B cell subpopulations in the pathogenesis of rheumatoid arthritis

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Gothenburg 2019

Cover illustration: Ragnhildur Stefánsdóttir

Al-one/ Einvera, 1997

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ISBN 978-91-7833-350-9 (PRINT)
ISBN 978-91-7833-351-6 (PDF)
http://hdl.handle.net/2077/58499

Printed in Gothenburg, Sweden 2019
Printed by BrandFactory

ABSTRACT

B cell depleting therapy has proven to be an effective treatment in rheumatoid arthritis (RA), a disease characterized by the presence of autoantibodies against citrullinated proteins (ACPA) and the Fc portion of IgG (rheumatoid factor, RF). This demonstrates the vital role B cells play in the disease. The aim of this thesis was to explore the role of B cell subpopulations in the pathogenesis of RA. Our interest in a specific B cell population arose with the discovery of murine autoreactive B cells, $CD21^{-/low}$ cells, which expressed low surface levels or lacked the complement receptor 2 (CD21). CD21 helps activate B cells, as it is a part of the B cell co-receptor complex.

In Studies I-III we analyzed B cell populations in human peripheral blood with the help of flow cytometry utilizing multiple cell markers. In Studies II-III, clinical as well as radiographic data was collected from RA patients.

In **Study I** we established that $CD21^{-/low}$ B cells are found in human peripheral blood and discovered that in healthy donors (HDs) this B cell population is mainly composed of memory B cells (MBCs) based on their phenotype and response to combined stimuli. In **Study II** we compared the B cell populations in peripheral blood of patients with established RA and HDs. We saw a higher proportion of a $CD21^{-/low}$ subpopulation, i.e. $CD21^{-/low}$ $CD27^{-}IgD^{-}$ (double negative, DN) in patients with autoantibodies (ACPA/RF) compared to HDs. Additionally, the frequency of $CD21^{-/low}$ DN cells was higher in ACPA/RF positive patients with more joint destruction compared to those with less, and the $CD21^{-/low}$ DN population correlated positively with the level of destruction. The $CD21^{-/low}$ DN population was highly enriched in the inflamed joints of RA patients and a third of the cells expressed RANKL, which stimulates osteoclastogenesis. In **Study III**, we compared the B cell populations in peripheral blood in newly diagnosed untreated RA patients and HDs. We observed that the proportion of $CD21^{+}CD27^{+}$ MBCs correlated positively with RF and ACPA titers. In addition, the frequency of $CD21^{+}$ DN cells and $CD21^{-/low}$ DN MBCs correlated positively with tender joint count and joint narrowing score respectively.

In conclusion, it seems that different MBCs have different roles in RA where $CD21^{+}$ $CD27^{+}$ MBCs appear to drive the autoantibody response, the $CD21^{+}$DN MBCs the joint inflammation and the $CD21^{-/low}$ DN MBCs the joint damage.

SAMMANFATTNING PÅ SVENSKA

B celler är en viktig del av vårt immunförsvar och kännetecknas av deras förmåga att producera antikroppar. Vid den autoimmuna ledsjukdomen reumatoid artrit (RA) har immunsystemet av okänd anledning börjat attackera lederna och ca 50-70% av patienterna har specifika antikroppar riktade mot egna vävnader, så kallade autoantikroppar. Terapi riktad mot B celler ger bra resultat i denna patientgrupp. Detta indikerar att B cellerna spelar en betydande roll vid RA.

Syftet med doktorandprojektet är att studera förekomst av olika subpopulationer av B celler hos patienter i samband med insjuknande i RA och under sjukdomsförloppet, samt att se om det finns samband mellan specifika B cells subpopulationer och kliniska symptom. I alla studierna användes flödescytometri för att analysera B cells subpopulationer med hjälp av flera cellmarkörer. Dessutom analyserades autoantikroppar (ACPA, RF), allmänna inflammationsmarkörer (SR, CRP), DAS28 och röntgen av händer och fötter i Studier II-III.

Tidigare hade man hos möss hittat en autoreaktiv B cells population som uttryckte lite eller helt saknade komplement receptor 2, CD21, på ytan ($CD21^{-/low}$ celler). CD21 är en viktig del av B cellens s.k. co-receptor komplex vilket påverkar aktiveringen av cellen. Vi ville studera $CD21^{-/low}$ celler hos friska människor samt RA patienter.

I **Studie I** påvisade vi $CD21^{-/low}$ celler i friska människor, och dessa celler liknade den tidigare beskrivna B cellspopulationen i möss. Största delen av dessa celler var minnesceller som indikerade att de inte behövde lika mycket stimulering som omogna celler för att aktiveras och deras respons var större. I **Studie II** jämförde vi B celler i friska kontroller och patienter med långvarig RA. RA patienter med autoantikroppar (ACPA, RF) hade en ökning av en subpopulation av $CD21^{-/low}$ celler som saknade både CD27 och IgD, därför kallade double negativa, DN. Andelen $CD21^{-/low}$ DN celler var högre i ACPA/RF positiva patienter med ökad leddestruktion jämfört med patienter med mindre. $CD21^{-/low}$ DN korrelerade också positivt med graden av leddestruktion i ACPA/RF positiva patienter. Dessutom utgjorde de en stor andel av B cellerna i ledvätska från RA patienter, där de uttryckte RANKL som stimulerar genes av osteoklaster, dvs. de celler som bryter ner ben. I **Studie III** jämförde vi B cells populationer i friska kontroller och patienter med nydiagnostiserad och obehandlad RA. Minnesceller som var $CD21^{+}$ och uttryckte $CD27^{+}$ korrelerade positivt med nivåer av autoantikroppar i blodet

och $CD21^{+}$ DN minnesceller korrelerade positivt med antalet ömma leder. I likhet med Studie II hittade vi ett samband mellan $CD21^{-/low}$ DN cellerna och leddestruktion. $CD21^{-/low}$ DN cellerna korrelerade positivt med graden av broskdestruktion.

Sammanfattningsvis har olika typer av minnesceller olika roller i RA, där $CD21^{+}$ $CD27^{+}$ celler verkar kopplade till autoantikroppstitrar, $CD21^{+}$ DN till ledinflammation och $CD21^{-/low}$ DN subpopulationen till leddestruktion.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Thorarinsdottir K***, Camponeschi A*, Cavallini N*, Grimsholm O, Jacobsson L, Gjertsson I, Mårtensson I-L. CD21$^{-/low}$ B cells in human blood are memory cells. *Clin Exp Immunol.* 2016; 185: 252-262.
*These authors contributed equally to the study

II. **Thorarinsdottir K**, Camponeschi A, Jonsson C, Nilsson J, Forslind K, Visentini M, Jacobsson L, Mårtensson I-L, Gjertsson I. CD21$^{-/low}$ B cells associate with joint damage in rheumatoid arthritis patients.
Submitted

III. **Thorarinsdottir K**, Forslind K, Agelii ML, Rudin A, Jacobsson L, Mårtensson I-L, Gjertsson I. Memory B cell subsets correlate with autoantibody titers, disease activity and joint damage in untreated early rheumatoid arthritis.
In Manuscript

CONTENT

ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
ANA	Anti-nuclear antibody
BCR	B cell receptor
bDMARD	Biologic disease-modifying antirheumatic drug
CDAI	Clinical disease activity index
CDR	Complementarity-determining regions
CLP	Common lymphoid progenitor
CR2	Complement receptor 2
CRP	C-reactive protein
csDMARD	Conventional synthetic disease-modifying antirheumatic drug
CTLA-4	Cytotoxic T-lymphocyte antigen-4
CVID	Common variable immunodeficiency
DAS28	Disease activity score 28
EGA	Evaluator global assessment
ESR	Erythrocyte sedimentation rate
Fab region	Fragment antigen-binding region
FACS	Fluorescence-activated cell sorting
Fc region	Fragment crystallizable region
GALT	Gut-associated lymphoid tissue
GC	Germinal center

HAQ	Health assessment questionnaire
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HD	Healthy donor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
Ig	Immunoglobulin
JAK	Janus kinase
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MBC	Memory B cell
MCP	Metacarpophalangeal
MHC	Major histocompatibility complex
mSHS	Modified Sharp van der Heijde score
MTP	Metatarsophalangeal
NSAID	Nonsteroidal anti-inflammatory drug
OPG	Osteoprotegerin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein-1
PGA	Patient global assessment

PIP	Proximal interphalangeal
PLO	Primary lymphoid organ
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor kappa-β
RF	Rheumatoid factor
SAC	*Staphylococcus aureus* Cowan 1
SE	Shared epitope
SF	Synovial fluid
SHM	Somatic hypermutation
SJC	Swollen joint count
SLC	Surrogate light chain
SLE	Systemic lupus erythematosus
SLO	Secondary lymphoid organ
TJC	Tender joint count
TNF	Tumor necrosis factor
TLR	Toll-like receptor
tsDMARD	Targeted synthetic disease-modifying antirheumatic drug
ueRA	Untreated early RA

1 INTRODUCTION

"The most disturbing yet revealing instance of the body's betrayal of itself is autoimmune disease," reflected Mark C. Taylor, a theologian, suffering from diabetes mellitus [1]. In fact, all through the first half of the 20th century the idea of autoimmunity was considered so serious and impossible that it wasn't accepted until in the 1950's. The concept of autoimmunity was also what sparked my interest in rheumatology. Autoimmunity remains an enigma for rheumatologists as can be seen in rheumatoid arthritis (RA). Daily we treat patients with RA and despite great progress in diagnosis and treatment we still can't fully explain why our patients develop the disease. Further research on pathogenesis and unveiling of disease mechanism is vital as with better understanding, the treatment targets become clearer leading to better choice of medication for the individual patient as well as new ideas for therapy or prevention. With the new age of biologic therapy, B cells have become a treatment target in many autoimmune diseases including RA. In this thesis my aim was to further investigate the role of B cells in the pathogenesis of RA.

1.1 RHEUMATOID ARTHRITIS

1.1.1 EPIDEMIOLOGY

One of the first descriptions of RA is from 1676 where Thomas Sydenham described a joint disease that differed from gout [2]. He described many features of the disease including its periodicity, hand deformities, disability and chronicity: *"it will last for months and years; nay, it will torment a patient throughout his miserable time."* [2] However, the modern term rheumatoid arthritis was not coined until 1859 by Alfred Baring Garrod, a physician at the West London Hospital. Until that time the disease had often been called rheumatic gout [2, 3]. RA is a chronic autoimmune disease that primarily involves the joints with a prevalence around 0.5-1%. The highest reported prevalence is in native American-Indian populations, around 5-7% and one of the lowest in rural Africa [4, 5]. There is a higher incidence in northern (20-50/100.000/year) compared to southern European countries (9-24/100.000/year) [5, 6]. Similar to many other autoimmune diseases, RA is more common in women (female to male ratio 3:1) [5, 7]. The patient can develop RA at any age with a peak incidence around the fifth decade of life although some more recent studies have suggested a later onset [5, 8].

1.1.2 ETIOLOGY

1.1.2.1 GENETIC HOST FACTORS

The etiology of RA is not completely understood but it is thought to be the result of a complex interplay between genetic and non-genetic host factors as well as environmental factors (Figure 1). Twin studies have revealed that around 50-60% of disease occurrences can be explained by genetic factors [4]. In the 1970s it was described that 70% of the RA patients carried a specific major histocompatibility complex (MHC) class II, i.e. Human leukocyte antigen (HLA)-DR4, compared to 30% of the controls. With further research it was discovered that the alleles of HLA-DR that carried the highest risk shared a common amino acid sequence (70-74) in the third hypervariable region of the DRβ chain which was called the shared epitope (SE) [9]. SE has been shown to have greater affinity for citrullinated compared to native self-peptides [10] and it is also associated with the presence of autoantibodies against citrullinated peptides (ACPA) [11]. This suggests that the SE plays a role in the activation of the adaptive immune response against citrullinated peptides. Citrullination i.e. the post-translational modification of arginine to citrulline, is not specific to RA but occurs during inflammation, endoplasmic reticulum stress and autophagy [12, 13]. Around 50-70% of RA patients present with serum autoantibodies, i.e. ACPA and/or autoantibodies against the Fc portion of IgG (rheumatoid factor, RF). The heritability of RA in these patients (seropositive) is around 40-65% whereas in patients without autoantibodies (seronegative) it is around 20% [14]. HLA genes explain around 40% of the genetic risk while the contribution of non-HLA genes is considerably smaller (5%) [15]. Among the non-HLA genes, the PTPN22 has one of the strongest risk associations with RA where polymorphisms in the gene is proposed to alter the threshold for activation in immune cells [15].

1.1.2.2 NON-GENETIC HOST FACTORS

The female to male ratio of 3:1 seen in RA is in line with other autoimmune diseases where women usually have an increased risk compared to men. Around 80% of patients with autoimmune diseases are women. The reason for this bias is not fully understood but sex hormones and sex chromosomes are thought to play a role [16]. Focus has been on the sex hormones that greatly influence the immune system e.g. tolerance mechanisms by impairing negative selection of autoreactive B cells [16]. The risk of RA is decreased during pregnancy and it is thought to be due to high levels of the immune suppressive hormone progesterone [16]. Regulation of immune responses via estrogen is more complicated as the levels of estrogen vary greatly through a woman's lifetime and the levels have contrasting effects with low and high

levels inducing a Th1 and Th2 response respectively [16]. How much the sex chromosomes contribute to the pathogenesis of RA is unclear. Women have two X chromosomes where the one is inactivated. However, the inactivation is often incomplete resulting in overexpression of genes [17]. Skewed silencing of X chromosome is more common in autoimmune diseases including RA but could be a consequence rather than a cause of disease [18, 19].

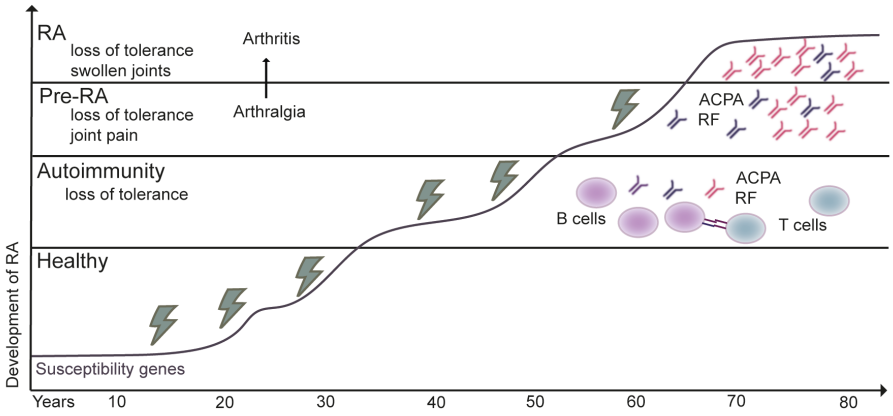

Figure 1. A general model of RA development: RA arises in a genetically susceptible individual after multiple non-genetic triggers (e.g. infections, sex hormones, smoking, exposure to silica) where at least one trigger induces post-translational modifications of proteins e.g. citrullination, which in turn activates an autoreactive immune system. The lightning symbolizes the multiple triggers by non-genetic factors needed for the development of RA. The nature of each "hit", when they occur and number needed is unknown. Pre-RA is when patient has autoantibodies and arthralgia (joint pain) but no arthritis (swollen joint).

1.1.2.3 ENVIRONMENTAL FACTORS

Smoking is the largest known environmental risk factor for RA and is estimated to account for around 20% of the total environmental risk [20]. The combination of smoking and two alleles of SE increases the risk of developing seropositive RA 21-fold compared to non-smokers carrying no SE genes [21]. There are also other agents affecting the lungs that can trigger ACPA positive RA [22] such as silica [23] and textile dust [24, 25]. Similar to smoking the combination of exposure to these irritants and being a carrier of SE greatly increases the risk of developing RA [25]. Other environmental factors, such as poor socioeconomic status [8] and infections have also been associated with the development of the disease [26]. An infectious trigger has long been suspected with candidate pathogens varying from *Proteus*

mirabilis [27] and Epstein-Barr virus[26, 28]. Other candidate pathogens are the bacteria found in periodontal disease, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans,* which could promote RA via their citrullination of peptides [15, 29]. However, a causal relationship between infection and RA has not been proven.

1.1.3 PATHOGENESIS

As mentioned above, around 50-70% of RA patients have autoantibodies specific for disease (RF and ACPA) and their presence is associated with more severe symptoms and joint damage [26]. These autoantibodies can appear up to at least 10 years before the debut of joint symptoms [30] but not all individuals with ACPA develop RA. Only around 20-40% of patients with arthralgia and autoantibodies, progress to RA within 2 years [31]. Taken together this implies that a breach of tolerance happens long before disease onset and that autoantibodies *per se* are not enough to cause disease, an additional trigger is needed. However what is also known is that the epitope diversity and concentration of ACPA increase over time suggesting that there is a progressive loss of tolerance [9].

The starting point of RA has been theorized to be in the lungs, as we know that smoking is a strong environmental risk factor. Supporting this is the presence of citrullinated peptides in the lungs in addition to increased levels of ACPA in bronchoalveolar lavage fluid compared to sera of ACPA positive patients suggesting local production of ACPA in the lungs [25, 32]. Another theory is that RA starts in the gums with the peptide-citrullinating bacteria i.e. *P. gingivalis* or *A. actinomycetemcomitans* acting as the main culprits [22]. Irrespective of the initial trigger and its localization it is thought that the modified peptide, now considered a foreign antigen, is carried to a secondary lymphoid organ (SLO) where B and T-cells are activated and autoantibodies are produced e.g. ACPA. ACPA can be pathogenic either directly by binding citrullinated epitopes and activating macrophages and/or osteoclasts [33, 34] or indirectly by acting through immune complexes binding to Fcγ receptors [35]. Ectopic lymphoid structures comprised of T and B cells, including ACPA positive B cells, appear in the synovium [9, 36, 37]. The thickness of the synovium expands where there is an accumulation of innate and adaptive immune cells as well as macrophages and activated fibroblasts [9]. This synovial tissue has been described as tumor-like as it infiltrates and erodes cartilage and bone [38]. Destruction of cartilage is mediated by matrix metalloproteinases and other enzymes secreted mainly by synovial fibroblasts. However, the degradation of bone is the work of osteoclasts, emerging from pre-osteoclasts stimulated by factors that e.g. act via receptor

activator of nuclear factor kappa-β (RANK) [9]. Its ligand, RANKL, (also known as osteoclast differentiation factor), is secreted or expressed on the surface of fibroblasts, T and B cells. The final result is chronic inflammation and destruction of multiple joints [26].

1.1.4 CLINICAL MANIFESTATIONS

The typical RA patient has an insidious onset of morning stiffness (lasting more than 1 hour) and swollen and tender joints as well as an increase in inflammatory markers such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) [26, 39, 40]. The disease typically is polyarticular (>4 joints simultaneously) affecting the small joints of the hand and feet in a symmetrical fashion [41]. The clinical picture varies greatly with some patients having mild disease to other having severe disease with multisystem inflammation [38]. Patients commonly have secondary Sjögren's syndrome (dryness in eyes and mouth) and rheumatoid nodules [42]. Disease activity and smoking are risk factors for severe extra-articular manifestations such as interstitial lung disease, vasculitis and neuropathy. These severe organ manifestation occur less frequently nowadays with better treatments [42]. In addition, RA patients have a higher prevalence of several comorbidities such as cardiovascular disease, infections, osteoporosis and lymphoma, which is in large part due to inflammation and also, e.g. for osteoporosis and infections in some part due to treatment [43, 44]. The higher risk of mortality in RA is largely explained by its comorbidities [45].

1.1.5 DIAGNOSIS AND DISEASE ACTIVITY

There is no single test that results in the diagnosis of RA. Rather, the diagnosis is based on a combination of clinical findings, laboratory results including immunological tests and radiographic hallmarks of RA. As the symptoms can overlap considerably with that of other rheumatic diseases an accurate diagnosis can be difficult to make. The classification criteria for RA from 1987 and 2010 are often used as tools to help in the diagnosis of RA. However, the criteria do not replace the rheumatologist's clinical knowledge, as the criteria were not developed for the purpose of diagnosis but for studies so that the study groups would be as homogenous as possible [40].

1.1.5.1 CLASSIFICATION OF RA

In 1987 a committee of the American Rheumatism Association revised the classification criteria for RA which are still in use (Table 1) [46]. However, with increasingly more effective treatments for RA it became important to make the diagnosis earlier in the disease process before irreversible damage occurred. Unfortunately, the 1987 American College of Rheumatology

(ACR) criteria had low sensitivity and specificity for diagnosing patients with early RA and new classification criteria were devised in 2010, ACR/European League Against Rheumatism (EULAR) 2010 criteria (Table 2) [26].

Table 1. *The 1987 ACR criteria*

1. Morning stiffness (at least 1 hour)
2. Arthritis of ≥3 joint areas (PIP, MCP, wrist, elbow, knee, ankle and MTP joints)
3. Arthritis of hand joints (≥1 swollen joints) (PIP, MCP or wrist)
4. Symmetrical arthritis
5. Rheumatoid nodules
6. Serum RF
7. Radiographic changes (hand or wrist)

Patients fulfilling at least 4/7 criteria are classified as having RA. Criteria 1-4 must have been present for at least 6 weeks. PIP, proximal interphalangeal; MCP, metacarpophalangeal; MTP, metatarsophalangeal.

Table 2. *The 2010 ACR/EULAR criteria*

1. Joint involvement (0-5)
- 1 large joint (0)
- 2-10 large joints (1)
- 1-3 small joints (large joints not counted) (2)
- 4-10 small joints (large joints not counted) (3)
- >10 joints (at least 1 small joint) (5)
2. Serology (0-3)
- Negative RF and negative ACPA (0)
- Low positive RF or low positive ACPA (2)
- High positive RF or high positive ACPA (3)
3. Acute-phase reactants (0-1)
- Normal CRP and normal ESR (0)
- Increased CRP or ESR (1)
4. Duration of symptoms (0-1)
- Less than 6 weeks (0)
- 6 weeks or more (1)

Target population: Patients who have at least one swollen joint that is not better explained by another disease. Points are shown in parentheses. Cutpoint for definite rheumatoid arthritis is 6 points or more. Patients can also be classified as having rheumatoid arthritis if they have: (a) typical erosions; (b) long-standing disease previously satisfying the classification criteria.

1.1.5.2 DISEASE ACTIVITY SCORES

To assess disease activity in RA several combined indices have been developed and below I describe some of these: Disease activity score 28-ESR (DAS28-ESR) is one of the most widely used both in clinical practice and in studies (Table 3). A similar score is the DAS28-CRP, where ESR has been exchanged for CRP. Another widely used index is the Clinical disease activity index (CDAI), which is a simpler variant than the DAS28 scores. With these instruments the rheumatologist can assess whether the patient is in remission or has low, moderate or high disease activity (Table 3).

Table 3. *Composite measures of disease activity*

Indices	Components	Cutpoints for disease activity			
		Remission	Low	Moderate	High
DAS28-ESR	TJC28, SJC28 ESR, PGA	≤2.6	>2.6 to ≤3.2	>3.2 to ≤5.1	>5.1
DAS28-CRP	TJC28, SJC28 CRP, PGA	≤2.6	>2.6 to ≤3.2	>3.2 to ≤5.1	>5.1
CDAI	TJC28, SJC28 PGA, EGA	≤2.8	>2.8 to ≤10	>10 to ≤22	>22

CDAI, Clinical disease activity index; DAS28, Disease activity score 28; EGA, Evaluator global assessment, ESR, erythrocyte sedimentation rate; PGA, Patient global assessment; SJC28, Swollen joint count 28; TJC28, Tender joint count 28.

1.1.6 PROGNOSIS AND TREATMENT

Factors predicting poor prognosis at disease onset are the presence of autoantibodies (RF and ACPA) [30, 47, 48], erosive joint changes on radiographs of hands and feet, high levels of inflammation measured (ESR, CRP) and multiple swollen joints. Studies have shown that patients treated early (<12 weeks) from debut are more likely to achieve remission than those that are treated later [49]. If improvement in disease activity is small after 3 months of treatment, the therapy is adapted and re-evalued 3 months later [26].

For a large part of the 20th century there were no effective treatments for RA. This was apparent when Dr. Walter Bauer, the president of the American Rheumatism Association (ARA), addressed the ARA meeting in 1947 with the paraphrased words of Wendell Holmes: " If most of the so-called specific rheumatic medicines were thrown into the Atlantic ocean, it would be much better for mankind and so much worse for the fishes" [50]. Nonsteroidal anti-

inflammatory drugs (NSAIDs) were then used for pain and stiffness as they are now. However they do not change the course of disease. There was a major breakthrough in 1948 with the discovery of corticosteroids, which gave patients a miraculous symptomatic improvement and disease-modifying effects [51, 52]. This discovery earned Kendall, Hench and Reichstein the Nobel Prize in 1950 [53]. However, it soon became apparent that the patients got serious side effects if they were treated with corticosteroids permanently and that other treatments were needed [54]. There are now many **d**isease-**m**odifying **a**nti**r**heumatic **d**rugs (DMARDs) in use for RA and they are divided into main two classes: synthetic and biologic DMARDs (bDMARDs). Synthetics are further divided into conventional (csDMARDs) and targeted (tsDMARDs) [26]. Use of csDMARDs has evolved empirically and their modes of action are still largely unknown e.g. methotrexate, which is the most commonly used csDMARD. The second major breakthrough in the treatment of RA came late in the 90's with the first bDMARD, the tumor necrosis factor (TNF) inhibitors that revolutionized the treatment of RA [55]. We now have other bDMARDs that target other cytokines (e.g. IL-6 and IL-1) and cells (B cells and T cells). The tsDMARDs, which are a relatively new drug category, include Janus kinase (JAK) inhibitors that seem to have a good effect in RA [26].

1.2 B CELLS

B (bursal or bone marrow-derived) cells are an essential part of the adaptive immune system and humoral immunity and are thought to have evolved in jawed vertebrates more than 500 million years ago [56]. As terminally differentiated plasma cells they are known for their ability to secrete antibodies. However, they can also be efficient antigen presenting cells and secrete cytokines [56].

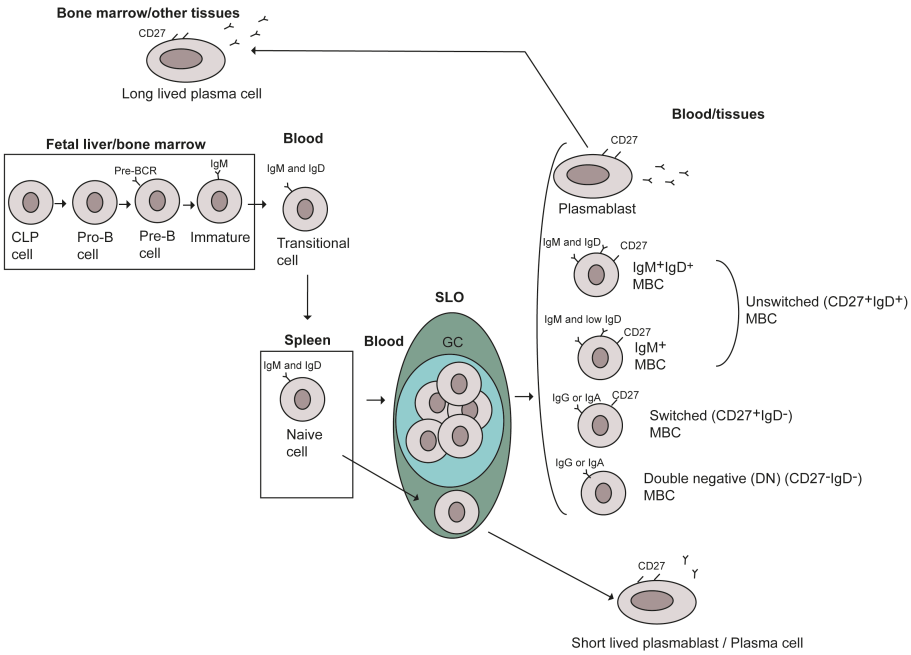

Figure 2. Human B cell development and differentiation. B cells arise from the common primary lymphoid progenitor (CLP) cells in the primary lymphoid organs (PLO) i.e. fetal liver and fetal/adult bone marrow. There they develop through pro-B and pre-B cell stages into immature cells [57]. They emerge from the PLO, as transitional cells and migrate to the spleen where they further differentiate into naive cells [58]. Naive cells circulate through blood and secondary lymphoid organs (SLO). Within SLO such as lymph nodes or spleen, naive B cells are activated and differentiate into memory B cells (MBCs) or plasmablasts that further differentiate into plasma cells. MBCs can migrate and reside in peripheral tissue. Plasma cells have a preference for bone marrow and GALT (gut associated lymphoid tissue) [58, 59]. GC, germinal center.

In mice, B cells can be divided into two populations: B1 and B2 cells, of which the former can be divided into B1a and B1b and the latter into follicular (FO) and marginal zone (MZ) B cells according to ontogeny and

anatomic distribution [60-62]. However this classification is debated in humans. B1 cells in mice develop mainly in the fetal liver and are thought to play a role in innate immunity. They go to pleural and peritoneal cavities as well as spleen and secrete natural antibodies that are the first line of immune defense in blood [57, 60]. In humans a $CD5^+$ B1-like population is found but it is unclear whether it is a human counterpart to the B1 population in mice [62]. Human B2 cells, also called follicular B cells, develop near the end of fetal life (in fetal liver) and all through adult life (in bone marrow) (Figure 2). MZ B cells in mice derive their name from their location in the marginal zone in the spleen [60]. Whether a true MZ B cell population exists in humans is debated. However, human IgM^+ IgD^{low} memory B cells (MBCs) are in many ways similar to the MZ B cells in mice and it has been proposed that they are the MZ B cell counterpart in humans [61].

1.2.1 B CELL RECEPTOR AND CO-RECEPTOR COMPLEX

1.2.1.1 THE B CELL RECEPTOR

B cells express a membrane-bound antibody that recognizes specific antigens. The antibody i.e. immunoglobulin (Ig), combined with α-and β-chains, which transmit signals to the cells core, comprise a B cell receptor (BCR) (Figure 3A) [56]. Antibodies exist in two forms; either expressed on the surface of B cells or secreted as soluble factors. They are composed of two identical heavy and light chains (Figure 3B).

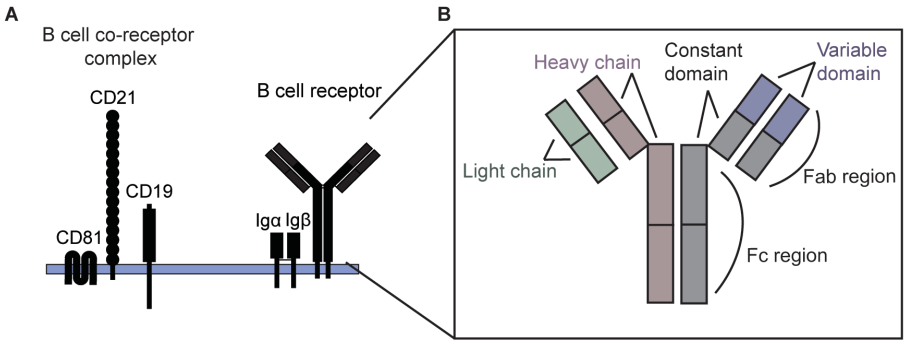

Figure 3. A. The BCR and the B cell co-receptor complex. B. Figure depicts antibody structure composed of light chains (green) and heavy chains (pink). Each light and heavy chain has a constant domain (grey) and a variable domain (purple). Fab region and Fc region are indicated with lines. Figure adapted from Thorarinsdottir et al. SJI [63].

The Fab portion of the antibody recognizes the antigen. The Fc region is important for the function of the antibody and can be of different isotypes or classes i.e. IgM, IgD, IgA, IgE or IgG. The variable domain has three regions that come in contact with the antigen, called the complementarity-determining regions (CDR1 to CDR3) with the CDR3 having the greatest diversity of the CDR regions.

1.2.1.2 THE B CELL CO-RECEPTOR COMPLEX

The BCR co-receptor, composed of CD19, CD81 and the complement receptor 2 (CR2, CD21), lowers the threshold of BCR activation. It is thought to occur via a co-ligation of the BCR and its co-receptor, which results in the phosphorylation of the CD19 tail by a BCR-associated tyrosine kinase and an amplification of the BCR signal to the cells core. It has been proposed to occur when the BCR and the BCR co-receptor bind simultaneously to an opsonized antigen with the BCR binding to the antigen and CD21 to a complement fragment on the surface of the antigen, respectively (Figure 4).

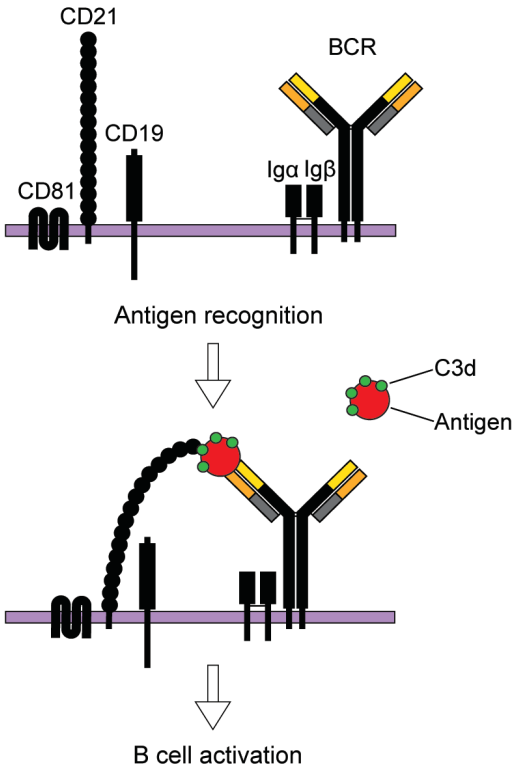

Figure 4. BCR and BCR co-receptor bind to an antigen and a complement fragment, respectively, leading to the activation of the B cell. Figure from Thorarinsdottir et al. SJI [63]

In addition to binding to complement fragments (C3d, C3dg and iC3b), CD21 has been proposed to bind the Epstein-Barr virus [64], the Fc-receptor for IgE (FcεRII or CD23) [65], prions [66] and interferon-α [67]. CD21 can be found on the surface of B cells, follicular dendritic cells, thymocytes and a subset of T cells. B cells go from expressing low levels to high levels of CD21 when they develop from transitional to naive cells [68]. They lose a substantial amount or all CD21 molecules when they differentiate further into plasmablasts and plasma cells [69]. In addition, the B cells continuously shed CD21 and this shedding is thought to increase upon activation of the B cells [70, 71]. Soluble CD21 can form biologically active complexes with CD23 and complement fragments but the function of these are outside the scope of this thesis [72, 73].

1.2.1.3 TOLL-LIKE RECEPTORS

B cells express pattern-recognition receptors (PRRs) including Toll-like receptors (TLRs) that bind conserved pathogen components (pathogen-associated molecular patterns, PAMPs) but can also bind self-antigens [74]. The TLRs recognizing bacteria and fungi are expressed on the cell surface and TLRs binding viral or microbial nucleic acids e.g. TLR7 and TLR9, are found on intracellular membranes [75].

1.2.2 B CELL DEVELOPMENT IN BONE MARROW

There are checkpoints during the B cell development in the bone marrow to ascertain that the B cells develop into harmless functioning cells i.e. that they have a diverse antibody repertoire, and do not recognize self-antigens [76]. This selection process entails testing the function of the BCR and its precursor, the pre-BCR. The common lymphoid progenitors (CLPs) give rise to progenitor (pro)-B cells that further develop into precursor (pre)-B cells[77]. At this stage the pre-B cell receptor composed of the μ heavy chain and the surrogate light chain (SLC) is tested. This checkpoint tests whether the heavy chain is functional and whether it can pair with the invariant SLC. Development of human B cells stop at this stage if the pre-BCR fails this test. However, the loss of the SLC in B cells in mouse only leads to a partial block [78]. Subsequently, the pre-B cells develop into a BCR-expressing immature cell where the next checkpoint tests whether the BCR is autoreactive. If the combination of light chains and heavy chains result in an autoreactive BCR the light chain undergoes new light chain rearrangements [79]. If the B cells continue to express high-affinity autoreactive BCRs the cells go into apoptosis (clonal deletion) or become unresponsive (clonal anergy) [57, 77]. Those that are not negatively selected migrate from the bone marrow as transitional cells. Only around 15% of the generated B cells in the bone marrow reach peripheral blood. The selection process in the bone marrow is

called central tolerance. In the periphery further selection takes place and is called peripheral tolerance [80].

1.2.3 TRANSITIONAL AND NAIVE B CELLS

1.2.3.1 TRANSITIONAL CELLS

The next tolerance checkpoint is at the transitional B cell stage and is also testing the BCR's ability to bind to self-antigens. BCR signaling is necessary for survival but too strong a signal at this stage leads to apoptosis and in humans a reduction from 40% to 20% in autoreactive cells is seen [80, 81]. The transitional cells comprise around 5% of the total B cell population in peripheral blood [82] whereas the proportions are much higher in cord blood [83]. These newly migrated cells travel to the spleen. Transitional B cells can be divided into three populations in an order of development: T1, T2, T3 [84].

1.2.4 NAIVE B CELLS AND FORMATION OF MEMORY AND ANTIBODY-SECRETING CELLS

The majority of the peripheral blood B cells are composed of naive cells that circulate through blood and SLO, with a lifespan of a few weeks [58]. They have decreased their surface expression of IgM and upregulated IgD compared to transitional cells [58] .

Immunological memory is the function of memory cells and antibody-secreting cells [85]. The primary response to an antigen forms the immunological memory, which upon re-encounter with the antigen will respond faster and in an amplified affinity-matured manner (secondary response). This usually means that the secreted antibodies are switched from IgM to IgG and/or IgA [85]. The B cell memory responses can be formed in a T-cell dependent (TD) or T-cell independent (TI) manner, which will now be shortly described, beginning with the TD response where the antigen is a protein. The naive B cell in the SLO binds its cognate protein antigen via the BCR and internalizes the antigen-BCR complex. After processing the protein the B cell presents the peptides via MHC class II molecule to an activated helper T cell at the border of the T-cell zone and the B cell follicle. The B cell receives co-stimulatory signals from the T cell i.e. CD40 ligand and cytokines [58, 85]. The activated B cell now either becomes an extra-follicular short-lived plasma cells, or forms a histological structure called the germinal center (GC) in the B cell follicle. The short lived plasma cell secretes mainly IgM but can later secrete isotype-switched antibodies [85]. However, in the GCs the B cells usually go through class-switch from IgM to IgG, IgA or IgE. In addition, the B cells clonally expand and their BCRs accumulate somatic

hypermutations (SHM) in their variable domain resulting in high-affinity BCRs. Both long-lived MBCs and long-lived plasma cells arise from the GCs [59, 68, 85].

The TI antigens are not proteins and they contain multiple epitopes, which can crosslink numerous BCRs. TI antigens can be divided into TI-1 and TI-2 antigens. TI-1 antigen, e.g. lipopolysaccharides (LPS), is a polyclonal activator as it also activates TLRs via TLR ligands, whereas TI-2 antigen e.g. polysaccharides, needs additional signals to activate the B cells [84, 86, 87]. The response to TI antigens usually does not generate MBCs but instead plasma cells that secrete low-affinity antibodies [58].

1.2.4.1 MEMORY B CELLS

The widest definition of a MBC is a cell that has been activated by an antigen inducing a response and that remains responsive to a second encounter with the same antigen. The MBCs as a group have typical and important characteristics such as: Class-switching, SHM, clonal expansion and a lifespan of decades. In addition they can proliferate and/or differentiate more efficient and faster than naive B cells upon stimulation [58, 84]. When looking at specific types of MBC they often do not possess all of those characteristics, e.g. there are IgM^+ MBCs that have none or few SHM [58].

In humans, the TNF superfamily member CD27 has become a universal marker of MBCs [88] but there are exceptions to this as not all MBCs express CD27. In peripheral blood the main types of MBCs are IgG^+ and IgA^+ $CD27^+$ MBCs. However, the majority of IgA^+ MBCs is localized in the mucosa-associated lymphoid tissue (MALT). The IgG^+ MBCs in humans are further divided into IgG1, IgG2, IgG3 and IgG4 (in a descending order of frequency). Although class-switched MBCs are thought of as typical MBCs, the IgM^+-only (i.e. $IgM^+IgD^{low/-}$) and $IgM^+IgD^+CD27^+$ MBCs constitute around 5% and 15% of the peripheral blood B cells respectively. IgD and IgE MBCs are hardly detectable in peripheral blood [58]. Approximately 20-25% of the IgG^+ MBCs [89] and some of the IgA^+ MBCs lack CD27 [90]. A large proportion of these cells seem to be GC derived and they are often derived from a common GC clone [58].

Antibody-secreting cells can be divided into plasmablasts and plasma cells where plasmablasts often are thought of as an intermediate stage to plasma cells [91]. Plasma cells can be either short-lived, found at extra-follicular sites, or long lived, residing in the bone marrow or gut-associated lymphoid tissue (GALT). In humans long-lived plasma cells have been found to live around 20 years but more studies are needed [59]. In contrast to MBCs the plasma cells

are terminally differentiated, do not proliferate and have usually downregulated their BCR [59].

1.3 CD21$^{-/LOW}$ B CELLS

In the following chapters the B cell populations and studies referred to are in humans, unless otherwise stated. The expression of CD21 varies on different B cell populations (Table 4). There is low expression on early transitional cells (T1) whereas naive cells and MBCs express CD21. However, plasmablasts and plasma cells lack or have low levels of CD21. Activation of a B cell can lead to lower expression levels [72]. In addition, different groups of researchers have reported an increase of CD21$^{-/low}$ B cell populations in various diseases albeit using different definitions of these populations in terms of markers. Nevertheless, these CD21$^{-/low}$ B cell populations share many similarities, as described below.

Table 4. *Cell surface markers on different B-cell populations*

	Surface markers and expression levels								Surface BCR		
B-cell population	CD21	CD19	CD20	CD10	CD27	CD24	CD38	CD138	IgM	IgD	IgG/A
T1	-/low	+	+++	+	-	+++	+++	-	+++	+	-
T2	+	+	+++	low	-	++	++	-	++	++	-
T3	+	+	+	-/+	-	+	+	-	+	++	-
Naïve	+	+	+	-	-	+	+	-	+	++	-
IgM IgD$^{-/low}$ MBCs	+	+	+	-	+	++	-/+	-	+++	+/-	-
CD27^{+}IgD^{-} MBCs	+	+	+	-	+	++	-/+	-	-	-	+
Plasmablast	-/low	low	-	-	+++	-	+++	+	-	-	-
Plasma cell[92]	-	- /+	-	-	+++	-	+++	+++	-	-	-

T1, T2, T3 are transitional cells. MBCs, memory B cells.

1.3.1 CD21$^{-/LOW}$ B CELLS IN MOUSE MODELS

Different CD21$^{-/low}$ B cell populations have been identified in mouse models. One of these was described in the surrogate light chain knockout (*SLC*$^{-/-}$) mouse model. This is a knockout mouse model where the genes of the SLC have been deleted leading to an absence of SLC on pre-B cells. In mice that lack the transmembrane region of the antibody μ heavy chain, B cell development is arrested at the pro-B cell stage in the bone marrow being

unable to develop into pre-B cells. The absence of SLC should also halt the development of B cells at the same stage, as SLC is used to test whether the heavy chain is functional. However, mice that lack one or all of the SLC components (VpreB1, VpreB2, λ5) do not show a complete block but rather an impairment at this stage, allowing some B cells to still develop [93]. Keenan et al. found that pre-B cells could even develop in the combined absence of SLC and bona fide light chains [94]. Nevertheless, despite lower B cell numbers, in the spleen the *SLC*$^{-/-}$ mice had an increased proportion of MZ B cells and an increase of a B cell population that lacked the markers characterizing FO, MZ and B1 cells, CD23, CD21 and CD43 respectively. This subset was therefore termed CD21^{-}CD23^{-} (hereafter called CD21$^{-/low}$ cells) [94]. Later work showed that in adult control mice, most of the CD21$^{-/low}$ cells were naive B cells but in the *SLC*$^{-/-}$ mice they were MBCs expressing mainly IgM [95]. The *SLC*$^{-/-}$ mice had an increase in IgM and IgG autoantibodies, predominantly IgG_{2b} and IgG_3. However, only the CD21$^{-/low}$ cells from *SLC*$^{-/-}$ mice and not control mice produced IgG autoantibodies upon stimulation with LPS. The MZ B cells were responsible for the IgM autoantibodies [96].

The frequency of CD21$^{-/low}$ cells increase with age in both the *SLC*$^{-/-}$ and control mice and their phenotype is similar to another CD21$^{-/low}$ B cell population that in 2011 was described simultaneously by Marrack and Cancro [97, 98]. This B cell population also accumulated with age, hence the name Age-associated B cells (ABCs). Their frequency varies between individual mice but for unknown reasons emerge sooner and with higher numbers in female mice [97-99]. The ABCs are found in peripheral blood, spleen and bone marrow but rarely in lymph nodes [99]. The two groups defined the ABCs differently. Marrack et al. used CD11c and CD11b as markers in combination with B220 whereas Cancro et al. relied on another combination of markers including CD19, CD43, CD93, CD21 and CD23 in order to distinguish the ABCs from immature, B1, MZ and FO B cells [97, 98]. The CD11c^{+}CD11b^{+}ABCs were competent antigen presenting cells and localized to the B/T cell border [97, 100]. Most ABCs were mature and unswitched and upon stimuli they differentiated into plasma cells and were prone to switch to IgG2a/c [97, 98, 101].

1.3.2 CD21$^{-/LOW}$ B CELLS IN HEALTH

We have characterized the CD21$^{-/low}$ B cell population in healthy donors (HDs) that will be further presented in Results, study I. Otherwise there are few studies performed on CD21$^{-/low}$ B cells in HDs. In 2005 a new MBC population was described in the tonsils of HDs. It constituted around 10% of

the B cell population in the tonsils and lacked the CD27 memory marker as well as CD21 and CD38 and was defined by its expression of the low affinity receptor for IgA i.e. Fc-receptor-like protein 4 (FcRL4) [102]. The FcRL4 was first thought be an inhibitory receptor but it seems be a modulator of response in that it can sensitize B cells to TLR9 stimuli culminating in NFκB activation [103]. In addition, it has been shown in a group of HDs (0-50 years) that the frequency of a $CD21^{-/low}$ population, defined as $CD21^{-/low}CD38^{low}$cells, increases slightly with age [82].

1.3.3 $CD21^{-/LOW}$ B CELLS IN DISEASE

1.3.3.1 INFECTIONS

$CD21^{-/low}$ B cells are increased in various chronic infections and have been described in virus infections such as Human immunodeficiency virus (HIV) [104, 105], Hepatitis C virus (HCV) [106, 107] and Hepatitis B virus (HBV) [108] infections as well as in tuberculosis (*Mycobacterium tuberculosis*) [109] and malaria (plasmodium species) [110].

Whether the $CD21^{-/low}$ B cell expresses CD27 differs between the diseases investigated. In HCV, tuberculosis and malaria a $CD21^{-/low}$ population lacking CD27 accumulate in infected individuals [104]. However, there is a clonal expansion of $CD21^{-/low}CD27^{+}$ cells in HCV patients with mixed cryoglobulinemia, a lymphoproliferative disorder with autoantibody production e.g. RF and autoimmune manifestations [111].

A $CD21^{-/low}$ B cell population that is $CD27^{-}$ and $CD11c^{+}$ can comprise over 15% of all B cells in the people living in malaria endemic areas [112]. The expression of CD11c is also found in $CD21^{-/low}$ B cell populations in HIV [104] and HCV [113]. Additionally, the $CD21^{-/low}$ populations in HIV, HCV and tuberculosis express FcRL4 [114], which could be due to TLR stimulation [115] or the pathogen itself [116]. In HCV, the proportion of $CD21^{-/low}$ B cells that express FcRL4 correlates positively with liver inflammation [117]. In contrast, the $CD21^{-/low}$ cells in malaria do not express FcRL4 but rather seem to express FcRL5 [110].

In these chronic infections the $CD21^{-/low}$ B cells have been called "exhausted" because in HIV, HCV and malaria they have a decreased proliferation and/or differentiation capacity after BCR stimulation. Nevertheless, the response varies between these diseases with the population in malaria being least responsive i.e. proliferating less than naive cells upon combined stimuli (anti-Ig, anti-CD40, IL-10 and IL-4 and TLR9-agonist) and not differentiating into plasma cells when stimulated with TLR9-agonist, *Staphylococcus aureus*

Cowan 1 (SAC), pokeweed mitogen and IL-10 [110]. In contrast, in HCV mixed cryoglobulinemia the cells proliferated upon combined stimuli (anti-Ig, CD40L, TLR9-agonist, IL-2 and IL-10) [111] and in HIV although they had diminished proliferative response when stimulated with anti-Ig, CD40L, IL-2 and-10, they could secrete antibodies upon polyclonal activation (SAC and TLR9 agonist) [104].

There is evidence pointing to the pathogen as the driving force behind the expansion of the $CD21^{-/low}$ population in these infections. Firstly, the frequency of $CD21^{-/low}$ B cells in HIV correlates to a certain extent with the viral load [105, 118]. Secondly, there is an enrichment of pathogen-specific B cells in the $CD21^{-/low}$ populations in HIV [104] and malaria [112, 119]. Thirdly, the frequency of $CD21^{-/low}$ cells decreases or disappears with successful treatment against tuberculosis and antiviral therapy in HIV and HCV [114]. Finally, the expansion of $CD21^{-/low}$ cells was only found in malaria-exposed individuals compared to age-matched controls in similar villages not exposed to malaria [120].

Taken together, the $CD21^{-/low}$ cells in aforementioned chronic infections usually have attenuated response to stimuli, often have upregulated characteristic cell surface markers i.e. CD11c and FcRL4 and in some of these infections the underlying pathogen seems to drive their expansion.

1.3.3.2 $CD21^{-/LOW}$ B CELLS IN IMMUNODEFICIENCIES

Common variable immunodeficiency (CVID), characterized by an absence of the switched antibodies IgG and IgA with/or without lack of IgM is the most common primary immunodeficiency in adults [121]. An expansion of $CD21^{-/low}$ B cells to more than 20% of all peripheral blood B cells defines a group Ia of CVID patients that are prone to get autoimmune cytopenia and splenomegaly [121]. Isnardi et al. showed that the $CD21^{-/low}$ cells in this patient group expressed FcRL4 and lacked CD27. In addition, they were shown to be IgM^{+}, enriched for autoreactivity and to exhibit reduced proliferation after stimuli with BCR and CD40L without losing the ability to secrete antibodies [122].

An increase of $CD21^{-/low}$ B cells, defined as $CD21^{-/low}$ $CD38^{-/low}$, has also been described in other immunodeficiencies e.g. Wiscott-Aldrich syndrome [123] and CTLA-4 haploinsufficiency-associated immune dysregulation [124].

1.3.3.3 $CD21^{-/LOW}$ B CELLS IN AUTOIMMUNITY

Chronic infections and autoimmune diseases share their chronic state of inflammation where the immune system is not able to rid the body of the antigen. Also similar to that in chronic infections, an increase of $CD21^{-/low}$ B

cells in peripheral blood has been described in autoimmune diseases e.g. systemic lupus erythematosus (SLE) [121], primary Sjögren's syndrome [125], multiple sclerosis [126] and IgG4-related disease [127]. One of the earliest studies of $CD21^{-/low}$ B cells in autoimmune diseases was on RA patients. It will be described later in chapter 1.4 B cells in RA.

In SLE there is an increase of a $CD21^{-/low}$ B cell population ($CD38^{-}$) during acute flares [128] and a similar population has been correlated to disease duration [129]. A $CD21^{-/low}$ B population (defined as $CD27^{-}$) has been identified in primary Sjögren's syndrome that expresses CD11c and correlates with lymphoproliferation [125]. In some of these autoimmune diseases the $CD21^{-/low}$ B cell population has been associated with autoreactivity, e.g. it was enriched in autoreactive clones in both SLE [128] and primary Sjögren's syndrome [125]. Comparable to the $CD21^{-/low}$ B cell population in chronic infections, it has a diminished proliferative capacity in primary Sjögren's syndrome as the $CD21^{-/low}$ did not respond to BCR stimulation only but proliferated upon stimulation with anti-Ig, CD40L and TLR9 agonist [125].

In summary, the $CD21^{-/low}$ B cells in autoimmune diseases have similar properties to the $CD21^{-/low}$ B cells found in chronic infections, i.e. they often express CD11c and exhibit attenuated response to stimuli. Additionally, in some of these autoimmune diseases the $CD21^{-/low}$ B cells are enriched with autoreactive clones. This indicates that the underlying disease (i.e. autoantigen) drives the expansion of $CD21^{-/low}$ B cells, which is again reminiscent of the pathogen driving the expansion of $CD21^{-/low}$ B cells in chronic infections.

1.3.3.4 $CD21^{-/LOW}$ B CELLS AFTER CHECK POINT INHIBITORS

Check point inhibitors are a new treatment strategy in cancer where the "breaks" are lifted of the immune system leading to an immune attack on the cancer cells. The "breaks" that are lifted, i.e. inhibited, are Programmed cell death protein 1 (PD-1) and Cytotoxic T-lymphocyte antigen-4 (CTLA-4). Interestingly, this has also resulted in autoimmune side effects, e.g. rashes, arthritis and inflammatory bowel disease. Analysis of the B cell compartment after treatment of melanoma patients with check point inhibitors revealed that a combination therapy with anti-PD-1 and anti-CTLA-4 or only anti-CTLA-4 resulted in a decrease in total B cells and an increase in a $CD21^{-/low}$ B cell population as well as plasmablasts. These $CD21^{-/low}$ B cells were $PD\text{-}1^{+}$ and proliferating ($Ki\text{-}67^{+}$). The changes in B cell count and composition coincided with the development of immune-related adverse events mainly affecting skin, colon and liver [130]. This suggests a possible role of the $CD21^{-/low}$ B cells in the autoimmune symptoms.

1.4 B CELLS IN RA

1.4.1 B CELLS IN PERIPHERAL BLOOD

1.4.1.1 PATIENTS WITH EARLY RA

There are a few studies that have analyzed the B cell compartment in peripheral blood of early RA patients, whereas there are studies on B cells in patients with established RA (see below 1.4.1.2). In studies on early RA the disease duration is usually less than 12 months. Figure 5 summarizes the main findings in studies on B cell populations in early and established RA.

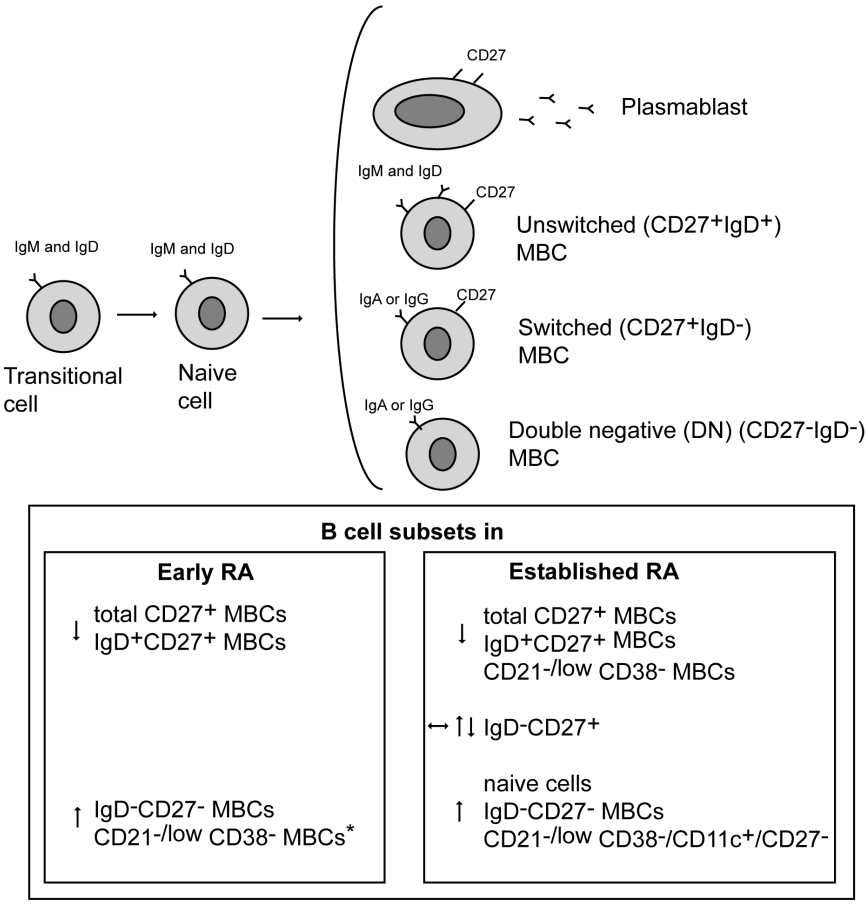

Figure 5. The main B cell populations in peripheral blood investigated in RA studies. Box describes results from studies comparing B cell populations in early RA (left) and in established RA (right) compared to HDs. Study was on untreated RA patients but disease duration was 2-26 months. MBC, memory B cell.*

Most of the studies on early RA show a decrease in the proportion and/or absolute count of the $CD27^+$ unswitched MBCs compared to HDs [131-133]. Some also detect a decrease in the frequency of total $CD27^+$ MBCs in these patients compared to HDs [131, 133]. This decrease is evident already a year prior to disease onset [134]. McComish et al. also looked at the $CD21^{-/low}$ B cells, defined as $CD21^{-/low}CD38^-$, in untreated RA patients and compared to HDs, observed a slight increase in the frequency of these cells, a frequency similar to that in treated patients with established disease [133]. In addition, one study has reported an increase in the frequency of $CD27^-IgD^-$ (double negative, DN) MBCs B cells in untreated early RA patients compared to HDs [135].

1.4.1.2 PATIENTS WITH ESTABLISHED RA

1.4.1.2.1 B cell populations

Studies have reported lymphopenia [136, 137] and/or B cell lymphopenia in established RA [137-142]. This does not seem to be specific for RA as it has also been found in other autoimmune joint diseases e.g. psoriatic arthritis [140]. There could be an association between B cell lymphopenia and disease duration as one study found a positive correlation between those two parameters [138].

Studies have reported a decrease in the proportion of $CD27^+$ unswitched MBCs in patients with established RA compared to HDs (Figure 5) [143-145]. However, there are contrasting reports regarding the proportion of $CD27^+$ switched MBCs [137-139, 143, 144, 146]. In addition, a number of publications have found an increase in DN MBCs in established RA patients compared to HDs [135, 136, 145]. One study reported an increase in DN MBCs and plasmablasts in patients with established compared to early RA [147].

An increase in activated ($CD86^+$) cells in both the naive and $CD27^+$ MBC subset in the blood of patients with RA compared to HDs has been described [148]. In line with this Adlowitz et al. reported an increase in activated cells, defined as $CD21^{-/low}$ cells in the switched and DN MBC populations [149]. Another $CD21^{-/low}$ population described as increased in older female RA patients is the $CD11c^+$ $CD21^{-/low}$ population that also correlated with age in the female RA patients [97]. McComish et al. also found an increase in $CD21^{-/low}$ $CD38^-$ cells in patients with established RA as they had found in untreated RA patients [133]. However, one of the most detailed studies on $CD21^{-/low}$ B cells in RA is the study by Isnardi et al. from 2010 [150]. In this paper they described an increase in an autoreactive $CD21^{-/low}$ B cell population in blood of RA patients compared to HDs. The researchers first investigated the $CD21^{-/low}$ B cell population in CVID patients where the patients had severely

decreased frequencies of MBCs. Because of this they defined the $CD21^{-/low}$ B cell population as $CD19^{+}CD27^{-}CD21^{-/low}$ in the CVID patients and went on to study the same B cell population in RA patients. They found the $CD27^{-}CD21^{-/low}$ to be increased in both CVID and RA patients compared to HDs. Around 30% of the $CD27^{-}CD21^{-/low}$ were $IgM^{low}IgD^{+}$ and 20% were switched. Because the Igs from this $CD21^{-/low}$ B cell population were devoid of SHM they deduced that the cells were naive. The IgMs from the $CD21^{-/low}$ cells displayed an increased frequency of autoreactive clones compared to $CD27^{-}CD21^{+}$ cells in both HDs and RA patients. In the RA patients a large fraction of the $CD21^{-/low}$ clones were highly HEp-2 reactive [150]. As in above-mentioned autoimmune diseases $CD27^{-}CD21^{-/low}$ cells from patients with RA did not respond well to BCR and CD40 costimulation. However, they did upregulate the activation markers CD25 and CD69 after TLR9 triggering. Gene expression analysis (microarrays) of the $CD21^{-/low}$ cells showed an upregulation of genes for inhibitory receptors as well as of CD11c [150].

1.4.1.2.2 Effect of treatment on B cell populations

It is difficult to know when looking at the B cell populations in established RA whether the changes seen in B cell populations compared to HDs, are due to the disease itself or other factors e.g. treatment with DMARDs. The most commonly used treatment is with the csDMARD methotrexate, where a trend of normalization of both the frequency and absolute number of B cells was observed four months into therapy [131], and steroid dose was positively correlated with the proportion of MBCs, especially switched MBCs [138]. Studies that have looked at the effect of bDMARDs and tsDMARDs are described below and summarized in Table 5.

An increase in the frequency and/or absolute number of the total B cell population has been reported after treatment with TNF- [138, 140], IL-6 receptor inhibitors [139] and JAK inhibitors [142]. With the JAK inhibitors this increase was seen relatively early, at 8-12 weeks but with a progressive reduction at later time points [142].

TNF inhibitors have been found to increase unswitched MBCs [143, 145] and switched MBCs (only detected in seronegative patients) [142] and decrease in activated $CD86^{+}$ cells [148] and proportions of DN MBCs [135]. An inverse association between the number of previous TNF inhibitors used and proportions of DN MBCs has also been described [138]. A high proportion of $CD27^{+}$ MBCs before treatment start with TNF inhibitors has been associated with good clinical response [138].

Table 5. *Effect of bDMARDs and tsDMARDs on total and MBC populations*

B cell populations	TNF inhibitors	IL-6 receptor inhibitors	JAK inhibitors	CTLA4-Ig	Anti-CD20 antibody
Total B cells	Increase	Increase	Increase	Not known	Decrease
Unswitched MBCs	Increase	Decrease	Not known	Decrease	Decrease
Switched MBCs	Increase (seroneg only)	Decrease	Not known	Decrease	Decrease
DN MBCs	Decrease	Decrease	Not known	Not known	Decrease

Studies on IL-6 receptor inhibitors have reported an effect on the DN MBCs (Table 5). IL-6 receptor inhibitors seem to decrease the frequency of total DN MBCs [135, 145] or a DN subset, i.e. IgA^+ DN MBCs [136]. A lower frequency of DN MBCs at baseline was associated with good clinical response to IL-6 receptor inhibitors [136]. One study reported a decrease in the proportion of switched and unswitched MBCs (DN proportions were not reported) [139].

A T cell inhibitor, CTLA-4 inhibitor (abatacept) seems to decrease the proportion of switched [141, 144] and unswitched MBCs (Table 5) [141].

Rituximab, a B cell depleting therapy, is an anti-CD20 monoclonal antibody. After rituximab treatment the reconstitution of B cells in blood occurs first with T1/T2 then T3 transitional cells and finally the naive. One of the first studies on the effect of rituximab on B cells in RA patients showed that patients with incomplete B cell depletion after one cycle of rituxmab had poorer outcome [151]. The B cells remaining in the blood after rituximab therapy i.e. those resistant to depletion, are mainly $CD21^{-/low}$ DN MBCs and large proportion of the DN MBCs was also $CD95^+$ [149]. Baseline characteristics that can indicate good response to treatment have been found to be a lower frequency of $CD27^+$ MBCs [137], an increased DN MBCs [152] and a lower proportion of $CD95^+$ cells in DN and switched MBCs [149].

Regarding relapse after rituximab treatment, a study reported that responders that relapsed early (week 24-40 after treatment) had a higher proportion of $CD27^+$ MBCs before starting rituximab treatment than responders with late relapse (week 48-133 after treatment) [153]. It has also been shown that patients relapsing at the time of B cell repopulation tended to show a higher frequency of MBCs at repopulation as compared with patients who relapsed later [154].

1.4.1.2.3 B cell populations and clinical parameters

Considering autoantibodies, one study found that titers of ACPA correlate positively with absolute numbers of plasmablasts and negatively with absolute numbers of switched and unswitched $CD27^+$ MBCs in peripheral blood [133]. An inverse correlation between RF titer and absolute numbers of IgM MBCs was also reported.[133]

Disease activity score (DAS 28) has been found to correlate positively with the absolute numbers and proportions of plasmablasts [133, 145] and the proportion of $CD86^+$ cells [132], and negatively with the absolute numbers of B cells [140] and circulating MBCs in peripheral blood [144]. In fact, high frequency of both DN and unswitched MBCs [138] has been associated with higher disease activity. In addition, there was a positive correlation between the frequency of unswitched MBCs and DAS28 after three months of treatment with IL-6R inhibitors [139].

Many studies do not find any effect of gender or age on B cell populations in peripheral blood in RA. Considering gender, there are conflicting results regarding this and proportions of MBCs [133, 138]. However, there are some studies that have seen a decrease in the absolute numbers of plasmablasts, transitional B cells and MBCs [133] as well as in the proportion of unswitched MBCs with age [138]. In contrast, increased disease duration has been linked to an increase in $CD27^+$ MBCs [146].

1.4.2 B CELLS IN THE JOINTS

The joints are the main target tissue in RA and previous work has shown that synovium and synovial fluid (SF) of RA joints are infiltrated by leukocytes such as granulocytes, monocytes, T and B cells [155]. SF leukocytes are typically granulocytes. In contrast, the synovium rarely contains granulocytes but e.g. T cells are abundant. In the synovium the B and T cells often aggregate in follicles and sometimes they form ectopic GCs [156]. It has been shown that some of these B cells are autoreactive that can recognize citrullinated proteins [36, 157].

In RA patients the B cells in SF express lower levels or lack CD21 [158, 159] but this does not seem to be disease specific as it is also seen in other non-RA arthritic diseases [159]. The B cells in RA SF seem to be mainly MBCs and predominantly IgD^-CD27^+ or IgD^-CD27^- [160-162]. In 2014, Yeo et al. found an enrichment of $FcRL4^+$ cells in the SF and synovium of RA patients compared to blood (13% vs. 0.4%). These cells were $CD21^{-/low}$ and there was an enrichment of $RANKL^+$ cells in the SF $FcRL4^+$ cell subsets compared to

FcRL4$^-$ cells. The authors suggested that these cells could be pathogenic. The FcRL4$^+$ cells were mainly switched (47%) or DN (41%) MBCs and they expressed higher levels of CD11c, CD80 and CD86 than FcRL4$^-$ cells. In addition, they produced TNFα suggesting a pro-inflammatory phenotype [160].

2 AIM

The overall aim of this thesis was to identify B cell subpopulations that associated with pathogenicity in RA, i.e. whether there were any correlations between different B cell subpopulations and disease progression or autoantibodies.

The specific research questions were addressed in three studies:

- Study I: What is the phenotype and function of $CD21^{-/low}$ B cells in healthy donors?
- Study II: Do $CD21^{-/low}$ B cells or other B cell populations associate with clinical parameters in patients with manifest RA?
- Study III: Do $CD21^{-/low}$ B cells or other B cell populations associate with clinical factors or serum autoantibodies in the blood of patients with untreated early RA?

3 PATIENTS AND METHODS

3.1 ETHICS

All three studies were approved by the local ethical committee. All patients (Studies II-III) signed a written informed consent before entering the studies. Where no personal information or identity was recorded, as when collecting peripheral blood from HDs (Studies I-II) and tonsil samples (Study I), no written consent or approval by the Human Research Ethics Committee was needed (Swedish law 2003: 460, paragraphs 4 and 13). All mothers were given oral and written information for cord blood samples and gave oral consent to participate in the study (Study I).

3.2 PATIENTS AND HEALTHY DONORS

STUDY I: In this study we collected peripheral blood from 18 healthy women, aged 24–30 (n=9) and 55–64 years (n=9). Cord blood samples from six healthy newborn infants born at term (≥38 gestational weeks) and tonsils from six healthy children, aged 1–9 years undergoing tonsillectomy due to enlarged tonsils, were collected at the Sahlgrenska University Hospital.

STUDY II: For peripheral blood samples we recruited 24 patients with manifest RA from the outpatient clinic at Sahlgrenska University Hospital, Gothenburg and Uddevalla Hospital, Sweden. The inclusion criteria were: female; Caucasian, aged 30-70 years; diagnosis of RA according to ACR 1987 criteria; disease duration 1-15 years. Exclusion criteria: Detectable autoimmune diseases other than RA; ongoing infection; cancer; previous or current treatment with B cell therapy. Disease activity was measured by swollen and tender joints, ESR, CRP and the composite index DAS28-ESR. In addition, ACPA and RF were measured. Disease severity was assessed according to radiographs of hands and feet. Eleven age- and sex-matched HDs were recruited. Patient and HD characteristics are shown in Table 6.

For SF eight RA patients (6 females and 2 males) with swollen knee joints were recruited from the outpatient clinic at Sahlgrenska University Hospital. Patient characteristics are shown in Table 7.

Table 6. *Clinical characteristics of patients and controls in Study II (peripheral blood analysis)*

Baseline characteristics	$ACPA^+/RF^+$ patients	$ACPA^-/RF^-$ patients	HDs (n=11)
Age (years)	52±12	57±10	48±16
Disease duration (years)	6±3	2 ±1	N/A
Tender joint count (0-28)	0.7±1.2	3.9 ± 4.2	N/A
Swollen joint count (0-28)	2.0±3.9	2.3±2.1	N/A
Patient global assessment of disease activity (0-100 mm, VAS)	29±23	33±25	N/A
CRP (mg/l)	4±4	4±3	N/A
ESR (mm/h)	9±7	13±9	N/A
DAS28-ESR score	2.29±0.84	3.2±1.0	N/A
Patient assessment of pain (0-100 mm, VAS)	25±18	30±18	N/A
HAQ (0-3)	0.52±0.47	0.63±0.53	N/A
Non-smoker	9 (53)	3 (43)	N/A
Current smoker	4 (24)	2 (29)	N/A
ACPA positive	15 (88)	0	N/A
RF positive	10 (59)	0	N/A
ANA positive	4 (24)	4 (58)	N/A
NSAIDs	10 (59)	5 (71)	N/A
Prednisolone	2 (12)	0	N/A
csDMARDs			
Methotrexate	16 (94)	5 (71)	N/A
Sulfasalazine	4 (24)	2 (29)	N/A
Hydroxychloroquine	1 (6)	0	N/A
TNF inhibitors	7 (41)	1 (14)	N/A

Data are denoted as number of patients as well as proportions (%) for categorical data and means ± standard deviation for continuous data. ANA, anti-nuclear antibodies; HAQ, health assessment questionnaire; N/A, not applicable.

Table 7. *Clinical characteristics of patients and controls in study II (used for SF and peripheral blood analysis*

Baseline characteristics	Patients							
	1	2	3	4	5	6	7	8
Age (y)	26	21	61	34	53	30	51	62
Gender	F	F	M	F	M	F	F	F
Disease duration (y)	6	16	37	3	21	2	10	16
Smoker	No	No	Yes	No	No	No	No	Ex
ACPA	pos	neg	pos	neg	pos	pos	pos	pos
RF	pos	neg	pos	neg	pos	pos	pos	pos
ANA	pos	pos	ND	neg	neg	neg	neg	neg
DMARDs	ETN	SZP	No	No	ETN	No	ETN	No
NSAID	Daily	No	No	As needed	No	No	As needed	No
Prednisolone (mg/d)	5 /d	No	5	5	No	No	No	7.5
Other diseases	No	No	ITP	No	No	No	No	Psoriasis

ETN, Etanercept; ITP, idiopathic thrombocytic purpura; ND, not done; SZP, Salazopyrin

STUDY III: Sixty patients with early untreated RA were recruited at the Rheumatology outpatient clinic at the Sahlgrenska University Hospital, Gothenburg, and the Rheumatology clinic at the University Hospital at Malmö and Lund. For comparison 28 age-and sex-matched donors were recruited. Inclusion criteria were: fulfilling the ACR/EULAR 2010 criteria for RA, ≥18 years old, at least 2 swollen joints and 2 tender joints, RF positive or ACPA positive or CRP ≥10 mg/ml, at least moderate disease activity (>3.2) by composite index disease activity score (DAS28)-CRP, duration of symptoms (retrospectively patient-reported joint pain) < 24 months, and no treatment with DMARDs or prednisolon. Patient characteristics are shown in Table 8.

Table 8. *Clinical characteristics of patients and controls in study III (peripheral blood analysis)*

Characteristics	RA patients (n=60)	HDs (n=28)
Age, yr	55 ± 15	55±15
Female, n (%)	44 (73)	17 (61)
Symptom duration, mo	6 ± 4	NA
Tender joint count, 0-28	10 ± 7	NA
Swollen joint count, 0-28	9 ± 5	NA
CRP,mg/l	25 ± 37	ND
ESR,mm/h	34 ± 28	ND
DAS28-CRP	5.07 ± 1.0	NA
DAS28-ESR	5.5 ± 1.1	NA
CDAI	30 ± 12	NA
Patient global assessment VAS in mm	57 ± 21	NA
HAQ-DI	1.04 ± 0.48	NA
Non-smoker, n (%)	27 (45)	ND
Ex-current smoker, n (%)	43 (5)	ND
ACPA ≥ 20 IU/ml, n (%)	47 (78)	ND
RF ≥ 20 IU/ml, n (%)	46 (77)	ND

Data are denoted as number of patients as well as proportions (%) for categorical data and means ± standard deviation for continuous data. NA, not applicable; ND, not done.

3.3 METHODS

3.3.1 RADIOGRAPHS OF HANDS AND FEET (STUDY II-III)

Radiographs of hands and feet were performed in routine care after disease duration of 7.1 ± 5.1 years. A certified assessor blinded to clinical data evaluated the radiographs. Joint damage in hands and feet was quantified using the modified Sharp van der Heijde score (mSHS, 0-488), scoring for degree of joint erosion (16 areas per hand, 6 areas per foot) and joint space narrowing (15 areas per hand and 6 areas per foot). The median score for ACPA/RF positive patients was 20; to provide the categories of less damaged (Low mSHS) or more damaged joints (High mSHS) the patients were split by the median mSHS score into those $<$ 20 and $\geq$ those 20 or above, respectively.

3.3.2 FLOW CYTOMETRY AND FLOW CYTOMETRY BASED METHODS (STUDY I-III)

The main methodology used in the present thesis is flow cytometry or fluorescence-activated cell sorting (FACS). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll in Study I-II. Whole blood was used in Study III. Non-specific binding to Fc-receptors was inhibited with mouse, rat and rabbit serum. To delineate the different B cell subpopulations the following markers were used for flow cytometry: CD19, CD21, CD27, IgD, IgG, IgM, CD24, and CD38 (Study I-III); IgA, CD10 and CD243 (ABCB1) (Study I). Additional cell markers were used to determine the role of the cells and activation status: CD40, CD62L, CD69, CD80, CD86, and CD95 (Study I) CD11c (Study I-II), and potential to mediate joint destruction: FcRL4, CXCR3, and RANKL (Study II).

Gating strategy of the main B cell subpopulations is shown in Figure 6. First gating was done on PBMCs (Study I-II) or whole blood (Study III) with a lymphocyte gate. Then singlets were gated, and thereafter the $CD19^{+}$ cells, i.e. B cells (Figure 6A). CD21 and CD23 were used in Study I and II to separate the $CD21^{+}$ and $CD21^{-/low}$ cells (Figure 6A). After Study I and II, we found that for our purposes it was not necessary to use CD23 in the definition of the cells. Therefore, in Study III we decided to exclude CD23 and analyze the cells based on their expression levels of CD21, i.e. as $CD21^{+}$ or $CD21^{low/-}$. The $CD21^{+}$ and $CD21^{l/low}$ cells were gated on separately using either CD38 vs. CD24 (Figure 6B) [163, 164] or the IgD vs. CD27 (Figure 6C) [164] strategy for subpopulations.

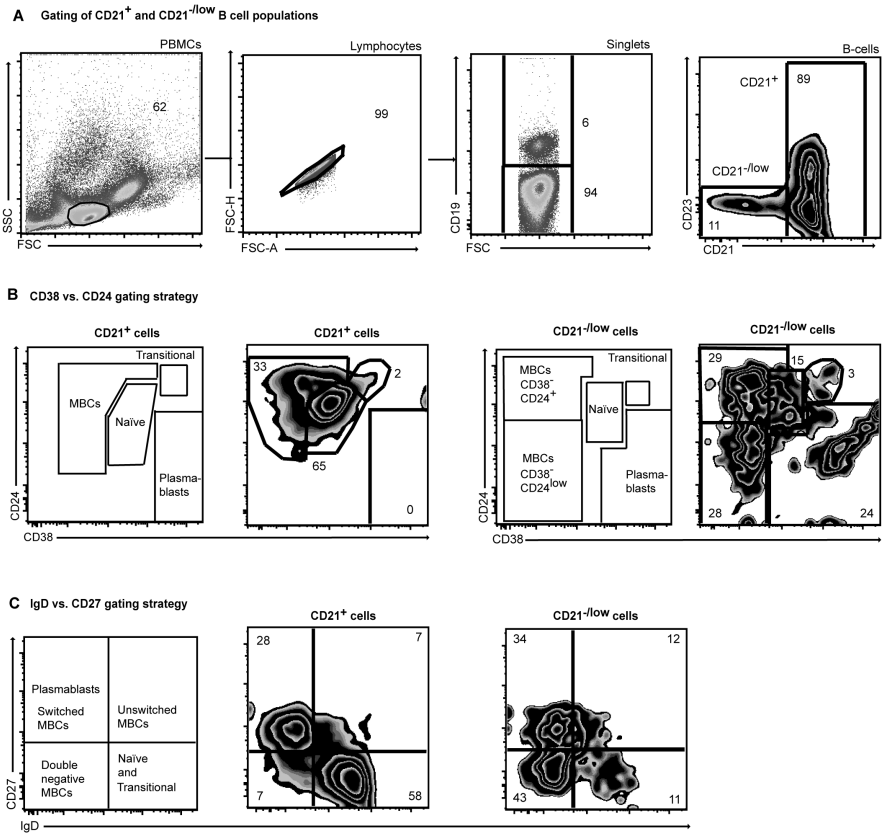

Figure 6. Gating strategies used in Studies I-III. A. Shows gating from lymphocytes to CD21^{+} vs. CD21$^{-/low}$ cells. Further gating of the CD21^{+} vs. CD21$^{-/low}$ cells is shown in B and C, where schematics and FACS plots are shown for B. CD38 vs. CD24 gating strategy and C. IgD vs. CD27 gating strategy. Sample from HD.

The CD38 vs. CD24 gating separates: Transitional (CD38^{++}CD24^{++}), naive (CD38+CD24+), MBCs (CD38^{+} that are either CD24^{+} or CD24^{low}) and plasmablasts (CD38^{++}CD24^{-}).

The IgD vs. CD27 gating separates the transitional and naive (CD27^{-}IgD^{+}), unswitched MBCs (CD27^{+}IgD^{+}), switched MBCs (CD27^{+}IgD^{-}) and DN MBCs (CD27^{-}IgD^{-}).

3.3.3 CELL SORTING (STUDY I)

Flow cytometry was also used for B cell sorting where we isolated $CD21^{+}CD27^{-}$ ($CD21^{+}$ naive and transitional cells) and $CD21^{+}$ $CD27^{+}$ ($CD21^{+}$ MBCs) and $CD21^{-/low}$ cells. First B cells were purified from PBMCs using immunomagnetic beads (purity 99%). The B cells were then stained with antibodies recognizing $CD19^{+}$, $CD27^{+}$ and $CD21^{+}$ and sorted on a Synergy cell sorter (purity >95%).

3.3.4 CD69 UPREGULATION ON CELL SURFACE (STUDY I)

We cultured PBMCs or B cells (purified as described in section 3.3.3) in 96-well plates and stimulated with the following single stimuli: TLR7/8 agonist (R848), TLR9 agonist (CpG), BCR agonist (anti-human IgA/IgG/IgM F(ab')2) and IL-2. CD69 upregulation was assessed after 3 h by flow cytometry.

3.3.5 PROLIFERATION AND DIFFERENTIATION OF B CELLS AND SUBPOPULATIONS (STUDY I)

Total B cells or subpopulations (purified as described in section 3.3.3) were labeled with CellTrace Violet Cell Proliferation Kit and cultured in 96 well plates. The cells were stimulated with different combinations of the stimuli used in 3.3.4: TLR7/8 agonist, TLR9 agonist, BCR agonist and IL-2. We assessed cell proliferation and plasmablast differentiation at day 5 by flow cytometry.

3.3.6 CHEMOKINE ANALYSIS (STUDY II)

To study the migration pattern of the synovial B cells the following chemokines were analyzed: CXCL-9, CXCL-10 and CXCL-11 in SF and blood plasma of patients with RA (Table 7). Analysis of the chemokines was performed using cytometry bead-based immunoassay (LEGENDplex™ Human Proinflammatory Chemokine Panel, BioLegend) according to the manufacturer's instructions. Samples were acquired with a FACSVerse equipped with FACSuite software (BD Bioscience, San Jose, USA) and analyzed by FCAP Array software (Soft Flow, Pecs; Hungary). Plasma and SF were diluted 1:2 in PBS and treated with heteroblock (HeteroBlock®, Omega Biologicals, USA) before analysis.

3.3.7 ELISA ANALYSIS OF RANKL/OPG (STUDY II)

Concentrations of osteoprotegerin (OPG) and RANKL in plasma and SF were measured using ELISA kits according to the manufacturer's instructions (Human Osteoprotegerin/TNFRSF11B Duoset ELISA, Human TRANCE/RANKL/TNFSF11 Duoset ELISA, R&D Systems, USA). Plasma and SF of patients (Table 7) were diluted 1:2 in PBS and treated with heteroblock (HeteroBlock®, Omega Biologicals, USA) before analysis.

3.3.8 STATISTICAL ANALYSIS (STUDY I-III)

Study I: Graph-Pad Prism version 6 was used for statistical analyses. Comparisons between two groups was analyzed using unpaired Mann-Whitney test and between >2 groups one-way analysis of variance (ANOVA) was used followed by Dunnett's multiple comparison test. $P<0.05$ was considered significant.

Study II: Comparisons of B cell populations in patients and HDs were compared using a two-tailed Mann-Whitney test or Kruskal-Wallis test. $P<0.05$ was considered significant. The correlations between B cell subpopulations and clinical factors were calculated using Spearman's rank test. In this explorative study we decided against applying multiple significance test such as Bonferroni as it would reduce the chance of type I error at the expense of type II error [165]. Statistical analyses were performed using GraphPadPrism version 7 and IBM SPSS Statistics version 23.

Study III: In large data set, multivariate factor analysis (SIMCA) can be applied to reveal patterns and relations between the analyzed factors. To investigate the associations between B cell subpopulation proportions (X-variables) and clinical diagnosis of RA (Y-variable) or clinical parameters (Y-variables) multivariate factor analysis (SIMCA-P+ software; Umetrics, Umeå, Sweden) was used. To investigate whether patients could be discriminated from HDs based on the various X-variables i.e. B cell populations, **o**rthogonal **p**rojections to **l**atent **s**tructures **d**iscriminant **a**nalysis (OPLS-DA) was used. VIP (variable importance for projection) values were used to identify X-variables that associated most strongly with the respective Y variable. When examining associations between a Y-variable (clinical parameters such as DAS28) and X-variables (B-cell populations) orthogonal projections to latent structures by means of partial least squares (OPLS) was used. Data was normalized using log transformation. In the analyses the column bars represent the importance of each X-variable to the Y-variable, i.e. the larger the bar is the stronger the contribution to the model is. The error bar represents the certainty of the contribution. The quality of the OPLS

models was assessed based on the parameters R2 (i.e., how well the variation of the variables is explained by the model), and Q2 (i.e., how well a variable can be predicted by the model).

Univariate analysis of B cell populations between groups was performed with a two-tailed Mann-Whitney test for comparisons of two groups and a Kruskal-Wallis test for comparisons of three groups (IBM SPSS Statistics version 23). For correlation analysis, a two-tailed Spearman's Rank correlation test was used (IBM SPSS Statistics version 23). A $P<0.05$ was considered statistically significant. Linear regression was used to adjust for age when looking at the association between $CD21^+$ DN and TJC28 as well as $CD21^-$DN and joint narrowing score.

4 RESULTS AND DISCUSSION

4.1 STUDY I: CD21$^{-/LOW}$ B CELLS IN HUMAN BLOOD ARE MEMORY CELLS

A study had reported that cells lacking CD21 and CD23, CD21$^{-/low}$ cells were autoreactive in *SLC*$^{-/-}$ mice but not in control mice [94]. In translational effort, we set out to determine whether we could define a similar B cell subpopulation in humans. In Study I we looked at the CD21$^{-/low}$ subset in cord blood and adult peripheral blood, characterizing a CD21^{-}CD23^{-} population, which we defined as the CD21$^{-/low}$ population. We also asked which B cell population these cells belonged to e.g. naive or MBCs (Figure 7).

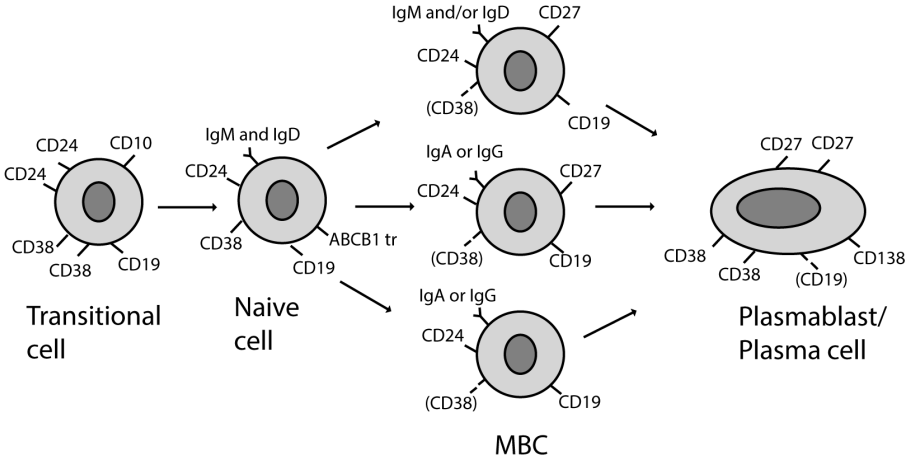

Figure 7. Main B cell populations in human peripheral blood: Transitional, naive and MBCs and plasmablasts/plasma cells. Typical cell surface markers are shown. Some plasmablasts/plasma cells express CD19 whereas others have low levels or lack CD19 [92]. ABCB1 tr., ABCB1 transporter.

When we looked at CD21$^{-/low}$ cells in peripheral blood of adults and cord blood we found large differences. The CD21$^{-/low}$ cells in cord blood were primarily CD10^{+} transitional cells but only 10% of the CD21$^{-/low}$ population in adults belonged to the transitional compartment. In fact, after excluding CD10^{+} cells i.e. transitional cells, a CD21$^{-/low}$ population was barely detectable in cord blood but comprised around 5% of the B cells in peripheral blood from adults (both in 24-30 and 55-64 years olds). This was in accordance with previous studies that show that MBCs are rare in the blood of infants until 2 years of age [166]. This also suggested that the CD21$^{-/low}$ cells

seen in adults were not transitional cells but belonging to a more mature B cell population.

We did not exclude transitional cells from further experiments, as they were so few amongst the CD21$^{-/low}$ cells. When we used the classical memory marker CD27 [88] we found that it was expressed by only 30% of CD21$^{-/low}$ cells in peripheral blood. This would indicate that the cells were mainly naive. However, 75% of CD21$^{-/low}$ cells in tonsils expressed CD27, thus indicating memory cells. To further delineate the phenotype we used multiple markers to characterize the phenotype and we also included tonsils for comparison. When we used the CD38 and CD24 gating strategy we found that the CD21$^{-/low}$ cells in adult peripheral blood were primarily CD38^{-} (85%), which indicated a memory cell population rather than naive B cell or plasmablasts (Figure 8).

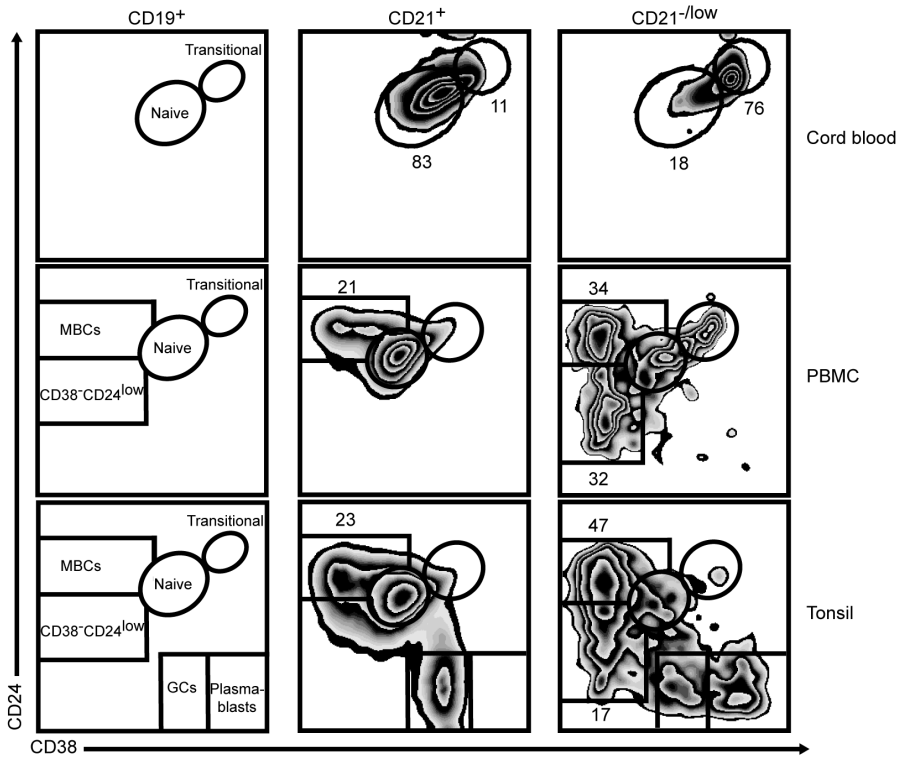

Figure 8. Gating strategy CD38 vs. CD24 is shown in the schematics. Representative samples from cord blood, adult peripheral blood (PBMCs) and tonsils are depicted. GCs, germinal center B cells.

Of the $CD21^{-/low}$ cells in peripheral blood around 15% were $IgM^{+}IgD^{dim}$, 17% IgG^{+} and 9% IgA^{+} % consistent with not being naive B cells. However, as there was still a large proportion that was $IgM^{+}IgD^{+}$ we wanted to see whether we could exclude the possibility that the $CD21^{-/low}$ cells belonged to naive cells. For this purpose we used expression of the marker ABCB1 transporter as it is only expressed on naive cells [167], and found that $CD21^{-/low}$ cells lacked the transporter. In addition, we found that the $CD21^{-/low}$ cells resembled MBCs more than naive cells ($CD21^{+}CD27^{+}$ and $CD21^{+}CD27^{-}$ respectively) when analyzing their expression of activation markers i.e. CD80 and CD95. The $CD21^{-/low}$ cells also showed an increased downregulation of CD62L compared to naive cells and MBCs, indicating previous activation. Taken together the phenotype of the $CD21^{-/low}$ cells using multiple markers indicated that they were mainly MBCs.

Next we wanted to investigate whether the $CD21^{-/low}$ cells behaved similar to MBCs. Planning this experiment, we anticipated that the cells would be less responsive to BCR stimuli because the lack of CD21 would probably raise the activation threshold for BCR activation. However, we were unsure how the $CD21^{-/low}$ cells would respond to other stimuli i.e. TLR agonists and cytokines. For this purpose we stimulated the cells in vitro with a single stimuli:

- IL-2 or,
- BCR agonist or,
- TLR7/8 agonist or,
- TLR9 agonist or,

We then measured the upregulation of CD69 on the cell surface as it is one of the earliest inducible leucocyte activation markers (3 hours) [168]. Again, we compared the $CD21^{-/low}$ cells to the $CD21^{+}$ naive and $CD21^{+}$ MBCs and found that the $CD21^{-/low}$ cells did not respond as well as the other populations to the stimuli (except for IL-2 single stimuli where none of the cell populations responded) (Figure 9). Our previous analysis of the $CD21^{-/low}$ cells phenotype had revealed that the $CD38^{-}$ could be divided into two groups, $CD24^{+}$ and $CD24^{low}$, where the $CD24^{+}$ cells were enriched for IgM^{+} IgD^{+} cells and the $CD24^{low}$ cells for $IgM^{-}IgD^{-}$ cells. This difference in phenotype was also reflected in their response, as mainly the $CD38^{-}$ $CD24^{low}$ subpopulation was unresponsiveness to any of the single stimuli (Figure 9).

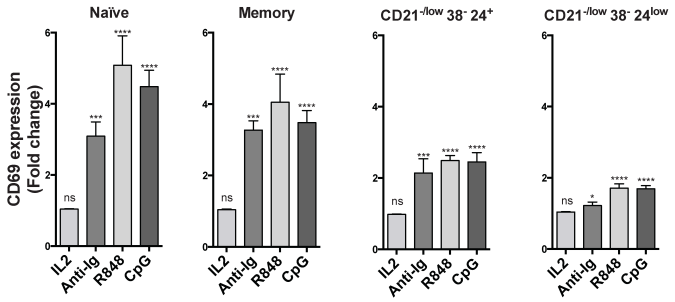

Figure 9. Expression of CD69 upon single stimuli is shown for CD21^{+} naive and memory cells and the CD24^{+} and CD24^{low} subsets of CD21$^{-/low}$ CD38^{-} cells. R848, TLR7/8 agonist; CpG, TLR9 agonist.

However, we had so far only used single stimuli and our measurement of response was only the upregulation of the CD69 marker. This did not tell us anything about the CD21$^{-/low}$ cells ability to proliferate and differentiate upon stimulation. We decided to test that in our next experiment. For stimulation, we chose to use the combination of IL-2 and a TLR7/8-agonist as it had induced the highest level of proliferation in total B cells. We sorted the cells into the following subpopulations CD21^{+}CD27^{+}, CD21^{+}CD27^{-} and CD21$^{-/low}$ cells before stimulation and culture for five days. The sorting was necessary as stimulation of the B cells for longer periods of time (as in this experiment) leads to downregulation of CD21 on all B cells, hence we would not be able to electronically gate on CD21-positive and negative cells [169]. It is known that MBCs respond with proliferation and differentiation to TLR agonists without any BCR stimulation but that the naive B cells need BCR stimulation as well [170]. Our results showed that the naive cells did not proliferate upon IL-2 and TLR7/8 agonist stimulation whereas a similar proportion of both CD21^{+} MBCs and CD21$^{-/low}$ cells proliferated (88% and 75%, respectively) (Figure 10A, upper row). This strongly suggested that the CD21$^{-/low}$ cells were MBCs. Further addition of a BCR agonist induced proliferation of all three cell populations: CD21^{+} naive and MBCs and CD21$^{-/low}$ cells (Figure 10B, upper and middle row). Again the response of CD21$^{-/low}$ cells was very similar to the MBCs but not to the naive cells (Figure 10B, upper and middle row). We also looked at the cells ability to differentiate in response to aforementioned combination stimuli. Fewer than 5% of naive cells differentiated after stimulation with either stimuli combination (Figure 10A and B, bottom row). Stimulating with IL-2 and TLR7/8 agonist induced around 20% of CD21^{+} MBCs and 10% of CD21$^{-/low}$ B cells to differentiate (Figure 10A, bottom row). Adding the BCR agonist to the stimuli increased the proportion differentiated in both CD21^{+} MBCs and CD21$^{-/low}$ cells to around 40% (Figure 10B, bottom row). Our conclusion was that the CD21$^{-/low}$

cells belong to the memory compartment based on their phenotype and response to stimuli.

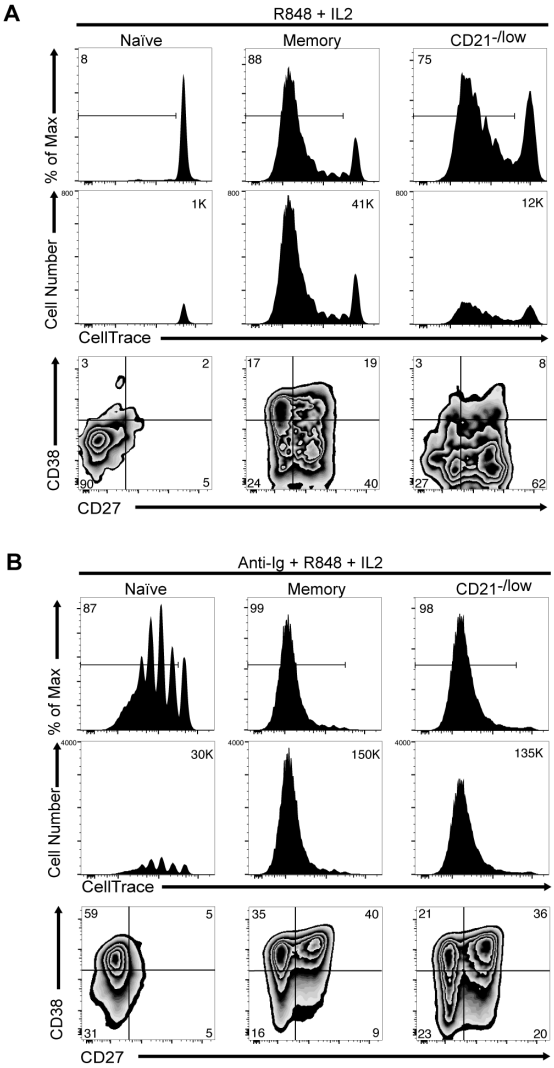

Figure 10. Proliferation and differentiation of B cell subpopulations upon stimuli with A. TLR7/8-agonist (R848) and IL-2. B. TLR-7/8 agonist, BCR-agonist and IL-2. Upper row demonstrates proliferation as a proportion of the maximum proliferation, middle row the absolute cell numbers. Bottom row shows the proportion of cells that has differentiated into plasmablasts ($CD27^{++}CD38^{++}$)

4.2 STUDY II: CD21$^{-/LOW}$ B CELLS ASSOCIATE WITH JOINT DAMAGE IN RHEUMATOID ARTHRITIS PATIENTS

Our results in Study I demonstrated the presence of a CD21$^{-/low}$ cell population in HDs. We thereafter set out to investigate the CD21$^{-/low}$ cells in autoimmune disease because of the findings that the CD21$^{-/low}$ cells in the *SLC*$^{-/-}$ mouse model but not in the control mice were autoreactive [94]. We chose to look in patients with RA because the disease is well defined in terms of disease activity and severity. We divided the RA patients into two groups based on the presence or absence of autoantibodies (ACPA and RF) and analyzed their B cell populations (Gating strategy is shown in Figure 11).

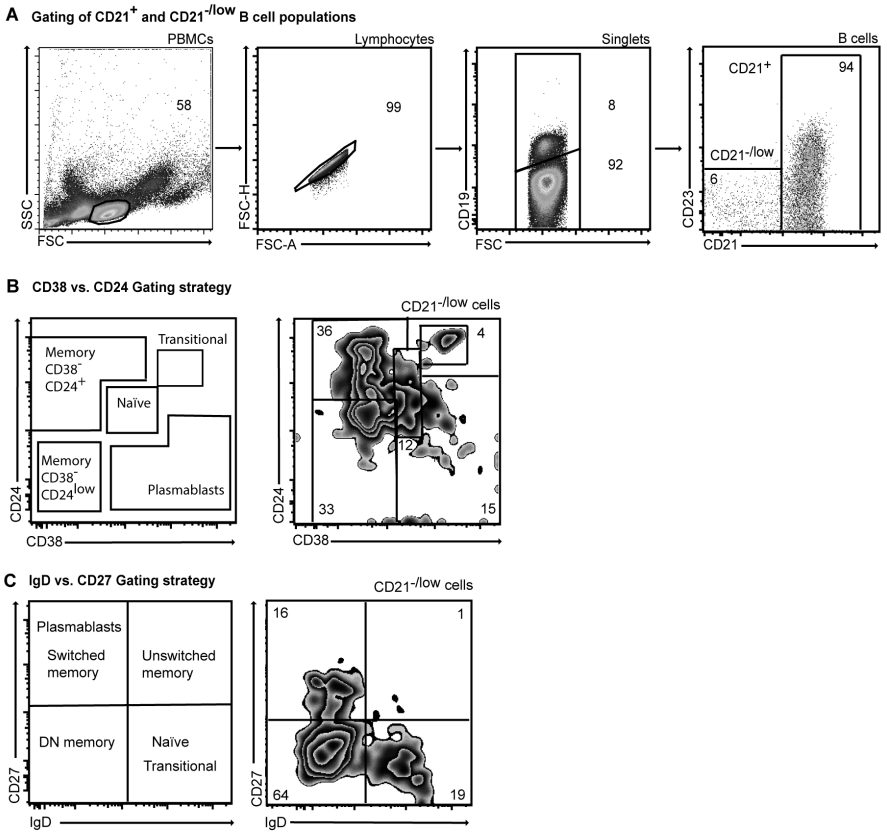

Figure 11. Gating strategy of B cell subpopulations is shown. A. First we gated on PBMCs for lymphocytes, then singlets and further CD19^{+} B cells. After dividing B cells into CD21^{+} and CD21$^{-/low}$ subsets, two main gating strategies were used: B. The CD38 and CD24 gating strategy C. The IgD and CD27 gating strategy.

There were no significant differences in the absolute numbers and proportions of $CD21^{-/low}$ B cells between the two RA patient groups and the HDs. Just as we had seen for HDs in Study I the phenotype of the $CD21^{-/low}$ cells in RA patients indicated that they were mainly MBCs as around 70% were $CD38^{-}$ and approx. 80% were either $CD27^{+}$ or $CD27^{-}IgD^{-}$. Additionally there was an overlap between the differentially defined memory subpopulations, such that 55% of the $CD21^{-/low}CD38^{-}CD24^{low}$ cells were also $IgD^{-}CD27^{-}$ ($CD21^{-/low}$ DN MBCs). The proportion of these two MBC populations $CD21^{-/low}$ $CD38^{-}CD24^{low}$ and $CD21^{-/low}$ DN were elevated in ACPA/RF positive patients relative to HDs. Differences between the ACPA/RF negative patients and HDs were not statistically significant.

Next we wanted to investigate whether these B cell subpopulations were associated with clinical parameters. We did not find any association between the different subpopulations and disease activity. However, it should be noted that the patients had mainly low-active disease so we were unable to compare low-activity versus high-activity. We found no correlations with disease duration or treatment (including present or previous TNF inhibitory therapy). Here it should also be taken into account that the groups were small. It is known that ACPA/RF positive patients have worse prognosis than ACPA/RF negative patients with an increased risk of developing joint erosions and deformities. As expected, joint destruction (measured by modified Sharp van der Heijde score (mSHS)) was greater in ACPA/RF positive patients than in ACPA/RF negative patients (median scores were 25 (IQR 12-35) and 15 (IQR 12-17) respectively). mSHS is a composite score combining an erosion score, quantifying bone loss and a joint narrowing score, measuring cartilage destruction. The ACPA/RF positive patients were divided into two groups at the median mSHS to produce Low mSHS (<20) and High mSHS (≥20). The proportion of $CD21^{-/low}$ B cells between the two groups showed that the $CD21^{-/low}$ DN population in patients with High mSHS was significantly expanded relative to the same population in both Low mSHS patients and HDs (Figure 12A). Moreover, the frequency of the $CD21^{-/low}$ DN MBCs was positively and significantly correlated with mSHS in ACPA/RF positive patients (Figure 12B). The erosion score is characteristic of RA but the joint narrowing score, i.e. the breakdown of cartilage, can be attributed to other causes than RA, such as osteoarthritis. Separating the two components of mSHS showed that the greater contribution to joint damage was derived from the erosion score for which the positive correlation with the $CD21^{-/low}$ DN population approached statistical significance, whereas the joint narrowing score did not (Figure 12C and data not shown). We interpreted our findings as a link between expansion of the $CD21^{-/low}$ DN MBC population and erosive joint damage in RA.

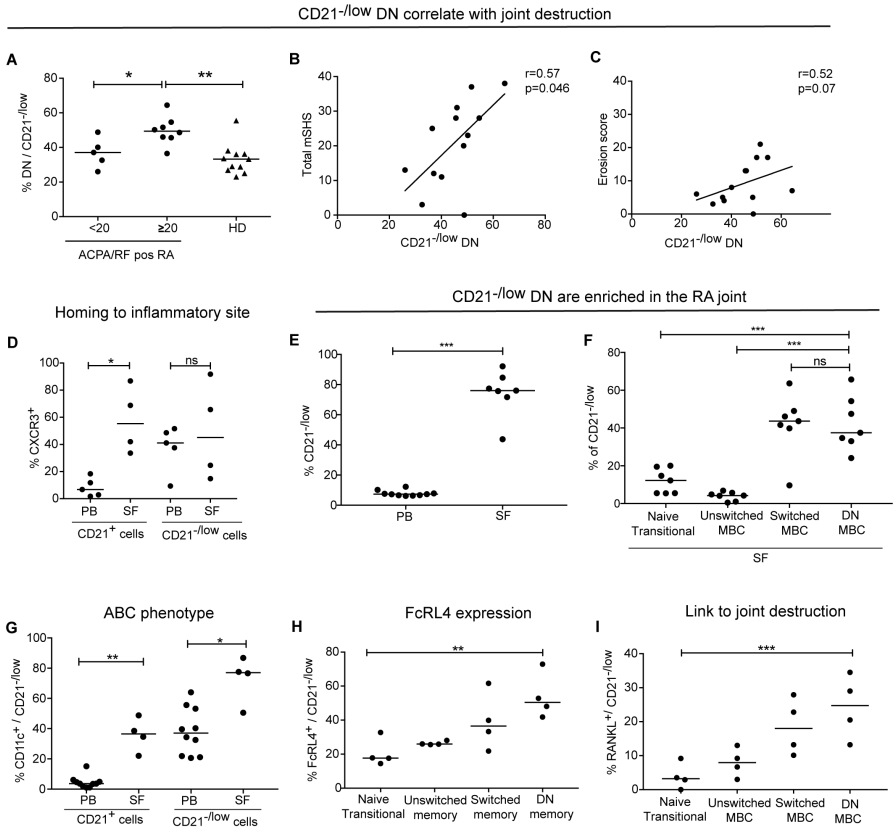

Figure 12. CD21$^{-/low}$ DN cells associate with joint damage. A. Proportion of CD21$^{-/low}$ DN in ACPA/RF positive RA patients with High mSHS (≥20) and Low mSHS (<20) as well as HDs. B-C. Correlation of the frequency of CD21$^{-/low}$ DN in ACPA/RF positive RA patients and B. total mSHS and C. Erosion score. D. CD21$^{-/low}$ DN cells express CXCR3 that indicates homing to inflammatory sites. E-F. CD21$^{-/low}$ DN cells are enriched in SF. G-I. CD21$^{-/low}$ DN express G. FcRL4 H. CD11c and I. RANKL

The next question we asked was whether any of the B cells recruited to the target organ, i.e. the joint, was CD21$^{-/low}$ and, if so, whether they were DN. In SF, we found that around 80% of the B cells were CD21$^{-/low}$ and that around half of these cells expressed CXCR3 supporting homing to inflammatory sites (Figure 12D). CXCR3 was also expressed on approximately half of the CD21^{+} cells in SF (Figure 12D). Additionally, the CD21$^{-/low}$ cells in SF were mainly composed of DN and switched MBCs (Figure 12E and F).

A marker that has been associated with the CD21$^{-/low}$ cells is CD11c [171]. There were large differences in the expression of CD11c between CD21^{+} and

$CD21^{-/low}$ cells. In blood it was barely detectable on $CD21^{+}$ cells whereas around 35% of the $CD21^{-/low}$ cells were $CD11c^{+}$ (Figure 12G). This difference became more pronounced in the SF, where the vast majority of the $CD21^{-/low}$ cells were $CD11c^{+}$ (including the DN) and around one third of the $CD21^{+}$ cells.

RANKL is essential for osteoclastogenesis, which leads to breakdown of bone and can thus contribute to joint destruction [172]. An $FcRL4^{+}$ B cell population expressing $RANKL^{+}$ has been described in synovial tissue and fluid from RA patients [160]. We found that few B cells in blood expressed FcRL4, which is in line with previous studies demonstrating that $FcRL4^{+}$ cells are found in tissues but are scarce in blood [102]. Furthermore, around 40% of the $CD21^{-/low}$ B cells in SF were $FcRL4^{+}$ and these were concentrated in the DN memory population of which around half were $FcRL4^{+}$ (Figure 12H). We also investigated whether the $CD21^{-/low}$ DN cells could possibly influence joint erosions and looked at their expression of RANKL. There was no detectable expression of RANKL on any B cell subpopulation in blood (data not shown), whereas around 30% of the $CD21^{-/low}$ DN cells and 20% of the switched MBCs ($CD27^{+}IgD^{-}$) in SF expressed RANKL (Figure 12I). The ratio of soluble RANKL/Osteoprotegerin (RANKL/OPG) can be used as a measure of the potential for bone erosion [173, 174]. We measured this ratio in blood and SF and found that it was constant for all subjects ruling out soluble RANKL as an agent for bone damage in RA.

In conclusion, the $CD21^{-/low}$ DN MBCs in ACPA/RF positive patients associate with joint damage based on its higher proportions in patients with higher mSHS score compared to those with lower scores. In addition the frequency of $CD21^{-/low}$ DN cells correlated with mSHS. This population is enriched in SF of RA patients and likely overlaps with previously described $FcRL4^{+}$ cells in SF. The $CD21^{-/low}$ DN MBCs express RANKL, further linking these cells to joint destruction.

4.3 STUDY III: MEMORY B CELL SUBSETS CORRELATE WITH AUTOANTIBODY TITERS, DISEASE ACTIVITY AND JOINT DAMAGE IN UNTREATED EARLY RHEUMATOID ARTHRITIS

In study II, we investigated RA patients with established RA, under treatment and with low disease activity. In Study III, we studied B cell populations in a cohort of untreated early RA (ueRA) patients. The advantages of studying untreated patients were that the patients had highly active RA and differences in B cell populations between ueRA patients and HDs could not be due to the effect of treatments. Thus, differences in B cell populations between patients and HDs would potentially be more clear and robust.

Analysis of the B cell subpopulations was done similarly to study II, where we utilized two main gating strategies i.e. CD38 vs. CD24 and IgD vs. CD27. For defining the $CD21^{-/low}$ cells we only used CD21 and not CD23, as it was now clear from Study I-II that $CD21^{-/low}$ could be defined without using CD23. This also allowed us to use other markers in its place that would provide more information, e.g. IgM and IgG for isotypes. Analysis using the aforementioned gating strategies generated many different B cell subsets, so in order to see whether any specific B cell population associated with RA and/or clinical parameters, we applied multivariate analysis. Age and gender were included in the analysis as possible confounders but did not affect the results. We found that the $CD21^{+}$ B cell populations with the strongest positive associations to untreated early RA (ueRA) were the naive and transitional cells, gated either as $CD27^{-}IgD^{+}$ (naive and transitional cells) or $CD38^{+}CD24^{+}$ (naive only cells) (Figure 13A). The strongest negative association to ueRA was with the MBCs ($CD38^{-}CD24^{+}$), both unswitched ($CD27^{+}IgD^{+}$) and switched ($CD27^{+}IgD^{-}$) MBCs. These associations were confirmed with univariate analyses (Figure 13B-E and data now shown). The decrease of MBCs seen here in ueRA is also observed in other autoimmune diseases [175-177]. What this decrease stands for is currently unknown but it could be due to MBCs leaving the circulation and homing to inflamed tissues e.g. the joints in RA. Unfortunately we did not have smoking data for the HDs so we could not include smoking in the analysis.

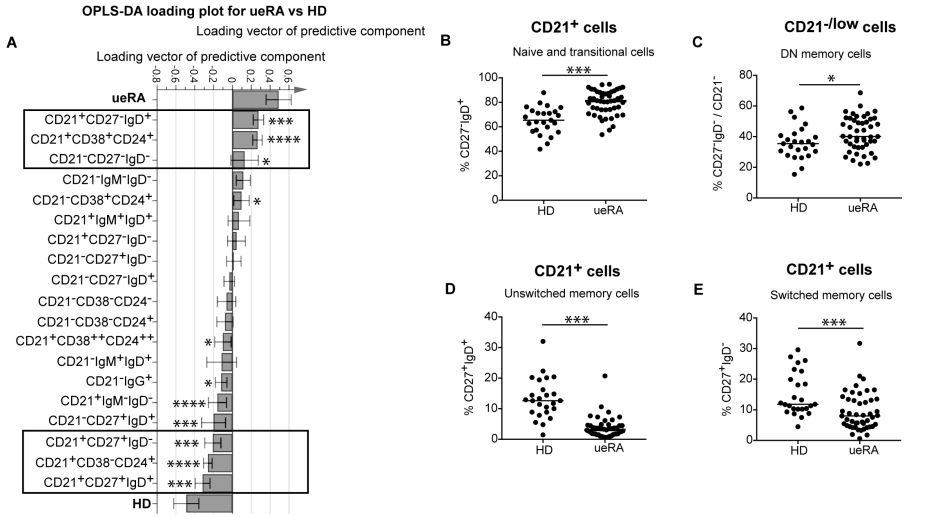

*Figure 13. B cell populations in ueRA patients and HDs. A. Plot shows associations between B cell populations in ueRA patients and HDs using multivariate analysis. Stars indicate significant differences between the two groups using univariate analysis. In B.-E. univariate analysis was used to compare the frequencies of B. CD21^{+} naive and transitional cells (CD27^{-}IgD^{+}), C. CD21$^{-/low}$ DN (CD27^{-}IgD^{-}) MBCs, D. CD21^{+} unswitched (CD27^{+}IgD^{+}) MBCs and E. CD21^{+} switched (CD27^{+}IgD^{-}) MBCs between ueRA patients and HDs. Line represents median. Mann-Whitney test. *<0.05, **<0.01, ***<0.001, ****<0.0001.*

Because we had found in study II that the CD21$^{-/low}$ DN (CD27^{-}IgD^{-}) population in patients with established RA was enriched in the inflamed joints and that this population correlated with the joint destruction, we were interested in its association also to ueRA. Unlike the unswitched and switched CD21^{+} MBC populations, which were reduced in ueRA, we found a positive association between ueRA and CD21^{-} DN MBCs that was confirmed in univariate analysis (Figure 13 A and C).

Our next question was whether the $CD21^+$ naive and transitional B cells were associated with disease activity. Using multivariate analyses, we saw a possible effect of smoking (Figure 14A), which was confirmed in univariate analysis (Kruskal-Wallis test) comparing the proportions of $CD21^+$ naive and transitional B cells in current, ex-smokers and non-smokers (74%, 75% vs. 83% respectively, with p=0.03). We therefore looked at the disease activity separately in these two groups of patients i.e. non-smokers and current/ex-smokers. In non-smokers, there was a negative correlation between the frequency of $CD21^+$ naive and transitional cells and disease activity measured by DAS28-CRP and TJC28, i.e. the higher the frequency of these B cells the lower the disease activity (Figure 14B and C). No significant correlations were found in RA patients that were ex-/current smokers.

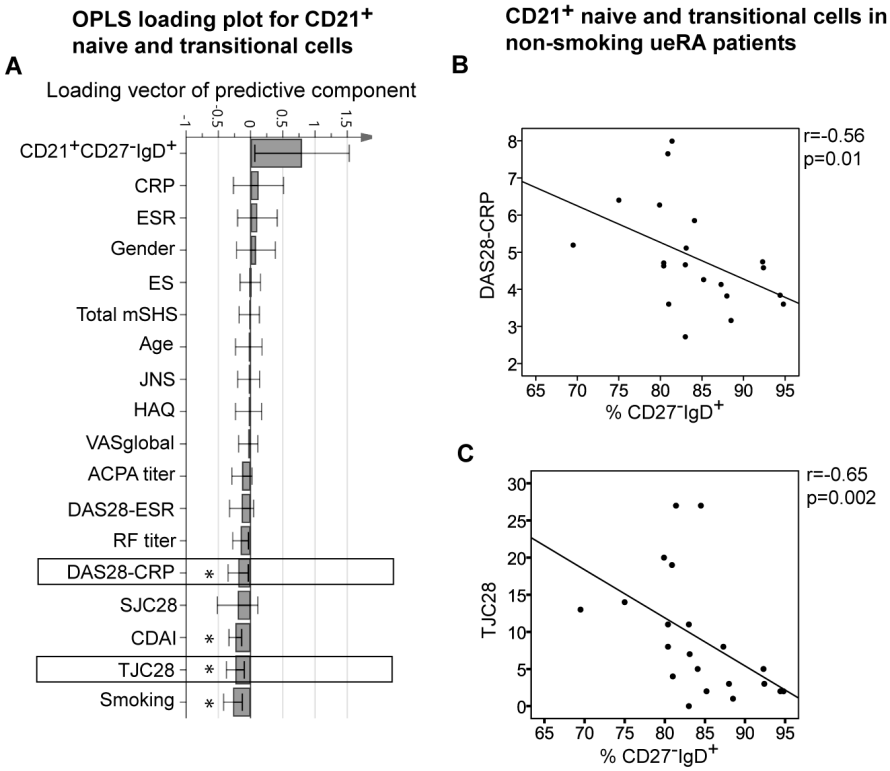

*Figure 14. The association between $CD21^+$ naive and transitional cells and clinical disease activity in RA patients. A. Multivariate analysis demonstrated association between $CD27^-IgD^+$ cells and smoking as well as disease activity that was confirmed in univariate analysis B-C. (Spearman test). *<0.05,*

This observation raised the question of whether there might be a correspondingly higher frequency of a specific memory B cells population correlating to disease activity. We found that the $CD21^{+}$–DN population was associated the clinical factors CDAI, DAS28-ESR, DAS28-CRP, SJC28, TJC28 as well as age (Figure 15A) and univariate analysis confirmed a positive correlation between $CD21^{+}$–DN and TJC28 (Figure 15B) that remained significant after adjusting for age with linear regression.

Keeping in mind our previous results from study II, where we revealed a possible role for $CD21^{-/low}$ DN B cells in joint damage in RA patients with established disease, we investigated a potential association with clinical parameters in ueRA. Using multivariate analyses, we found that $CD21^{-/low}$ DN cells were positively associated with joint narrowing score (JNS), total erosion score (mSHS) and age, as well as negatively associated with TJC28 and ACPA titers (Figure 15C). After adjusting for age, a significant positive correlation between frequency of $CD21^{-/low}$ DN cells and joint narrowing score remained (Figure 15D), linking the enhanced presence of this B cell population with joint damage.

Smoking seemed to influence our multivariate models of $CD27^{+}$ MBCs and this was confirmed in a univariate analysis for $CD21^{+}CD27^{+}$ unswitched MBCs comparing non-smokers vs. ex/current smokers (2 % vs. 4 % respectively, $p<0.05$). Therefore, to look at whether there were differences in $CD27^{+}$ MBCs depending on autoantibody status we looked separately in non-smokers and ex-/current smokers. As almost all of the ex-/current smokers were RF and/or ACPA positive we could only investigate the effect of autoantibody status in non-smokers. We found significantly higher frequencies of $CD21^{+}$ switched MBCs in ACPA/RF positive non-smoking ueRA patients compared to ACPA/RF negative RA (7% vs. 4%, $p =0.035$).

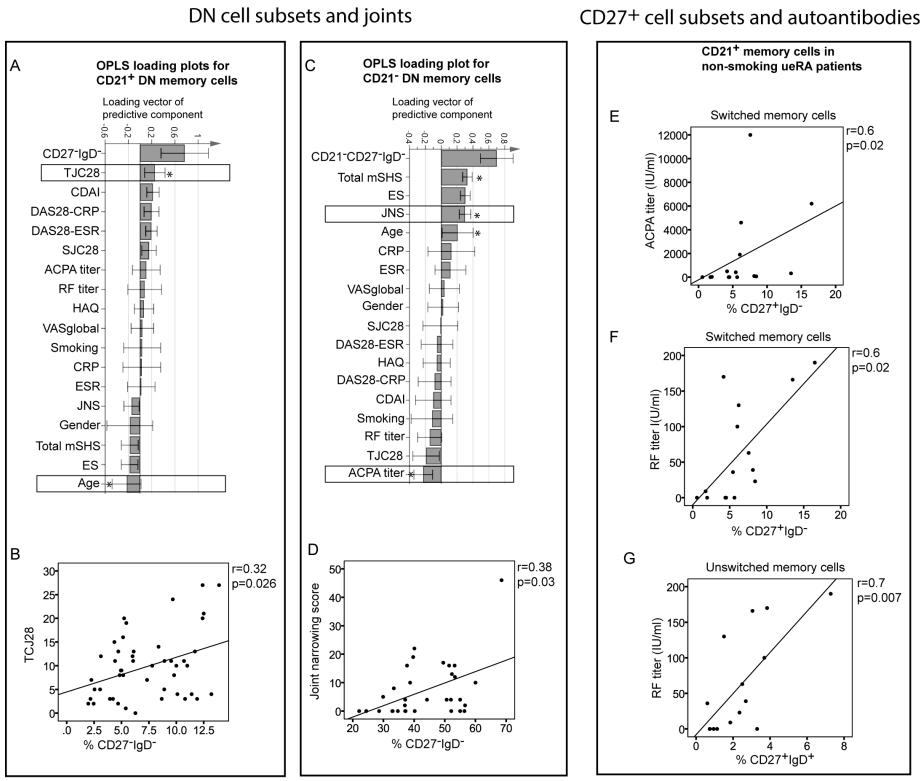

Figure 15. Figure shows the associations found in study III between B cell populations and measures of disease activity, severity and autoantibodies.

Few studies have shown correlations between B cell populations and autoantibody titers. In this study we found that in non-smokers, but not ex-/current smokers, there was a positive correlation between $CD21^+$ switched MBCs and both ACPA and RF titer (Figure 15E and F) and between $CD21^+$ unswitched MBCs and RF titer (Figure 15G) linking both switched and unswitched MBCs to the autoantibody production. Possible mechanism behind these associations could be that in RA a proportion of the $CD21^+$ unswitched MBCs becomes IgM^+IgD^+ plasmablasts secreting RF (which is mainly IgM) and additionally, a proportion of the $CD21^+$ switched MBCs becomes IgM^+ plasmablasts secreting RF or IgG^+ plasmablasts secreting ACPA (which is mainly IgG).

5 CONCLUSION

The main findings in the thesis are as follows:

- The $CD21^{-/low}$ B cell population is mainly MBCs both in HDs and RA patients. Similar to other $CD21^{-/low}$ populations described in autoimmune disease, the $CD21^{-/low}$ DN MBCs express CD11c and FcRL4. However, in the other diseases they are not necessarily DN i.e. $CD27^{-}IgD^{-}$.
- In ueRA patients we conclude that the $CD21^{+}$ DN B cells play a role in joint inflammation and the $CD21^{+}$ $CD27^{+}$ MBCs in autoantibody production. These conclusions are based on our findings that these cell populations correlated positively with tender joint count and with autoantibody titers respectively.
- Smoking is associated with an increase in the proportion of $CD27^{+}$ MBCs suggesting that it affects the memory population of B cells. An observation that suggests that smoking should be taken into account when designing B cell studies.
- The main conclusion is that $CD21^{-/low}$ DN MBC population may contain pathogenic clones that lead to joint damage via RANK/RANKL interactions. This is supported by our findings that:
  - $CD21^{-/low}$ DN MBCs are increased in ACPA/RF positive patients with established RA compared to HDs. Furthermore, they are increased in ACPA/RF positive RA patients with more joint destruction compared to those with less. Moreover, the $CD21^{-/low}$ DN MBCs correlated positively with the level of joint destruction.
  - $CD21^{-/low}$ DN MBCs express a homing marker i.e. CXCR3 for inflammatory sites and are greatly enriched in the joints of RA patients.
  - In the inflamed RA joint, a third of the $CD21^{-/low}$ DN MBCs express RANKL further tying them to joint erosions as RANKL stimulates the maturation of osteoclasts, which leads to bone erosions.
  - In ueRA the $CD21^{-/low}$ DN MBCs correlated positively with joint narrowing score.

6 FUTURE PERSPECTIVES

Our main findings suggest that different B cell populations are associated with disease activity, disease severity and autoantibodies in RA patients.

In early RA patients the next step is to see whether the correlations we have seen continue after the start of therapy. Do the $CD21^{+}CD27^{+}$ MBCs follow changes in autoantibody titers? Do the DN cell populations associate with joint damage and inflammation in the patients after 1 year? After 2 years? Another important question is whether any of these B cell populations can predict treatment response to biologic therapy and whether the B cell populations can differentiate between the different treatments, e.g. patients that have a high frequency of DN B cells should receive treatment X instead of treatment Y.

We want to look further at the function of the $CD21^{-/low}$ cells in RA patients and to see whether they respond differently to stimuli than $CD21^{-/low}$ cells in HDs. It would be interesting to test the different combination of TLR7/TLR9 agonists, BCR agonists and IL-2, as well as other cytokines. What would also provide more clues about the role of $CD21^{-/low}$ cells is information about what they secrete upon stimuli i.e. pro-inflammatory cytokines, anti-inflammatory cytokines, autoantibodies or RANKL. It would also be important investigate whether the $CD21^{-/low}$ cells in peripheral blood migrate to the inflamed joints. This could be done indirectly by looking at the B cell clones in peripheral blood and joints and see if any clones are shared between the two compartments.

The new cancer treatment with checkpoint inhibitors gives a new insight into the triggering of autoimmunity. We would like to study the B cell populations, especially the $CD21^{-/low}$ population in these patients, and to see whether any specific B cell population could predict treatment response. It would also be interesting to investigate whether any B cell population could predict the type of reaction the patients develop e.g. arthritis versus gut inflammation.

ACKNOWLEDGEMENTS

I would like to thank:

My supervisor, Inger, for being a fantastic supervisor and a very caring and generous person. I've learned so many things from you, including new Swedish words. Admittedly, I feel a bit bad that I only had time to teach you these very few and carefully selected Icelandic words: "Bleeessaður" (Tjeeeenare in Swedish) and "Örlítið grœnmeti" (A little bit of vegetables). You are a true mentor and a friend.

My assistant supervisor, Lill, for all your invaluable help, great advice and time. You taught me that the devil is in the details. I really hope that your meticulousness has rubbed off on me (crossing fingers).

Lennart, my other assistant supervisor, for all support through the years.

My co-workers in Inger's group (Karin, Monica, Charlotte, Sofia, Jenny, Shan, Lollo, Berglind): You are all such nice, wonderful and talented people that it is a pleasure to come to work. I couldn't have asked for a better group. Extra thanks to Monica for her help with SIMCA (our statistical program) and Charlotte for becoming my Siamese twin in the lab for a while. Thank you!

Alessandro, who is one of those people that are always enthusiastic and happy to help. Thank you for all your assistance.

Linda Bergqvist, Anna-Karin Lundell and Kristina Forslind for your help and expert advice.

All my co-authors.

All my co-workers at the Department of Rheumatology and Inflammatory Research. Thank you for your help!

My co-workers at the Rheumatology Department at Sahlgrenska University Hospital.

My friends in Kliniska Forskarskolan.

All the patients at the Rheumatology Department!

My family and friends for all the support and love.

The two best little girls in the world, my daughters, Lena Run and Klara Sif. Then there is my husband Aron who is my greatest support (he even offered to read this thesis but I suspect that he was perhaps more interested in reading the acknowledgements). You're the best.

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