IgG1 and APCA sialylation in individuals, in which asymptomatic autoimmunity preceded into clinical RA. The same was observed with the degree of galactosylation, which also decreased in both total IgG1 and ACPAs prior to onset of arthritis. Fucose content, however, was found to be not significantly changed in the different groups (Figure 30).
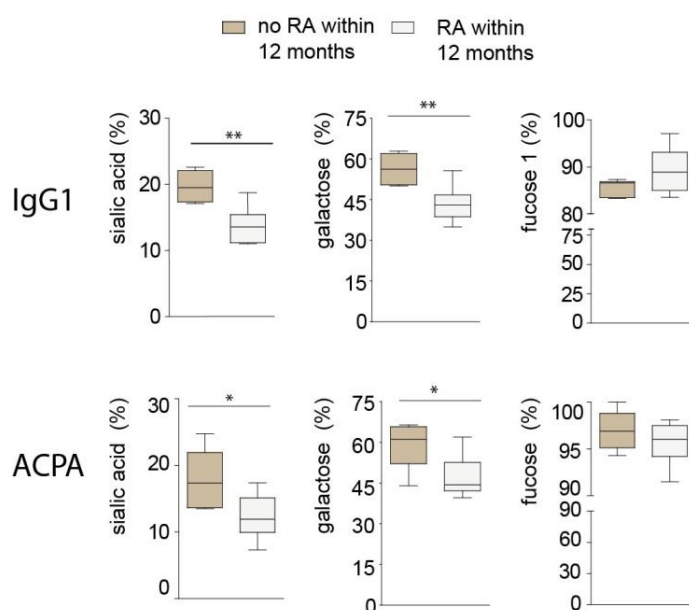
Figure 30: Decrease in sialylation precedes development of clinical RA
Comparison of the sialylation, galactosylation and fucosylation at Asn297 of total IgG1 and ACPA-specific IgG1 from asymptomatic ACPA-positive individuals that did not develop RA or developed RA within the following year (each group n=6). Error bars represent SEM * $P \leq$ 0.05, ** $P \leq$ 0.01, *** $P \leq$ 0.001; Student's *t* test. Figure 30 was adapted from Pfeifle *et al.* 2017[1]

Likewise, we analysed glycan-content on IgGs isolated from healthy controls, asymptomatic autoimmune "pre-RA" and active RA patients by mass-spectrometry and again found the tendency of a decreased sialylation and galactosylation in patients with active RA (Figure 31).
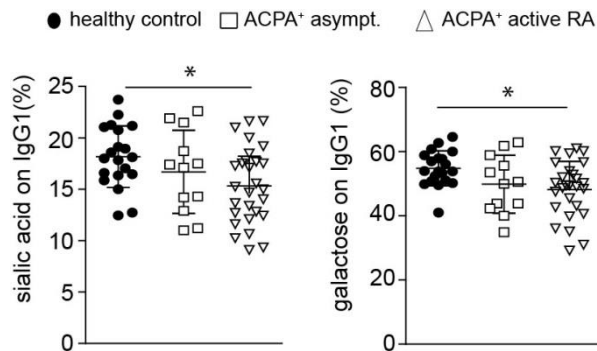

Figure 13: Sialic acid content decreases during progression of RA

Mass spectrometry-based analysis of the sialic acid and galactose content at Asn 297 of total IgG from healthy controls (HC), ACPA-positive asymptomatic individuals that have not yet developed RA (ACPA+ asympt.) or ACPA-positive active RA patients (ACPA+ active RA). Mean values were presented as bars and error bars represent SEM * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; Ordinary One-Way ANOVA. Figure 31 was adapted from Pfeifle *et al.* 2017[1].

Next we asked the question, if this observed changes in glycosylation also reflected the inflammatory capacity of the IgGs. To address this question, we differentiated human monocyte-derived dendritic cells (MoDCs) and coincubated those for 24h with heat-aggregated IgG immune complexes generated from healthy individuals and ACPA+ asymptomatic pre-RA and active RA patients. Subsequently, we analysed TNFα, IL-6 and IL-8 secretion in the supernatant.
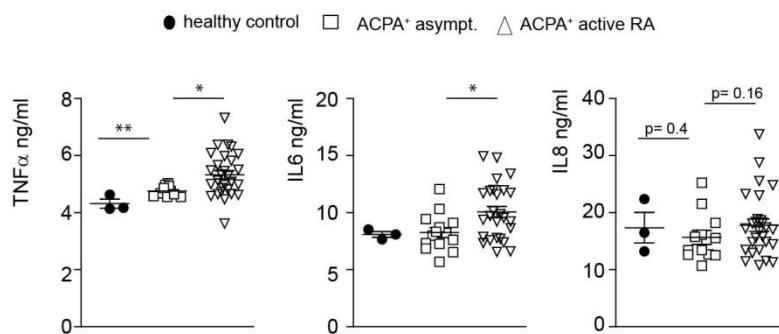
Figure 14: IgG activity parallels disease activity

Determination of the inflammatory activity of heat aggregated IgG immune complexes (IC) generated from the indicated groups of donors. ICs were incubated with *in vitro* generated human MoDCs 14 hours before the levels of indicated cytokines was measured in the supernatant. Error bars represent SEM *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Student's *t* test. Figure 32 was adapted from Pfeifle *et al.* 2017

Strikingly, IgG derived from RA patients displayed a drastically higher *in vitro* activity, reflected by their capacity to induce higher secretion of TNFα and IL-6. IL-8 secretion also tended to be increased, yet it was significant (Figure 32).

Since both sialic acid and galactose was found to be decreased in active RA, we aimed to determine, if IgG activity is also dependent on sialic acid content in human disease. Therefore, we treated IgGs derived from ACPA[+] asymptomatic "pre-RA" with neuraminidase and compared untreated and desialylated ICs in regard to their capacity to induce the secretion of pro-inflammatory cytokines.
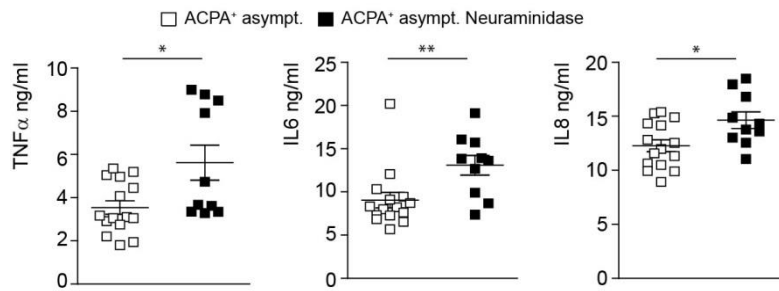
Figure 15: IgG activity correlates with desialylation
Determination of the inflammatory activity of heat aggregated ICs from ACPA-positive asymptomatic individuals before and after neuraminidase-mediated removal of sialic acid (n= 3, 10, 26; p≤0.02). ICs were incubated with *in vitro* generated human MoDCs 14 hours before levels of indicated cytokines was measured in the supernatant. Error bars represent SEM *P < 0.05, **P < 0.01, ***P < 0.001; Student's *t* test. Figure 33 was adapted from Pfeifle *et al.* 2017[1].

IgG desialylation lead to increased IgG activity, manifested in significantly increased TNFα, IL-6 and IL-8 secretion. These results confirmed that IgG sialylation is involved in regulation of IgG activity (Figure 33).

IV.   DISCUSSION

Rheumatoid arthritis is an intricate autoimmune disease. Even though research has identified many cells and cytokines associated with RA the pathogenesis still remains elusive to a considerable extend.

While it is now well accepted that the highly inflammatory Th17 subset and autoantibody producing B cells are key players of RA pathogenesis [53] several issues remain puzzling. On the one hand it has been clearly demonstrated, that the IL-23/Th17 axis is crucially involved in RA development [42, 236]. Th17 cells in peripheral blood, as well as the Th17 hallmark cytokine IL-17 in synovial fluids were found to be increased in patients with RA compared to osteoarthritic controls [240, 243]. Furthermore, *in vitro* studies demonstrated that Th17 cell cytokines like IL-17 can induce neutrophil recruitment, osteoclastogenesis and bone resorption in human RA bone explant cultures [64, 239, 275, 276].

Furthermore, IL-21 and IL22 have also been demonstrated to promote osteoclastogenesis in RA or increase RANKL expression of fibroblast-like synoviocytes (FLSs) [277, 278, 279]. In experimental arthritis the genetic loss of IL-23 in *Il23a*$^{-/-}$ mice led to a complete protection against CIA [42, 241, 250]. Again, also *Il17a*$^{-/-}$ and *Il22*$^{-/-}$ mice developed a less severe arthritis [241, 250], and IL-21R$^{-/-}$ mice are resistant to K/BxN arthritis due to a failure in IgG production [280].

However, despite the good evidence for the involvement of the IL-23/Th17 axis in RA, it has remained unclear, where, when and how the IL-23/Th17 axis contributes to autoimmune arthritis.

In this work, we dissected the pathogenesis of experimental arthritis into its two main parts. The initiation phase was defined as the period between immunization and onset of arthritis. During this time activated T cells instruct naive B cells to produce autoantibodies. The second phase was defined as "autoantibody mediated effector phase", which reflects full blown arthritis after induction of CIA or can be mimicked by injection of appropriate serum- or autoantibodies in the CAIA and K/BxN transfer models. During these two phases, we initially studied the effect of IL-23 depletion.

4.1 IL-23 GOVERNS THE PRIMING PHASE BUT NOT THE EFFECTOR PHASE OF AUTOIMMUNE ARTHRITIS

The data presented in this work demonstrated that *Il12a*$^{-/-}$ mice are not protected against arthritis. This was true for models depending on a breach of tolerance as well as in the K/BxN serum transfer model, where arthritis is initiated by inflammatory effector mechanisms inside the joints. We even found a tendency of *Il12a*$^{-/-}$ mice to develop more severe arthritis in the K/BxN serum transfer model (Figure 10). This is in accordance with recent publications which presented data supporting a protective role of IL-12/Th1 and IFNγ [281].

In contrast, we found that IL-23 was crucial for the pathogenesis of arthritis in the collagen-induced arthritis (CIA, Figure 6) model and in K/BxN mice (Figure 7). However, IL-23 neutralization was ineffective in passive arthritis models like the collagen-antibody induced arthritis model (Figure 8A) and the of K/BxN serum transfer arthritis (Figure 8B/C).

These findings together with the data presented in this work, strongly argue for a crucial role of the IL-23/Th17 axis during the priming phase of arthritis. However, the IL-23/Th17 axis is dispensable during the chronic, autoantibody dependent effector phase.

This suggestion is in line with a previous study showing that therapeutic targeting of IL-23 during the early phase of CIA suppressed arthritis, but failed to improve active arthritis when performed at later time points [42, 251]. Interestingly, it has also been shown, that Th17 cells do not predominate within arthritic joints in human and mouse models [244, 282]. Additionally, a recent study on human RA and IL-23 showed that RA patients exhibit higher IL-23 serum levels than healthy controls. However, no correlations were found between IL-23 and the DAS28 score[283], providing additional indications that the IL-23/Th17 is crucial in the priming phase but dispensable during the autoantibody dependent inflammatory phase of RA.

4.2   Iᴌ-23 ᴜɴʟᴏᴄᴋs ᴘᴀᴛʜᴏɢᴇɴɪᴄ ᴀᴄᴛɪᴠɪᴛʏ **OF IɢG**

One major function of CD4$^+$ T helper cells lies in orchestrating the humoral immune response. Interestingly, recent studies have demonstrated that Th17 cells are able to provide effective B cell help [281, 284] and that Th17 cytokines regulate germinal center reactions [74, 285, 286]. Furthermore, Th17 cells were found in germinal centers [287]. However, only few studies have investigated their specific role in humoral responses.

Humoral responses can be regulated on several levels. Therefore, we analysed the impact of IL-23 neutralization on germinal center formation, germinal center B cell proliferation, autoantibody titers, and IgG subtype usage and affinity maturation.

In this work we detected no influence of IL-23-activated Th17 cells on germinal center formation and germinal center B cell proliferation (Figure 12). Both $Il23a^{-/-}$ and wildtype mice developed normal germinal centers and there was also no difference in serum autoantibody titres nor in their capacity to generate high affine IgG against model antigens (Figure 13/14).

But humoral responses can also be tuned by altering the inflammatory capacity of IgG. Consequently, we analysed the capacity of CII-IgG from wildtype and $Il23a^{-/-}$ to trigger pro-inflammatory cytokine release from innate immune cells. ICs composed out of global serum IgG showed no difference between wildtype and $Il23a^{-/-}$ derived IgG fractions in regard to pro-inflammatory activity. However, ICs composed out of CII-specific IgG from $Il23a^{-/-}$ mice exhibited a significantly decreased capacity to induce TNFα, IL-6 and CXCL1 (Figure 11). These pro-inflammatory cytokines are typically associated with arthritis development [204, 288]. Likewise, IL-23 depletion during K/BxN arthritis dramatically reduced the capacity of K/BxN serum to induce arthritis in recipient mice without altering IgG titres (Figure 12).

Notably, changes in the IgG sialylation (Figure 17) and therefore also in the IgG activity (Figure 15), was only present in the autoantigen-specific fraction of IgG. Total IgG ICs showed only a marginal capacity to provoke the production of inflammatory cytokines by BM-DCs. Furthermore, we found that treating developing plasma cells with IL-23 alone had no suppressive effect on $st6gal1$ expression (Figure 23). Also Th17 cells without activation via IL-23 were not capable to supress $st6gal1$ in developing plasma cells (Figure 23) Interestingly, it was shown in a previous study with RA patients that sialylation was similarly decreased in ACPA compared to pooled serum antibodies [227], which is in line with our findings in mice. Moreover, it is well-established that ACPA glycosylation changes prior to the shift from asymptomatic autoimmunity

to active RA [226].Together, these findings suggest that IL-23 acts via the Th17 axis through an antigen-specific T cell-B cell interaction during the development of autoreactive plasma cells inside the germinal centers.

4.3   IL-23 REGULATES IgG ACTIVITY BY MEDIATING ASIALYLATION OF IgG

IgG activity is regulated through varying glycans attached on asparagine 297 of the IgG heavy chains. This glycans consist of a heptasaccharide core structure and several branching residues [173, 184]. Terminal sialylation has become the most interesting glycan modification so far, since the presence of this residue renders an IgG molecule anti-inflammatory [178, 192].

The content of IgG sialylation was the only difference between arthritic wildtype mice and CIA-resistant $Il23a^{-/-}$ mice (Figure 17). Galactosylation and fucosylation did not differ between wildtype and $Il23a^{-/-}$ mice and also Fc sialylation showed no difference when we analysed total IgG and unspecific IgG fractions, respectively. However, wildtype mice displayed significantly decreased sialylation of autoantigen-specific (α-collagen-type II) IgG. Interestingly, this decrease of CII-IgG sialylation in wildtype mice preceded the development of arthritis (Figure 11). The importance of this finding is highlighted by the recent finding, that the sialylation of antigen-specific IgG is able to convert arthritogenic IgG into IgG that inhibits CIA, while sialylation of unspecific IgG had no beneficial effect[192].

The importance of regulation of the IgG sialylation degree on CII-IgG through IL-23 has been also confirmed by the finding that enzyme-mediated addition of terminal sialic acid decreased the pro-inflammatory activity in an *in vitro* IgG activity assay (Figure 18). Conversely, the enzymatic cleavage of terminal sialic acid diminished the anti-inflammatory potential of $Il23a^{-/-}$ derived CII-ICs (Figure 19). Furthermore, enzymatic digestion of sialic acid lead to a regain of arthritogenic activity of IgG isolated from αIL-23 treated K/BxN mice and subsequently to a dramatically increase in arthritis severity during K/BxN-IgG transfer. (Figure 20)

The immunosuppressive capacity of sialylated IgG has been subject of many discussions. In this work, ICs composed of hyper-sialylated CII-specific IgGs led to a decreased production of pro-inflammatory cytokines from bone marrow-derived dendritic cells, potentially suggesting a decreased binding capacity unto activating FcγRs.

This is in line with previous findings, which showed a reduced binding efficiency of sialylated IgG to activating FcγRs [166, 169]. Furthermore preferential binding to type II FcRs (SIGNR-1, CD23) as proposed by Ravetch *et al.*, may account for the strong anti-inflammatory effects of the serum derived from αIL-23 treated K/BxN mice. Sialylation of the biantennary glycan attached in Asn297 has been shown to increase the conformational flexibility leading to "closed conformations" of CH2 domains. This leads to a decreased binding capacity to type I FcRs while increasing the affinity for inhibitory type II FcRs [166, 192, 289, 290]. Furthermore, complement binding may be involved, since Fc sialylation was shown to impair IgG-mediated complement activation and cytotoxicity [291].

4.4   IL-23 UNLOCKS IGG PATHOGENICITY VIA TH17 CELLS

To investigate the mechanism controlling antibody sialylation by IL-23, we analysed the expression of β-galactoside alpha-2,6-sialyltransferase 1(*st6gal1*) in splenic plasma cells. During the development of CIA and before onset of arthritis, we found suppressed *st6gal1* expression in splenic plasma cells (PCs) on mRNA and protein level. *Il23a*^{-/-} mice, in turn, were unable to downregulate *st6gal1* (Figure 21/22A). These increased st6ga1l levels, remained stable until the late chronic phase of CIA and were found to be increased in plasma cells isolated on day 50 from spleen and bone marrow (Figure 22B). The Exchange of collagen type II with ovalbumin (OVA) as an alternative antigen enabled tracking of antigen-specific plasma cells by intracellular staining with OVA-A488. Consistently, antigen-specific plasma cells derived from *Il23a*^{-/-} mice displayed an increased *st6gal1* expression compared to plasma cells derived from WT mice (Figure 22C).

This data demonstrate that the IL-23-dependent downregulation of *st6gal1* is antigen-specific (also shown by the differences observed in the sialylation of CII-specific IgG), but not dependent on CII as particular antigen.

Antigen-specific regulation of glycosylation suggests CD4^{+} T helper cell involvement. Interestingly, a number of observations argue for CD4^{+} T cells to play a dominant role during RA pathogenesis. This includes the increase of CD4^{+} T cells during RA, the effectiveness of T cell-targeting therapies and genetic association studies with HLA-DRβ1 [30, 233, 235]. However, the exact mechanism by which CD4^{+} T cells contribute to RA has remained enigmatic [247].

This data also supports the idea, that CD4^{+} Th cells are mainly involved in the initiation phase of arthritis. The fact, that *Il23a^{-/-}* mice displayed an increased *st6gal1* expression in their plasma cell compartment and increased sialylation of CII-specific IgG strongly suggest Th17 cells to be involved, since IL-23 is critically for the terminal differentiation of pathogenic Th17 cells [269, 270].

Culturing of developing plasma cells in the presence of IL-23-activated Th17 cells resulted in a remarkable downregulation of *st6gal1* expression in developing plasma cells (Figure 23). Interestingly, sustained IL-23 signaling seems to be necessary for maintaining the pathogenicity of Th17 cells. IL-23 activation during *in vitro* differentiation alone was not sufficient to suppress *st6gal1* in developing plasma cells (Figure 23A). Intriguingly, Th17 cells accumulated inside secondary lymphatic organs during the prodromal phase of experimental arthritis (Figure 9). Furthermore, immunofluorescence analysis located RORγt[+] and IL-17^{+} Th17 cells inside germinal centers and in close proximity to developing plasma cells (Figure 24).

This work strongly associates the *st6gal1*-regulated IgG pathogenicity to the IL-23-activated CD4^{+} Th17 subpopulation. Additionally, our experiments suggest IL-23 to be mainly involved in the priming phase of arthritis. This conclusion is further supported by the fact that Th17 cells were found to increase during the course of disease in secondary lymphatic tissues like spleen and lymph nodes, while hardly any Th17 cell could be detected in inflamed paw of arthritic mice (Figure 9). Moreover, this explains the ineffectiveness of IL-23 inhibition during the antibody-dependent phase of chronic joint inflammation [251, 252, 253].

Recent findings demonstrate that IL-23 drives the pathogenic behaviour of Th17 cells. Actually, IL-23 is dispensable for the differentiation of Th17 cells *in vitro* and *in vivo*, implicating that these "immature" Th17 cells may be able to fulfil basic functions, but are incapable to defeat certain pathogens like *M.tuberculosis* or do not contribute to autoimmune disease [44]. Th17 seem to develop their potent inflammatory capacities only in the presence of IL-23 [43, 269].

This could also be the reason for the unchanged germinal center reaction, regular IgG class-switch and normal affinity maturation observed in *Il23a^{-/-}* mice. While these factors are regulated by the basic Th program, the inflammatory activity of IgG has to be licenced by terminally differentiated Th17 cells.

Therefore, we suggest that Th17 cells, which received the IL-23 communicated "permission to be pathogenic" [237] are able to license developing plasma cells to downregulate the *st6ga1l*, which

leads to the production of desialylated autoantibodies and subsequently to autoimmune inflammation.

4.5 ST6GAL1 SUPPRESSION BY TH17 CELLS IS IL-21 AND IL-22 DEPENDENT

Th17 cells differentiate in the presence of small concentrations of TGF-β and high concentrations of pro-inflammatory cytokines like IL-6 and IL-21, which lead to the upregulation of IL-23Rα [37, 52, 54, 237, 292]. IL-23 exposure is crucial for Th17 cells to obtain their full inflammatory capacity [292]. IL-23 induces Blimp-1 and thereby upregulates and/or activates the production of Th17 pathogenicity factors like GM-CSF, IL-21 and IL-22 [57, 270, 292, 293]. Taken together, recent research demonstrates that even though IL-23 has been initially identified to initiate IL-17 expression from T cells [294], it controls important effector functions beyond the expression of the so called "hallmark cytokine" of Th17 cells.

Interestingly, our data suggest that in spite of increased expression of IL-17 during the course of arthritis, B cells did not respond to Th17 cell derived IL-17 in terms of *st6gal1* regulation (Figure 25). This finding is in accordance with several studies, demonstrating that even though *Il17*^{-/-} mice showed supressed arthritis [241], IL-17 was not shown to be involved in T cell-B cell interactions [70]. In contrast, blockade of IL-21 and IL-22 , both Th17 cytokines which are regulated through IL-23 [57], abrogated the Th17-mediated suppression of *st6gal1* expression (Figure 25). Consistently, IL-22^{+} Th17 cells increased inside secondary lymphatic organs during the prodromal phase of arthritis (Figure 26).

Particularly IL-22 has been discussed to exert pleiotropic effects. *IL-22*^{-/-} mice display supressed arthritis susceptibility [250] and increased IL-22 serum levels correlate with a more erosive form of RA in patients [83]. In contrast, injection of IL-22 i.p. after the priming phase of CIA had a suppressive effect on inflammation (by inducing IL-10)[295]. Consistently, synovial inflammation has been shown to be independent of IL-22 [249]. Interestingly, IL-22 has been reported to be upregulated in spleen and serum during the priming phase of arthritis and produced mainly by CD4^{+} T cells. During later time points IL-22 expression increased inside the paws [295].

Since it has been published that the IL-22-receptor (IL-22Rα1) is not expressed on lymphocytes, we investigated the *il22ra1* mRNA and protein expression in naïve B cells, developing plasma cells and in B cells isolated during collagen-induced arthritis. Although we could not detect IL-

22Rα1 mRNA in naïve B cells, we found it to be upregulated during B cell development into plasma cells on both mRNA and protein level. Furthermore, IL-22Rα1 surface expression increased on splenic plasma cells during the prodromal phase of arthritis (Figure 27). This is in accordance with an already published increase of IL-22Rα1 in total spleen during CIA [80, 82].

IL-21, on the other hand, is well known for its important effects on developing plasma cells [74]. IL-21 receptor expression on B cells has been shown to be required for the development of arthritis [274].

Taken together, our finding suggests the regulation of IgG activity via IL-21 and IL-22. IL-23 permits Th17 cells to acquire a pathogenic phenotype that involves secretion of IL-21/22 and interaction with developing plasma cells resulting in suppression of *st6gal1*. However, to finally resolve the impact of each cytokine *in vivo*, and to uncover the "when" and "how", further research has to be done. B cell knockouts of IL-22Ra1 and IL-21R during arthritis could help to clarify these points. *In vivo* profiling of IL-21/22-producing Th17 cells by using immunofluorescence staining and specific neutralization of IL-21/22 during the course of arthritis will help to understand when these Th17 mediated events occur. Further *in vitro* studies should clarify if additional signals from Th17 cells are involved.

Two publications may be mentioned that challenge our hypothesis. Firstly Cobb *et al.* [296]demonstrated that antibody secreting cells do not exclusively control the sialylation dependent anti-inflammatory function of IgG. The authors claimed that IgG is predominantly regulated inside the bloodstream in an antigen unspecific manner [296]. However, extracellular IgG sialylation in the bloodstream is difficult to imagine due to the structural encrypted sialic acid residues of N-glycans. Additionally, these findings present no explanation for the specific decrease in IgG sialylation of autoantigen-specific IgG in arthritic mice (Figure 17) and human RA patients [185, 223, 224, 227].

Another publication analysing RORγt[gfp/gfp] mice reported IL-17^{+}RoRγt[+] to be dispensable during KRN-T cell transfer arthritis [297, 298]. RORγt is known as master regulator of Th17 cells and global inhibition of RORγt has been shown to block several autoimmune diseases including arthritis [47, 299].

Here it is important to state that RORγt is not the only transcription factor associated with Th17. Multiple other transcription factors have been shown to be important for Th17 differentiation, which include BATF, STAT3, IRF4, RUNX1, IκBζ, and RORα [48, 248, 300, 301, 302, 303]. RORγt expression per se does not enforce Th17 pathogenicity [304] and overexpression of RORγt in CD4^{+} T cells has also been linked to anti-inflammatory effects [305].

4.6 CONCLUDING REMARKS

It is well established that autoantibodies against citrullinated proteins may precede autoimmune disease for several years. Much has been speculated about a second "hit" from microbial factors or environmental influences that promote a shift of asymptomatic ACPA[+] autoimmunity into inflammatory disease. However, so far it has been unclear how this switch is mediated.
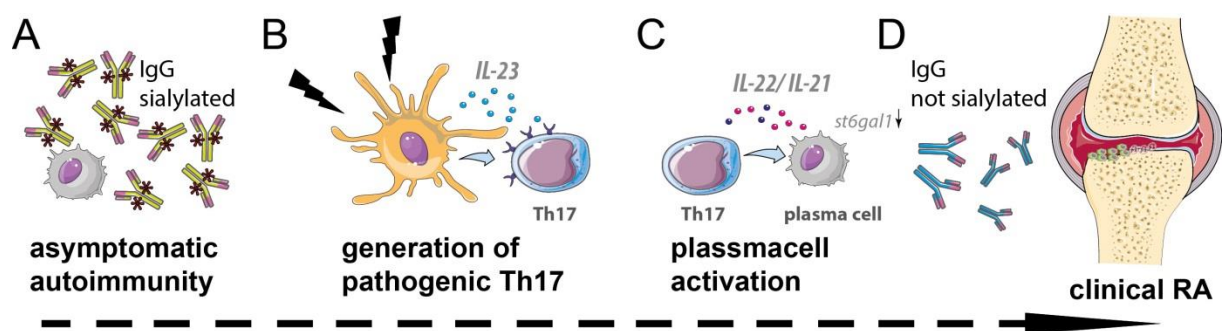
Schematic overview of how IL-23 licenses Th17 cells to induce pathogenic IgG production in plasma cells during the progression of autoimmune arthritis

In this work, we demonstrate that the activation of the IL-23/Th17 axis mediates the suggested "second hit" on developing plasmablasts. IL-23-activated Th17 cells activate the full proinflammatory potential of IgG through suppressing *st6gal1* expression in developing plasma cells in an IL-21/IL-22 dependent manner. Subsequently, decreased IgG sialylation leads to more active IgG and thereby to joint inflammation.

Our data also suggest that the IL-23/Th17 only plays a subordinate role during the antibody dependent effector phase of RA. Therefore, targeting the IL-23/Th17 axis might be more effective as a preventive therapy or as part of a therapeutic strategy aiming to inhibit a renewed pro-inflammatory activity in the humoral immune compartment.

4.7 A DECREASE IN PLASMABLAST ST6GAL1 ACTIVITY AND SIALYLATION PARALLELS CLINICAL ONSET OF ARTHRITIS

Also human RA seems to be a Th17 cell and B cell-dependent disease. Increased Th17 cell frequencies are associated with RA [248] and T cell targeting therapies support the idea that T cells are critically involved in the RA pathogenesis [306]. However, targeting IL-23 during active RA was disappointing [252]. Autoreactive plasmablasts have been identified in blood and in inflamed joints and depletion of B cells ameliorates RA. Autoreactive ACPA antibodies have been shown to be highly specific for RA but may precede joint inflammation for up to ten years [226, 307]. However during this phase of autoimmunity, ACPA display an anti-inflammatory glycosylation profile.

Intriguingly, during the shift from asymptomatic autoimmunity to chronic autoimmune inflammation ACPA diversifies and increases in serum levels. Importantly, this is paralleled by increases in the frequencies of asialylated and agalactosylated ACPA-glycoforms[185, 227]. Indeed, agalactosylated and asialylated ACPA have been mechanistically implicated in the initiation of RA and seem to directly contribute to the destruction processes of bone [219, 222].

To determine if the regulatory mechanism identified in experimental arthritis also reflect mechanisms in human RA, we established a method that enabled us to determine α2,6-sialyl-transferase activity inside human plasmablasts of the peripheral blood. Evaluation of the surface sialic acid content revealed that indeed, decreased α2,6-sialyltransferase activity of plasmablasts paralleled arthritis development (Figure 29). Additionally, we showed on IgG level that terminal sialic acid and galactose content decreased in ACPA[+] asymptomatic individuals prior to the appearance of clinical RA (Figure 30). This findings are in line with recently published data [226].

This decrease in the sialic acid content of IgG was also linked to an increase in the pro-inflammatory activity of ACPA[+] IgG isolated from RA patients (Figure 32/33). This demonstrates, that human α2,6-sialyltransferase expression regulates IgG activity. Additionally, it can be suggested that IgG-sialylation in humans is also already imprinted during germinal center reactions, since even plasmablasts in the peripheral blood differed in α2,6-sialyltransferase activity. Although anti CII-IgG of arthritic mice and antibodies of active RA displayed similar alterations in sialylation, in humans these changes were milder. However, our data may underestimate the true decrease in sialylation of autoreactive antibodies in humans, since the functional autoantigens of RA patients are not yet fully defined.

Noteworthy, although RA patients demonstrate a reduced galactosylation and a reduction in sialylation within their IgGs, during CIA we only found a reduced sialylation. This is consistent with recent findings. It may be possible, as Ohmi *et al.* proposed, that these findings are due to "chronic exposure" of plasma cells to an inflammatory environment [192]. Another possibility would be, that in humans, additional factors exist which control IgG glycosylation.

The data presented in this work does not definitely prove the role of IL-23 in human RA. However, it offers an explanation for several well-known findings. ACPA also precedes RA development and the ACPA fraction similarly shows specific changes in their glycan profile that differs from the Fc glycans of total serum IgG. Changes in ACPA-Fc glycosylation have been correlated with disease activity [185, 226]. Furthermore, even though Th17 cells are crucial participants in the pathogenesis of RA, neutralization of IL-23 during active disease has proven to be ineffective. This strongly argues for a similar early involvement of IL-23-activated Th17 cells in RA.

V.   ABBREVIATIONS

AAL	aleuria Aurantia lectine
ACPA	anti-citrullinated protein antibodies
ACR	American College of Rheumatology
ADCC	antibody dependent cellular cytotoxicity
AHR	aryl hydrocarbon receptor
αIL-23	anti-IL-23 antibody
anti-CarP	anti-carbamoylated protein antibodies
APC	antigen presenting cell
APRIL	"a proliferation inducing ligand"
Asn	aspargine
b4galt1	β-1,4-galactosyltransferase 1
BAFF	B cell activating factor of the TNF family
BATF3	basic leucine zipper transcription factor
Bcl-6	B cell lymphoma protein 6
BCR	B cell receptor
BLIMP	B lymphocyte-induced maturation protein-1
BM	bone marrow
BM-DC	bone marrow-derived dendritic cell
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
CIA	collagen-inducedarthritis
CII	type II collagen
cTEC	cotrical thymic epithelial cell
CXCL8	C-X-C motif ligand 8
DAS28	disease activity score 28
DC	dendritic cell
DCIR	dendritic cell immunoreceptor
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DC-STAMP	dendritic cell-specific transmembrane protein
dg	deglycosylated
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxid
DN	double negative
DNA	deoxyribonucleic acid
ds	desialylated
DZ	dark zone
EAE	experimental autoimmune encephalomyelitis
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EULAR	European League Against Rheumatism

Fab	antigen-binding fragment
FACS	fluorescence-activated cell sorting
Fc	crystalizable fragment
FcRn	neonatal Fc receptor
FCS	foetal calf serum
FcγR	Fcγ-receptor
Foxp3	forkhead-box-protein P3
fuc	fucose
G6PI	glucose-6-phosphate isomerase
gal	galactose
GATA-3	Trans-acting T cell-specific transcription factor
GC	germinal center
GlcNAc	N-acetylglycosamine
GM-CSF	granulocyte macrophage colony-stimulating factor
HC	heavy chain
HLA	human leukocyte antigen
HRP	horseradish peroxidase
i.p.	intraperitoneal injection
i.v.	Intravenous injection
IC	IgG complex
IFN-γ	Interferon gamma
Ig	immunoglobulin
IgL	immunoglobulin light chain
IL	interleukin
IMDM	Iscove's modified dilbecco's medium
IRF4	Interferon regulatory factor 4
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
IVIG	intravenous IgG
KC	cytokine induced neutrophil chemoattractant
KSCN	potassiumthiocyanate
LC	light chain
LCA	*lens culinaris agglutinin*
Lfa	lymphotoxin alpha
log	logarithm
LPS	lipopolysaccharide
MHC	major histocompatibility class
mRNA	messenger RNA
MZ	marginal zone
N	aspargine
NOD	non-obese diabetic
NP	4-hydroxy-3-nitrophenylacetyl
NP-CGG	NP-gamma globulin
OD	optical density
OVA	ovalbumin
PAMP	pathogen associated molecular patterns

PBS	phosphate buffered saline
PBST	PBS-Tween
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1
PFA	paraformaldehyde
PNA	Peanut agglutinin
PNGase F	Peptide -N-Glycosidase F
PRR	pathogen recognition receptors
RA	rheumatoid arthritis
RF	rheumatoid factor
RNA	ribonucleic acid
RORα	RAR-related orphan receptor alpha
RoRγ	RAR-related orphan receptor gamma
RPMI	Roswell Park Memorial Institute medium
RT	roomt temperature
SDS	sodium dodecyl sulfate
sem	standard error of mean
SHM	somatic hyper-mutation
sial	sialyation
SNA	*sambucus nigra lectin*
St6gal1	β-galactoside α2,6 sialyltransferase 1
STAT	signal transducer and activator of transcription
Taci	transmembrane activator and CAML interactor
Tbet	T-box transcription factor
TBS	Tris-buffered saline
TBST	TBS-Tween
TCR	T cell receptor
TD-antigens	T cell dependent antigens
Tfh	follicular T cells
TGF	transforming growth factor
Th	T helper cell
TI-antigens	T cell independent antigens
TNFa	Tumor necrosis factor-alpha
TRA	tissue restricted antigens
TRAP	tartrate-resistant acid phosphatase
Tregs	regulatory T cells
Tris	tris(hydroxymethyl)aminomethane
Tween 20	Polysorbat 20
UDP	uridindiphosphat
x g	centrifugation speed in g

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"Not to us, O Lord, not to us! But to your name bring honor, for the sake of your loyal love and faithfulness." Psalm 115:1 (NET Bible®)

Soli Deo Gloria

VIII. CURRICULUM VITAE

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Place of Birth:	Reutlingen
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Scientific expertise

2010-2017 **PHD THESIS AT THE** Department of Internal Medicine 3, Institute for Clinical Immunology,University of Erlangen-Nuremberg, Germany. Supervisors: Prof. Georg Schett and Dr. Gerhard Krönke. Main subject: "The IL23/TH17 axis unlocks autoantibody activity and unmasks autoimmune disease"

2010 **MASTER THESIS AT THE** Department of Dermatology, Laboratories of DC Biology, University Hospital of Erlangen, Friedrich-Alexander-University of Erlangen-Nuremberg Germany. Supervisor: Prof. Diana Dudziak. Project: "miRNA expression-profiles of dendritic cell subpopulations."

2007-2008 **RESEARCH ASSISTANT AT THE** Institute for Clinical Microbiology, Friedrich-Alexander-University of Erlangen-Nuremberg Germany

2008 **BACHELOR THESIS AT THE** Institute for Clinical Microbiology, Friedrich-Alexander-University of Erlangen-Nuremberg Germany. Supervisor: Prof. Wolfgang Hillen. Project: "TetR induction through tetracycline derivates."

Education

2010 **MASTER OF SCIENCE IN CELL- AND MOLECULAR BIOLOGY** AT THE Friedrich-Alexander University, Erlangen-Nuremberg, Germany. Overall mark: 1.5

2008 **BACHELOR OF SCIENCE IN BIOLOGY** AT THE Friedrich-Alexander University, Erlangen-Nuremberg, Germany. Overall mark: 2.0

2004 **HIGHER EDUCATION ENTRANCE QUALIFICATION** Theodor-Heuss Wirtschafts-Gymnasium, Reutlingen, Germany. Overall mark: 1.9

2001   SECONDARY SCHOOL CERTIFICATE Freibühschlue, Groß-Engstingen, Germany. Overall mark: 1.5

Languaes

German   native language

English   fluent

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Congress participations

2013   **33rd European Workshop for Rheumatology Research** (EWRR) in Prague, Czeck Republic
Poster presentation

2015   **1st International Symposium on B cells: Immunity and Autoimmunity** in Erlangen, Germany

2016   **ACR/ARHP Annual Meeting** in Washington, DC, United States of America
Poster presentation

2016   **First interdisciplinary congress on IL-17** in Munich, Germany

Awards/Bursaries

2016   Novartis bursary (4000€) for attending proster presentation at the 2016 ACR/ARHP Annual Meeting , Washington, DC, United States of America

2016   Novartis bursary (1000€) for presenting poster at the 1st interdisciplinary congress on IL-17

Publications

Pfeifle R, Rothe T, Ipseiz N, Scherer HU, Culemann S, Harre U*, et al.* Regulation of autoantibody activity by the IL-23-TH17 axis determines the onset of autoimmune disease. *Nat Immunol* 2016

Harre U, Lang SC, Pfeifle R, Rombouts Y, Frühbeißer S, Amara K*, et al.* Glycosylation of immunoglobulin G determines osteoclast differentiation and bone loss. *Nat Commun* 2015, **6**.