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BEYOND CONVENTIONAL CARE: DEVELOPING NOVEL THERAPEUTIC APPROACHES TO COMBAT ARTHRITIS

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Cover illustration: A mouse neutrophil (Ly-6G Hoechst) interacting with mouse IgG2b immune complexes is captured by a Zeiss LSM800 confocal microscope. Photographer: Zhongwei Xu.

Beyond conventional care: Developing novel therapeutic approaches to combat arthritis

Thesis for Doctoral Degree (Ph.D.)

By

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To my family

致我的家人

Life finds a way - Jurassic Park

Popular science summary

Countless pathogens exist in the universe, and when they enter the body of an animal, they can cause infections. Through the course of evolution, animals have developed immune system ("plague-freeing system" in Chinese) to defend these foreign invaders. Invertebrates, like insects and worms, possess relatively simple immune systems. In contrast, vertebrates, like humans and birds, have developed a more sophisticated immune system. This complex system, equipped with T cells and B cells, can adaptively tailor its response to combat specific novel invaders. This is achieved by the expansion of specific B cells (producing antibodies) and T cells, which can then rapidly clear the pathogen, thereby increasing the chances of survival for the host.

In the vast majority of individuals, the immune system is well educated to distinguish foreign pathogens from the body's own tissues before puberty. However, in few individuals, the immune system may mistakenly perceive one's own organs as threats and subsequently initiate an attack. This kind of immune attack leads to autoimmune diseases. For example, type-I diabetes arises from an attack in the pancreas, while rheumatoid arthritis (RA) develops due to an attack on the joints.

RA remains incurable as its root cause is still elusive. If not adequately managed, RA patients will experience life-long joint symptoms such as pain and dysfunction, which can eventually lead to disability. Therefore, it is crucial to understand why the immune system mistakenly starts to attack joints. With this knowledge, we can devise strategies to prevent such attacks in at-risk individuals. And if the attack is already underway, we can determine how best to halt or alleviate it.

In study I, we generated several autoantibodies to induce arthritis in mice. We found that a receptor, FCGR3, is both critical and sufficient for cartilage antibody induced arthritis. The absence of its counterpart, FCGR2B, can even override the effect of complement activation (which amplifies arthritis).

In study II, we engineered an artificial autoantibody, denoted R69-4, based on the observations that similar antibodies did not induce but protect against arthritis in mice. R9-4 was designed to target a specific sequence (F4) on type-II collagen (COL2), a protein abundant in cartilage. Interestingly, we found that R69-4 also binds to various other proteins in synovial fluid, and further prevents arthritis progression by blocking the function of FCGR3 on neutrophils. Given its strong efficacy in suppressing arthritis, R69-4 may contribute to the management of RA, particularly in patients who are resistant to multiple treatments.

In study III, we delved into the role of two genes, *Ncf1* and *Fcgr2b*, in the development of arthritis, given the significant influence of genetic factors on RA. We

discovered that both genes affect T cell tolerance, a mechanism to prevent mistaken attack on the body's own tissues. Interestingly, each gene employs a unique mechanism in this process.

In study IV, we intentionally created a mouse strain with self-reactive B cells that target another specific sequence (C1) on COL2. We anticipated that these mice would develop spontaneous arthritis because of the production of autoantibodies by these B cells. However, the animals were protected by these special B cells. We further discovered that these self-reactive B cells could activate regulatory T cells and enhance T cell tolerance through a regulatory molecule called CD72. This process effectively suppressed C1 specific autoimmunity rather than other types of autoimmunity.

科普摘要

自然界中遍布众多病原体，一旦入侵体内便可能引发感染。为了应对这些威胁，各种动物都进化出了各自的免疫系统（“免除疫病”）来抵御这些入侵者。无脊椎动物，如昆虫和软体动物，有一套相对简单的免疫系统；脊椎动物，如人类和鸟类，则进化出了相对复杂的免疫系统。这套复杂的系统拥有 T 细胞和 B 细胞，能够对任何新型病原体作出反应。这一过程主要依赖特定 B 细胞（产生抗体）和 T 细胞的快速增殖，从而迅速清除特定病原体，大大提升了生存几率。

绝大多数人免疫系统在成年前都已成熟，能够精准区分病原体和自身组织。少数人免疫系统可能误判，将自身器官当作威胁，进而产生错误攻击。这类错误即导致所谓的自身免疫性疾病。例如，当自身免疫攻击胰腺时，可能会导致 1 型糖尿病；而这种反应针对关节时，则可能诱发类风湿性关节炎 （RA）。

RA 确切病因目前仍不清楚，因此尚无法根治。其主要症状为关节肿痛和功能障碍，若不及时治疗，疾病进展最终可导致残疾。因此，理解免疫系统为何会针对关节发起错误攻击是解决 RA 的关键。发现病因有助于预防此类错误的发生，还可以为已经发病的患者提供更有效的治疗和管理方法。

在研究一中，我们制备了多种针对关节的自身抗体，用于在小鼠中诱导关节炎。我们发现一个受体，FCGR3，在抗体介导的关节炎疾病模型中至关重要。当与其配对的受体 FCGR2B 缺失的情况下，自身抗体甚至可以绕过补体激活（炎症放大）直接诱发关节炎症。

在研究二中，我们设计了一种人工自身抗体（R69-4），因为我们发现类似的自身抗体在小鼠中不仅不致病，反而对关节有保护作用。R69-4 被设计来结合二型胶原上一段特定的氨基酸序列（F4），而二型胶原是软骨中最重要的组成蛋白。我们发现 R69-4 同时还可结合关节滑液中的多种蛋白，从而通过抑制中性粒细胞 FCGR3 的功能阻止关节炎的发生和进展。因其良好的抗炎作用，R69-4 可能对 RA 有一定治疗潜力，尤其是对多种药物耐药的患者。

在研究三中，我们探索了自身免疫相关基因 *Ncf1* 和 *Fcgr2b* 在关节炎发病过程中的作用。众所周知，基因对 RA 发病至关重要。我们发现两个基因都会增强 T 细胞耐受（一种预防自身免疫的机制），但却通过不同途径产生影响。

在研究四中，我们特意创建了一个新的小鼠种系。考虑到这些小鼠拥有针对二型胶原另一段氨基酸序列（C1）的自身反应性 B 细胞，我们原本预期这个 B 细胞克隆产生的自身抗体会引发关节炎。然而，这些小鼠不仅未发病，反而受到了这些自身反应性 B 细胞的保护。进一步研究发现，这些 B 细胞可以激活调节性 T 细胞，并通过一个调节蛋白（CD72）增强 T 细胞耐受，从而抑制 C1 相关的自身免疫反应。

Abstract

Rheumatoid arthritis (RA) is an autoimmune disorder without a definitive cure. Although RA is driven by systemic autoimmunity, its most pronounced manifestation is organ-specific inflammation, particularly synovitis in joints. Persistent synovitis results in progressive joint damage and deformity, ultimately compromising joint function. The etiology of RA is multifaceted, intricately intertwining genetic, environmental, and immunological elements. While autoreactive agents have traditionally been viewed as pathogenic contributors to the development of arthritis, our research, utilizing multiple experimental arthritis models, has pinpointed several pivotal autoreactive mediators, which are surprisingly regulatory.

In study I, we established a cartilage antibody induced arthritis (CAIA) model. The deficiency of Fc gamma receptor (FCGR) 2B enables swift onset of CAIA within a 12-hour time frame, and overrides the resistance arising from complement C5 deficiency. Notably, our results highlight that FCGR3 is essential and sufficient for CAIA development. The role of FCGR4 remains to be further elucidated.

In Study II, we engineered a range of recombinant antibodies targeting the F4 epitope on type-II collagen (COL2). One of these antibodies, denoted R69-4, not only prevented the onset of CAIA, but also effectively suppressed the established disease. Further screening revealed that R69-4 binds to numerous targets in the synovial fluid (SF), including the complement C1q. As a result, R69-4 markedly dampens FCGR3 signaling in SF neutrophils, thereby interrupting neutrophil self-orchestrated recruitment. Given this efficacy, R69-4 emerges as a promising therapeutic candidate for RA, particularly during its acute stage.

In study III, we introduced mutations to the immunodominant T cell epitope of COL2. A mutation resulting in higher affinity to major histocompatibility complex class II (MHC II) confers resistance to collagen-induced arthritis (CIA). However, the absence of either FCGR2B or neutrophil cytosolic factor 1 (NCF1) disrupts this tolerance. In particular, the deficiency of NCF1 leads to a reduction of regulatory T cells (Tregs), and a decrease of autoimmune regulator (AIRE) expression in medullary thymic epithelial cells (mTECs).

In Study IV, we identified a subset of autoreactive B cells that are ubiquitously present across species. These B cells target the C1 epitope on COL2. Transferring these C1 B cells effectively suppressed arthritis of recipient mice in an antigen-specific manner. We further discerned that the suppressive efficacy of C1 B cells stems from the activation of Tregs and the functional integrity of CD72. In RA patients, we noted a reduced frequency of C1 B cells, possibly attributed to their differentiation into plasma cells. Interventions that can reverse this transition may contribute to preventing the onset of RA.

List of scientific papers

I. **Zhongwei Xu**, Àlex Moreno-Giró, Danxia Zhao, Alexander Krämer, Rajan Kumar Pandey, Bingze Xu, Susanna Lundström, Rikard Holmdahl. *Fcgr2b* and *Fcgr3* are the major genetic factors for cartilage antibody induced arthritis, overriding the effect of Hc encoding complement C5. *In revision*

II. **Zhongwei Xu**, Bingze Xu, Susanna Lundström, Àlex Moreno-Giró, Danxia Zhao, Myriam Martin, Erik Lönnblom, Qixing Li, Alexander Krämer, Lei Cheng, Bibo Liang, Dongmei Tong, Roma Stawikowska, Anna Blom, Gregg Fields, Roman Zubarev, Rikard Holmdahl. A subset of type-II collagen-binding antibodies prevents experimental arthritis by inhibiting FCGR3 signaling in neutrophils. *Nat Commun* 14, 5949 (2023)

III. Qijing Li, Jianghong Zhong, Huqiao Luo, Vilma Urbonaviciute, **Zhongwei Xu**, Chang He, Rikard Holmdahl. Two major genes associated with autoimmune arthritis, Ncf1 and Fcgr2b, additively protect mice by strengthening T cell tolerance. *Cell Mol Life Sci.* 79, 482 (2022)

IV. Mike Aoun, Ana Coelho, Alexander Krämer, Amit Saxena, Pierre Sabatier, Christian Beusch, Erik Lönnblom, Manman Geng, Nhu-Nguyen Do, **Zhongwei Xu**, Jingdian Zhang, Yibo He, Laura Romero Castillo, Hassan Abolhassani, Bingze Xu, Johan Viljanen, Joanna Rorbach, Gonzalo Fernandez Lahore, Inger Gjertsson, Alf Kastbom, Christopher Sjowall, Jan Kihlberg, Roman Zubarev, Harald Burkhardt, Rikard Holmdahl. Antigen presenting autoreactive B cells activate regulatory T cells and suppress autoimmune arthritis in mice. *J Exp Med.* 220(11) (2023)

Manuscripts not included

I. Laura Romero-Castillo, Nhu-Nguyen Do, Outi Sareila, Bingze Xu, Viktoria Hennings, **Zhongwei Xu**, Carolin Svensson, Ana Oliveira-Coelho, Zeynep Sener, Vilma Urbonaviciute, Olov Ekwall, Harald Burkhardt, Rikard Holmdahl. Human MHC class II and invariant chain knock-in mice mimic rheumatoid arthritis with allele restriction in immune response and arthritis association. *Manuscript*

II. Alexander Krämer, Susanna L Lundström, Àlex Moreno Giró, Taotao Li, Ana Coelho, **Zhongwei Xu**, Bingze Xu, Roman A. Zubarev, Rikard Holmdahl. Fc sialylation has no effect on the pathogenicity of arthritogenic antibodies. *Manuscript*

III. Jie Su, Jussi Kupari, Ming-Dong Zhang, Bingze Xu, Dmitry Usoskin, Alejandro Gonzalez Alvarez, **Zhongwei Xu**, Rikard Holmdahl, Patrik Ernfors. Arthritis pain caused by a sustained type I interferon signaling. *Manuscript*

Contents

List of abbreviations

(Alphabetic)

ACPA	anti-citrullinated peptide antibodies
ACR	American College of Rheumatology
AIRE	autoimmune regulator
axSpA	axial spondyloarthritis
bDMARD	biological disease-modifying anti-rheumatic drugs
BMDM	bone marrow-derived macrophage
C5aR	C5a receptor
CAIA	cartilage antibody induced arthritis
CarP	carbamylated protein
CBA	cytometric bead array
CCP	cyclic citrullinated peptide
CFA	complete Freund's adjuvant
CIA	collagen induced arthritis
COL2	Type-II collagen
COMP	cartilage oligomeric matrix protein
CRP	C-reactive protein
csDMARD	conventional synthetic disease-modifying anti-rheumatic drugs
CTLA4	cytotoxic T-lymphocyte-associated protein 4
DAS28	disease activity score 28
DMARD	disease-modifying anti-rheumatic drugs
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FACS	fluorescence-activated cell sorting
FC	flow cytometry
FCGR	Fc gamma receptor
GAS	group A streptococcus
GC	germinal center
GCA	giant cell arteritis
GPI	glucose-6-phosphate isomerase
GPIA	$GPI_{325-339}$ peptide induced arthritis
GWAS	genome-wide association study

HLA	human leukocyte antigen
HRCT	high-resolution computed tomography
HSCT	hematopoietic stem cell transplantation
i.d.	intradermal
i.p.	intraperitoneal
i.v.	intravenous
IBD	inflammatory bowel disease
IC	immune complex
IF	immunofluorescence
IFA	incomplete Freund's adjuvant
IHC	immunohistochemistry
ILC	innate lymphoid cells
ILD	interstitial lung disease
IP	immunoprecipitation
JAK	Janus kinase
KI	knock-in
KO	knockout
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LPS	lipopolysaccharide
MAC	membrane attack complex
MHC	major histocompatibility complex
MIP	mannan induced psoriasis
MRI	magnetic resonance imaging
mTEC	medullary thymic epithelial cell
MTX	methotrexate
NCF1	neutrophil cytosolic factor 1
NET	neutrophil extracellular trap
OMERACT	Outcome Measures in Rheumatology
PAD	peptidyl arginine deiminase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1
PsA	psoriatic arthritis
PVNS	pigmented villonodular synovitis
qPCR	real-time quantitative polymerase chain reaction
RA	rheumatoid arthritis

RBC	red blood cell
RF	rheumatoid factor
ROS	reactive oxygen species
sDFR	sustained drug-free remission
SE	shared epitope
SF	synovial fluid
SLE	systemic lupus erythematosus
SS	Sjögren's syndrome
T2T	treat-to-target
TCR	T cell receptor
TNF-α	tumor necrosis factor alpha
Treg	regulatory T cell
US	ultrasonography
WT	wide type

1 Introduction

1.1 Autoimmune diseases

The immune system is an intricate network primarily designed to defend the host against invaders such as bacteria, fungi, viruses, and parasites. A well-balanced immune system, underpinned by precise regulation, is crucial for health maintenance. However, when this balance tips to either end of the spectrum, the host faces challenges. With a compromised immune system, the host becomes vulnerable to opportunistic infections, whereas an overactive immune system predisposes the host to autoimmunity, potentially leading to autoimmune diseases.

The prevalence of autoimmune diseases reaches up to 5% in Western countries [1], placing a significant disease burden on healthcare systems. Given their chronic nature, these diseases necessitate life-long management, further emphasizing their profound impact. Rheumatology clinics play an essential role in this landscape. At these specialized clinics, patients with autoimmune symptoms receive their diagnoses of conditions such as rheumatoid arthritis (RA), axial spondyloarthritis (axSpA), psoriasis and psoriatic arthritis (PsA), Sjögren's syndrome (SS), giant cell arteritis (GCA), or systemic lupus erythematosus (SLE), among others.

Currently, there is no definitive cure for any autoimmune diseases in clinical practice. The conventional care, primarily using immunosuppressive agents, focuses on the long-term management of signs and symptoms arising from autoimmunity. The prognosis of multiple autoimmune diseases has markedly improved with the introduction of biological antagonists that target key mediators or pathways in inflammation. Despite these significant advancements, a considerable number of cases remain refractory to treatment. Therefore, while achieving remission for every patient is still the foremost target at this stage, the ultimate goal remains a cure.

In an ideal scenario, autoimmune diseases can be cured by permanently eliminating autoreactive clones of immune cells responsible for the pathogenesis. An attempt utilizing high-dose chemotherapy, followed by a fresh reinstallation of immunity through autologous hematopoietic stem cell transplantation (HSCT), has shown feasibility in patients with severe autoimmune diseases resistant to conventional therapies. However, this aggressive approach, often reserved for cases of refractory or relapsed leukemia, yielded only a 43% rate of progression-free survival without relapse beyond 5 years [2]. Furthermore, it also carries a notable risk of transplant-related mortality [3], which limits its potential for broader clinical application in the vast majority of patients with autoimmune diseases. Hence, there remains a high demand to carry out extensive research, to devise safe and highly effective medications for these diseases.

1.2 Rheumatoid arthritis

1.2.1 Curiosity about the unknown

RA is one of the most common autoimmune diseases. In fact, it is an ancient disease with evidence tracing back to around 2700 BC. This assertion is supported by the discovery of a fully wrapped Egyptian mummy unearthed by Flinders Petrie. Upon examination, his team recorded typical signs of joint deformity, most pronounced in the mummy's hands, but also present in other joints including the temporomandibular joints [4,5]. Around 400 BC, Greek physician Hippocrates, often hailed as the "father of modern medicine", described a diseased condition bearing a resemblance of RA [6]. Subsequently, numerous physicians documented similar descriptions, though often with insufficient details to definitively identify the disease. Until 1800, French medical student Augustin-Jacob Landré-Beauvais described a new form of gout disease differentiated from "primary asthenic gout" in his dissertation with a tentative and cautious tone, which was later acknowledged by modern medicine as the first description of RA [7]. However, the terminology "rheumatoid arthritis" itself was introduced by Alfred Baring Garrod several decades later (1859) [8], following his pioneering work in understanding the role of uric acid in genuine gout disease. Before then, the condition resembling RA was termed as "rheumatic gout".

The definition of RA has been refined over the centuries, greatly informed by technological advances in radiology, serology, and experimental medicine. In 1895, German physicist Wilhelm Röntgen stumbled upon a terrifying type of radiation, which is capable of penetrating almost everything [9]. He termed it X-ray (for the sake of brevity) and soon captured the iconic radiographic image of his wife's hand, clearly displaying a ring on one of her fingers using this radiation. This discovery quickly led medical professionals to explore the use of X-ray for various purposes, including attempting to visualize articular deformities. However, X-ray provided limited insights to discriminate RA from other joint diseases, or to establish a connection between RA and the immune system. In 1940, Norwegian physician Erik Waaler identified rheumatoid factor (RF), a type of anti-IgG antibody. He made the discovery after observing that serum from an RA patient aggregated sheep red blood cells (RBCs) that were opsonized by IgG [10], leading to the proposal of the autoimmune nature of RA. Yet, during that era, the concept of autoimmunity that the immune system could attack one's own tissues, was not widely embraced.

A significant shift came in 1956 when American physician Ernest Witebsky and his colleagues provided compelling evidence favoring autoimmunity. They injected extracts of thyroid glands from donor rabbits into recipient rabbits and observed specific autoantibody response as well as structural damage in the thyroid glands of the recipients [11,12]. His constructive work later formed the foundation of utilizing experimental evidence

to recognize autoimmune diseases, the so-called Witebsky postulates. In the same year, American doctor Carl M. Pearson succeeded in establishing experimental arthritis by the injection of adjuvant into rats [13]. In the early 1970s, high levels of immune complexes (ICs) were detected in the bloodstream [14], synovium [15], and synovial fluid (SF) [16,17] of RA patients. In 1977, another major advancement came from American doctor David D. Trentham. His team induced chronic arthritis that resembles all stages of RA, by injecting rats with heterogeneous type-II collagen (COL2) emulsified in adjuvant [18]. This is the first time that RA-like disease was established in animals using cartilage component, which later became the best-known animal model for RA, collagen induced arthritis (CIA). In centuries, these groundbreaking findings have revolutionized our understanding of RA, and firmly ascertained that RA is an autoimmune disease.

In 1987, the American College of Rheumatology (ACR) established the first widely-recognized criteria for RA [19], marking a significant milestone in standardizing the definition and diagnosis of RA. This initiative united the research community in their efforts to combat the disease. In 1998, Dutch researchers introduced a panel of citrulline-containing peptides, and discovered that antibody response to this panel had a sensitivity of 76% and a specificity of 96% for diagnosing RA [20]. This critical finding solidified the link between RA and anti-citrullinated peptide antibodies (ACPAs), greatly enhancing our understanding and the early diagnosis of the disease [21]. Recognizing the diagnostic value of ACPA, two prominent rheumatology organizations, European League Against Rheumatism (EULAR) and ACR, collaboratively incorporated it into the RA classification criteria in 2010 [22], and are routinely updating their RA management guidelines.

1.2.2 Integration of the known

From 1955 to 2015, RA was estimated to affect roughly 0.46% of the global population [23]. Its worldwide distribution exhibits a geographic pattern, with different populations showing prevalence rates ranging from 0.1% to 2% [24]. This variation arises from a combination of both genetic and environmental risk factors [25]. For instance, distinct ethnic groups residing in the same region can display large difference in RA prevalence [26], underscoring a profound influence of genetic predisposition. Numerous studies have identified significant risk loci for RA, including human leukocyte antigen (HLA)-DRB1, PTPN22, IL6ST, CCR6, IL2RA, CCL21, and TNIP2, among others [27-30]. Notably, HLA-DRB1 is seen as one of the most significant loci. Some specific DRB1 haplotypes, such as HLA-DRB1*0401, HLA-DRB1*0405, and HLA-DRB1*0101, were shown to be associated with a higher prevalence of ACPA [31], increased vulnerability to RA [32], and greater disease severity [33]. These associations were initially hypothesized to arise from a shared epitope (SE) at HLA-DRβ1 positions 70-74 [34], which were thereafter corrected to HLA-DRβ1 positions 11 and 13, as well as multiple HLA gene products other than HLA-DRβ1 [35]. Beyond genetic determinants, environmental factors such as smoking [36-38] may also be associated with RA susceptibility.

Individuals with one or more above risk factors may be predisposed to develop RA. In clinical settings, RA patients are categorized into seropositive or seronegative subtypes, mainly based on the presence of RF and ACPA. Seropositive RA is the more common subtype, with autoantibodies often detectable years before clinical onset [39]. The precise trigger for this abrupt joint attack, transitioning the pathophysiological condition from asymptomatic autoimmunity to acute synovitis, remains elusive. At its onset, RA is characterized by typical inflammation of symmetric joints (swelling, redness, warmth, pain, and dysfunction), primarily small joints of hands. If left untreated, RA typically persists due to its chronic and fluctuating nature, with time leading to joint damage and deformity.

Apart from joint disorders, established RA is associated with multiple comorbidities affecting other systems. Firstly, RA individuals have an elevated risk of cardiovascular diseases, and therapies such as methotrexate (MTX) or tumor necrosis factor alpha (TNF-α) inhibitors lowered this risk [40]. Secondly, certain malignancies, especially hematologic cancers, are more common in RA individuals, which is unlikely due to the adverse effects from RA treatments [41,42]. Thirdly, being a chronic condition, RA often correlates with inferior mental health [43]. Lastly, interstitial lung disease (ILD) stands out as one of the most prevalent and grave extra-articular comorbidities of RA. It is estimated that severe ILD affects approximately 2% to 8% of RA patients [44], often necessitating hospitalization or even intensive care. Individuals with these conditions face a mortality rate 2 to 10 times higher than RA population without ILD [45]. Even among outpatients, high-resolution computed tomography (HRCT) has detected fibrosing alveolitis in 19% of RA patients [46], which has the potential to progress to severe ILD. These findings offer support to the hypothesis that at least a subset of RA cases may originate from malfunction of mucosal immunity in the lung [47], which could be caused by smoking [48].

Overall, RA is a heterogenous disease among individuals, and its progression is driven by a complex network involving various contributors.

B cell lineage is a distinguished contributor for RA because of the presentation of self-antigens and the production of autoantibodies. Similar to other clones that combat infections by producing high-affinity antibodies, ACPA-producing B cells also undergo somatic hypermutations during affinity maturation, which in turn promotes antigen spreading [49]. The presence of ectopic germinal center (GC) formation within RA synovium highlights the crucial role of these recruited B cells in promoting local chronic inflammation [50]. Notably, the formation and function of these ectopic GCs depend on the secretion of CXCL13 [51] and the interaction with $CD8^+$ T cells [52]. A sharp increase of autoantibodies during the peri-onset period [39] indicates that these B cell clones may be associated with the initial joint attack in RA.

T cells, especially $CD4^+$ T helper cells, also play a critical role in RA pathogenesis. The robust link between some specific HLA haplotypes and RA susceptibility was first

reported by American physician Peter Stastny [53], and was later corroborated by multiple methodologies including genome-wide association study (GWAS) [54]. HLA refers to human major histocompatibility complex (MHC) molecules that are critical in presenting self-antigens to T cells in RA development. Citrulline-specific Th1 cells have been shown to populate in joints of RA patients and retreat upon therapies using biological agents [55,56], demonstrating the vital role of this T cell subset. In contrast, Th17 cells are less frequent in joints but somewhat associated with a refractory phenotype [57]. Notably, regulatory T cells (Tregs) within RA synovium failed to suppress the proliferation of effector T cells compared to their peripheral counterparts [58], implying a joint-specific loss of suppressive capacity of Tregs.

Macrophages are the predominant producer of TNF-α in RA synovium [59]. The introduction of TNF-α antagonists, which revolutionized RA management, has demonstrated an exclusively critical role of this cytokine in RA pathogenesis. Within RA synovium, there are two distinct subsets of macrophages with differing roles. Indeed, bone marrow-derived macrophages (BMDM, MHC-II$^+$), located within synovial sub-lining layer, significantly promote the development of arthritis. Depleting this subset of macrophages, plus circulating monocytes, has been shown to mitigate experimental arthritis [60]. On the other hand, embryo-derived synovial macrophages (CX3CR1$^+$, MHC-II$^-$), residing within the lining layer (**Figure 1**), exhibit an anti-inflammatory phenotype. These macrophages create a protective barrier that physically seclude the joint, and are able to replenish their population independently of circulating monocytes [61]. Depleting this subset disrupts the synovial lining barrier, leading to increased neutrophil influx and heightened inflammation in experimental arthritis [61].

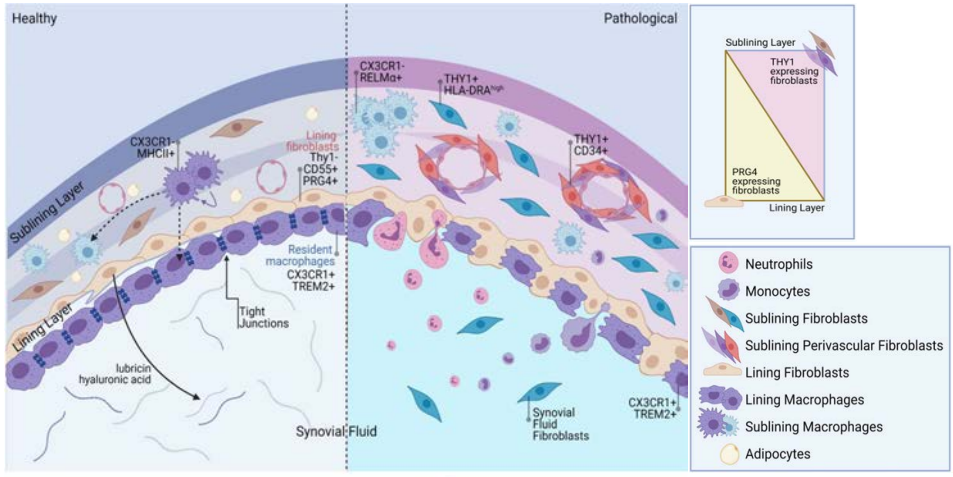

Figure 1. Architecture of synovium under healthy and inflammatory conditions (Reprinted from Marsh LJ, et al. *Immunol Rev*. 2021 [62])

Synovial fibroblasts represent another fundamental cell type within the organized synovial architecture. Over recent years, the fundamental role of synovial fibroblasts has become increasingly evident. When synovial fibroblasts from RA patients are implanted into immunodeficient mice, they demonstrate the ability to migrate towards implanted cartilage tissue, leading to its destruction [63]. This suggests that these fibroblasts can spread arthritis to previously unaffected joints. Single-cell RNA sequencing has identified two distinct fibroblast subsets ($FAP\alpha^+$) in the synovium, with one subset expressing THY (CD90) and the other expressing PRG4. Analogous to macrophages, these fibroblasts also have differing locations and functions (**Figure 1**). However, neither bears regulatory potential in RA. Specifically, the $CD90^-$ fibroblasts, situated within the synovial lining layer, primarily mediate bone and cartilage erosion, with minimum contribution to inflammation. In contrast, the $CD90^+$ fibroblasts, located within the sub-lining layer, predominately drive inflammation rather than joint damage [64]. The progression of arthritis by these $CD90^+$ fibroblasts is largely influenced by endothelium-derived Notch signaling, particularly Notch3 signaling [65].

Neutrophils are instrumental in host defense against acute infections as well as in sterile inflammation. They are consistently the first cells to arrive at sites of inflammation. The recruitment of neutrophils is fine-tuned, epitomizing the concept of inflammation resolution where "the end is programmed from the beginning" [66]. In the context of RA, neutrophils have a multifaceted role in disease progression. At disease onset, neutrophils largely contribute to "visible" inflammation, manifesting as joint redness and swelling. SF supernatants taken from RA patients can swiftly activate bloodstream neutrophils, prompting them to initiate reactive oxygen species (ROS) burst [67]. Neutrophils recruited into SF also exhibit an activated phenotype, characterized by a high basal level of intracellular ROS production [68]. Subsequent research indicates that SF neutrophils drive inflammation through mechanisms beyond just elevated ROS output. They also have amplified chemokine production, enhanced formation of neutrophil extracellular traps (NETs), and delayed apoptosis, among other responses [69]. Notably, accelerated NETosis in RA could serve as a source of citrullinated autoantigens [70], thereby contributing to the generation of ACPA through citrullinated antigen presentation from synovial fibroblasts to T cells [71].

In addition to the players mentioned above, other cell subsets, such as osteoclasts and their precursors [72,73], chondrocytes [74], and innate lymphoid cells (ILCs) [75], etc., all contribute to the development of RA. The intricate communication network among these entities involves various cytokines, chemokines, receptors, enzymes, and costimulatory molecules, which are also fundamental to the progression of RA. Therapeutic strategies that target specific cell subsets or block certain signaling pathways have demonstrated substantial advancements in RA management, underscoring the importance of these components.

1.2.3 Application of the knowledge

Since spontaneous remission is both rare and unpredictable for RA patients, early diagnosis and intervention are essential in improving long-term prognosis, especially in preventing irreversible joint damage [76].

1.2.3.1 Diagnosis

According to the ACR/EULAR 2010 classification criteria [22], to define RA in clinical practice, three requirements must be met:

a. Presence of synovitis in ≥ 1 joint.
b. No alternative diagnosis better explaining the synovitis.
c. Achievement of a total score ≥ 6, determined by evaluating number and site of involved joints (0–5), serological abnormality (0–3), elevated acute-phase response (0–1), and symptom duration (0–1).

The presence of synovitis is typically assessed through both clinical examination and supplementary diagnostic tests. When recognize the signs of synovitis, which commonly manifest as joint swelling, warmth, pain, tenderness, and reduced range of motion, rheumatologists will proceed to order tests to diagnose the patient. General inflammation can be indicated by blood tests such as the erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP), though these markers are not specific. While magnetic resonance imaging (MRI) stands as the gold standard for visualizing active synovitis[77], its application in clinical settings is often limited due to its high costs, time-consuming procedures, and limited accessibility in many clinics. In comparison, ultrasonography (US) offers a convenient, cost-efficient, and reliable alternative. Compared to MRI scanning, US has a sensitivity ranging from 0.64-0.91 and a specificity ranging from 0.60-0.94 in different joints [78]. Additionally, it detects not only synovitis, but also synovial hypervascularity [79] and bone erosions [80]. Even in RA individuals at very early stage, US can detect a significant amount of subclinical synovitis [81]. To enhance its diagnostic accuracy and standardize its application, EULAR and Outcomes Measures in Rheumatology (OMERACT) jointly introduced a stepwise approach [82], which further improved the reliability of US in identifying synovitis.

However, it is often challenging to use single modality to exclude alternative diseases other than RA causing synovitis. Conditions such as psoriatic arthritis [83], infectious arthritis [84], and pigmented villonodular synovitis (PVNS) [85] all have the potential to perpetuate synovitis. Therefore, rheumatologists need to consider a combination of factors for an accurate diagnosis. This includes the patient's disease history (e.g., arthritis duration), clinical examination (e.g., joint symmetry), complications (e.g., skin rash), laboratory tests (e.g., autoantibodies), and imaging results (e.g., US or MRI). In rare

instances, arthroscopy and synovial biopsy might be required to clarify the diagnosis and to guide treatment.

Besides the number of affected joints, serology serves as another crucial determinant in patient scoring. As per the ACR/EULAR 2010 classification criteria [22], the positivity of RF or ACPA is deemed as abnormal and significantly contributes to the final score. While the method for detecting RF can vary by region, ACPA measurement primarily relies on the anti-cyclic citrullinated peptide (CCP) test. Presently, the 2nd-generation CCP test (CCP2) is regarded as the standard test for detecting ACPA due to its high sensitivity and specificity [86]. Some research indicates that the 3rd generation of CCP (CCP3) kit might offer slightly higher sensitivity [87,88], and has predictive value in pre-RA cohort [89]. However, its specificity may be somewhat compromised [88,90]. To ascertain whether diagnosed RA is coexisting with other joint diseases, it is sometimes necessary to conduct additional tests, like blood uric acid levels, to exclude "asthenic gout" from "rheumatic gout".

In the context of seronegative RA, accurately diagnosing based on the classification criteria can be challenging. Even with undetectable RF or ACPA, more than half of the cases traditionally defined as seronegative have been found to possess autoantibodies against other epitopes [91]. These autoantibodies are also highly specific in seropositive RA patients. Apart from IgM RF and anti-CCP2 IgGs, a significant portion of seronegative patients test positive for IgA/IgG RF, ACPA fine-specificity, or anti-carbamylated protein (Anti-CarP) antibody [92]. Moreover, A national cohort study revealed that approximately 8.8% of seronegative RA cases were initially misdiagnosed , later being correctly identified as PsA, axSpA, or inflammatory bowel disease (IBD) related conditions [93]. Collectively, these findings imply that traditionally defined seronegative RA might not be genuinely autoantibody-free. Such a diagnosis demands more comprehensive investigation.

1.2.3.2 Conventional care

In RA patients lacking appropriate management, the rate of disability has been observed to rise in correlation with the disease's duration. In a Chinese population, the disability rate increased from 5.4% for those with RA < 1 year, to 61.3% for those with the disease ≥ 15 years [94]. In contrast, proactive administration of disease-modifying anti-rheumatic drugs (DMARDs) significantly reduced disability in RA patients, despite using only conventional synthetic DMARDs (csDMARDs) but not biological DMARDs (bDMARDs) [95]. Notably, early treatment was shown to have superior long-term prognosis compared to delayed treatment [96,97]. As such, once a diagnosis is confirmed, treatment should be promptly commenced based on a shared decision-making approach between rheumatologists and patients.

The treat-to-target (T2T) algorithm is the foundational principle for RA management according to both EULAR and ACR recommendations [98,99]. This strategy emphasizes two primary targets. Initially, the aim is to achieve at least 50% of improvement after 3 months of treatment. Subsequently, the goal is, by the end of 6 months of treatment, to reach remission in patients with early RA, and to attain low disease activity in long-standing disease. The T2T principle to induce remission typically follows a specific sequence. According to EULAR recommendations, DMARD-naïve patients should be administered with a single csDMARD (anchor drug MTX preferable), in combination with short-term glucocorticoids. If the desired target is not reached, MTX can be combined (or replaced) with another csDMARD for those without poor prognosis factors, or a bDMARD for those with such factors. If the target remains unachieved, the replacement of other bDMARDs or Janus kinase (JAK) inhibitors can be considered.

Multiple cohorts have demonstrated the superiority of the T2T approach over routine care. The NOR-VEAC 2.0 cohort study revealed that, after 2 years of treatment, 66.4% of patients with early RA achieved disease activity score 28 (DAS28) remission following the T2T principle, whereas only 47.9% reached DAS28 remission following routine care [100]. It is worth noting that a majority of these patients were treated with MTX monotherapy. In line with these findings, the RA BIODAM cohort also showed results favoring the T2T approach [101]. On another note, numerous high-quality trials have proven that induction therapies combining MTX with a bDMARD offer faster control of disease, higher remission rates, improved function, and an enhanced quality of life compared to the MTX monotherapy [102-105]. Research also indicates that the opportunity to achieve sustained drug-free remission (sDFR) diminishes rapidly over time in patients starting slow-acting monotherapy [106]. As such, inducing remission as swiftly as possible should be the top priority in any treatment strategy. We eagerly anticipate an upcoming era when guidelines will prioritize optimal induction therapies over economic considerations, at least for those with moderate to high disease activity. Furthermore, faster induction of remission could allow for DMARDs tapering in more individuals with sDFR, which might not necessarily lead to higher overall costs [107].

After achieving sustained clinical remission for over a year, it might be feasible to consider tapering DMARDs. Tapering these drugs offers potential benefits to patients, as these immune-suppressive agents, especially bDMARDs, carry risk of adverse effects in a dose-dependent manner [108]. This strategy can also reduce costs for healthcare systems. However, despite considerable variability in study results, tapering either csDMARDs or bDMARDs resulted in an elevated incidence of disease flare [109]. Specifically, in patients under sustained remission using csDMARDs only, tapering csDMARDs to half dose resulted in an increase of flare rate from 6% to 25% over a 12-month period [110]. In the same cohort, completely discontinuing the half dose of csDMARDs further raised the rate of flare from 16.7% to 38.5% over another year [111]. Likewise, tapering bDMARDs increased the

rate of flare from 5% to 63% in patients under sustained remission using bDMARDs [112], but fortunately, 88% of the flared patients regained remission after resuming the full dose of bDMARDs [112]. Collectively, these studies demonstrate that while tapering DMARD doses can result in flares for some individuals, it is still feasible to sustain clinical remission in a significant portion of patients. Yet, from a radiographic standpoint, the vast majority of patients in clinical remission exhibit active synovitis [113], which suggests potential ongoing disease progression. As such, a more stringent criterion might be necessary before contemplating DMARDs tapering (e.g., patients without radiographic progression over a year). It would be crucial to accurately identify which patients are suitable for tapering, and to devise a reliable method for predicting flares in those tapering their DMARDs.

In summary, the current evidence supports the principles for RA conventional care: to initiate prompt intervention, to achieve early remission, to maintain routine follow-up, and to implement careful tapering.

1.2.3.3 Beyond conventional care

The quote, "To cure sometimes, to relieve often, to comfort always" by American physician Edward Livingston Trudeau, serves as the motto for medical students at the university where I received my medical training. This is to remind physicians to always remember caring about the needs and feelings from patients. In terms of research, the ultimate aspiration of scientists is undoubtedly to find cures. Regarding RA, as we discussed previously, certain attempts, such as treating it as a hematopoietic tumor by freshly reinstalling immunity, failed to cure the disease. There could be two hypotheses to explain these results. The first hypothesis is that the core causative agents are still shrouded in mystery. These entities survive the high-dose chemotherapy and may re-initiate the disease *de novo* by priming the re-introduced fresh immune system. The other is that the re-introduced immune system *per se*, is hyperreactive and more prone to initiate systemic autoimmunity in general. As such, a complete cure might remain out of reach until the etiology of RA is fully deciphered.

Due to the lack of a definitive cure, there is a pressing need to refine the therapies to relieve. Over recent decades, scientists have made significant strides, identifying several pathways crucial to RA pathogenesis. The introduction of antagonists targeting these key mediators have revolutionized the management and prognosis of RA. These biologics, including TNF-α inhibitors, IL-6 inhibitors, and JAK inhibitors, have been incorporated into the T2T algorithm.

Research into targeting other pivotal pathways continues alongside the above agents. For instance, IL-17, mainly secreted by Th17 cells, was previously viewed as a promising therapeutic target. However, IL-17 inhibitors have shown limited clinical efficacy in RA, especially when compared to their effectiveness in other autoimmune diseases [114].

Nevertheless, IL-17 inhibitors did provide remission for RA patients who responded inadequately to TNF-α inhibitors [115]. This corroborates the notion that IL-17 may be linked to a more refractory phenotype [57]. Apart from IL-17 inhibitors, antagonists targeting IL-1 [116] or IL-12/IL-23 [117] did not display comparable efficacy as TNF-α inhibitors either. In contrast, agents blocking co-stimulation using a decoy receptor (CTLA4-Ig) [118] has demonstrated profound efficacy in inducing clinical remission. Similarly, stimulating checkpoint programmed cell death protein 1 (PD-1) with the newly developed monoclonal antibody, peresolimab, exhibited marked efficacy in RA treatment [119].

In addition to targeting these vital molecules, focusing on specific cell subsets has emerged as a promising strategy in RA treatment. For instance, depleting B cells has been shown to induce notable remission in RA patients [120]. Notably, this good efficacy was found to correlate with the expression of a B cell lineage signature in the synovium [121]. Besides, an upregulated fibroblast signature in synovium was revealed to contribute to multidrug resistance [122], which suggests that targeting synovial fibroblasts could be a potential approach for RA treatment, especially for refractory cases. Several attempts on animals have delivered encouraging results [64,123,124].

Besides the push to develop new therapeutic strategies, to optimize RA management is equally important. The advent of AlphaFold [125] has not only pioneered a new era in protein structure prediction, but also galvanized researchers from diverse fields to leverage the capabilities of machine learning. In the context of RA, a multitude of deep learning algorithms have demonstrated remarkable accuracy in gauging clinical outcomes based on medical history [126], predicting responses to multiple DMARDs [122,127-130], and foreseeing sustained remission [131] and disease flare [132] during treatment. However, the high variability across these studies poses challenges to their generalization and broader applicability. Therefore, an urgent call for standardized guidelines is necessary to align the global scientific community. It is thrilling to envisage a future, where machine learning algorithms, in real-time, screen therapeutic targets, conduct *in silico* trials, predict treatment outcomes, tailor personalized strategies, assess individual feedback, synthesize global subjects, and eventually, just tell you what to take after breakfast.

1.3 Experimental arthritis

Despite the upcoming era of artificial intelligence, experimental arthritis models are still among the foremost tools to investigate RA pathogenesis. As the etiology has not been fully disclosed, to perfectly replicate the disease in animals poses a challenge. Many induced models primarily target the articular cartilage, initiating chondritis first, followed by secondary synovitis. This sequence contrasts with the onset of RA in humans, which is marked by primary synovitis that bears potential to lead to secondary chondritis. This distinction is supported by the absence of anti-COL2 antibodies during the early synovitis stage in RA patients [133], most of whom demonstrate an ACPA response instead.

However, once arthritis is established, these animal models do effectively mimic both the acute and chronic phases of RA. Here we focus on some widely used mouse models.

1.3.1 Collagen induced arthritis

The CIA model has become the gold standard among RA mouse models. Mouse strains with susceptible MHC haplotypes, such as $H2^q$, can develop autoimmunity when injected with heterogeneous COL2 emulsified in an adjuvant. This model, utilizing the molecular mimicry between heterogeneous COL2 and endogenous COL2, depicts a typical progression of autoimmunity, encompassing phases of priming, onset, and chronicity. The injected foreign COL2 primes T cells and B cells, driving the expansion of COL2-specific T cell clones, as well as the affinity maturation of B cells to produce highly specific antibodies against COL2. Some of these T cell clones and antibodies subsequently cross-react to endogenous COL2 in articular cartilage, leading to joint inflammation. This procedure closely mirrors the onset of rheumatic heart disease, where an initial infection with group A streptococcus (GAS) triggers immune responses that inadvertently target cardiac valves. This mistaken targeting is attributed to molecular mimicry between bacterial proteins, such as the M protein, and several cardiac proteins, including cardiac myosin, tropomyosin, keratin, laminin, and vimentin [134].

While the CIA model encapsulates many aspects of RA pathogenesis, it still lacks certain key elements, such as HLA-DRB1 and ACPA. Humanized mouse strains with transgenic HLA-DR1 or DR4 demonstrated susceptibility to CIA [135,136], making these strains more human-physiological to mimic RA. Furthermore, immunization with peptidyl arginine deiminase (PAD) isoforms elicited the production of ACPA in various mouse strains, including the HLA-DRB1*0401 knock-in (KI) mice [137–139]. However, these mice did not display signs of arthritis. Further refinement is required to develop a strain that replicates human RA more accurately. Conversely, introducing too many human alleles into mice might push them away from a mouse-physiological status to a human-artificial one, at least perceived by the mouse's immune system.

1.3.2 K/BxN serum transfer arthritis

When studying innate immunity in RA for research purposes, the K/BxN serum transfer arthritis model emerges as one of the top choices. Interestingly, this model was fortuitously discovered when KRN mice were crossed with NOD mice [140]. The KRN strain carries a transgenic T cell receptor (TCR) that can recognize a peptide derived from glucose-6-phosphate isomerase (GPI), a ubiquitous enzyme residing in almost all cell types. Meanwhile, the NOD strain possess an MHC class II molecule that can present the GPI peptide to T cells. Consequently, offspring that carry both the specific TCR and MHC-II molecule, develop spontaneous arthritis, accompanied by high titers of autoantibodies targeting GPI [141]. In the context of RA, GPI-specific antibodies are however not prevalent, appearing in about 12% to 29% of patients [142]. This prevalence does not significantly differ

from that in PsA, axSpA, or undifferentiated arthritis. However, the emergence of GPI-specific antibodies correlates with a more aggressive phase of RA [142,143], suggesting their high arthritogenic potential.

Encouragingly, the transfer of K/BxN serum induces arthritis in many inbred strains of recipient mice[144], underscoring its wide applicability. Specifically, purified IgGs from K/BxN serum, rather than the non-IgG leftover, induced arthritis as effectively as the full serum [145]. This indicates that the antibodies in K/BxN serum are the primary agents triggering arthritis. The mechanism by which GPI-specific antibodies initiate arthritis has been meticulously characterized. Despite no overexpression of GPI in joints, its deposits have been found lining the articular cartilage surface [146]. This phenomenon explains how organ-specific inflammation arises from a systemic immune response to a universally present protein. Following serum transfer, the extensive formation of *in situ* ICs surpasses the clearance capacity of synovial scavengers, thereby leading to perpetuated neutrophil recruitment. During this process, the activation of C5a receptor (C5aR) was identified as crucial for the release of lipid LTB_4, which further mediates early neutrophil recruitment. Once infiltrated into joints, neutrophils can orchestrate their own recruitment by producing IL-1β, conditioning on the activation of their Fc gamma receptor (FCGR) 3 by deposited ICs (**Figure 2**) [147,148]. The vast number of neutrophils recruited to joints significantly contribute to joint destruction by producing ROS, NETs, and collagenase (MMP-8). Concurrently, the resolution of inflammation involves neutrophil programmed death via apoptosis throughout the process [66].

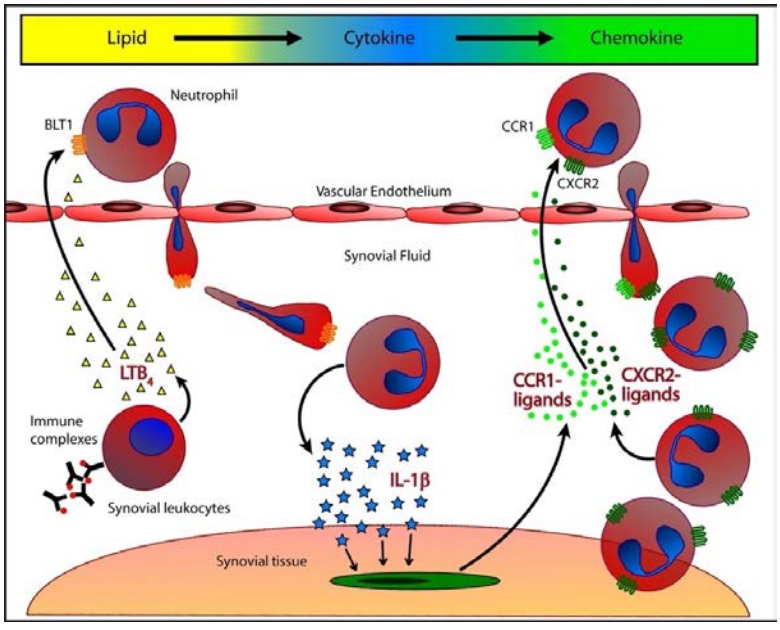

Figure 2. Neutrophil recruitment in autoantibody mediated arthritis
(Reprinted from McDonald B, et al. *Immunity*. 2010 [149])

1.3.3 Cartilage antibody induced arthritis

Similar to GPI-specific antibodies in humans, COL2-specific antibodies are also found in a minority of RA patients (3%-27%) [150]. These antibodies correlate with active inflammation as well [150]. Such discoveries pave the way for the potential induction of arthritis using anti-COL2 antibodies. While a singular monoclonal antibody may only trigger mild arthritis, a cocktail containing several clones has demonstrated the capability to induce severe arthritis across various mouse strains [151]. In addition to COL2, antibodies targeting cartilage oligomeric matrix protein (COMP), another structural protein in cartilage, have also exhibited arthritogenic properties [152].

An optimized cocktail targeting cartilaginous proteins can effectively induce what is termed as cartilage antibody induced arthritis (CAIA). In comparison to the K/BxN serum transfer arthritis, CAIA models offer several advantages. First, the monoclonal antibodies are produced *in vitro*, eliminating the need for animal sacrifice. Second, the precise dosing of the cocktail in CAIA ensures consistent results across different animals, providing a standardized reference for researchers globally. Third, the flexibility of CAIA allows for the combination of monoclonal antibodies with varied isotypes and specificities, whereas the antibodies in K/BxN serum are specific for GPI and are predominantly of the IgG1 isotype [141]. Last, CAIA antibodies exclusively target cartilage tissue, without the potential to primarily impact other systems.

In terms of mechanisms, CAIA models bear many similarities as the K/BxN serum transfer model, since both induce arthritis through autoantibodies targeting the surface of articular cartilage. However, they still have some minor differences concerning FCGR involvement and complement activation. CAIA models, depending on the IgG isotypes used in the cocktail, may involve variations in the dominance of FCGRs. In contrast, the K/BxN serum, which primarily contains IgG1 antibodies that do not bind to FCGR4 [153], heavily relies on the involvement of FCGR3 [148,154]. Regarding complements, C5 or C5aR deficiency quenched inflammation in both models. However, while C6 deficiency mitigated arthritis in CAIA by 65% [155], it did not exhibit any discernible effect in K/BxN serum transfer arthritis [156]. These observations suggest that both models rely on the release of anaphylatoxin C5a, but the formation of the membrane attack complex (MAC) due to complement activation contributes to the development of CAIA only. This may be attributed to the inability of mouse IgG1 antibodies to activate complement [157].

1.3.4 $GPI_{325-339}$ peptide induced arthritis

When studying adaptive immunity in RA for research purposes, the $GPI_{325-339}$ peptide induced arthritis (GPIA) model serves as a viable option. Immunization using full human GPI protein induced arthritis in various mouse strains in a B-cell dependent manner [158]. Subsequent screening pinpointed the sequence spanning 325-339 as one of the predominant T cell epitopes on GPI. Immunization with this human $GPI_{325-339}$ peptide

primed Th17 cells and eventually induced arthritis [159]. Further investigations also highlighted the role of Th1 cells in GPIA. Besides, a deficiency in ROS, resulting from an neutrophil cytosolic factor 1 (NCF1) mutation, dramatically escalated disease severity [160].

Outside the adaptive immune system, complement C5 has been proven pivotal in GPIA in wide type (WT) mice, but dispensable in mice with an NCF1 mutated background [160]. Moreover, while the $GPI_{325-339}$ peptide does trigger the production of specific antibodies to GPI, their levels remain minimum compared to those induced by the full GPI protein [159,160]. This indicates that the role of these autoantibodies might be somewhat constrained in initiating arthritis.

2 Research aims

The development of RA involves a complex interplay that initiates and sustains autoimmunity following the breakdown of immune tolerance. Within this intricate network, autoreactive B cells and T cells play a crucial role in setting the activation threshold. T cells interact with self-antigens, infiltrate into synovium, and secrete key cytokines, significantly contributing to arthritis development. Assisted by T cells, B cell lineage produces highly specific IgG autoantibodies targeting citrullinated proteins, COL2, and GPI, etc. Some of these antibodies considerably advance arthritis development through the activation of FCGRs. However, not all autoreactive elements are pathogenic. In our research, we have observed that an autoreactive B cell clone (COL2 C1) and an autoantibody (COL2 F4) are regulatory rather than arthritogenic, in the development of experimental arthritis.

In **study I**, we aimed to determine the relative importance of FCGRs and complement C5 in cartilage antibody induced arthritis.

In **study II**, we aimed to investigate the protective mechanism of a series of recombinant antibodies targeting COL2 F4 epitope in experimental arthritis.

In **study III**, we aimed to explore the mechanism of NCF1 and FCGR2B in regulating T cell tolerance in experimental arthritis.

In **study IV**, we aimed to decipher the protective mechanism of COL2 C1 antigen-specific B cells in experimental arthritis.

3 Materials and methods

3.1 Antibody preparation

Monoclonal antibodies used to induce CAIA were produced through hybridoma technology. A hybridoma cell merges characteristics of both a plasma cell and a tumor cell, enabling it to produce antibodies with sustained longevity. In essence, a prime clone was identified via subcloning and then advanced to large-scale antibody production. After 3 weeks, supernatants were harvested. Monoclonal antibodies were subsequently purified using affinity chromatography with protein-G columns. Once eluted, the antibodies were dialyzed against phosphate buffered saline (PBS), concentrated to 20 mg/mL, and preserved at 4 °C until administration.

Recombinant antibodies were produced using the Expi293F cell line. In brief, plasmid constructs containing sequences encoding either heavy chain or light chain were transformed into DH5α chemical competent cells (E. coli) to amplify the plasmids. After the extraction and purification of plasmids, the Expi293F cells were transfected using a transfection reagent with plasmids encoding both chains in a balanced ratio. Supernatants were collected after 5 days, and subsequent steps paralleled the method as described above. For the R69 antibodies, a glycine solution was chosen for dialysis instead of PBS.

3.2 *In vivo* models

CIA models were induced by intradermally (*i.d.*) injecting 100 μg of bovine or rat COL2 emulsified in complete Freund's adjuvant (CFA) into the base of tail in susceptible mouse strains. A boost using 50 μg of bovine or rat COL2 emulsified in incomplete Freund's adjuvant (IFA) was carried out in some experiments on day 21 or 35 after the immunization.

CAIA models were induced by intravenously (*i.v.*) injecting a cocktail containing 2-4 monoclonal antibodies. The specific doses of the cocktail were detailed in different experiments. A boost using 25 μg of lipopolysaccharide (LPS) was intraperitoneally (*i.p.*) introduced in some mouse strains but not in the *Fcgr2b*$^{-/-}$ mice.

GPIA models were induced by *i.d.* injecting 10 μg of $GPI_{325-339}$ peptide emulsified in CFA into the base of tail in susceptible mouse strains. No boost was administrated.

Mannan induced psoriasis (MIP) models were induced by *i.p.* injecting 20 mg of mannan into *Ncf1*$^{*/*}$ mice. No boost was administrated.

Experimental autoimmune encephalomyelitis (EAE) models were induced by *i.d.* injecting 100 μg of MOG_{78-96} peptide or MOG_{1-125} protein emulsified in CFA. Two boosts were *i.p.* administrated with 200 ng of Bordetella pertussis toxin.

3.3 *In vitro* assays

Mice were genotyped by polymerase chain reaction (PCR) followed by electrophoresis, or real-time quantitative polymerase chain reaction (qPCR). Relative antibody titers were measured by enzyme-linked immunosorbent assay (ELISA). Antibody specificities were determined by bead-based flow immunoassay (Luminex). Cytokines, chemokines, and complements were quantified using cytometric bead array (CBA). Potential targets were screened by immunoprecipitation (IP), followed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). RNA transcriptions of certain genes were relatively quantified by qPCR.

Blood, SF, splenic, and bone marrow samples were phenotyped by flow cytometry (FC). SF neutrophils were visualized by confocal microscopy. Intracellular ROS burst was measured by FC with dihydrorhodamine 123 as the indicator. T cell recall response was examined by ELISpot. Antigen-specific B cells were enriched by tetramer staining, followed by fluorescence-activated cell sorting (FACS).

Joint tissue sections were visualized by optical microscopes after histological staining. Specific targets in tissues were stained and visualized by immunohistochemistry (IHC), or immunofluorescence (IF) followed by confocal microscopy.

3.4 Ethical considerations

In the natural food chain hierarchy, many predators seem to lack empathy towards their preys. Through a series of evolutionary mutations, humans emerged and rapidly ascended to the top of this chain. Some of these evolutionary changes embedded genes in our DNA that influence one's location in the spectrum from self-interest to empathy. In this spectrum, neither extreme is inherently wrong, since empathy embodies humanity, while self-interest ensures survival.

Survival was the top priority in the Stone Age when humans faced a high risk of extinction. Today, this risk has become extremely low but not zero. External threats, like massive asteroid impacts, are largely unpredictable, while internal ones, such as devastating diseases, are potentially preventable. This reality underpins the rationale to develop medicine, aiming to maximize the chances of survival.

Empathy can be displayed in two forms. Emotional empathy refers to the ability to feel other's feelings, whereas cognitive empathy is about to understand other's feelings. Cognitive empathy appears to be less genetically determined than emotional empathy [161], which indicates that a person may not inherit the capacity to understand other's feelings, without education. Regrettably, our current generation seems to possess limited capacity to understand other's feelings, even after education. For instance, many people lament the use of chimpanzees in experiments, but do not express the same concern for

creatures like fruit flies. Yet, these flies have significantly contributed to pioneering human's genetics, a subject to study why different DNA combinations determine your weight to be 50 kg or 50 g. It would be intuitive for people to feel the distress of chimpanzees, perhaps through their screams and tears, but nevertheless difficult to imagine the pain of fruit flies when they are sacrificed in research. On the other hand, this poor cognitive empathy promotes the survival of humans. The limited capacity to understand the animals' feelings, fosters lenient ethical standards, allowing for animal experiments in medical research.

In all circumstances, the rules of animal experiments must be strictly regulated. The principles of 3R (Replacement, Reduction and Refinement) serve as an excellent model. These principles emphasize the importance of strategies, when possible, to replace animals with alternative methods, to reduce the number of animals used, and to refine procedures to minimize their suffering. Within the scope of this thesis, we rigorously adhered to the 3R principles. For example, we utilized the CAIA model instead of the K/BxN serum transfer model, precluding the need to sacrifice numerous donor mice. Moreover, we excluded LPS from the standard protocol when inducing CAIA in *Fcgr2b*$^{-/-}$ mice, thus reducing the suffering to the animals.

Yet, all these efforts are driven by the underlying intention of using animals to secure our own survival. Is this rational, and correct? We should not answer this question since it is ridiculous to be both an athlete and judge simultaneously. While history will be the ultimate judge of our actions, one may wonder: will there even be a history of humans if we no longer rule the world?

"I genuinely understand and feel your sorrow and anger, your honor. We were extremely self-centered to perform experiments on your kind, to advance our own health. But as you know, our primary drive was survival, not causing harm. We endeavored to conduct research *in vitro* wherever possible, and when *in vivo* studies were inevitable, we took measures to minimize suffering. We provided food, water, and security for the included individuals. We compensated the suffering ones with optimal conditions. We never intended to eradicate your kind, but fostered your prosperity instead." This could be our testimony, should rodents one day take over the world, and convict us in their courts.

4 Results

4.1 Study I: FCGRs in CAIA

In our previous research endeavors, we fine mapped the mouse genome in CIA. We pinpointed two loci, containing genes encoding FCGRs and C5, are of vital importance for the development of CIA. Using *Fcgr2b*$^{-/-}$ mice, we established a swift CAIA model within a 12-hour time frame. This model then enabled us to determine the significance of these two loci in CAIA. Surprisingly, the knockout (KO) of FCGR2B heightened the expression of FCGR3 on myeloid cells. Mice deficient in FCGR3 failed to develop CAIA, highlighting the critical role of this receptor. The blockade of FCGR3 drastically mitigated the disease severity. Delving deeper, we found that blocking FCGR3 led to diminished FCGR4 levels, and vice versa. Both antagonists notably curbed arthritis. However, in CAIA mice receiving placebo, FCGR3 levels were reduced on SF neutrophils compared to their counterparts in blood, while FCGR4 levels remained unaltered. We then utilized a cocktail composed solely of IgG1 autoantibodies, interacting exclusively with FCGR2B and FCGR3. The results showed that the IgG1 cocktail rapidly induced CAIA in *Fcgr2b*$^{-/-}$ mice, but not in *Fcgr3*$^{-/-}$ mice, cementing sufficiency of FCGR3 for CAIA development. Additionally, after backcrossing FCGR2B deficient mice with C5 deficient ones, we observed a rapid induction of CAIA in the doubly deficient mice, but not in those with just C5 deficiency. This implies that the FCGR2B KO overrides the resistance derived from C5 deficiency.

4.2 Study II: Protective autoantibodies

In prior research, we determined that autoantibodies to the F4 epitope on COL2 appear regulatory rather than arthritogenic. Through phage display, we generated a range of recombinant antibodies targeting this epitope. One candidate, R69-4, emerged as the most effective in suppressing arthritis. Testing in various animal models revealed that R69-4 completely suppressed CAIA, offered partial protection against CIA, but lacked efficacy in GPIA or MIP. This suggests that its protective capacity is limited to antibody induced arthritis. Further analysis revealed that R69-4 does not disrupt the dynamics of arthritogenic antibodies or their cartilage-binding ability. While it effectively halted cartilage degradation, it did not suppress chondrocytes from releasing collagenases. Our attention then turned to the immune system, finding that R69-4 inhibited the expansion of neutrophils during acute arthritis. Characterizing SF showed a halt in IL-1β secretion and the suppression of its preceding FCGR3 signaling by R69-4. *In vitro* assays demonstrated its capability to rapidly exhaust FCGR3 on SF neutrophils, triggering a significant ROS burst. This effect was notably reduced in protein-free PBS compared to protein-rich culture media. Binding assays suggested R69-4 can bind to neutrophil FCGR3 via ligand-receptor interaction, rather than through specific binding. This might indicate a pre-complexing of R69-4 before it interacts with neutrophils. Extensive screening for its potential targets

identified numerous candidates, including complement C1q. IHC showed that R69-4 stains not only cartilage but also various other tissues.

4.3 Study III: NCF1 and FCGR2B in T cell tolerance

Initially, we found that mice possessing a point mutation on COL2 (*Col2*^{266E}) are entirely resistant to CIA, though not to GPIA. This mutation is situated within a major T cell epitope of COL2. Upon the immunization of COL2, these mice exhibited minimum development of autoreactive T cells targeting COL2. Yet, when backcrossed with NCF1 mutated or FCGR2B deficient strains, the subsequent *Col2*^{266E}*.Ncf1*$^{*/*}$ and *Col2*^{266E}*.Fcgr2b*$^{-/-}$ offspring became susceptible to CIA once again. This indicates that the absence of either NCF1 or FCGR2B breaks this tolerance. Subsequent observations highlighted an increase in autoreactive T cells against COL2 due to either of these deficiencies. The NCF1 mutation, in particular, led to a decreased frequency of Tregs, and a reduced expression of autoimmune regulator (AIRE) in both medullary thymic epithelial cells (mTECs) and thymus B cells. In contrast, the FCGR2B deficiency did not result in these phenotypic changes. The underlying mechanism by which FCGR2B deficiency breaks T cell tolerance is a promising avenue for future research.

4.4 Study IV: Protective autoreactive B cells

In this study, we identified a universal presence of autoreactive B cells targeting the COL2 C1 epitope across multiple species. Remarkably, C1 B cells bypass typical negative selection processes like clonal deletion, receptor editing, and anergy. Mice with the KI of the heavy chain of an antibody, CB20, also targeting the C1 epitope, exhibited elevated frequency of C1 B cells. Surprisingly, these mice are resistant to CIA, although not to EAE. Transferring C1 B cells into recipient mice suppressed CIA, suggesting a potential anti-inflammatory role for these antigen-specific B cells. C1 B cells conferred protection against CIA, in an IL-10 independent manner, differentiating them from typical regulatory B cells. Deep dives into their function revealed an elevation in Treg frequencies and heightened CD44 and Ki-67 expressions in Tregs. Subsequent experiments established that Treg activation necessitates direct contact with C1 B cells, underscoring their role as antigen-presenting B cells. Single-cell RNA sequencing highlighted a distinctive transcriptional signature in C1 B cells, notably with elevated CCR7 and CD72 transcription levels. FC analysis validated the increased expression of the two markers upon activation. Notably, specific blockade of CD72 blunted the arthritis-suppressing ability of C1 B cells and reversed resistance to CIA of the corresponding mice. This underscores a CD72-dependent mechanism by which C1 B cells suppress antigen-specific autoimmune response.

5 Discussion and Conclusions

In this thesis, we provide a comprehensive characterization of several pivotal mediators in experimental arthritis, with a primary focus on factors that counteract autoimmunity. Notably, we have found that certain elements, including some autoreactive B cells and autoantibodies, traditionally viewed as pathogenic, possess regulatory functions. These discoveries prompts a re-evaluation of the prevailing belief that autoreactive agents are solely responsible for causing autoimmune diseases.

In **study I**, we introduced a rapid CAIA model, which manifests within hours in mice bearing an FCGR2B deficiency. This deficiency also overrides the resistance derived from the absence of complement C5. This observation underscores the significance of FCGRs over complements in determining the threshold for immune activation in antibody mediated arthritis. Utilizing this model, we determined that FCGR3 is both essential and sufficient for CAIA development. While FCGR4 demonstrated involvement in CAIA mediation, it is premature to draw conclusive statements. This is primarily because specific blockade of FCGR4 considerably disrupts FCGR3 function [162]. One definitive observation is that FCGR4 does not mediate IgG1 induced immune response. Upon reviewing literature related to K/BxN arthritis, we discerned major consistencies between the K/BxN serum transfer model and CAIA. Both models employ autoantibodies targeting cartilage, and the downstream pathways adhere to the same patterns. The autoantibodies targeting GPI in K/BxN serum are predominantly of the IgG1 isotype [141], which does not bind to FCGR4 and is poor to activate complement [157], therefore FCGR3 becomes necessary but MAC formation appears dispensable. A CAIA cocktail composed solely of IgG1 antibodies should mimic all these characteristics of the K/BxN serum transfer model. When IgG2a and IgG2b isotypes are included in CAIA cocktail, FCGR4 and MAC formation would join. In summary, this study reinforces the notion that in antibody mediated immune responses, the diversity of FCGRs exists primarily to mediate the different effects of various IgG isotypes, thereby facilitating precise regulation of downstream pathways.

In **study II** [163], we engineered a range of recombinant antibodies targeting the F4 epitope of COL2. Among these, R69-4 stood out as a promising candidate. This antibody notably suppressed the development of antibody mediated arthritis. Initially, we hypothesized that this suppression might be attributed to R69-4 blocking binding sites for certain degradative enzymes. However, subsequent tests negated this hypothesis. The blockade of the F4 epitope *per se* does not offer profound protection, as demonstrated by the experiment using the Fc glycan mutated version (R69-4-N297G). Yet, the protective potential requires the cooperation from the Fc side. We further ascertained that R69-4 has the potential to rapidly exhaust neutrophil FCGR3, preferably when complexed. As shown in **Study I**, blocking FCGR3 overwhelmingly mitigated arthritis in

CAIA. Screening synovial proteins revealed several potential targets that can complex R69-4. Although C1q can contribute to these distinct ICs, the protective efficacy of R69-4 is independent of complement activation. In summary, these observations suggest that the protective potential of R69-4 arises from the ICs themselves, but not through depleting its targets. This also hints at the possible existence of "checkpoint epitopes" in peripheral tissues. In cases where an autoimmune response mistakenly targets one's own organs, these checkpoint epitopes might become exposed, triggering the production of specific regulatory autoantibodies that counteract the initial autoimmune attack.

In **study III** [164], we introduced two mutations to the immunodominant T cell epitope on COL2. This epitope is recognized by the MHC II molecule A^q in CIA-susceptible mouse strains carrying the $H2^q$ haplotype, as well as by HLA-DRβ1*0401 in humans [165]. The epitope mutation, enhancing binding affinity to MHC molecules, significantly increased resistance to CIA. Conversely, the mutation leading to T cell non-recognition predisposed mice to autoimmunity. This resistant strain serves as a valuable mouse model for exploring T cell tolerance regulation, especially since such investigations are not feasible in WT mice, as autoreactive T cells induced by heterogeneous COL2 cannot recognize the endogenous COL2 peptide of the immunized mice. To enable arthritis induction, we further introduced an *Ncf1* mutated or *Fcgr2b* KO background into this resistant strain, which with either deficiency effectively broke the tolerance. Notably, the NCF1 deficiency led to a reduction in Treg frequency and diminished AIRE expression, whereas the FCGR2B deficiency did not have these effects. In summary, we show that mutations on the immunodominant T cell epitope of COL2, resulting in enhanced MHC II affinity, provide a robust T cell resistance. However, this resistance can be broken by NCF1 or FCGR2B deficiencies, paving the way for autoreactive T cell activation and subsequent arthritis onset.

In **study IV** [166], we described that a subset of antigen-presenting B cells specific for the C1 epitope on COL2, are suppressive instead of arthritogenic. This is evident by the resistance of KI mice populated with these C1 B cells to CIA, and the potent suppression on CIA when transferring C1 B cells into recipient mice. We noted that C1 B cells consistently bypassed negative selection across all tested species and entered the periphery. Their regulatory role involves the activation of antigen specific Tregs, which subsequently dampen inflammation. The regulatory capability of C1 B cells hinges on two critical factors: C1 related inflammation and the functional integrity of CD72. Notably, a marked decrease in C1 B cells was observed in RA patients, suggesting that the differentiation of C1 B cells into plasma cells may play a role in RA pathogenesis. In summary, these findings suggest that C1 B cells may undergo positive selection to counterbalance undesirable autoimmune reactions. This aligns with our findings from **Study II**, where certain autoantibodies exhibited regulatory properties. Nevertheless, the action of these suppressive B cells is likely to precede that of regulatory antibodies.

6 Points of perspective

In this thesis, we present four studies that map the procedure of immune responses in the reverse order of occurrence, primarily emphasizing the wisdom of immune system to prevent excessive inflammation or autoimmune diseases. We characterized the functions of an autoreactive but suppressive B cell clone (**Study IV**), two genes promoting T cell tolerance (**Study III**), a subset of regulatory autoantibodies (**Study II**), and key FCGRs in the terminal effector phase (**Study I**). Together, these investigations pave the way for fresh perspectives in subsequent research and bear potential clinical implications.

Drawing on the results of **Study IV**, it becomes crucial to explore the transition in the BCR repertoire when a healthy individual begins producing ACPA, marking a shift from a healthy state to pre-RA. An exhaustive analysis of all autoreactive B cell clones might help pinpoint key regulatory clones. Inhibiting the differentiation of these regulatory clones into plasma cells could potentially halt the progression to the pre-RA phase. Additionally, fostering the clonal expansion of these suppressive B cells may become another promising therapeutic approach for treating RA. From the insights gathered in **Study III**, it is evident that T cell tolerance is influenced by a multitude of factors. The combined impact of genetic and environmental determinants sets the threshold for T cell activation. In this context, we have introduced a promising mouse model to further investigate T cell tolerance. Augmenting T cell tolerance could potentially impede the infiltration of autoreactive clones into the synovium, further preventing the transition from the pre-RA phase to onset. Based on the findings from **Study II**, certain autoantibody clones can prevent the onset of arthritis induced by other arthritogenic antibodies. Moreover, even after onset, these regulatory autoantibodies can persistently suppress inflammation. They may act as a checkpoint to further inhibit autoimmunity, succeeding the suppressive B cells detailed in **Study IV**. But they should not have any overlap in the target epitopes. Furthermore, the promising antibody candidate, R69-4, holds potential for RA treatment, especially during its acute phase. As elucidated in **Study I**, the robust suppressive capability of FCGR2B has been validated. This receptor may act as the final checkpoint mechanism against autoimmunity. Its absence overrides complement activation, leading to swift arthritis onset due to autoantibodies. Strategies to amplify FCGR2B or inhibit its counterparts could offer potential avenues for alleviating RA.

Taken together, these studies suggest that there might be negative regulatory mechanisms at every stage of immune activation. These regulators seem to be universal in immunity, regardless of the targeted subjects as infections, tumors, or one's own tissues. By pinpointing these negative regulators in the immune system, we can polish this weapon to fight enemies, while simultaneously, minimizing mistakes in harming allies.

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