Autoreactive B cells in rheumatoid arthritis

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Chapter VII

Summarizing discussion

Introduction

B cell autoreactivity is an inevitable consequence of two major random events during B cell development which are crucial for the purpose of adaptive humoral immunity: to produce high antibody diversity that recognizes infinitely diverse pathogenic molecular patterns. These two events are B cell receptor (BCR) gene segment recombination of immature B cells in the bone marrow and BCR somatic hypermutation in the germinal centre. Immune regulations including BCR editing and clonal deletion that induce B cell tolerance towards self-antigens take place to prevent B cells from attacking the body.[1] Immunological tolerance is, however, not unassailable. In fact, approximately 14-20% mature naïve B cells are autoreactive in healthy individuals.[2-4] Despite the explicit danger of having autoreactive B cells, these cells may increase the diversity pool of BCR which can undergo subsequent somatic hypermutation directed against pathogens. Indeed, some broadly neutralizing protective antibodies also have some degree of autoreactivity.[5] Therefore, regulation of autoreactive B cell activation is essential to prevent these cells from incurring autoimmune diseases.

The important roles of (autoreactive) MBC in the disease process of many autoimmune diseases are highlighted by the clinical benefits of the anti-CD20, B cell-depleting monoclonal antibody rituximab in treating rheumatoid arthritis (RA), pemphigus vulgaris, anti-neutrophil cytoplasmatic antibody (ANCA)-associated vasculitides and anti-muscle-specific kinase (MuSK)-positive myasthenia gravis.[6-9] However, how autoreactive B cells exert their pathogenicity in human autoimmune disease is not completely understood. RA is one of the most common autoimmune diseases which is hallmarked by chronic joint inflammation. The majority of RA patients harbour autoreactive anti-citrullinated proteins antibodies (ACPA) which are disease-specific and can prognosticate severe joint erosion in established RA. As described in **chapter 1**, the presence of ACPA years before RA onset demonstrates that autoimmunity against citrullinated self-antigens precedes autoimmune disease. In some individuals, the ACPA response matures before the onset of RA, as evidenced by epitope spreading, class switching and glycosylation changes. In most patients with established RA, the ACPA response does not change despite medication and clinical remission. It is therefore paramount to understand the characteristics of ACPA-expressing B cells in different phases of RA.

To this end, we made use of biotinylated, second generation cyclic citrullinated peptides (CCP2) conjugated to fluorophore-labelled streptavidin molecules.[10] By

using such streptavidin tetramers, Kerkman, *et al* showed that ACPA-expressing B cells mainly display a memory phenotype and circulate at a frequency of approximately 0.01% of total B cells in the peripheral blood of RA patients.[11] To further study these rare autoreactive MBC we also compared them to well-characterized, protective MBC against tetanus toxoid (TT) in both quiescent and activated states. In the studies described in this thesis, we aimed to understand how ACPA-expressing MBCs evolve in different phases of RA, to deliniate the potential pathogenic roles of these cells in RA, to learn how these cells resist immune tolerance regulation and to develop strategies that selectively deplete or suppress ACPA-expressing MBC.

ACPA-expressing B Cells in RA

Evolution of ACPA-expressing MBC

To better understand the involvement of ACPA-expressing MBC in the pathogenesis of RA, we characterized these cells from ACPA-positive individuals with joint pain (arthralgia) at-risk for developing RA, immunosuppressor-naïve early onset RA and immunosuppressor-treated established RA. To compare, TT-specific MBC in both quiescent and activated states were also characterized using flow cytometry. Our results, described in **chapter 2**, showed that a fraction of ACPA-expressing MBCs in all tested RA phases were proliferative. In arthralgia individuals, the activation markers of ACPA-expressing MBC resembled that of TT-specific MBC in a quiescent state. In contrast, ACPA-expressing MBCs from early onset RA were highly activated which phenocopied recently boosted TT-specific MBC. Moreover, ACPA-expressing MBC remained persistently activated and proliferative years after the onset of disease and upon therapy with conventional synthetic disease modifying anti-rheumatic drugs (csDMARD). This observation was the opposite of recently boosted TT-specific MBC which gradually returned to a quiescent state. Additionally, the persistently activated phenotype of ACPA-expressing MBC suggests that these cells may receive continuous BCR and CD40 triggering. In chronic infections such as Human Immunodeficiency Virus (HIV), Hepatitis C Virus (HCV) and malaria, unresolved and persistent antigen activation is associated with the expansion of atypical MBC populations which express multiple inhibitory receptors including FcRL4. These atypical MBCs are hyporesponsive to in vitro BCR restimulation and are therefore considered "exhausted".[12,13] In contrast, ACPA-expressing MBC from RA patients did not upregulate multiple inhibitory

receptors other than Fas and CD22, as described in **chapter 3**. This finding is interesting because these cells may encounter citrullinated self-antigens ad infinitum in the body. Therefore, knowledge on how ACPA-expressing MBC maintain their activated nature in established RA is important to develop measures to suppress these cells. Future studies regarding phenotypic changes of ACPA-expressing MBC upon therapeutic interventions that specifically target BCR signaling, cytokine receptor signaling or B-T cell interaction may enlighten this important clinical question. Moreover, single cell sequencing and phosphoproteomics study of these autoreactive MBC will not only give clues on the factors that support these aberrant cells but could also uncover (novel) targets of therapy.

Another relevant finding in **chapter 2** was that the activated phenotype of ACPA-expressing MBC did not correlate with systemic inflammation parameters, including a composite disease activity score or single measures such as the erythrocyte sedimentation rate. In line, some RA patients who underwent treatment-induced, clinical remission upon treatment with csDMARDs still harboured activated, ACPA-expressing MBC. Additionally, ACPA positivity is a strong predictor of relapses in RA patients who undergo remission.[14] These observations indicate that csDMARDs may mainly suppress inflammatory processes down-stream of activated ACPA-expressing MBC. A quiescent ACPA-expressing MBC response may therefore serve as an immunological treatment goal for RA therapy. This hypothesis remains to be tested.

The study reported in **chapter 2** was cross-sectional and therefore not suitable to draw conclusions on how and when ACPA-expressing MBCs developed their activated state. Longitudinal studies following the characteristics of ACPA-expressing B cells, microbiome alterations and infection histories of ACPA-positive healthy individuals over time may provide answers to when these cells become activated and which environmental factors or life events trigger this event.

Pathogenic roles of ACPA-expressing B cells

B cells are not only the producers of antibodies but also activators of T cells and immunoregulators through cytokine production. As described in **chapter 2**, both in early onset and established RA, ACPA-expressing MBC displayed upregulation of T cell-activating ligands including HLA-DR, CD80 and CD86. The upregulation of these markers was absent in ACPA-expressing MBC from arthralgia individuals. In an arthritis mouse model, CD80/86 expression on B cells was found to be essential in the activation of autoreactive T cells and the induction of arthritis.[15] In fact,

CD80/86 blocker abatacept is effective in treating RA and the clinical benefits are more profound in ACPA-positive patients.[16,17] Moreover, CD80/86 is also able to stimulate other CD28-expressing cells including neutrophils and eosinophils which have pro-inflammatory capacities.[18] These data indicate that ACPA-expressing MBC in RA are well-equipped to stimulate T cells as well as other pro-inflammatory immune cells and suggest that the interaction between ACPA-expressing B cells and other immune cells could be essential in the pathogenesis of RA.

ACPA-expressing B cells are enriched in RA synovial fluid. The data described in **chapter 2** showed that despite low numbers of B cells in RA synovial fluid, ACPA-expressing B cells were 15 times more concentrated in this compartment than in the peripheral blood. This finding demonstrates that ACPA-expressing B cells may preferentially migrate towards inflicted joints and persist to stay in this compartment. In RA synovial fluid, most ACPA-expressing B cells differentiated into plasmablasts. Interestingly, the frequency of ACPA-expressing MBC in the blood correlated positively with the abundance of plasma ACPA-IgG, suggesting that these MBC might continuously differentiate into ACPA-secreting plasmablasts in the inflicted joints. Therefore, it is clinically important to understand the factors that stimulate ACPA-expressing MBC migration to the synovial compartment. Insights to this question could be translated into treatment to prevent ACPA-positive healthy individuals from developing RA by inhibiting the migration of activated, pathogenic autoreactive B cells into the joint.

In **chapter 2**, we also showed that ACPA-expressing MBCs were prominent producers of pro-inflammatory and pro-angiogenic cytokines including IL-8, IL-6, TNF-α and VEGF upon BCR and CD40 ligation. In RA synovial fluid, where citrullinated antigens are abundant and inflammation is rampant, ACPA-expressing B cells produced IL-8 spontaneously. IL-8 is a chemoattractant for neutrophils, the most abundant immune cells in RA synovial fluid.[19] We showed that IL-8 produced by ACPA-expressing B cells was functional in vitro, supporting the role of ACPA-expressing B cells in inducing and/or maintaining inflammation in RA joints. These findings highlight the interconnection between ACPA-expressing B cells and neutrophils. Neutrophils from RA patients have enhanced propensity to form neutrophil extracellular trap (NET) that contains citrullinated self-antigens.[20] IL-8 production by B cells is driven by antigen specificity. Therefore, NET-activated, ACPA-expressing B cells may produce IL-8 which in turn recruits more neutrophils. The new entrant neutrophils subsequently could be stimulated by CD80/86 as well as ACPA and consequently form more NETs. As the RA synovial

fluid microenvironment promotes the long-term survival of ACPA-expressing B cells[21], these cells may maintain longstanding influx and activation of short-lived neutrophils. This vicious circle may drive the chronicity of joint inflammation in RA.

In conclusion, ACPA-expressing B cells may exert their pathogenicity in RA by migrating to the joints, activating pro-inflammatory immune cells including T cells, promoting persistent influx of neutrophils via secretion of IL-8 and producing autoantibodies as well as pro-inflammatory cytokines in this compartment.

How ACPA-expressing B cells breach immune checkpoints

B cell development is highly controlled by a series of regulatory mechanisms which safeguard the normal function of humoral immune responses. One of the regulations is the negative feedback from antigen-IgG immune complexes (IC) directed to antigen-specific MBC through crosslinking of BCR and inhibitory Fc-gamma-receptor IIB (CD32B). This feedback is essential to dampen antigen-specific B cells from developing into (autoreactive) plasma cells, thereby providing space to other antigen-specific plasma cells in the limited bone marrow niche. Correspondingly, CD32B-knock out mice display hypergammaglobulinemia and susceptibility to autoimmunity and anaphylactic reactions.[22] Our data described in **chapter 2** showed that ACPA-expressing MBC down-regulated their CD32 expression which may allow these cells to circumvent inhibitory signals from ACPA-containing IC.

As downregulation of other immune checkpoint receptors (ICR) on B cells in mouse models also leads to B cell dysregulation as well as autoantibody formation, we sought to assess the expression of seven other relevant ICRs on ACPA-expressing MBC from RA patients, as described in **chapter 3**. We observed that the percentage of ACPA-expressing MBC expressing CD5, PECAM-1, CD200R, LAIR-1 and FcRL4 resembled that of total MBC. These data highlight the diversity of ACPA-expressing MBC, at least with regard to the expression of these markers.

One surprising observation we found was the upregulation of the apoptotic receptor Fas on ACPA-expressing MBC. Deficiency of this receptor leads to autoantibody formation and lethal lymphoproliferation.[23] In contrast, ACPA-expressing MBC were proliferative and activated despite overexpressing Fas. Notably, the overexpression of Fas and downregulation of CD32 on ACPA-expressing MBC resembled the phenotype of TT-specific MBC two weeks post-booster vaccination. One stark difference was that the frequency of ACPA-expressing MBC in the blood

was much lower than that of recently boosted TT-specific MBC. Moreover, the proliferative phenotype of ACPA-expressing MBC was not reflected by a higher frequency of these cells in the blood compared to quiescent TT-specific MBC. There are at least two possible explanations to this paradox. First, ACPA-expressing MBC may preferentially migrate to tissues and differentiate into plasmablasts. This hypothesis is supported by enrichment of ACPA-expressing B cells in the synovial fluid and by the positive correlation between the frequency of peripheral ACPA-expressing MBC and circulating ACPA-IgG.

A second explanation would be that Fas expression on ACPA-expressing MBC may induce apoptosis on the cells which did not get sufficient BCR signaling. Apoptotic signaling from Fas can be blocked by concurrent BCR signaling.[24] BCR signaling of ACPA-expressing MBC is triggered by citrullinated autoantigens that are present in the synovium.[25] Moreover, ACPA-expressing MBC activation is enhanced and prolonged by the glycan group on the BCR.[26] Additionally, due to the enhanced CD19 expression described in **chapter 2**, these cells may have a low BCR activation threshold which permits slight signal to trigger downstream effects. This hypothesis may explain why the vast majority of ACPA-expressing B cells harbours glycan groups at their BCR variable domain as this glycan group could enhance BCR signaling which counteracts apoptotic signal from Fas. This hypothesis can be tested by overexpressing Fas and CD19 in ACPA-expressing B cell clones with or without the glycan group. If the hypothesis is correct, ACPA-B cells with the glycan groups could have a survival advantage upon Fas ligation compared to those without.

In conclusion, we found no general downregulation of ICR on ACPA-expressing MBC with the exception of CD32B. CD32B downregulation may help ACPA-expressing MBC to maintain their activated and proliferative phenotype despite numerous circulating ACPA. In contrast, ACPA-expressing MBC highly express Fas and CD22. Whether the downstream signaling of these ICRs is intact remains to be studied. The expression of Fas and CD22 may either help ACPA-expressing MBC to circumvent activation-induced cell death and/or shape the ACPA-expressing MBC response through deletion of those cells that do not bear BCR-variable domain glycan groups. Either way, the complex function of Fas and CD22 may serve as promising targets to inhibit ACPA-expressing B cells as RA treatment.

Other Interesting Markers

Single cell sequencing of ACPA-expressing B cells holds promise to provide an in-

depth view on the nature and evolution of these autoreactive cells. Yet, not only that this technique remains challenging especially for the study of such rare cell populations, sequencing will also only detect changes in mRNA levels which do not necessarily translate into protein expression. Therefore, directed exploratory experiments to examine the expression of important markers using flow cytometry or mass cytometry still prove to be useful. These markers include B cell development markers, receptors for T cell-dependent stimulation, complement receptors, other check-point receptors, anti-apoptotic proteins, homing markers, survival receptors, possible effector ligands, and signalling proteins (Table 1). Information of the expression of these proteins will provide a more complete picture of ACPA-expressing B cells, including how these cells survive and remain activated, their migration capacity and their pathogenicity in RA.

Table 1. Interesting markers to test on autoreactive B cells

No	Marker	Function	Ref
A. B cell developmental markers Hypothesis: Autoimmune disease patients may have reduced numbers or dysfunctional "regulatory" B cells.			
1	CD24	• Newly emigrated "transitional" B cells express CD24hi CD38hi • CD19+ CD24hi CD38hi are reported to be IL-10-producing "regulatory" B cells. Their function is impaired in SLE.	27
2	CD38	• An ADP-Ribosyl Cyclase-1 producing cyclic ADP-ribose, an intracellular calcium ion mobilizing messenger. • A receptor for CD31 (PECAM-1) • Newly emigrated "transitional" B cells express CD24hi CD38hi • CD19+ CD24hi CD38hi are reported to be IL-10-producing "regulatory" B cells. Their function is impaired in SLE.	27
3	BLIMP-1	• Pax-5 transcription repressor, crucial for plasma cell differentiation	28
4	Tbet	• T-bet expression in B cells promotes autoantibodies appearance in murine lupus model • Age-associated B cells express T-bet	29, 30
5	PD-L2	• In mouse, CD80+PD-L2+ memory B cells differentiate rapidly into antibody-forming cells but do not generate germinal centres.	31

6	IRF4	• Interferon regulatory factor 4 controls plasma cell differentiation • IRF4 is required for the activation of Ig lambda germline transcription and recombination	32, 33
B. Receptors for T cell-dependent stimulation Hypothesis: Enhanced expression of receptors for T cell-dependent stimuli on autoreactive B cells may be beneficial to compete with other B cells for a limited amount of stimuli.			
1	IL21R	• IL-21 induces the expression of Blimp-1, a transcription factor crucial for plasmablast/cell differentiation	34
C. Complement receptor Hypothesis: Enhanced expression of activating molecules on autoreactive B cells may be beneficial for their survival Alternative hypothesis: Down-regulation of activating molecules may balance out BCR signalling hyper-reactivity which can induce apoptosis			
1	CD21	• Complement receptor 2 • A part of CD19/CD21/CD81 complex which regulate BCR signalling threshold • BCR-CD21 co-ligation promotes antiapoptotic molecule Bcl-2 which protects human B cells from Fas-mediated apoptosis. • RA patients have an increased frequency of CD21 (-/lo) naïve B cells, the majority of which express germline autoreactive antibodies and are anergic	35-37
2	CD81	• A part of CD19/CD21/CD81 complex which regulate BCR signalling threshold	35-37
D. Immune checkpoint receptors Hypothesis: Enhanced expression of inhibitory molecules on autoreactive B cells may balance out BCR signalling hyper-reactivity which can induce apoptosis Alternative hypothesis: Down-regulation of inhibitory molecules may be beneficial for their survival			
1	CD279 PD-1	• Programmed cell death-1 is also expressed on human B cells and inhibits B cell activation cascade	38
2	CD72	• CD72 inhibits B cell response to the endogenous TLR7 in lupus mouse model	39

E.	**Anti-apoptotic proteins**		
Hypothesis: ACPA-expressing B cells upregulate anti-apoptotic proteins to balance out the hyperactivated feature.			
1	BCL-6	• BCL-6 is a master regulator of germinal centre • Promoter of human IL-8 contains a match to the BCL-6 consensus site	40, 41
2	BCL-XL	• Self-reactive B cells overexpressing Bcl-xL escape negative selection and are tolerized by clonal anergy and receptor editing • BCL-XL is able to enhance the secretion of IL-8 in human melanoma lines and glioblastoma	42-44
3	BCL-2	• Suppressor of apoptosis • Reduces apoptosis in germinal centre B cells which resulted in low frequency of V gene somatic hypermutation. • Defects in Bcl-2-regulated cell death signalling were reported to cause or correlate with autoimmunity	45
F.	**Homing Markers and Adhesion Molecules**		
Hypothesis: the rare frequency of ACPA+ B cells in the peripheral blood may be due to homing of these cells to inflammatory or other tissues.			
1	CD195 CCR5	• The frequency of CCR5+ Memory B cells is increased in RA compared to healthy donor. Moreover CCR5+ B cells are enriched in the synovial tissue of RA patients.	46
2	CCR10	• A mucosal and inflamed skin homing receptor • Binds to chemokines CCL28 and CCL27	47
3	CD62L	• Important for lymphocyte homing to lymph nodes and binds to carbohydrates, e.g. Peripheral lymph node addressin (PNAd) expressed by endothelial cells in the peripheral lymph nodes • TLR2 or TLR9 activation causes rapid loss of CD62L expression which leads B cell migration to the spleen and away from lymph nodes	48, 49
4	Beta7 Integrin	• Mucosal homing receptor	49, 50
5	CD11c	• CD11c/CD18 binds to fibrinogen, a substrate of citrullination • CD11c positivity on B cells is increased after chronic stimulation	51, 52
6	CD18	• CD11c/CD18 binds to fibrinogen, a substrate of citrullination	51

G.	**B cell survival receptors**		
Hypothesis: ACPA-expressing B cells may survive and remain activated due to enhanced receptors for survival factors			
1	TACI (CD267)	• A BAFF and APRIL (a proliferation inducing ligand) receptor • TACI-deficient mice develop fatal autoimmune glumerulonephritis	53-55
2	BAFFR (CD268)	• A BAFF (B-cell activating factor) receptor • Belimumab, an antibody against BAFF is clinically beneficial for SLE patient	53, 54
3	CD269 (BCMA)	• BCMA (B cell maturation antigen) is a BAFF receptor. Binds to TNF-superfamily, leads to NF-kB activation. • Increased expression in activated B cells in lupus	56
H.	**Signalling phospho-protein**		
1	SYK pY352	• Syk phosphorylated on tyrosine 352 residue is capable of downstream signalling	57, 58
2	PTPN-6 (SHP1) pY536	• Phosphorylation at tyrosine 536 residue enhances phosphatase activity	57, 59
3	STAT-3 pY705	• Upon IL-6 or growth factor stimulation, Stat3 is activated via phosphorylation at tyrosine 705 residue	60
4	Phospho-BCL-2	• Phosphorylation of BCL-2 leads to reduction of its anti-apoptotic function.	
I.	**Potentially pathogenic B cell markers**		
1	CD70	• CD70+ B cells exaggerate autoimmune encephalomyelitis in mouse models • Blockade of the CD27-CD70 pathway ameliorates disease manifestations in murine collagen-induced arthritis	61, 62
2	CD253 RANKL	• Activates osteoclast	63
3	ZAP70	• ZAP-70^{+} B cells are enriched in synovial fluid B cells in RA, but also present in blood. • ZAP-70 expression increases survival and is associated with inflammatory and autoimmune phenotype in RA.	64

Antigen-Specific Targeting of ACPA-expressing MBC

Selective targeting of autoreactive B cells is an attractive approach for therapeutic interventions in autoimmune diseases. This approach may deliver better clinical outcome than rituximab while limiting the immunosuppressive side effects. Extensive strategies to target autoreactive B cells were described in **chapter 1**. The most unique feature of ACPA-expressing B cells is their recognition of post-translationally modified self-antigens, particularly citrullinated antigens. In chapters 4, 5 and 6 we explored the possibility of antigen-specific targeting of ACPA-expressing B cells by conjugating cyclic citrullinated peptides (CCP) with a ribosome inactivating protein saporin (**chapter 4**), with CD22 ligands (CD22L, **chapter 5**) or with the Bruton's Tyrosine Kinase (BTK) inhibitor acalabrutinib (**chapter 6**). Various strategies including a sequential prodrug strategy to prevent circulating ACPA from neutralizing the therapeutic molecule (**chapter 4**), a multivalent strategy using polymeric scaffolds (**chapter 5**) and monovalent antigen-drug strategy (**chapter 6**) were also explored. In **chapter 4**, we addressed one of the possible major challenges in antigen-directed immunotherapy, i.e. neutralization of the drug by circulating autoantibodies. To this end, we designed a prodrug which contained inactivated biotinylated CCP bound to a saporin-conjugated streptavidin tetramer. The inactivated CCP failed to bind both monoclonal ACPA and patient-derived polyclonal ACPA due to the introduction of a carboxy-p-nitrobenzyl (CNBz) group at the side chain of the citrulline residue. Using nitroreductase to release the CNBz group, the citrulline side chain was completely restored to bind ACPA and ACPA-expressing B cells. We showed that nitroreductase-treated CNBz-CCP-saporin was as effective as CCP-saporin to induce apoptosis in a ACPA-B cell clone but not in a tetanus-specific control B cell clone. Moreover, CNBz-CCP-saporin failed to induce toxicity on any of the cell lines. As a following step, we plan to attach nitroreductase to a CD20-targeting antibody so that the prodrug can only be activated in the vicinity of B cells. Subsequently, the activated CCP-saporin binds to the ACPA BCR, followed by internalization by ACPA-expressing B cells which allows saporin to exert its cytotoxicity intracellularly. This strategy will serve as a proof-of-concept on sequential antigen-specific targeting of autoreactive B cells. In this study, we used potentially immunogenic, bacterial-derived proteins namely streptavidin and nitroreductase. Streptavidin functions to multimerize the antigenic peptides while nitroreductase is essential for activating the prodrug. In vivo, these proteins may compromise the therapeutic value of the drug and therefore alternative methods and/or materials need to be sought out. Alternative strategies can be found in **chapter 1**.

We then explored, in **chapter 5**, the use of multivalent scaffold polyisocyanopeptides (PIC) to deliver inhibitory signals from CD22L to ACPA-expressing B cells. This more direct strategy, compared to the one used in **chapter 4**, was chosen because polymers may be more potent in binding to the BCR. Also, such scaffolds can be readily used as carriers for cell modulating compounds. We conjugated CD22L into CCP-PIC constructs to specifically inhibit ACPA-expressing B cell clones. As a result, CD22L-containing CCP-PICs inhibited ACPA-specific BCR signaling pathway more effectively than a combination of separate CD22L-PICs and CCP-PICs. Whether this system still can work in the presence of circulating ACPA remains to be studied. If the presence of circulating ACPA hinders the CCP-CD22L-PIC function in inhibiting ACPA-expressing B cells, the produg strategy described in **chapter 4** might also be combined with the CCP-CD22L-PIC strategy.

Lastly, in **chapter 6** we explored the possibility to selectively target ACPA-expressing B cells using CCP conjugated with acalabrutinib, a BTK inhibitor. BTK is an essential kinase which conveys BCR signaling. Previously, it was shown that B cells from ACPA-positive RA patients have increased BTK activity compared to B cells from ACPA-negative RA patients.[65] Furthermore, inhibiting BTK leads to reduced disease activity and histological damage in mouse models of RA.[66,67] Looking back on our results in **chapter 3**, we hypothesized that by inhibiting BCR signaling, ACPA-expressing MBC may become more vulnerable to Fas-induced apoptosis. In **chapter 6** we synthesized CCP-acalabrutinib conjugates. This construct selectively inhibited the proliferation of an immortalized, ACPA-expressing B cell line. Surprisingly, the CCP-acalabrutinib conjugates failed to inhibit intracellular BTK, possibly due to their monomeric antigen in the drug conjugate which was not internalized via BCR. These findings suggested that covalent CCP-acalabrutinib conjugates can specifically inhibit ACPA-expressing B cell lines in a BTK-independent manner. Correspondingly, high doses of acalabrutinib, which inhibited BTK completely, failed to recapitulate the effect of CCP-acalabrutinib on these cells. While these data suggest that the immortalized, ACPA-expressing B cell line used may not be an optimal cell line for the study of the effects of BTK inhibition on autoreactive B cells, the “off targets” of CCP-acalabrutinib conjugates in these immortalized, ACPA-expressing B cells may be interesting and remain to be discovered. Moreover, CCP multivalency and an addition of low pH-sensitive linker between antigen and acalabrutinib may improve the antigen-BTK inhibitor drug construct.

Concluding Remarks

In conclusion, this thesis explored the evolution and nature of ACPA-expressing B cells in RA. In individuals with arthralgia considered to be at-risk to develop RA, ACPA-expressing MBC resembled quiescent TT-specific MBC, except for an already elevated expression of the proliferation marker Ki-67. In early onset, immunosuppressor-naïve RA, ACPA-expressing MBCs were highly activated, resembling recently boosted TT-specific MBC. Furthermore, in established, csDMARD-treated RA, ACPA-expressing MBCs retained their hyperactivated phenotype despite showing less of Ki-67 and HLA-DR expression compared to the cells from immunosuppressor-naïve RA. These characteristics were in contrast to those of recently boosted TT-specific MBCs, which gradually returned to their quiescent phenotype. In established RA, ACPA-expressing MBCs expressed T cell-stimulating ligands such as HLA-DR, CD80 and CD86; were able to excessively secrete pro-inflammatory cytokines such as IL-8, IL-6 and TNFα upon stimulation; were enriched in arthritic synovial fluid; differentiated into plasmablasts in this compartment; and spontaneously produced neutrophil chemotactic factor IL-8. These findings showed the potential pathogenic roles of ACPA-expressing B cells in human RA, notably by orchestrating both innate and adaptive immune responses to attack the joints where citrullinated self-antigens are prevalent.

To understand how ACPA-expressing B cells remain hyperactivated for long duration, the expression of immune checkpoint receptors (ICRs) on these cells were examined. The downregulation of ICRs promotes the production of autoantibodies and B cell dysregulation. In established RA, ACPA-expressing MBC downregulated the expression of CD32 which may help these cells to circumvent the inhibitory signals from ACPA-IgG immune complexes. Surprisingly, ACPA-expressing MBC did not downregulate seven other ICRs tested in our studies. In contrast, ACPA-expressing MBC highly expressed the apoptotic receptor Fas and the sialic-acid binding, inhibitory receptor CD22. The expression of Fas and CD22 on ACPA-expressing MBC may shape the ACPA immune response and promote the survival of these cells.

Due to strong evidence of the central roles of ACPA-expressing B cells in RA etiopathogenesis, these cells are prime targets for therapy or prophylactic vaccines

against RA. Citrullinated antigen-directed therapy strategies were also explored in this thesis. Conjugating CCP with immunomodulators including CD22L and acalabrutinib led to specific inhibition of the function and survival of an ACPA-expressing B cell line *in vitro*. A possible challenge in antigen-directed therapy is the presence of large numbers of circulating ACPA that may prevent the medicinal product to bind to ACPA-expressing B cells. A sequential prodrug strategy may solve this problem by chemically and reversibly inactivating CCP so that it does not bind to circulating ACPA. The prodrug is then activated only on the surface of ACPA-expressing B cells which leads to specific binding of the drug to the BCR of ACPA-expresing B cells. Subsequently, the antigen-drug conjugate will be internalized and exert its cytotoxicity specifically on ACPA-expressing B cells. The first evidence for the feasibility of such an approach on ACPA-expressing B cells is presented in this thesis.

How ACPA-expressing B cells evolve from the initial breach of immunological tolerance into cells that actively promote the development of RA is not yet clearly understood. Moreover, how ACPA-expressing B cells thrive and migrate towards the joints remains to be studied. Insights into these questions may eventually help to develop preventative as well as therapeutic measures for patients with ACPA-positive RA.

References

1. Nemazee D. Mechanisms of central tolerance for B cells. *Nature Reviews Immunology* 2017; **17**(5): 281-94.

2. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. *Science (New York, NY)* 2003; **301**(5638): 1374-7.

3. Tiller T, Tsuiji M, Yurasov S, Velinzon K, Nussenzweig MC, Wardemann H. Autoreactivity in human IgG+ memory B cells. *Immunity* 2007; **26**(2): 205-13.

4. Watanabe A, Su KY, Kuraoka M, et al. Self-tolerance curtails the B cell repertoire to microbial epitopes. *JCI Insight* 2019; **4**(10).

5. Yang G, Holl TM, Liu Y, et al. Identification of autoantigens recognized by the 2F5 and 4E10 broadly neutralizing HIV-1 antibodies. *The Journal of experimental medicine* 2013; **210**(2): 241-56.

6. Edwards JC, Szczepanski L, Szechinski J, et al. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *The New England journal of medicine* 2004; **350**(25): 2572-81.

7. Joly P, Mouquet H, Roujeau JC, et al. A single cycle of rituximab for the treatment of severe pemphigus. *The New England journal of medicine* 2007; **357**(6): 545-52.

8. Guillevin L, Pagnoux C, Karras A, et al. Rituximab versus azathioprine for maintenance in ANCA-associated vasculitis. *The New England journal of medicine* 2014; **371**(19): 1771-80.

9. Hehir MK, Hobson-Webb LD, Benatar M, et al. Rituximab as treatment for anti-MuSK myasthenia gravis: Multicenter blinded prospective review. *Neurology* 2017; **89**(10): 1069-77.

10. Cossarizza A, Chang HD, Radbruch A, et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). *European journal of immunology* 2019; **49**(10): 1457-973.

11. Kerkman PF, Fabre E, van der Voort EI, et al. Identification and characterisation of citrullinated antigen-specific B cells in peripheral blood of patients with rheumatoid arthritis. *Annals of the rheumatic diseases* 2016; **75**(6): 1170-6.

12. Pérez-Mazliah D, Ndungu FM, Aye R, Langhorne J. B-cell memory in malaria: Myths and realities. 2020; **293**(1): 57-69.

13. Kardava L, Moir S, Wang W, et al. Attenuation of HIV-associated human B cell exhaustion by siRNA downregulation of inhibitory receptors. *The Journal of clinical investigation* 2011; **121**(7): 2614-24.

14. Rech J, Hueber AJ, Finzel S, et al. Prediction of disease relapses by multibiomarker disease activity and autoantibody status in patients with rheumatoid arthritis on tapering DMARD treatment. *Annals of the rheumatic diseases* 2016; **75**(9): 1637-44.

15. O'Neill SK, Cao Y, Hamel KM, Doodes PD, Hutas G, Finnegan A. Expression of CD80/86 on B cells is essential for autoreactive T cell activation and the development of arthritis. *Journal of immunology (Baltimore, Md :* 1950) 2007; **179**(8): 5109-16.

16. Sokolove J, Schiff M, Fleischmann R, et al. Impact of baseline anti-cyclic citrullinated peptide-2 antibody concentration on efficacy outcomes following treatment with subcutaneous abatacept or adalimumab: 2-year results from the AMPLE trial. *Annals of the rheumatic diseases* 2016; **75**(4): 709-14.

17. Emery P, Burmester GR, Bykerk VP, et al. Evaluating drug-free remission with abatacept in early rheumatoid arthritis: results from the phase 3b, multicentre, randomised, active-controlled AVERT study of 24 months, with a 12-month, double-blind treatment period. *Annals of the rheumatic diseases* 2015; **74**(1): 19-26.

18. Mehrishi JN, Szabó M, Bakács T. Some aspects of the recombinantly expressed humanised superagonist anti-CD28 mAb, TGN1412 trial catastrophe lessons to safeguard mAbs and vaccine trials. *Vaccine* 2007; **25**(18): 3517-23.

19. Edwards SW, Hallett MB. Seeing the wood for the trees: the forgotten role of neutrophils in rheumatoid arthritis. *Immunol Today* 1997; **18**(7): 320-4.

20. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Science translational medicine* 2013; **5**(178): 178ra40.

21. Kerkman PF, Kempers AC, van der Voort EI, et al. Synovial fluid mononuclear cells provide an environment for long-term survival of antibody-secreting cells and promote the spontaneous production of anti-citrullinated protein antibodies. *Annals of the rheumatic diseases* 2016; **75**(12): 2201-7.

22. Takai T, Ono M, Hikida M, Ohmori H, Ravetch JV. Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. *Nature* 1996; **379**(6563): 346-9.

23. Hao Z, Duncan GS, Seagal J, et al. Fas receptor expression in germinal-center B cells is essential for T and B lymphocyte homeostasis. *Immunity* 2008; **29**(4): 615-27.

24. Rothstein TL, Wang JK, Panka DJ, et al. Protection against Fas-dependent Th1-mediated apoptosis by antigen receptor engagement in B cells.

Nature 1995*;* **374**(6518): 163-5.

25. Matsuo K, Xiang Y, Nakamura H, et al. Identification of novel citrullinated autoantigens of synovium in rheumatoid arthritis using a proteomic approach. *Arthritis Res Ther* 2006*;* **8**(6): R175.

26. Kissel T, Ge C, Hafkenscheid L, et al. Surface Ig variable domain glycosylation affects autoantigen binding and acts as threshold for human autoreactive B cell activation. *Sci Adv* 2022*;* **8**(6): eabm1759.

27. Blair PA, Norena LY, Flores-Borja F, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity* 2010*;* **32**(1): 129-40.

28. Klein U, Dalla-Favera R. Unexpected steps in plasma-cell differentiation. *Immunity* 2007*;* **26**(5): 543-4.

29. Rubtsova K, Rubtsov AV, Thurman JM, Mennona JM, Kappler JW, Marrack P. B cells expressing the transcription factor T-bet drive lupus-like autoimmunity. *The Journal of clinical investigation* 2017*;* **127**(4): 1392-404.

30. Rubtsova K, Rubtsov AV, Cancro MP, Marrack P. Age-Associated B Cells: A T-bet-Dependent Effector with Roles in Protective and Pathogenic Immunity. *Journal of immunology (Baltimore, Md :* 1950*)* 2015*;* **195**(5): 1933-7.

31. Zuccarino-Catania GV, Sadanand S, Weisel FJ, et al. CD80 and PD-L2 define functionally distinct memory B cell subsets that are independent of antibody isotype. *Nature immunology* 2014*;* **15**(7): 631-7.

32. Johnson K, Hashimshony T, Sawai CM, et al. Regulation of immunoglobulin light-chain recombination by the transcription factor IRF-4 and the attenuation of interleukin-7 signaling. *Immunity* 2008*;* **28**(3): 335-45.

33. Klein U, Casola S, Cattoretti G, et al. Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. *Nature immunology* 2006*;* **7**(7): 773-82.

34. Ozaki K, Spolski R, Ettinger R, et al. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *Journal of immunology (Baltimore, Md :* 1950*)* 2004*;* **173**(9): 5361-71.

35. Isnardi I, Ng YS, Menard L, et al. Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. *Blood* 2010*;* **115**(24): 5026-36.

36. Tedder TF, Inaoki M, Sato S. The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. *Immunity* 1997*;* **6**(2): 107-18.

37. Mongini PK, Jackson AE, Tolani S, Fattah RJ, Inman JK. Role of complement-binding CD21/CD19/CD81 in enhancing human B cell protection from Fas-mediated apoptosis. *Journal of immunology (Baltimore, Md : 1950)* 2003; **171**(10): 5244-54.

38. Thibult ML, Mamessier E, Gertner-Dardenne J, et al. PD-1 is a novel regulator of human B-cell activation. *International immunology* 2013; **25**(2): 129-37.

39. Akatsu C, Shinagawa K, Numoto N, et al. CD72 negatively regulates B lymphocyte responses to the lupus-related endogenous toll-like receptor 7 ligand Sm/RNP. *The Journal of experimental medicine* 2016; **213**(12): 2691-706.

40. Toney LM, Cattoretti G, Graf JA, et al. BCL-6 regulates chemokine gene transcription in macrophages. *Nature immunology* 2000; **1**(3): 214-20.

41. Basso K, Dalla-Favera R. BCL6: master regulator of the germinal center reaction and key oncogene in B cell lymphomagenesis. *Advances in immunology* 2010; **105**: 193-210.

42. Gabellini C, Gomez-Abenza E, Ibanez-Molero S, et al. Interleukin 8 mediates bcl-xL-induced enhancement of human melanoma cell dissemination and angiogenesis in a zebrafish xenograft model. International journal of cancer Journal international du cancer 2017.

43. Gabellini C, Castellini L, Trisciuoglio D, Kracht M, Zupi G, Del Bufalo D. Involvement of nuclear factor-kappa B in bcl-xL-induced interleukin 8 expression in glioblastoma. *Journal of neurochemistry* 2008; **107**(3): 871-82.

44. Fang W, Weintraub BC, Dunlap B, et al. Self-reactive B lymphocytes overexpressing Bcl-xL escape negative selection and are tolerized by clonal anergy and receptor editing. *Immunity* 1998; **9**(1): 35-45.

45. Tischner D, Woess C, Ottina E, Villunger A. Bcl-2-regulated cell death signalling in the prevention of autoimmunity. *Cell death & disease* 2010; **1**: e48.

46. Nanki T, Takada K, Komano Y, et al. Chemokine receptor expression and functional effects of chemokines on B cells: implication in the pathogenesis of rheumatoid arthritis. *Arthritis research & therapy* 2009; **11**(5): R149.

47. Lundell AC, Rabe H, Quiding-Jarbrink M, et al. Development of gut-homing receptors on circulating B cells during infancy. *Clinical immunology (Orlando, Fla)* 2011; **138**(1): 97-106.

48. Morrison VL, Barr TA, Brown S, Gray D. TLR-mediated loss of CD62L focuses B cell traffic to the spleen during Salmonella typhimurium infection. *Journal of immunology (Baltimore, Md : 1950)* 2010; **185**(5): 2737-46.

49. Mei HE, Yoshida T, Sime W, et al. Blood-borne human plasma cells in

steady state are derived from mucosal immune responses. *Blood* 2009; **113**(11): 2461-9.

50. Farstad IN, Halstensen TS, Lazarovits AI, Norstein J, Fausa O, Brandtzaeg P. Human intestinal B-cell blasts and plasma cells express the mucosal homing receptor integrin alpha 4 beta 7. *Scandinavian journal of immunology* 1995; **42**(6): 662-72.

51. Nham SU. Characteristics of fibrinogen binding to the domain of CD11c, an alpha subunit of p150,95. *Biochemical and biophysical research communications* 1999; **264**(3): 630-4.

52. Karnell JL, Kumar V, Wang J, Wang S, Voynova E, Ettinger R. Role of CD11c+ T-bet+ B cells in human health and disease. *Cellular immunology* 2017; **321**: 40-5.

53. Thien M, Phan TG, Gardam S, et al. Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. *Immunity* 2004; **20**(6): 785-98.

54. Navarra SV, Guzman RM, Gallacher AE, et al. Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. *Lancet* 2011; **377**(9767): 721-31.

55. Seshasayee D, Valdez P, Yan M, Dixit VM, Tumas D, Grewal IS. Loss of TACI causes fatal lymphoproliferation and autoimmunity, establishing TACI as an inhibitory BLyS receptor. *Immunity* 2003; **18**(2): 279-88.

56. Kim J, Gross JA, Dillon SR, Min JK, Elkon KB. Increased BCMA expression in lupus marks activated B cells, and BCMA receptor engagement enhances the response to TLR9 stimulation. *Autoimmunity* 2011; **44**(2): 69-81.

57. Chen Z, Shojaee S, Buchner M, et al. Signalling thresholds and negative B-cell selection in acute lymphoblastic leukaemia. Nature 2015.

58. Carsetti L, Laurenti L, Gobessi S, Longo PG, Leone G, Efremov DG. Phosphorylation of the activation loop tyrosines is required for sustained Syk signaling and growth factor-independent B-cell proliferation. *Cellular signalling* 2009; **21**(7): 1187-94.

59. Zhang Z, Shen K, Lu W, Cole PA. The role of C-terminal tyrosine phosphorylation in the regulation of SHP-1 explored via expressed protein ligation. *The Journal of biological chemistry* 2003; **278**(7): 4668-74.

60. Wang Y, van Boxel-Dezaire AH, Cheon H, Yang J, Stark GR. STAT3 activation in response to IL-6 is prolonged by the binding of IL-6 receptor to EGF receptor. *Proceedings of the National Academy of Sciences of the United States of America* 2013; **110**(42): 16975-80.

61. Francosalinas G, Cantaert T, Nolte MA, Tak PP, van Lier RA, Baeten DL. Enhanced costimulation by CD70+ B cells aggravates experimental autoimmune encephalomyelitis in autoimmune mice. *Journal of neuroimmunology* 2013; **255**(1-2): 8-17.

62. Han BK, Olsen NJ, Bottaro A. The CD27-CD70 pathway and pathogenesis of autoimmune disease. *Seminars in arthritis and rheumatism* 2016; **45**(4): 496-501.

63. Yeo L, Lom H, Juarez M, et al. Expression of FcRL4 defines a pro-inflammatory, RANKL-producing B cell subset in rheumatoid arthritis. Annals of the rheumatic diseases 2014.

64. Tolusso B, De Santis M, Bosello S, et al. Synovial B cells of rheumatoid arthritis express ZAP-70 which increases the survival and correlates with the inflammatory and autoimmune phenotype. *Clinical immunology (Orlando, Fla)* 2009; **131**(1): 98-108.

65. Corneth OBJ, Verstappen GMP, Paulissen SMJ, et al. Enhanced Bruton's Tyrosine Kinase Activity in Peripheral Blood B Lymphocytes From Patients With Autoimmune Disease. *Arthritis & rheumatology (Hoboken, NJ)* 2017; **69**(6): 1313-24.

66. Wu H, Huang Q, Qi Z, et al. Irreversible inhibition of BTK kinase by a novel highly selective inhibitor CHMFL-BTK-11 suppresses inflammatory response in rheumatoid arthritis model. *Scientific reports* 2017; **7**(1): 466.

67. Haselmayer P, Camps M, Liu-Bujalski L, et al. Efficacy and Pharmacodynamic Modeling of the BTK Inhibitor Evobrutinib in Autoimmune Disease Models. *Journal of immunology (Baltimore, Md : 1950)* 2019; **202**(10): 2888-906.

68. Vergroesen RD, Slot LM, Hafkenscheid L, et al. B-cell receptor sequencing of anti-citrullinated protein antibody (ACPA) IgG-expressing B cells indicates a selective advantage for the introduction of N-glycosylation sites during somatic hypermutation. Ann Rheum Dis. England; **2018**: 956-8.