Expression and function of complement- and Toll-like receptors in human B cells under physiological and autoimmune conditions -linking innate and adaptive immunity

Mariann Kremlitzka

Supervisor:
Professor Anna Erdei DSc

Doctoral School of Biology
Head: Professor Anna Erdei, DSc

Immunology PhD Programme
Head: Professor Anna Erdei, DSc

Department of Immunology, Institute of Biology
Eötvös Loránd University, Budapest, Hungary

2014
Table of Contents

List of abbreviations.................................................................................................................. 4

1. Introduction .............................................................................................................................. 6
   1.1. B cells and autoimmunity ................................................................................................. 7
       1.1.1. Development and function of B cells ........................................................................... 7
       1.1.2. B cell tolerance and autoimmunity ............................................................................. 10
       1.1.2.1. Rheumatoid arthritis ............................................................................................. 11
       1.1.2.2. The role of B cells in rheumatoid arthritis ............................................................... 11
       1.2. Regulation of B cell functions by cross-talk between complement- and Toll-like receptors ................................................................................................................................. 14
           1.2.1. The complement system .......................................................................................... 14
       1.2.2. Toll-like receptors ...................................................................................................... 16
   1.3. Complement- and Toll-like receptors on human B cells ................................................. 17
       1.3.1. Complement receptor type 1 (CR1/CD35) ................................................................ 17
       1.3.2. Complement receptor type 2 (CR2/CD21) ................................................................ 18
       1.3.3. Expression and role of CR1 and CR2 on human B cells under physiological and autoimmune conditions ....................................................................................................................... 19
       1.3.4. Toll-like receptors on B cells under physiological and autoimmune conditions ...... 21
   1.4. Cross-talk between the complement system, TLRs and adaptive immunity ............... 23

2. Aims ........................................................................................................................................ 26

3. Materials and methods ........................................................................................................... 27
   3.1. Materials .......................................................................................................................... 27
   3.2. Methods ............................................................................................................................ 31

4.1. Results I............................................................................................................................... 36
   4.1.1. RA patients at different stages of disease have similar frequencies of B cell subsets as healthy controls ................................................................................................................................ 37
   4.1.2. Naive B cells up-regulate CR1 and down-regulate CR2 expression during differentiation to memory cells both in healthy donors and RA patients ................................................................................................................................. 38
   4.1.3. CR1 inhibits the proliferation of B cells derived from active RA patients ............... 40
   4.1.4. CR1 clustering prevents B cell differentiation to plasmablasts .................................. 40
4.1.5 CR1 mediates inhibition of Ig production by B cells of healthy donors and active RA patients ................................................................. 43
4.2. Results II. .............................................................................................................. 45
  4.2.1. CR1 inhibits both BCR- and TLR9-induced proliferation of tonsillar B cells........... 46
  4.2.2. CR1 inhibits BCR- and TLR9-induced up-regulation of activation markers and cytokine production .................................................................. 48
  4.2.2. CR1 inhibits BCR- and TLR9-induced up-regulation of activation markers and cytokine production .................................................................. 48
  4.2.3. Effect of CR1 clustering on BCR- and TLR9-induced plasmablast formation........... 51
  4.2.4. Syk is the major signaling molecule involved in CR1-induced inhibition of B cell activation ........................................................................ 53
4.3. Results III. ............................................................................................................ 55
  4.3.1. Binding of CpG to resting human B cells induces dose- and time-dependent phosphorylation of Syk and Lyn .................................................... 56
  4.3.2. The role of Syk and Lyn in the CpG-induced activation, proliferation and cytokine production of B cells ........................................................................ 58
  4.3.3. Syk regulates CpG-driven B cell differentiation ..................................................... 61
  4.3.3. Syk regulates CpG-driven B cell differentiation ..................................................... 61
  4.3.4. Inhibition of Syk prevents the synergistic activation of MAPKs induced by the BCR and CpG .......................................................................................... 63
  4.3.5. Extracellular CpG ODN induces Syk activation in B cells ...................................... 65
  4.3.6. Inhibition of Syk does not influence the uptake of CpG oligonucleotides .............. 68
  4.3.7. Syk regulates CpG-driven up-regulation of TLR9 expression in B cells ................. 70
5. Discussion ............................................................................................................... 72
References .................................................................................................................. Error! Bookmark not defined.
Summary .................................................................................................................... 95
Összefoglalás .............................................................................................................. 97
Acknowledgements .................................................................................................... 99
List of publications .................................................................................................... 100
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AEC</td>
<td>3-amino-9-ethyl carbazole</td>
</tr>
<tr>
<td>AET</td>
<td>2-aminoethyl-iso-tiouronium</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AutoAb</td>
<td>Autoantibody</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell-activating factor</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCP</td>
<td>Cyclic citrullinated peptide</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>cpm</td>
<td>Count per minute</td>
</tr>
<tr>
<td>CR1</td>
<td>Complement receptor type 1</td>
</tr>
<tr>
<td>CR2</td>
<td>Complement receptor type 2</td>
</tr>
<tr>
<td>CSR</td>
<td>Class switch recombination</td>
</tr>
<tr>
<td>DAS28</td>
<td>Disease activity score for 28 joints</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme-linked immunosorbent spot</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FDC</td>
<td>Follicular dendritic cell</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FO B cells</td>
<td>Follicular B cells</td>
</tr>
<tr>
<td>GKN</td>
<td>Glucose–potassium–sodium buffer</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IC</td>
<td>Immune complex</td>
</tr>
<tr>
<td>IFN&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>int</td>
<td>Intermediate</td>
</tr>
<tr>
<td>LHR</td>
<td>Long homologous repeat</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Mf</td>
<td>Macrophage</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>Min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>MZ</td>
<td>Marginal zone</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Plasma cell</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
</tr>
<tr>
<td>rh</td>
<td>Recombinant human</td>
</tr>
<tr>
<td>R_{MFI}</td>
<td>Relative mean fluorescence intensity</td>
</tr>
<tr>
<td>SCR</td>
<td>Short consensus repeat</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SHM</td>
<td>Somatic hypermutation</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>Th17</td>
<td>IL-17 secreting helper T cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor-alfa</td>
</tr>
</tbody>
</table>
1. Introduction

Protection against infections is the major physiologic function of the immune system, which warrants the integrity of our body. The immune system consists of cells and molecules that mediate a coordinated response against foreign substances. Tolerance to self antigens (Ags) is an elementary property of the normal immune response. Failure of self-tolerance caused by inappropriate selection of immune cells or tissue injury can result in reactions against autologous Ags, triggering an autoimmune response.

During the development of an appropriate humoral immune response, B cell receptor (BCR)-mediated signals are strictly controlled by the balanced signaling through complement receptor type 1 (CR1/CD35), type 2 (CR2/CD21) and Toll-like receptors (TLRs). TLR ligands and complement-containing immune complexes (ICs) may alter effector B cell functions as well as the fate of self-reactive cells, making plausible that alteration in the expression and/or function of IC-binding receptors may contribute to the development of autoimmune disorders.

In this thesis I focus on the expression and functions of CR1, CR2 and TLR9 on human B cells. It will be discussed how these receptors connect the innate and adaptive arms of the immune response through binding of ICs containing complement fragments- and nucleic acid.
1.1. B cells and autoimmunity

1.1.1. Development and function of B cells

B cells are central components of the humoral immune response, being responsible for the production of serum immunoglobulins (Igs). Development of B cells can be divided into two main stages (Figure 1). The initial steps of B cell development are „antigen-independent” and take place in the bone marrow. B cells express clonally distributed surface Igs, which are the Ag recognition units of the BCR. Igs arise from the combinatorial rearrangement of variable (V), diversity (D) and/or joining (J) gene segments of the Ig loci. This is a random process that generates a diverse BCR repertoire that binds a variety of molecular structures, recognizing both self and non-self structures. Although the frequency of autoreactive B cells significantly declines during the transition from the immature into the mature B cell stage, some of them are able to escape the selection process in the bone marrow and get into the periphery as potentially self-reactive B cells.

B lymphocytes leave the bone marrow as IgM+CD10+ transitional cells and undergo several maturation stages as they migrate through the blood into peripheral lymphoid tissues. The secondary lymphoid organs provide a microenvironment suitable for the efficient presentation of Ags to B cells. Ag-recognition is an indispensable element of B cell expansion in the periphery: B cells which do not encounter Ag undergo apoptosis. If both Ag and additional secondary signals are present in the form of cytokines, follicular dendritic cells (FDCs) and T cell contacts, B lymphocytes give rise to a memory population as well as antibody (Ab)-secreting plasma cells (PCs). This strict regulation of B cell activation prevents non-specific priming of bystander cells and decreases the possibility of an exaggerated reaction against self and non-self structures.

Two main populations of B lymphocytes exist: B-1 and B-2 cells (Figure 1). B-1 cells are IgMintIgD-CD21int lymphocytes, produce natural Abs and localize mainly in the peritoneal cavity and gut-associated lymphoid tissues. In contrast to B-2 cells, B-1 cells are derived from the fetal-liver and they possess self-renewing capacity. Two subsets have been identified based on the expression of CD5: B-1a cells are CD5+, whereas B-1b cells do not express CD5. Their repertoire appears to be directed towards highly conserved structures, such as phosphoryl-choline, DNA and nuclear proteins. These molecules contain repeating epitopes which are able to engage multiple surface Igs, thereby induce Ag-specific B cell responses without additional T cell help. However, this process does not result in somatic mutation,
germinal center (GC) reaction and the formation of immunological memory, although some exceptions have recently been reported.\textsuperscript{11}

Among mature B-2 cells, two populations can be phenotypically identified: marginal zone (MZ) B cells and follicular (FO) B cells.\textsuperscript{12} They derive from the bone marrow and localize in the MZ of the spleen or in B cell follicles of secondary lymphoid tissues, respectively. Similar to B-1 cells, MZ B lymphocytes belong to the innate arm of immunity, respond mainly to T-independent Ags and produce natural Abs.\textsuperscript{13} MZ B cells are defined as $\text{IgM}^\text{high}$, $\text{IgD}^\text{low}$ and $\text{CD21}^\text{int}$ lymphocytes. They reside at the interface of the circulation and lymphoid tissues which - together with the expression of polyreactive BCRs and high levels of TLRs - enable them to cross over the classical boundaries between the innate and adaptive immune responses.\textsuperscript{14}

In contrast to B-1 cells and MZ B cells, FO B cells respond mainly to T-dependent protein Ags and require the participation of T helper (Th) cells for their activation. FO B lymphocytes are characterized as $\text{IgM}^\text{low}$, $\text{IgD}^\text{high}$, $\text{CD23}^+$ cells.\textsuperscript{12} Upon Ag encounter and cognate effector T cell-B cell interaction, they start to proliferate and differentiate into short-lived PCs or enter into GC reaction.\textsuperscript{15} In GCs, B cells expand, undergo somatic hypermutation (SHM) and isotype-switching.\textsuperscript{16} The GC reaction results in Ag-specific B cells with high-affinity BCRs, which access the memory B cell compartment.\textsuperscript{17} Upon Ag re-challenge, memory B cells rapidly expand and differentiate into long-lived PCs under the cognate control of memory T cells.\textsuperscript{18,19} Long-lived PCs will home to the bone marrow or migrate into inflamed tissues where they produce high affinity Abs.\textsuperscript{20}

B cells perform several immunological functions and have been considered mainly as positive regulators of the immune response. Indeed, the most prominent function of B cells is Ab secretion. Abs can be produced naturally, without prior activation, called natural Abs or in response to foreign Ags. Beside Ig secretion and Ag presentation for T cells,\textsuperscript{21} B cells can produce various cytokines and thus can influence the immune response in several manners.\textsuperscript{22-24} These diverse functions of B cells enable them to be central players in the regulation of immune processes and underlie their role in the maintenance of self-tolerance and induction of autoimmunity.
Figure 1. B cell development and populations

The figure shows the broad delineation of B cell developmental stages and B lymphocyte subsets. CLP indicates common lymphoid progenitor; SHM, somatic hypermutation; and CSR, class switch recombination. (Modified after LeBlen TW and Tedder TF; 2008; Blood)
1.1.2. B cell tolerance and autoimmunity

Autoimmunity is caused by the failure of an organism to recognize its own constituent parts as self and comprises an immune response against its own cells and tissues. Autoimmune disorders are classified into two types: organ-specific (like insulin-dependent diabetes) and systemic (such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)) diseases. In most cases the combination of different genetic and environmental factors leads to the dysregulation of the normal immune functions and contributes to disease activity.

Autoimmunity appears when the mechanisms that control self-tolerance are unable to eliminate or inactivate pathogenic autoreactive cells. Several checkpoints control B cell selection, both in the bone marrow and in the peripheral lymphoid tissues. High-affinity recognition of self-Ags by immature B cells in the bone marrow may result in elimination (clonal deletion) or inactivation (clonal anergy) of the developing lymphocytes. Alternatively, self-reactive B cells may change their Ag receptor specificity by a mechanism called receptor editing. These processes represent only the first checkpoints in the establishment of self-tolerance which drop dramatically the number of autoreactive B cells. Defects in these early selection routes have been observed in various autoimmune diseases, like SLE and RA. Non-self-reactive, immature B lymphocytes migrate to the periphery where they may encounter autoantigens not present in the bone marrow. As a result, B cells undergo a second self-tolerance checkpoint, where high-avidity recognition of self-Ags results in their deletion while low-avidity interactions lead to anergy or ignorance. Nevertheless, a certain part of B cells retain their capacity to recognize self-Ags and secrete IgM autoantibodies (autoAbs), which may have protective function by elimination of apoptotic cells. SHM in the GC may also induce the generation of potentially harmful self-reactive lymphocytes, which may be rendered functionally inactive or may die by apoptosis without cognate T cell help.

Genetic polymorphism of a large number of genes that regulate B cell activation and differentiation, as well as environmental factors may allow autoreactive B cells to survive and therefore may induce autoimmunity. Some of these mutations have been identified in mouse models of autoimmune diseases and later confirmed in human disorders. Over-expression of B cell co-receptors, like CD19 and CR2 or deficiency of molecules that have inhibitory function, such as SHP-1 and FcγRIIb may all affect the regulation of self-reactive BCR-signaling, thus predispose to autoimmunity. One of the best characterized IC-binding receptors on B cells is the inhibitory FcγRIIb. Polymorphisms, which cause decreased
FcγRIIb expression and inhibitory function have been proven to associate with SLE.\textsuperscript{38,39} Similarly, increased survival of autoreactive B cells due to elevated serum levels of B cell-activating factor (BAFF) has been confirmed in RA, SLE and Sjögren’s syndrome and made this molecule a potential target in autoimmune diseases.\textsuperscript{40,41}

Therapeutic benefit of B cell depletion has refocused their role in autoimmunity (Figure 2).\textsuperscript{42} Beyond autoAb production B cells exert several cellular functions, including autoantigen presentation,\textsuperscript{43} secretion of inflammatory cytokines and the formation of ectopic GCs.\textsuperscript{44,45} The emerging picture shows that B cells, autoAbs and T cells are all important components of abnormal immune responses that lead to self-recognition and tissue damage in autoimmune patients, with their relative contribution changing during disease progression. Autoimmune diseases where B cell functions are closely correlated with disease activity include SLE, RA, type 1 diabetes, and multiple sclerosis.\textsuperscript{46} Understanding the overlapping roles of B cells as mediators of autoimmune diseases will facilitate the development of more precisely directed therapies with broader clinical efficacy and may improve current depletion strategies that remove all B cells.

1.1.2.1. Rheumatoid arthritis

RA is a systemic autoimmune disease characterized by chronic inflammation of the synovium, cartilage injury and bone erosion. It affects approximately 0.5-1 % of the population with dominance in females and causes suppressed erythropoesis, morning stiffness and formation of extra-articular nodules. In contrast to the normal synovium, infiltrating lymphocytes are commonly seen in the inflamed tissue.\textsuperscript{47,48} Although the pathogenesis of RA is not fully understood, the presentation of autoantigens on certain MHC molecules is likely to be a key event for the activation of CD4\textsuperscript{+} T cells with subsequent effect on stimulation of B cells and macrophages (Mfs).\textsuperscript{49,50} RA is associated with the production of pro-inflammatory cytokines like interferon-gamma (IFNγ), tumor necrosis factor-alfa (TNFα), interleukin (IL)-1 and IL-6 in the joint, leading to pannus formation and bone erosion.\textsuperscript{51}

1.1.2.2. The role of B cells in rheumatoid arthritis

The presence of autoAbs and the clinical improvement of patients after B cell depleting therapies (like Rituximab treatment) underline the role of B cells in the pathogenesis of RA. B lymphocytes play several critical roles in the pathogenesis of autoimmune diseases (Figure 2). They produce autoAbs, which contribute to IC-formation and complement activation in the joint. By the expression of different adhesion and costimulatory molecules parallel with the
ability of autoantigen presentation, B cells provide help for T cell activation. Furthermore, activated synovial B cells secrete several cytokines and chemokines which may induce leukocyte infiltration, regulation of T cell functions and formation of ectopic lymphoid structures.

**Autoantibodies in RA**

RA patients produce several different autoAbs which are either RA-associated (present in RA but also in other autoimmune diseases, like rheumatoid factors (RFs)) or RA-specific Igs (which seem to be present only in RA like the antibodies to cyclic citrullinated peptides (CCPs)). RF and anti-CCP Abs are considered as clinically useful prognostic markers in RA.\(^{52}\) Serum levels of both these Abs correlate with disease activity, meaning that patients with a more active disease have higher titers of RF and anti-CCP.\(^{53}\) The exacerbated production of autoAbs may lead to pathological conditions through formation and deposition of ICs, by facilitated Ag uptake and by modulation of Ag presentation. All these effector functions of Abs have been proven to contribute to the development of RA.\(^{54}\)

**B cells as antigen presenting cells (APCs)**

B cells internalize Ags through their BCR, which is 1.000-10.000-fold more effective than pinocytosis. Therefore, B cells are able to present Ags available at very low concentration, like autoantigens. The critical role of B cells in RA as APCs was proved in an elegant study.\(^{55}\) Takemura et al. showed that B cells are indispensable for the activation of ectopic GC-derived T cells in the rheumatoid synovium, since adoptively transferred CD4\(^+\) T cells functioned selectively in tissues with B cell clusters, but could not be triggered in synovium lacking B cell infiltrates.\(^{56}\)

**Pro-inflammatory cytokine secretion**

Activated B cells secrete pro-inflammatory cytokines including IL-4, IL-6 and IFN-\(\gamma\).\(^{23}\) B cell-derived inflammatory mediators modulate the migration of dendritic cells (DCs), activate Mfs, and regulate T-cell functions; therefore they are implicated in the pathogenesis of RA.\(^{23,57}\)

**Regulatory B cells**

B lymphocytes are also involved in the inhibition of inflammatory immune responses, which is carried out by a population called regulatory B cells (Bregs).\(^{22}\) Bregs were first identified in animal models of different autoimmune diseases, where absence of B cells led to
enhanced inflammation and adoptive transfer of IL-10-secreting B lymphocytes reduced the severity of the ongoing autoimmune processes.\textsuperscript{58,59} The contribution of Bregs to human diseases was first proposed by the observation that depletion of B cells can exacerbate certain autoimmune diseases.\textsuperscript{60} The relevance of Bregs in human RA has been proven by Flores-Borja et al.. They showed that patients with RA have fewer CD19\textsuperscript{+}CD24\textsuperscript{high}CD38\textsuperscript{high} Bregs which are not able to inhibit Th17 cell differentiation or induce the development of regulatory T cells. These data suggest that in active RA patients, improper number and function of Bregs may contribute to autoimmunity.\textsuperscript{61}

\textit{Ectopic germinal center formation}

Synovial inflammation in RA is closely related to the formation of ectopic lymphoid microstructures. They are similar to the GCs of secondary lymphoid organs and are functionally competent, allowing affinity maturation of autoreactive IgGs.\textsuperscript{45} These extranodal GCs are ideal sites for Ag presentation and activation of autoreactive cells, making it conceivable that ectopic GCs may play a role in the pathology of RA.\textsuperscript{62,63}

\begin{center}
\textbf{Figure 2. Roles of B cells under physiological and autoimmune conditions}
\end{center}

The figure summarizes B cell functions in health and disease. AutoAb and pro-inflammatory cytokine production, as well as presentation of autoAgs to T cells play an important role in the pathogenesis of many autoimmune diseases.\textsuperscript{(Modified after Silverman GJ and Carson DA; 2003; Arthritis Research & Therapy)
1.2. Regulation of B cell functions by cross-talk between complement- and Toll-like receptors

Formation of ICs during ongoing infections represents an essential part of the normal immune response. TLR ligands and complement-containing ICs may influence effector B cell functions as well as the fate of autoreactive cells through binding to their cognate receptors. All these makes plausible that changes in the expression and/or function of IC-binding receptors may be involved in the pathogenesis of autoimmune disorders.

1.2.1. The complement system

The complement system is one of the major humoral components of innate immunity, consisting of more than forty different plasma and cell surface proteins. Complement has an important role in the discrimination between healthy and injured tissues, in disposal of ICs and apoptotic cells, as well as in inflammatory processes. The multiple interconnections among complement proteins and immune cells provide an excellent mechanism to protect the organism against infections and support the restoration of damaged tissues. However, dysregulation of this “well-balanced” system may lead to the pathogenesis of various diseases, like SLE and RA, making it plausible that modulation of complement activity could represent a viable strategy for the treatment of autoimmunity.\textsuperscript{64,65}

Complement activation occurs via three distinct pathways, known as the classical, alternative and lectin pathways (Figure 3). Upon activation, these pathways result in the formation of instable C3-convertases, C4bC2a (classical and lectin pathways) and C3bBb (alternative pathway). Both enzyme complexes are able to cleave the α-chain of the central component C3, generating C3a and C3b. While C3a act as an anaphylatoxin, binding of C3b to biological surfaces leads to opsonization for phagocytosis. Alternatively, C3b may bind to an already existing C3-convertase, which gains the ability to cleave C5 and thus activate the terminal complement pathway. This leads to inflammation by the generation of C5a and cell lysis through the assembly of the membrane attack complex.\textsuperscript{66-68}

To preserve integrity of the host, the complement system is strictly regulated by membrane-bound and soluble regulators. These regulators (such as CR1 or Factor H) facilitate either the dissociation of the C3/C5-convertases or serve as co-factors for Factor I-mediated cleavage of C3b. Cleavage of C3b into iC3b results in the interruption of the complement cascade. At the same time the limited proteolysis of C3 generates ligands for complement receptors.\textsuperscript{69} Binding of C3 fragment-containing ICs to CR1 or CR2 on human B cells
provides an important feedback signal for the adaptive immunity, thereby connects the innate and acquired arms of the immune response.\textsuperscript{70,71}

**Figure 3. Activation and regulation of the complement system**

The figure illustrates the activation of the complement cascade. Complement regulators are shown in red. MBL indicates mannose-binding lectin; MASP, MBL-associated serine protease; MAC, membrane attack complex; DAF, decay accelerating factor; MCP, membrane cofactor protein
1.2.2. Toll-like receptors

TLRs belong to the well-characterized pattern recognition receptors (PRRs), which recognize different pathogen-associated molecular patterns (PAMPs), such as lipids, carbohydrates and nucleic acids. More than 10 members of TLRs have been identified in humans; each of them has specific ligand(s) that they can detect (Figure 4). TLR4 recognizes the lipopolysaccharide (LPS) of bacteria, whereas TLR2 with TLR1 or TLR6 detects peptidoglycan and lipopeptides. TLR3 and TLR5 are activated by double-stranded ribonucleic acids (RNAs) of viruses and flagellin, respectively. TLR7/8 sense single-stranded viral RNAs, and TLR9 recognizes CpG-rich viral and bacterial nucleic acids.

TLRs involved in the recognition of molecular structures unique to bacterial and fungal cells (TLR1, TLR2, TLR4, TLR5 and TLR6) are localized on the cell surface and induce the production of inflammatory cytokines. Those TLRs which sense viral and bacterial nucleic acids (TLR3, TLR7, TLR8 and TLR9) reside mainly in intracellular compartments and are responsible for immune responses against intracellular pathogens by the production of type I IFNs.

![Figure 4. Toll-like receptors](image-url)

The figure illustrates human TLRs and their ligands. TLRs, expressed by B cells are shown in red. (Modified after Pone et al.; 2010; Critical Reviews in Immunology)
1.3. Complement- and Toll-like receptors on human B cells

Many common PAMPs, such as bacterial nucleic acids, LPS or zymosan act both as TLR ligands and activators of complement. Once activated, the complement cascade generates C3 cleavage products (such as C3b, iC3b and C3d), which are covalently attached to the activating agent and serve as ligands for various complement receptors including CR1 and CR2 on B cells. In addition to this, human B cells express a diverse repertoire of TLRs, in which TLR1, TLR7, TLR9 and TLR10 are predominant. Binding of ICs, containing ligands for both complement receptors and TLRs links complement activation and innate immunity to B cell biology, thereby provides important feedback signals for the adaptive immune response.

1.3.1. Complement receptor type 1 (CR1/CD35)

CR1 is an approximately 200kDa I-type membrane glycoprotein expressed by several cell types, including erythrocytes, B cells, thymocytes, monocytes, Mfs, neutrophil and eosinophil granulocytes, FDCs, Kupffer cells and podocytes.\(^75\),\(^76\) The extracellular part of its most common size F allotype consists of 30 short consensus repeats (SCRs). The amino-terminal 28 SCRs can be arranged into four long homologous repeats (LHRs), A-D, each consisting seven SCRs.\(^77\) It binds activated fragments of C3 and C4, such as C3b and C4b and with lower affinity, iC3b. CR1 belongs to the complement regulators and possesses decay accelerating activity for the C3/C5-convertases of the classical and alternative pathways. Additionally, it serves as a cofactor for Factor I-mediated cleavage of C3b, thus blocks further activation of the complement cascade.\(^78\) The first three SCRs in LHR-A, LHR-B and LHR-C are able to bind C3b and C4b. SCR1-3 in LHR-A binds preferentially C4b and weakly C3b, while SCR8-10 in LHR-B and SCR15-17 in LHR-C bind mainly C3b and also, even if with lower affinity, C4b. LHR-A contains the decay-accelerating activity site for C3-convertases, whereas SCRs in LHR-B and LHR-C exert cofactor activity.\(^78\),\(^79\)

On erythrocytes, CR1 plays an elemental role in the elimination of C3b/C4b-opsonized ICs which are transported to the liver and spleen for degradation.\(^80\) This transfer is facilitated by complement- and Fc receptors expressed on erythrocytes and tissue resident phagocytes.\(^81\),\(^82\) On monocytes and neutrophil granulocytes, the receptor induces phagocytosis and is involved in the presentation of complement-tagged ICs.\(^83\) Furthermore, CR1 is indispensable for the retention of C3b/C4b-opsonized antigens on FDCs in GCs, thus facilitates the maintenance of immunological memory.\(^84\),\(^85\)
Although the role of CR2 in various B cell functions is relatively well established, much less is known about the precise role of CR1 in these cells. Earlier studies on human B cells are controversial, most likely due to the different experimental conditions, the origin of cells and Abs, reacting with distinct epitopes of CR1. Daha et al. reported that pokeweed mitogen-induced stimulation of B cells together with anti-CR1 monoclonal Abs results in enhanced Ab production. Proving these results, Weiss et al. demonstrated that triggering of CR1 positively regulates the specific Ab response to low doses of Ag. These studies considered anti-CR1 Abs as ligands that mediated stimulatory effect on certain B cell functions. In contrast to this, Tsokos et al. reported that the natural CR1 ligand, C3b or its fragments mediate inhibitory effects on human B cell responses. In agreement, data presented by our group indicated that CR1 exerts negative modulatory effect on human B cell functions. Treatment of B cells with aggregated C3, which mimics multimeric C3b and binds to CR1, strongly and dose-dependently inhibits the anti-IgM-induced proliferation as well as Ca2+ mobilization of resting tonsillar B cells. Moreover, cross-linking of the BCR and CR1 were shown to lower the number of IgG anti-DNA producing PCs.

1.3.2. Complement receptor type 2 (CR2/CD21)

CR2 is a surface glycoprotein expressed on B cells and FDCs in mice with a somewhat broader tissue distribution in humans (etc. on a subset of T cells). It is composed of 15 (on B cells) or 16 (on FDCs) SCRs, which are generated by alternative splicing of a single exon. The first two most membrane-distal SCRs are involved in binding of the C3 fragments. CR2 binds C3d and C3dg (with lower affinity iC3b, too), the final cleavage products of C3 and represents a key link between innate and adaptive immunity. CR2 has also been reported to be the receptor for the immunoregulatory protein CD23, the Epstein-Barr virus. On FDCs, similarly to CR1, its expression is important for trapping C3 fragment-tagged Ags, resulting in efficient Ag binding and retention of ICs, which is central to the formation of immunological memory and long-term B cell responses. Mice, deficient in CR2, show defects in Ab response, GC formation and generation of memory B cells, which results from the lack of CR2 on both B cells and FDCs. In humans, CR2 is initially expressed on IgM+IgD− immature cells, although its expression on a small subpopulation of pre-B cells has also been described. CR2 is expressed almost on all mature peripheral human B lymphocytes, however it is lost during the differentiation step toward plasma cells.

CR2 functions as a co-receptor for the BCR. On human B cells it appears either in a trimolecular complex of CD19/CD21/CD81 or associated with CR1 and probably a few exist
alone.\textsuperscript{101,102} In the trimolecular complex, CR2 functions as the ligand binding unit whereas CD19 mediates signal transduction.\textsuperscript{103,104} It is well accepted that C3d, the natural ligand of CR2 acts as a molecular adjuvant, which bridges innate and adaptive immunity. The positive effect of CR2 on various B cell functions has been reported in several experiments, both in mice and humans. Dempsey et al. demonstrated that C3d-coupled Ag is 1.000- and 10.000-fold more effective in the induction of adaptive immunity than Ag alone.\textsuperscript{105} The critical role of CR2 in B cell immunity, especially at lower Ag concentration was proven in human studies too. Mongini et al. showed that co-engagement of membrane IgM and CR2 may be particularly important in eliciting an immune response to moderately multivalent Ags.\textsuperscript{106} In addition to its ability to amplify Ag-induced B cell activation, CR2 was proven to be able to rescue peripheral B cells from surface IgM-mediated apoptosis, to enhance Ag processing and presentation of C3d-bound particles, to modulate the expression of costimulatory molecules and to stabilize the BCR in lipid rafts.\textsuperscript{107-110}

1.3.3. Expression and role of CR1 and CR2 on human B cells under physiological and autoimmune conditions

The involvement of B cell activation, inappropriate stimulation and regulation of complement has all been implicated in the pathogenesis of autoimmune diseases.\textsuperscript{64,111,112} Contribution of CR1 and CR2 to the initiation and maintenance of tissue damage was examined in various animal models. Although several similarities exist regarding the general structure and certain functions of CR1 and CR2 in men and mice, differences in the genetic background, ligands and tissue distribution (Figure 5) warn us to interpret results obtained in animal studies with great care. While murine CR2 shows structural and functional homology to human CR2 and has a similar expression pattern, human CR1 is functionally different from murine CR1 and has opposite function as CR2. It is known that C3b and C3d applied in multimeric form enhance the division of mouse B cells. While human CR2 lowers the threshold of B cell stimulation, clustering of human CR1 has been proven to exert negative regulatory function on B cell activation.\textsuperscript{89,90} Therefore, in humans, depending on the overall structure of ICs and the presence of C3 and C4 cleavage fragments, CR1 and CR2 may affect the fate of B lymphocytes differently.

Expression of CR1 and CR2 on human B cells has been studied in a number of human autoimmune diseases. Previous studies reported decreased expression of CR1 on B lymphocytes of patients with SLE and RA: peripheral B cells of autoimmune patients showed a marked reduction in CR1 density compared to control subjects.\textsuperscript{35,113-115} Mechanisms which
are responsible for the decreased expression of complement receptors may be due to both inherited and acquired factors. Enhanced proteolytic cleavage, reduced transcription and IC overload have all been reported as a reason for reduced CR1 and CR2 expression in SLE and RA.\textsuperscript{116-118} The level of CR1 transcription has been shown to correlate conversely with circulating ICs and this phenomenon may lead to down-regulation of receptor expression and subsequent breakdown in IC clearance.\textsuperscript{113,119}

Our group assessing CR1 and CR2 expression on different B lymphocyte subpopulations of healthy donors and SLE patients revealed that under physiological conditions, CD27\textsuperscript{+}CD19\textsuperscript{+} memory B cells express higher level of CR1 than CD27\textsuperscript{-}CD19\textsuperscript{+} naïve cells. Its expression however is markedly decreased in both IgM\textsuperscript{+} and switched memory B cells of SLE patients. Reduction of CR1 expression was more prominent in patients with active SLE compared with their inactive counterparts. In contrast to CR1, no difference was found in the expression of CR2 on naïve and memory B cells of healthy individuals. However, reduced expression of CR2 was observed on both B cell subpopulations in SLE patients.\textsuperscript{120} These results confirmed that the expression of the inhibitory CR1 and the activatory CR2 is differentially regulated during the development of human B cells, a phenomenon, which could influence the maintenance of peripheral B cell tolerance. Therefore, lower expression of CR1 in SLE patients may break the tolerance by reducing the activation threshold of B cell stimulation.

Prokopec et al. have recently reported that the expression of CR1 and CR2 is lower on the B cells of RA patients. They did not find any association between the expression of CR1/CR2 and disease activity which highlights that down-regulation of receptors on B cells may contribute to initiation of autoimmunity rather than affecting disease severity. The simultaneous alteration of receptors may represent an imperfect B cell regulation that plays a major role in the initiation and maintenance of pathological autoAb production and arthritis processes.\textsuperscript{36}

The functional consequences of decreased complement receptor expression in human diseases have been studied barely. In one of these studies, the response of autoreactive B cells to an anti-IgD Ab - Epstein-Barr virus gp350 protein conjugate was investigated. Despite the fact that B cells of SLE patients expressed half as many surface CR2 as normal B cells, both peak responses and the percentage of responding cells were significantly increased in the former.\textsuperscript{121} Most recent evidence suggests that complement receptors also regulate the
elimination of self-reactive B cells, thus complement deficiency will result in lack of normal B cell tolerance.\textsuperscript{122}

These data further strengthen the notion that altered receptor expression likely contributes to the breakdown of self-tolerance which may lead to pathological autoAb production and autoimmune diseases.

![Figure 5. Main differences between mouse and human CR1 and CR2](image.png)

The figure illustrates the main differences between mouse and human CR1 and CR2 in respect of their ligand-binding sites, structures, tissue distribution and functions.

(Modified after Erdei et al.; 2009; Molecular Immunology)

1.3.4. Toll-like receptors on B cells under physiological and autoimmune conditions

TLRs sense microbial infection and engage several mechanisms that control the initiation of the adaptive immune response. DCs are the key cell types that couple TLR-mediated innate immune recognition to the initiation of T and B cell activation.

B lymphocytes possess a somewhat extraordinary status in the immune system because they express both clonally rearranged Ag-binding receptors and nonclonal PRRs, most notably TLRs. Human B cells express a diverse repertoire of TLRs, in which TLR1, TLR7, TLR9 and TLR10 are the most abundant. Stimulation of B cells with various TLR ligands (like Pam3CSK4 for TLR1/2; imiquimod for TLR7/8; or CpG oligodeoxynucleotides (ODNs)
for TLR9) results in up-regulation of activation markers, as well as proliferation, cytokine and Ab production.\textsuperscript{123-125} B1 cells and MZ B cells are particularly sensitive to TLR ligands and induce T cell-independent immediate Ab responses.\textsuperscript{14,126} The signal pathway mediated by the engagement of TLRs is known to involve myeloid differentiation factor 88 (MyD88) -induced nuclear factor-κB (NF-κB) activation and synergizes with the BCR-induced signals at the level of mitogen-activated protein kinases (MAPKs).\textsuperscript{127-130} Coupling BCR stimulation to TLR expression and function endows the human system with a highly specific immune response, since it allows focusing of innate signals only on Ag-stimulated B cells.\textsuperscript{131,132}

The effect of TLR signaling on B cell activation is also evident in situations where chromatin-Ig complexes (tagged with complement fragments as well) inappropriately activate B cells through synergistic BCR and TLR stimulation and lead to autoimmune diseases.\textsuperscript{133} In recent years, it has become increasingly evident that TLRs play an elemental role in triggering adaptive immunity and autoimmune responses. In the context of B cells, this is best established for TLRs that detect nucleic acids, including TLR7 and TLR9.\textsuperscript{134}

TLR7 and TLR9 might induce autoimmune diseases through several possible mechanisms. First, autoreactive B cells have been reported to have increased level of TLR7 and TLR9.\textsuperscript{135-137} Under normal circumstances, the localization of these TLRs in endosomal compartments prevents the recognition of self nucleic acids and induction of autoimmunity. Modifications of vertebrate RNA and deoxyribonucleic acid (DNA) provide an additional level of specificity which also reduces the probability of TLR activation.\textsuperscript{138,139} Inappropriate clearance of chromatin- or RNA-containing ICs released from dying cells was reported as a major reason for autoAb production in both SLE and RA.\textsuperscript{133,140-142} Although vertebrate nucleic acids are considered as poor ligands for TLRs, modified RNA and DNA may have high immunostimulatory potential and therefore may induce autoimmunity through processes dependent on both the BCR and endosomal TLR9.\textsuperscript{143} The exact molecular mechanisms mediated via the synergistic BCR/TLR engagement in autoimmune patients remain poorly understood. One current model highlights that, following binding of nucleic acid-containing Ags, BCRs are internalized into the cells and Ag complexes are delivered to TLRs localized in the endosomal/lysosomal compartments (Figure 6). Viglianti et al. showed that the proliferative response of autoreactive RF\textsuperscript{+} B cells to mammalian chromatin-containing ICs results from the simultaneous engagement of the BCR and TLR9. They found that the presence of autoAbs is indispensable for the activation of RF-specific B cells by hypomethylated CpG motifs, which further strengthens the critical role of BCR in the
transport of chromatin into TLR9-containing endosomes. BCRs that directly bind autoAg, either RNA or DNA, provide the same delivery system. It is noteworthy that the effects of TLRs on B cell functions are not restricted to secretion of autoAbs, but can also stimulate the production of pro-inflammatory cytokines, which can influence the activation of T cells and amplify inflammatory responses.

The role of TLRs in induction and spreading of autoimmunity is further supported by data demonstrating higher expression of membrane-bound BAFF on autoreactive B cells after TLR9 activation. Increased expression of BAFF in parallel with the anti-apoptotic effects of CpG ODN could overcome the clearance of apoptotic cells and result in B cell-mediated pathological processes. Additionally, positive regulatory effect of CpG-containing DNA on autoAb production was confirmed by results demonstrating TLR9-induced extracellular signal-regulated kinase (ERK) activation, which normally acts to prevent B cell secretion of autoAbs.

Considering the dual role of both B cells and TLRs in autoimmune diseases, these results suggest that TLR7/9 inhibitors have the potential to act synergistically with BCR pathway inhibitors to diminish IC-driven autoAb production. Indeed, chloroquine-related compounds - which both impair phagolysosomal functions and are TLR antagonists - are used as valuable therapeutics in both SLE and RA and emphasize the usefulness of combined B cell- and TLR-associated therapeutic agents in the treatment of rheumatic diseases.

1.4. Cross-talk between the complement system, TLRs and adaptive immunity

The complement system and TLRs are two effector arms of innate immunity, providing a first-line host defence against invading pathogens. Although these two systems often are studied as different entities, many PAMPs such as CpG, zymosan and LPS are able to activate both parts of the innate immune system. In this context, cross-talk between complement and TLRs is essential to appropriate coordination of the early innate response to infections. Apart from generating an immediate inflammatory reaction against invading pathogens, the activation of complement and TLRs also functions to initiate and shape the adaptive immune response. For instance, complement and TLRs have long been recognized as natural adjuvants for Ab production, and recent works have suggested a role for those in T-cell immunity too. Raby et al. reported a marked positive regulatory effect of TLR2, TLR4 and TLR9 activation on cell sensitivity to C5a. The enhanced C5a response of PBMCs after pre-exposure to different TLR ligands was attributed
to a decreased expression of the inhibitory C5 receptor-like 2. Song et al. demonstrated in two elegant studies, that coincidental activation of complement and TLR2/6, TLR4 or TLR9 in mice leads to elevated IL-6 secretion (released mainly from Mfs), and results in increased Th17 differentiation from stimulated naïve CD4+ T cells. The positive modulatory effect of TLR2/6 and TLR4 on complement was C5a receptor (C5aR)-dependent, whereas C3a receptor (C3aR) played a more important role than C5aR in regulating TLR9 signaling. Interaction between complement and TLRs can occur both ways. While, as described previously, the complement-derived anaphylatoxins, C3a and C5a increased TLR-induced pro-inflammatory cytokine production, other studies have reported that TLR activation may induce complement protein synthesis (like Factor B) and effector functions. Collectively, these results demonstrate that complement and TLR interaction may not only strengthen the hosts’ innate immune response but also their adaptive immunity to infections.

Considering the role of complement, TLRs and B cells in autoimmunity, synergistic activation of the two innate systems and subsequent priming of humoral immunity may be detrimental under pathological conditions. The basis for this paradigm in the context of B lymphocytes is illustrated in Figure 6. In several autoimmune diseases, like SLE and RA, the accumulation of apoptotic debris - due to increased apoptosis and/or decreased removal of the dead cells - may result in complement activation through the classical or alternative pathway. The apoptotic debris is enriched in self-RNA and DNA, which is capable of activating TLR7 and TLR9 in plasmacytoid DCs and B cells, particularly if it forms ICs with pre-existing autoAbs. The binding of autoAbs to complement-Ag complexes or dead cells further enhances the activation of complement and uptake of immune complexes by APCs. Activation of DCs by TLR ligands results in cytokine and chemokine production and triggers them to present self-Ags to autoreactive T cells. Activation of T cells provides help to B lymphocytes and supports further autoAb production and cytokine secretion.

The exposure of B cells to elevated levels of ICs containing self-Ags and ligands for complement- and Fc receptors also stimulates them directly via both TLR9 and the BCR. BCR-mediated signals are strictly controlled by IC-binding receptors (CR1, CR2, and FcγRIIb) which influence the final outcome of B cell activation. Decreased levels of inhibitory receptors (like CR1 and FcγRIIb) or elevated expression of co-activators (like CR2 and TLR7/9) in autoimmune diseases can lead to pathological processes, resulting in tissue damage and increased production of apoptotic debris, creating a vicious cycle of autoreactive cell activation.
In summary, complement and TLRs are two important components of innate immunity. Crosstalk between these two systems to enhance the immediate immune reaction to pathogens and to induce a more robust adaptive immune response has clear benefits from an evolutionary point of view. In other conditions, however, synergistic augmentation of the inflammatory response may be harmful. Understanding the mechanisms of such interactions may help to develop novel therapeutic approaches to prevent or treat inflammation under pathological conditions.

Figure 6. Cross-talk between the complement system, TLRs and adaptive immunity

The figure illustrates the development of inflammatory responses after the release of RNA/DNA from apoptotic cells. Binding of ICs, containing ligands for both complement receptors and TLRs might link complement activation and innate immunity to B cell biology and tolerance.
2. Aims

Complement and TLRs are two effector arms of innate immunity. In addition to generating an immediate inflammatory reaction against invading pathogens, the activation of complement and TLRs also functions to initiate and shape the adaptive immune response. The importance of complement- and Toll-like receptors in regulating B cell responses has been demonstrated in several autoimmune animal models. The pathogenic potential of these receptors in human autoimmune diseases however is not well-characterized. The main goal of my thesis is to gain more knowledge about the regulatory role of complement- and Toll-like receptors in B cell immunity under physiological and autoimmune conditions.

Our aims were

1. To compare the expression pattern of complement receptor type 1 (CD35) and type 2 (CD21) on CD19⁺CD27⁻ naive, CD19⁺CD27⁺ memory B cells and CD19⁺CD27high plasmablasts of healthy individuals and RA patients.

2. To reveal the effect of CR1 clustering on the BCR-induced proliferation and antibody production of B cells derived from healthy donors and RA patients.

3. To describe how ligand-induced clustering of CR1 modulates CpG (TLR9) -induced functions of human B cells.

4. To reveal how CR1 clustering affects BCR- and TLR9-induced signaling events.

5. To get a deeper insight into the mechanism of CpG-induced activation of human B cells.
3. Materials and methods

3.1. Materials

3.1.1. Buffers and media

RPMI medium 1640:
- RPMI powder (dissolved in 1000 ml distilled water), Sigma
- NaHCO$_3$, 2 mg/ml
- Sodium-piruvate, 0.22 mg/ml
- L-glutamine, 2mM
- Streptomycin, 0.1 mg/ml
- Penicillin, 100 U/ml

Tetramethylbenzidine (TMB) buffer (pH5.5) for ELISA:
- Na-acetate, 0.1M

Phosphate buffered saline (PBS):
- NaCl, 8g/l
- KCl, 0.2 g/l
- Na$_2$HPO$_4$·H$_2$O, 1.4 g/l
- KH$_2$PO$_4$, 0.2 g/l

FACS buffer:
- PBS
- FCS, 1%
- Na-azide, 0.1%

Sample buffer (5X) for SDS-PAGE:
- Tris-HCl, 313mM
- Glycerol, 50v/v%
- SDS, 5w/v%
- 2-mercaptoethanol, 5v/v%
- bromophenol blue, 0.002%

Running buffer for SDS-PAGE:
- Tris, 3 g/l
- SDS (10%), 10 ml/l
- Glycine, 14.4 g/l
- Distilled water up to 1000 ml

Transfer buffer for Western Blot:
- Tris, 3 g/l
- Glycine, 14.4 g/l
- Distilled water up to 1000 ml

Lysis buffer:

- Tris, 20mM
- NaCl, 150mM
- EDTA, 1mM  EGTA, 1mM
- Triton X-100, 1%
- β-glycerophosphate, 1mM
- Distilled water up to 1000 ml

Tris buffered saline (TBS)

- Tris, 2.42 g/l
- NaCl. 8 g/l

3-amino-9-ethyl carbazole (AEC) solution for ELISPOT:

- AEC, 100mg
- N,N-dimethyl formamide, up to 10 ml

AEC buffer for ELISPOT (pH 5.0):

- Acetic acid (0.2M), 148 ml
- Sodium acetate (0.2M), 352 ml
- Distilled water up to 1000 ml

2-aminoethyl-iso-thiouronium (AET):

- NaCl, 9g
- AET, 40g
- Distilled water up to 1000 ml (pH 8.5)
### 3.1.2. Antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Isotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-hCD19-APC</td>
<td>mouse IgG1</td>
<td>Immunotools</td>
</tr>
<tr>
<td>anti-hCD27-PE</td>
<td>mouse IgG2b</td>
<td>Calltag Laboratories</td>
</tr>
<tr>
<td>anti-hCD38-APC</td>
<td>mouse IgG1</td>
<td>Immunotools</td>
</tr>
<tr>
<td>anti-hCD20-FITC</td>
<td>mouse IgG1</td>
<td>Immunotools</td>
</tr>
<tr>
<td>anti-hCD21-FITC</td>
<td>mouse IgG1</td>
<td>Immunotools</td>
</tr>
<tr>
<td>anti-hCD35-FITC</td>
<td>mouse IgG1</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>anti-hCD19-APC</td>
<td>mouse IgG1</td>
<td>Immunotools</td>
</tr>
<tr>
<td>anti-mIgG-Alexa647</td>
<td>goat</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>anti-hCD80-biotin</td>
<td>mouse IgG1</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>anti-hCD86-FITC</td>
<td>mouse IgG1</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>anti-hCD40-APC</td>
<td>mouse IgG1</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>anti-hTLR9</td>
<td>mouse IgG1</td>
<td>Santa Cruz Bitechmotechnology</td>
</tr>
<tr>
<td>anti-hTLR9</td>
<td>rat IgG2a</td>
<td>Santa Cruz Bitechmotechnology</td>
</tr>
<tr>
<td>anti-LAMP1</td>
<td>rabbit IgG</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>streptavidin-PerCP-Cy5.5</td>
<td></td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>anti-pSyk</td>
<td>rabbit</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>anti-CD79A</td>
<td>rabbit</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>anti-pp38</td>
<td>rabbit</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>anti-pp44/42 MAPK</td>
<td>rabbit</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>anti-B-actin</td>
<td>rabbit</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>anti-pLyn</td>
<td>rabbit</td>
<td>Abcam</td>
</tr>
<tr>
<td>anti-SHP-2</td>
<td>mouse IgG1</td>
<td>BD Transduction Laboratories</td>
</tr>
<tr>
<td>anti-hTLR9</td>
<td>mouse IgG1</td>
<td>Santa Cruz Bitechmotechnology</td>
</tr>
<tr>
<td>anti-hCD35</td>
<td>mouse IgG1</td>
<td>Santa Cruz Bitechmotechnology</td>
</tr>
<tr>
<td>anti-hCD79A</td>
<td>mouse IgG1</td>
<td>Abcam</td>
</tr>
<tr>
<td>anti-mIgG-HRP</td>
<td>goat</td>
<td>Santa Cruz Bitechmotechnology</td>
</tr>
<tr>
<td>anti-nIgs-HRP</td>
<td>goat</td>
<td>Dako</td>
</tr>
<tr>
<td>anti-hlgM</td>
<td>mouse IgG1</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>anti-hlgG</td>
<td>mouse IgG1</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>anti-hlgM-HRP</td>
<td>mouse IgG1</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>anti-hlgG-HRP</td>
<td>mouse IgG1</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>anti-hlgG/A/M</td>
<td>goat F(ab')₂</td>
<td>J acne ImmunoResearch</td>
</tr>
<tr>
<td>anti-mIgG</td>
<td>goat F(ab')₂</td>
<td>J ac e ImmunoResearch</td>
</tr>
<tr>
<td>anti-goat IgG</td>
<td>rabbit F(ab')₂</td>
<td>J ac e ImmunoResearch</td>
</tr>
</tbody>
</table>

h: human, m: mouse, p: phospho, HRP: horseradish peroxidase,
FITC: fluorescein isothiocyanate, PE: phycoerythrin, APC: allophycocyanin
Lysosome-associated membrane protein 1 (LAMP1)
### 3.1.3. Reagents

#### Cytokines and reagents

<table>
<thead>
<tr>
<th>Name</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>rh IL-10</td>
<td>Immunotools</td>
</tr>
<tr>
<td>rh IL-2</td>
<td>Immunotools</td>
</tr>
<tr>
<td>rh soluble CD40 ligand</td>
<td>Immunotools</td>
</tr>
<tr>
<td>H&lt;sup&gt;3&lt;/sup&gt;-thymidine</td>
<td>American Radiolabeled Chemicals</td>
</tr>
<tr>
<td>Tetramethylbenzidine (TMB)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Sigma</td>
</tr>
<tr>
<td>Carboxyfluorescein succinimidyl ester (CFSE)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Bovine serum album (BSA)</td>
<td>Immunotools</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride (PMSF)</td>
<td>Jackson ImmunoResearch</td>
</tr>
<tr>
<td>Fetal calf serum (FCS)</td>
<td>Gibco</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>

*rh, recombinant human*

#### ODNs

<table>
<thead>
<tr>
<th>Name</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG ODN2006</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>GpC ODN2006</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>CpG ODN2006-biotin</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>CpG ODN2006-A488</td>
<td>Integrated DNA technologies</td>
</tr>
</tbody>
</table>

#### Inhibitors

<table>
<thead>
<tr>
<th>Name</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syk inhibitor IV (BAY61-3606)</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Syk inhibitor II</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Src kinase inhibitor I</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>MyD88 inhibitor</td>
<td>Invivogen</td>
</tr>
</tbody>
</table>
3.2. Methods

3.2.1. Patients and controls

Blood samples were obtained from 23 healthy individuals, aged 43 ± 15 years (18 women, 5 men). From RA patients, aged 52.8 ± 10.3 years, blood of 26 active, 11 moderately active and 10 inactive patients (42 women, 5 men) were obtained from the Institute of Rheumatology and Physiotherapy, Hungary. All of the patients fulfilled the criteria of a definitive diagnosis of RA, suggested by the American College of Rheumatology. Patients were grouped based on the disease activity score for 28 joints (DAS28). They were considered as active with DAS28>5.1, moderately active with 3.2<DAS28<5.1 and inactive with DAS28<3.2. No patients underwent biological therapy during the period of the experiments. Patients were treated with the following drugs: non-steroid anti-inflammatory drugs, steroid anti-inflammatory drugs (prednisolone, methylprednisolone) and disease modifying anti-rheumatoid drugs (methotrexate, leflunomide, chloroquine, sulfasalazine). All patients examined for functional assays were in active disease state (average DAS28: 6.17). The study was approved by the local ethical committee (Institutional Review Board of National Institute of Rheumatology and Physiotherapy) and written informed consent was obtained from all participating subjects.

Tonsils from children undergone routine tonsillectomy were obtained from the Saint Istvan and Saint Laszlo Hospital in Budapest, Hungary after obtaining written informed consent according to the Declaration of Helsinki.

3.2.2. B cell preparation and culture conditions

3.2.2.1. B cell preparation from blood

Peripheral blood mononuclear cells (PBMCs) from healthy individuals and active RA were isolated by Ficoll-Hypaque gradient centrifugation (GE Healthcare). B cells were purified by RosetteSep B cell isolation Kit (StemCell Technologies) through negative selection according to the manufacturer’s instructions. CD19+ B cell purity was higher than 90% in each case as measured by flow cytometry.

3.2.2.2. B cell preparation from tonsils

Tonsillar mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation. After rosetting with AET-treated sheep red blood cells, B cells were isolated by centrifugation over Ficoll-Hypaque solution. Separated B cells were further fractionated into low- and high-
density populations on a Percoll (Sigma-Aldrich) gradient. CD19⁺ B cell purity was higher than 95% in each case.

3.2.3. Assays for B cell functions

Blood-derived and high-density („resting”) tonsillar B cells were cultured at 2x10⁵ cells/well in 100 µl RPMI 1640 medium supplemented with 10% FCS and 50 µg/ml gentamycin in 96-well microtiter plates (Costar). Cells were stimulated with 5 µg/ml F(ab')₂ of goat anti-human IgG/A/M and/or with distinct amounts of CpG ODN2006 applied at suboptimal concentration. Cultures were analyzed for cell viability by propidium iodide (PI) exclusion. In experiments with Syk inhibitor, data were normalized to the number of living cells. Detailed description of culturing conditions of individual experiments is provided in the Figure legends.

3.2.3.1. Proliferation assay

Cell proliferation was assessed either by H³-thymidine incorporation or with the CFSE method as described earlier.²⁹,¹⁶⁶ Briefly, 48 hours after stimulus, cells were pulsed with 1 µCi/well ³H-thymidine for the last 16 hours of culture. Incorporated radioactivity was measured using a Wallac 1409 liquid scintillation beta counter (Wallac). Alternatively, cells were labeled with 0.5µM CFSE and proliferation was measured after 72 hours by flow cytometric measurement of CFSE dye dilution.

3.2.3.2. Cytokine measurement assay

Secreted IL-6, IL-10, IFN-γ and TNF-α levels were measured 48 hours after stimulus from pooled cell culture supernatants by the FlowCytomix technology according to the instruction of manufacturer (eBioscience, human IL-6, IL-10, IFN-γ and TNF-α Simplex Kit).

3.2.3.3. Measurement of plasmablast differentiation by flow cytometry

To induce plasmablast differentiation, B lymphocytes were cultured in the presence of 50 ng/ml rh IL-2, 50 ng/ml rh IL-10 and 100 ng/ml soluble CD40L or 50 ng/ml rh IL-6. After 7 days of culture, the number of CD19lowCD20lowCD27highCD38⁺ plasmablasts was determined by flow cytometry. Briefly, cells were washed twice in ice-cold PBS than stained with the following antibodies: anti-human CD20-FITC, anti-human CD27-PE, anti-human CD19-APC and anti-human CD38-APC. Measurements were performed using a FACSCalibur flow cytometer (BD Biosciences). Data of 100,000 cells were collected and % of plasmablasts was counted in the living B cell population.
3.2.3.4. Detection of antibody production by ELISA

To induce plasmablast differentiation, B lymphocytes were cultured in the presence of 50 ng/ml rh IL-2, 50 ng/ml rh IL-10 and 100 ng/ml sCD40L or 50 ng/ml rh IL-6. After 7 days of culture, IgM and IgG production was measured by ELISA. Briefly, microtiter plates (Costar) were coated with 3 µg/ml anti-human IgM or IgG at 4°C, overnight. After washing, the plates were incubated with the cell culture supernatants for 2 hours at 37°C. As standard, human IgM or IgG isolated from human serum was used at different concentrations. After washing, the assays were developed with HRP-conjugated anti-human IgM (1:1000) or IgG (1:2000). For visualization, TMB was used as chromogen.

3.2.3.5. Detection of ASCs by ELISPOT

To measure the number of IgM and IgG Ab secreting cells after 7 days of culture, ELISPOT assays were performed on 96-well ELISPOT plates (Millipore) coated with 5 µg/ml anti-human IgM or IgG. After washing and blocking with PBS containing 1% FCS, the plates were incubated with 2×10⁵ activated cells for 24 hours at 37°C. After washing, the assay was developed with HRP-conjugated anti-human IgM (1:1000) or IgG (1:2000). For visualization, AEC was used as chromogen. Data are shown as mean number of ASCs ± SD of duplicate samples.

3.2.4. Flow cytometry

Immunofluorescence measurements were performed using a FACSCalibur flow cytometer and the CellQuest software (BD Biosciences). On the basis of forward (FSC) and side scattering (SSC) properties, lymphocytes were differentially counted. Cell death was visualized by PI exclusion. A total of 4×10⁵ PBMCs or 1×10⁶ high-density tonsillar B cells were washed in PBS, and were stained using the appropriate antibodies according to the manufacturer’s instruction. For intracellular measurements, cells were fixed and permeabilized with BD Cytofix/Cytoperm solution (BD Biosciences). To reduce nonspecific Ab binding, cells were treated with Fc receptor blocking reagent (Miltenyi Biotec). Isotype-matched FITC-, PE- and APC-conjugated mouse or rat Igs were used as negative controls for non-specific staining. After incubation on ice for 30 minutes, cells were washed and resuspended in 200µl FACS buffer. Relative mean fluorescence intensity (R$_{MFI}$) was obtained by dividing the mean fluorescence intensity (MFI) of the sample with the isotype-matched control. Detailed description of each flow cytometry experiment is provided in the Figure legends.
3.2.5. Laser scanning confocal microscopy

CpG ODN uptake and its colocalization with TLR9 and LAMP-1 were visualized by confocal microscopy. Briefly, B cells were stained with CpG-Alexa Fluor 488 for 30 minutes at 37°C in the presence or absence of 10nM Syk inhibitor IV. After fixation and permeabilization, cells were stained with mouse anti-human TLR9 and rabbit anti-human LAMP-1 for 30 minutes at 4°C. After extensive washing, cells were labeled with anti-mouse Alexa Fluor 647 and anti-rabbit Alexa Fluor 555. Analysis was carried out using an Olympus FLUOView 500 laser-scanning confocal microscope (Hamburg, Germany). Fluorescence and DIC images (1024x1024 pixels) were acquired using a 60x oil-immersion objective. Images were processed by ImageJ software (http://rsbweb.nih.gov/ij).

3.2.6. Isolation of human C3, generation of C3b-like C3 (aggregated C3)

Human C3 was isolated from freshly drawn serum by fast protein liquid chromatography (FPLC) as described by Basta and Hammer. Purified C3 was collected, concentrated and dialyzed against PBS. To minimize IgG contamination, the C3 preparation was incubated with Protein G beads (ThermoScientific). The purity of C3 was checked by SDS-PAGE and Coomassie blue staining. C3 fractions were stored at -20°C until use. Aggregated C3 was generated by incubating isolated human C3 at 63°C for 20 minutes.

3.2.7. Western Blot analyses

To assess B cell activation induced by surface bound CpG ODN, 24-well microtiter plates (Costar) were coated with 5 µg/ml neutravidin (ThermoScientific) and different concentrations of biotin-labeled CpG ODN2006 or PBS alone (untreated cells). After overnight incubation at 4°C and extensive washing, 2×10^6 high-density B cells were added to the plates and stimulated for 30 minutes at 37°C.

To analyze the effect of Syk inhibition on the synergistic stimuli of BCR and CpG, 2×10^6 B cells/sample were activated with 0.1 µg/ml F(ab')_2 fragment of goat anti-human IgG/A/M for 2 minutes and/or 1 µg/ml CpG for 10 minutes in the presence or absence of 10nM iSyk IV.

To analyze TLR9 expression after CpG stimulation, 4×10^5 high-density B cells/well were activated with 2 µg/ml CpG in the presence or absence of 5nM iSyk IV or vehicle control. After 48 hours, 10^6 living cells/activation were sorted using a BD FACSARia III cell sorter (BD Biosciences).
The effect of different inhibitors on CpG-induced phosphorylation of Syk was measured by pre-treatment of $2 \times 10^6$ B cells/sample either with 5µM MyD88 inhibitor, 10nM Src kinase inhibitor I, 10nM Syk inhibitor IV or vehicle (DMSO) for 30 minutes at 37°C. After incubation, cells were left untreated or activated with 1 µg/ml CpG.

Detailed description of each individual experiment is provided in the Figure legends.

In each case, after cell activation and extraction in 1% Triton X-containing lysis buffer (containing 2 mM sodium-orthovanadate, 1mM PMSF, 2 µg/ml aprotinin, 2 µg/ml pepstatin, 5 µg/ml pepstatin), samples were centrifuged at 15,000g for 15 minutes at 4°C. Isolated total protein concentration was measured by Bicinchoninic Acid Kit (Sigma Aldrich). 50 or 100 µg protein per sample was boiled in reducing sample buffer, separated on 10% polyacrylamide gel and transferred to nitrocellulose membrane (Bio-Rad Laboratories). After blocking with 5% milk powder (SantaCruz Biotechnology) for 1 hour, blots were incubated with the relevant primary Abs overnight, at 4°C. Membranes were then incubated with HRP-conjugated secondary Abs. Bound Abs were visualized by the enhanced chemiluminescent method (Millipore) and analyzed with the ImageJ software. Relative intensity (representing the phosphorylation level of individual signaling molecules) was calculated by dividing the peak densitometry values of the target protein by the peak values of the loading control.

3.2.11. Statistical analysis

Statistical differences were assessed by Permutation test or Student’s t-test where appropriate, using PrismSoftware, version 4.0 (GraphPad Software). Values of $p < 0.05$ were considered statistically significant.
4.1. Results I.

Expression and function of CR1 and CR2 in healthy controls and SLE patients

The functional role of complement receptors was investigated in several animal models of collagen induced arthritis, furthermore a prominent role of CR1 and CR2 in the regulation of human B cell functions has been demonstrated too.\textsuperscript{168,169} It is important to emphasize however, that the results obtained in experiments with mice can be adapted to human systems only with great care due to major differences in the immune system – including the expression and function of complement receptors - of these species (see Introduction). Prokopec et al. showed that B cells of RA patients express lower level of CR1 and CR2 than healthy individuals.\textsuperscript{36} However they didn’t investigate how the distribution of various B cell subsets (expressing different levels of complement receptors under physiological conditions\textsuperscript{120}) may contribute to the overall reduction of CR1 and CR2 on B cells.

Our aims were therefore to reveal the expression pattern of CR1 and CR2 on different B cell subsets of healthy controls and RA patients being at different stages of the disease, and to investigate the functional consequence of the decreased expression of CR1 on B cells in rheumatoid arthritis.
4.1.1. RA patients at different stages of disease have similar frequencies of B cell subsets as healthy controls

Recently Catalán et al. showed that the frequency of B cells in the blood of healthy individuals and RA patients is similar, such as the percentages of human B cell subsets of healthy and autoimmune individuals. Since in this study patients were not analyzed at different stages of the disease, we aimed to clarify whether there is any difference regarding the percentage of various B cell subsets in the inactive, moderately active and active RA patients. As seen in Figure 7, the percentage of CD19<sup>+</sup>CD27<sup>-</sup> naive and CD19<sup>+</sup>CD27<sup>+</sup> memory B cells is highly dispersed in each group, and there is no significant difference regarding the frequency of the distinct B cell subsets (p>0.05) when various groups of RA patients and control subjects are compared. Furthermore there is no difference in the appearance of CD19<sup>low</sup>CD27<sup>high</sup> plasmablasts in the distinct groups investigated here.

**Figure 7. Frequency of B cell subsets of healthy individuals and RA patients**

B cell subsets were identified as CD19<sup>+</sup>CD27<sup>-</sup> naive, CD19<sup>+</sup>CD27<sup>+</sup> memory B cells and CD19<sup>low</sup>CD27<sup>high</sup> plasmablasts by flow cytometric analysis of peripheral mononuclear cells. The relative distribution of naïve B cells (A), memory cells (B) and plasmablasts (C) in the gated CD19<sup>+</sup> B lymphocyte population of active (n=26), moderately active (n=11), inactive (n=10) RA patients and healthy individuals (n=23) is shown. Data are expressed as mean percentage (%) of the different B cell subsets (Permutation-test, n<sub>p</sub>P>0.05).


4.1.2. Naive B cells up-regulate CR1 and down-regulate CR2 expression during differentiation to memory cells both in healthy donors and RA patients

Earlier studies showed that the expression of CR1 and CR2 is lower in the B cells of RA patients than in healthy individuals,\(^3^6\) but no data are available so far regarding the distribution of these complement receptors on different B cell subpopulations. To reveal this, we analyzed the expression of CR1 and CR2 in the following B cell subsets: CD19\(^+\)CD27\(^-\) naive, CD19\(^+\)CD27\(^+\) memory B cells and CD19\(^\text{low}\)CD27\(^\text{high}\) plasmablasts. We found that memory B cells of both the active RA patients and healthy individuals express significantly higher level of CR1 than naive B cells (p<0.05), while in the case of RA patients being at a moderately active or inactive stage, this elevation did not reach statistical significance (p>0.05) (Figure 8A). As demonstrated in the figure, CR1 expression on plasmablasts is significantly lower in each group when compared to naive and memory cells (p<0.05). Expression of CR2 however, changed in the opposite way; it decreased during differentiation to memory B cells (Figure 8B). The reduction was significant (p<0.05) in healthy donors as well as in active and inactive RA patients. As found in the case of CR1, expression of CR2 on plasmablasts is very low in contrast to the other B cell subsets (p<0.001), confirming earlier results.\(^1^0^0,1^2^0\) These data demonstrate that the expression of these two antagonistic complement receptors is regulated differentially during the development of human B cells.
Figure 8. Up-regulation of CR1 and down-regulation of CR2 during the differentiation to memory B cells

Expression of CR1 (A) and CR2 (B) was analyzed in three subsets of lymphocytes: naive B cells, memory B cells and plasmablasts. (B cell subsets were identified as described in the legend to Figure 7.) Cells of active (n=26), moderately active (n=11), inactive (n=10) RA patients and healthy individuals (n=23) were analyzed after staining with the following antibodies: FITC-conjugated anti-CD35 (CR1) or FITC-conjugated anti-CD21 (CR2), PE-conjugated anti-CD27 and APC-conjugated anti-CD19. Data are expressed as the R_MFI intensity of CR1 and CR2 normalized to the FSC value of the different B cell subsets (Permutation-test, *P>0.05; **P<0.05).
4.1.3. CR1 inhibits the proliferation of B cells derived from active RA patients

It has been described earlier that clustering CR1 via its ligand inhibits BCR-induced activation of B cells obtained from healthy individuals. Since the expression of this complement receptor is significantly reduced on the B cells of RA patients, it was important to investigate whether the inhibitory function of CR1 is still maintained. Therefore separated B cells of active RA patients were stimulated via their BCR in the presence of aggregated C3, the natural multimeric C3b-like ligand of CR1, and \(^{3}\)H-thymidine incorporation was measured. As seen in Figures 9A and B, the C3b-like ligand exerted a strong and dose-dependent inhibition on the BCR-induced proliferation. It is important to note that CR1-ligation alone had no effect at all. To further confirm the role of CR1, the experiments were carried out using the CR1-specific monoclonal Ab To5, clustered by anti-mouse IgG. As seen in Figures 9C and D, the antibody-mediated cross-linking of CR1 had a similar effect as the multimeric ligand, namely it caused a strong inhibition of B cell proliferation of both healthy donors and active RA patients. It should be mentioned that we did not see any difference between the proliferative capacity of B cells derived from healthy donors and active RA patients – confirming the results of Prokopec et al.\(^{36}\)

4.1.4. CR1 clustering prevents B cell differentiation to plasmablasts

In the next step we set out to investigate, whether CR1 cross-linking also affects plasmablast formation. To this end, freshly isolated B cells were cultured in the presence of anti-human IgG/M/A antibody, IL-2, IL-10, soluble CD40L and aggregated C3 – as detailed in the legend of Figure 10. After 7 days cells were washed and stained to monitor the expression of CD20 and CD27. Among the CD19\(^{+}\) cells, plasmablasts were identified by their CD20 and CD27 expression. As shown in Figure 10, cells of healthy donors and active RA patients cultured without any stimulatory agent contained very few CD20\(^{+}\)CD27\(^{high}\) plasmablasts (ranging between 1-5\%). Stimulation by anti-IgG/M/A alone induced little growth and differentiation, however addition of IL-2, IL-10 and CD40L caused a significant increase in the number of plasmablasts (ranging between 10-15\%). As it is demonstrated, ligation of CR1 by aggregated C3 dose dependently inhibited the differentiation of B cells, which was induced by anti-IgG/M/A and cytokine stimuli (Figure 10A). Again, it has to be emphasized that the lower expression of CR1 in RA patients did not influence the inhibitory function of the receptor (Figure 10B).
Figure 9. CR1 clustering inhibits BCR-induced proliferation of B cells derived from healthy donors and active RA patients

Isolated B cells were activated with the F(ab')2 fragment of goat anti-human IgG/M/A antibody (5 µg/ml) in the presence or absence of different concentrations of heat-aggregated C3 (Ai-ii and Bi-ii) or the CR1-specific Ab To5 cross-linked with F(ab')2 fragment of goat anti-mouse Ig (Ci-ii and Di-ii). As control, cells were cultured in medium, in the presence of heat-aggregated C3 only, or with an isotype-matched control mouse IgG. Cells were harvested after pulsing with 1 µCi/well H3-thymidine for the last 16 hours of culture. (Ai, Bi, Ci and Di) Data shown are mean ± SD cpm of triplicate cultures and one representative of five independent experiments with similar results is shown. (Aii, Bii, Cii and Dii) Data showing percent of inhibition are mean % of inhibition ± SEM of five independent experiments (Permutation-test, *P>0.05; †P<0.05).
Figure 10. CR1 clustering inhibits plasmablast differentiation

B cells isolated from healthy individuals (A) and active RA patients (B) were stimulated with the F(ab’)2 fragment of anti-human IgG/M/A (5 µg/ml) in the presence or absence of 50 ng/ml rh IL-2, 50 ng/ml rh IL-10, 100 ng/ml soluble CD40L and various concentrations of aggregated C3. As control, cells cultured in medium, cytokines or heat-aggregated C3 only were used. The number of CD20⁺CD19<sub>low</sub>CD27<sub>high</sub> plasmablasts was assessed after 7 days by flow cytometry.

(Ai and Bi) Data show mean frequency of CD19<sub>low</sub>CD20⁺CD27<sub>high</sub> cells ± SD of duplicate samples. One representative of four independent experiments with similar results is shown in the case of both healthy donors (Ai) and active RA patients (Bi).

(Aii and Bii) Data showing percent of inhibition are mean % of inhibition ± SEM of four experiments (Permutation-test, *P<0.05).
4.1.5 CR1 mediates inhibition of Ig production by B cells of healthy donors and active RA patients

Since we found that B cell differentiation to plasmablasts is strongly inhibited by CR1-clustering, next we set out to investigate whether the major B cell function, namely Ig-production is also affected by the complement-derived ligand. To define this, supernatants of B cells cultured as described in the previous paragraph were collected at day 7, and measured for the amount of secreted IgM and IgG. As illustrated in Figure 11, B cells of healthy donors produced little amounts of IgM (Figure 11A) and IgG (Figure 11B) without BCR-stimulation or in the presence of various cytokines. The highest amount of antibody was produced when cells were cultured in the presence of anti-human IgG/M/A and IL-2, IL-10 and soluble CD40L. A similar effect was observed in the case of B cells isolated from active RA patients (Figures 11C and 11D). CR1-clustering inhibited Ab secretion strongly in each case, and the effect of the complement protein was dose-dependent. In the case of active RA patients, the amount of secreted Abs (both IgG and IgM) was lower; however, the inhibitory effect of the aggregated C3 was still significant. Interestingly, in the patients’ B cells the BCR-stimulus alone also induced Ab production, which might be due to the altered signaling threshold of autoimmune B cells.¹⁷¹
Figure 11. Inhibition of BCR-induced IgM and IgG production by CR1 clustering

Isolated B cells of healthy individuals (A and B) and active RA patients (C and D) were activated with the F(ab')$_2$ fragment of anti-human IgG/M/A (5 µg/ml) in the presence or absence of 50 ng/ml rh IL-2, 50 ng/ml rh IL-10, 100 ng/ml soluble CD40L and various concentrations of aggregated C3. As control, cells cultured in medium, cytokines or the CR1-ligand only were used. Supernatants of cultured cells were collected on day 7 and the amount of secreted IgM (A and C) and IgG (B and D) was determined by ELISA.

(Ai, Bi, Ci and Di) Data are expressed as mean ± SD µg/ml secreted antibody of duplicate cultures. Results of one representative of four independent experiments with similar results are shown in the case of both healthy donors (A and B) and active RA patients (C and D).

(Aii, Bii, Cii and Dii) Data showing percent of inhibition are mean % of inhibition ± SEM of four experiments (Permutation-test, *P>0.05; *P<0.05).
Cross-talk between BCR, TLR9 and CR1

The complement system and TLRs represent two effector arms of innate immunity, providing a first-line host defense against invading pathogens. It is well-established that their separate activation functions to initiate and shape the adaptive immune response. However, much less is known about the way how the coincidental activation of these two systems influences the final outcome of B cell stimulation.

Therefore we investigated how ligation of CR1 via its ligand, aggregated C3 influences the TLR9-induced functions of B lymphocytes.
4.2.1. CR1 inhibits both BCR- and TLR9-induced proliferation of tonsillar B cells

It has been described earlier that clustering of CR1 via its ligand inhibits BCR-induced activation of B cells obtained from healthy individuals\textsuperscript{89} and in the simultaneous presence of anti-human IgG/M/A and CpG an enhanced proliferation can be observed\textsuperscript{128,131}. However, it has not been investigated so far how CR1 cross-linking influences TLR9-induced B lymphocyte functions. To evaluate the potential modulatory effect of this complement receptor, high-density tonsillar B cells were activated via their BCR with 5 µg/ml anti-human IgG/M/A, via TLR9 with 0.5 µg/ml CpG or with both of these stimuli in the presence or absence of aggregated C3. As seen in Figure 12, the C3b-like ligand exerted a significant and dose-dependent inhibition on the BCR- as well as on the BCR+TLR9-induced proliferation of B cells. A negative regulatory effect of CR1 on the TLR9-induced division was also observed, however, the degree of inhibition was smaller in this case. To further confirm the role of CR1, the experiments were carried out using the CR1-specific monoclonal Ab To5, clustered by anti-mouse IgG. As seen in Figure 12, the Ab-mediated crosslinking of CR1 had a similar effect as the multimeric ligand, namely it caused a strong inhibition of B cell proliferation. These results suggest that CR1 is a potent inhibitor of both the BCR and/or TLR9-induced proliferation of B cells.
Figure 12. CR1 clustering inhibits BCR- and TLR9-induced proliferation of B cells

$2 \times 10^5$ resting B cells were activated with the F(ab')$_2$ fragment of anti-human IgG/M/A Ab (5 µg/ml), with CpG ODN2006 (0.5 µg/ml) or with both of these stimuli in the presence or absence of different concentrations of heat-aggregated C3 or the CR1-specific antibody (To5) clustered by anti-mouse IgG. As control, cells were cultured in medium or with an isotype-matched control mouse IgG. Cells were harvested after pulsing with 1 µCi/well H$_3$-thymidine for the last 16 hours of culture.

(A) Data shown are mean ± SD cpm of triplicate cultures and one representative of five independent experiments with similar results is shown.

(B) Results showing percent of inhibition are mean % of inhibition ± SEM of five independent experiments (Permutation-test; *P<0.05).
4.2.2. CR1 inhibits BCR- and TLR9-induced up-regulation of activation markers and cytokine production

Activation of B cells through TLRs induces up-regulation of costimulatory molecules and enhances Ag presentation to T cells. Therefore we went on to test the effect of CR1 cross-linking on the TLR9 agonist-induced changes in the phenotype of B lymphocytes. Resting tonsillar B cells were activated via BCR with 5 µg/ml anti-human IgG/M/A, via TLR9 with 0.5 µg/ml CpG or with both of these stimuli in the presence or absence of aggregated C3 and changes in the expression of CD40 and CD86 were monitored. Figure 13 shows that CR1 clustering by the C3b-like ligand decreased the BCR- as well as the BCR+TLR9-induced surface expression of CD40 and CD86. In accordance with the results of the proliferation assay, ligation of CR1 inhibited the CpG-induced up-regulation of costimulatory molecules to a smaller extent.

Since CpG-stimulated B cells are known to produce several cytokines and chemokines, next we set out to investigate whether treatment of B cells with the C3b-like ligand of CR1 influences the production of certain B cell-derived cytokines. We found that the secretion of the regulatory IL-10 (Figure 14A), as well as the pro-inflammatory IL-6 (Figure 14B) and IFN-γ (Figure 14C) was diminished after clustering CR1 via its natural ligand.

These results show that CR1 exerts a negative modulatory effect on the BCR- and/or CpG-induced activation and cytokine production of human B lymphocytes.
Figure 13. CR1 clustering inhibits BCR- and TLR9-induced up-regulation of costimulatory molecules on human B cells

2×10^5 high-density B cells were activated for 48 hours with the F(ab’)2 fragment of anti-human IgG/M/A Ab (5 µg/ml), with CpG ODN2006 (0.5 µg/ml) or with both of these stimuli in the presence or absence of different concentrations of heat-aggregated C3. As control, cells were left untreated.

(Ai and Bi) Data show expression of CD40 and CD86, respectively. Data shown are mean ± SD R_MFI of duplicate cultures and one representative of four independent experiments is shown.

(Aii and Bii) Results showing percent of inhibition are mean % of inhibition ± SEM of three independent experiments (Permutation-test; °P<0.05; *P<0.05).
Figure 14. CR1 clustering inhibits BCR- and TLR9-induced IL-10, IL-6 and IFN-γ production of human B cells

B cells were activated for 48 hours with the F(ab’)_2 fragment of anti-human IgG/M/A Ab (5 µg/ml), with CpG ODN2006 (0.5 µg/ml) or with both of these stimuli in the presence or absence of different concentrations of heat-aggregated C3. As control, cells were left untreated. The figure shows the amount of secreted IL-10 (Ai), IL-6 (Bi) and IFN-γ (Ci). Results of the Flow Cytomix assay illustrate the amount of pg/ml secreted cytokines of pooled duplicate samples. One representative experiment of two is shown. Data in panels Aii, Bii and Cii represent mean % of inhibition ± SEM of two independent experiments (Permutation-test, *P<0.05).
4.2.3. Effect of CR1 clustering on BCR- and TLR9-induced plasmablast formation

In the next step we set out to investigate, whether CR1 cross-linking also affects TLR9-induced plasmablast formation. To this end, resting tonsillar B cells were activated via their BCR with 5 µg/ml anti-human IgG/M/A, via TLR9 with 0.5 µg/ml CpG or with both of these stimuli in the presence of IL-2, IL-6 and IL-10. Cells were either treated or not with aggregated C3. After 7 days cells were washed and stained to monitor the expression of CD20, CD27 and CD38. Among the CD19+ cells, plasmablasts were identified as CD20-\text{low}, CD27^{\text{high}} \cdot CD38^{\text{high}} lymphocytes. As seen in Figure 15, cells cultured only in the presence of cytokines contained very few CD20^{\text{low}} \cdot CD27^{\text{high}} \cdot CD38^{\text{high}} plasmablasts (ranging between 1-5%). However, stimulation by anti-IgG/M/A or with CpG caused an increase in the number of plasmablasts (ranging between 5-30%). As it is demonstrated in Figure 15, ligation of CR1 by aggregated C3 dose-dependently inhibited the differentiation of B cells, which was induced by anti-IgG/M/A and the cytokine mixture. In contrast to this, cross-linking of CR1 did not affect the TLR9-induced differentiation of B lymphocytes. After the simultaneous activation with anti-IgG/M/A and CpG, interestingly a 20-30% increase in the number of CD20^{\text{low}} \cdot CD27^{\text{high}} \cdot CD38^{\text{high}} cells could be observed as a consequence of CR1 clustering. Moreover - in contrast to other B cell functions - we could not detect any synergism between BCR and TLR9 in the process of plasmablast formation.
Figure 15. Effect of CR1 clustering on TLR9 and BCR+TLR9-induced plasmablast formation of human B cells

$2 \times 10^5$ B cells were activated for 7 days with the F(ab')$_2$ fragment of anti-human IgG/M/A Ab (5 µg/ml), with CpG ODN2006 (0.5 µg/ml) or with both of these stimuli in the presence of 50 ng/ml IL-10, IL-2 and IL-6. Cells were treated or not with different concentrations of heat-aggregated C3.

(A) The percentage of CD20$^{\text{low}}$CD27$^{\text{high}}$CD38$^{\text{high}}$ plasmablasts was determined at day 7 by flow cytometry. Data show mean frequency of CD20$^{\text{low}}$CD27$^{\text{high}}$CD38$^{\text{high}}$ cells ± SD of duplicate samples. One representative experiment of five is shown.

(B) Data show mean % of activation ± SEM of five independent experiments (Permutation-test; nsP<0.05; *P<0.05).
4.2.4. Syk is the major signaling molecule involved in CR1-induced inhibition of B cell activation

Stimulation of B cells via the BCR and TLR9 is known to initiate the synergistic activation of MAPKs and NF-κB, resulting in enhanced proliferation as well as up-regulation of costimulatory molecules and isotype switching.\textsuperscript{127-129} Recently it has been suggested that CpG-induces phosphorylation of membrane proximal kinases, such as the src kinase Lyn and the spleen tyrosine kinase (Syk),\textsuperscript{174,175} which highlights a more upstream interaction point between BCR- and TLR9-mediated signaling pathways. To find out at which level of the signaling cascade CR1 exerts its inhibitory function, resting B cells were activated via their BCR with 0.1 µg/ml anti-human IgG/M/A, via TLR9 with 0.5 µg/ml CpG or with both of these stimuli in the presence or absence of aggregated C3. Changes in the phosphorylation of key signaling molecules were investigated by Western blot assays. As seen in Figure 16, the phosphorylation of one of the key MAPKs, p38 was significantly enhanced when resting tonsillar cells were activated via the BCR in the presence of CpG, confirming earlier results. Treatment of B cells with aggregated C3 however, resulted in decreased induction of this MAPK. To test whether CR1 influences the BCR- and TLR9-induced signaling already at an earlier step of activation, we monitored the phosphorylation of Syk and the src kinase Lyn after CR1 ligation. Figure 16 shows that treatment of B cells with the C3b-like ligand reduces the BCR-, TLR9- and BCR+TLR9-induced phosphorylation of Syk and Lyn too, highlighting that CR1 exerts its inhibitory effect already at the initial signaling events during B cell stimulation.
Figure 16. CR1 clustering inhibits BCR- and TLR9-induced signaling events

Phosphorylation level of Syk, Lyn and p38 of 2×10^6 high-density B cells/lane that were left untreated (Lane 1), activated with 0.1 µg/ml F(ab')_2 fragment of goat anti-human IgG/A/M (Lanes 2 and 5), 1 µg/ml CpG (Lanes 3 and 6) or by both stimuli simultaneously (Lanes 4 and 7) in the presence (Lanes 5-8) or absence (Lanes 1-4) of 40µg/ml aggregated C3.

(A) Immunoblots show results of one representative experiment out of three.

(B) Graphs show results of summarized densitometric analysis of pSyk (i), pLyn (ii) and pp38 (iii) as mean relative intensity ± SEM of three independent experiments (Permutation-test, ^asP>0.05; ^P<0.05).
4.3. Results III.

Syk integrates BCR-, TLR9-, and CR1-mediated signals in B cells

Syk is indispensable for CpG-induced activation and differentiation of human B cells

The signal pathway mediated by the engagement of TLR9 classically involves MyD88-induced NF-κB activation. Although the important role of Syk in immunoreceptor-mediated signaling pathways has been established earlier and its role in TLR-associated innate immunity has raised attention too, their possible interaction has not been studied yet. Jabara et al. showed that after ligation of TLR9 by CpG ODNs, DOCK8 links TLR9 to a src kinase-Syk cascade which is essential for TLR9-driven B cell proliferation and differentiation. Furthermore studies using the human monocytic THP-1 cell line and mouse splenic B cells proved that CpG ODN induces phosphorylation of src-family kinases in a TLR9-independent manner, resulting in cell adhesion and motility. This signaling cascade intersects the conventional TLR9-MyD88 pathway by promoting the tyrosine phosphorylation of TLR9 and the recruitment of Syk to this receptor. It was also established that Syk-mediated BCR triggering is a prerequisite for optimal induction of TLR9-induced signaling in human B cells. However it remains unclear whether Syk can directly be activated in the course of the TLR9 signaling cascade or not. Therefore we decided to reveal how Syk is involved in TLR9-induced activation and functions of human B cells.
4.3.1. Binding of CpG to resting human B cells induces dose- and time-dependent phosphorylation of Syk and Lyn

Upon recognition of CpG-rich sequences in the endosome of human B cells, TLR9 initiates a conserved signaling cascade through the recruitment of MyD88, providing a mechanism for innate immunity to regulate the adaptive immune response. Our aim was to reveal how the activation and function of human B cells are influenced by the TLR-agonist bound to the cell surface, and whether Syk is involved in this process.

First we set out to investigate whether this tyrosine kinase is involved in the CpG-initiated activation of B lymphocytes. As seen in Figures 17Ai and Aii, treatment of resting tonsillar B cells with increasing amounts of CpG ODN2006 caused a dose-dependent phosphorylation of Syk and the membrane proximal src kinase Lyn. We also show that the phosphorylation of these kinases is time-dependent (Figures 17Bi and Bii). These results extend and further strengthen earlier observations obtained using blood derived cells, showing Syk phosphorylation upon CpG treatment of B cells.

Surface Igs are able to interact with phosphorothioate ODNs by charge-charge interactions. To rule out the possibility that in resting human B lymphocytes CpG activates the src-Syk kinase pathway by binding to cell surface Ig, the phosphorylation of Ig alfa (CD79A) was investigated, since activation of Syk through the BCR involves binding of its SH2 domain to phosphorylated tyrosines within this signaling moiety of the BCR. In contrast to the anti-Ig stimulus, we detected no phosphorylation of the Ig alfa after the addition of CpG (Figure 17C), suggesting that the ODN-induced activation of Syk in resting human B cells does not occur via cell surface BCR.
Figure 17. Binding of CpG to resting tonsillar B cells induces dose- and time-dependent phosphorylation of Syk and Lyn independent of the BCR.

(A and B) $2 \times 10^6$ resting B cells/sample were activated with different concentrations of CpG for 30 minutes (Ai-ii) or with 1 µg/ml CpG for different time intervals (Bi-ii). (Ai and Bi) Immunoblots show results of one representative experiment out of three. (Aii and Bii) Graphs show summarized densitometric analysis of Syk and Lyn phosphorylation as mean relative intensity ± SEM of three independent experiments (Permutation-test, *P>0.05; **P<0.05).

(C) Phosphorylation of CD79A in high-density B cells. Untreated cells (Lane 1), cells activated with 2.5 µg/ml F(ab')$_2$ fragment of goat anti-human IgG/A/M (Lane 2), with 1 µg/ml CpG (Lane 3) or via both stimuli simultaneously (Lane 4). Immunoblot shows one representative experiment of three.
4.3.2. The role of Syk and Lyn in the CpG-induced activation, proliferation and cytokine production of B cells

Next we set out to explore whether phosphorylation of Syk and Lyn is indispensable for CpG-induced B cell functions. To test this, high-density B cells were stimulated with suboptimal amount of CpG ODN2006 in the presence or absence of different concentrations of Syk and src kinase (Lyn) inhibitors, followed by assessing various functions of the cells. Based on preliminary experiments, we used the inhibitors in nanomolar concentrations. Since Syk relays tonic B cell survival signals and cell viability decreases in the presence of the inhibitor, dead cells were excluded from all measurements using propidium iodide, and data were normalized to the number of living cells. In accordance with earlier results, CpG ODN2006 induced a robust proliferation of human B cells, in contrast to the control GpC ODN. However, treatment of cultured cells with nanomolar concentrations of Syk inhibitor IV (iSykIV) resulted in a strong and dose-dependent decrease of the CpG-induced proliferation (Figures 18A and B). These results were confirmed by employing another Syk inhibitor (Syk inhibitor II, iSykII) as well as a src kinase inhibitor (Figure 18A). Due to its strong effect, we used the highly specific Syk inhibitor IV in further experiments.

We went on to test the effect of Syk inhibition on the TLR agonist-induced changes in the expression of costimulatory molecules. Consistent with our results shown previously, Syk inhibitor IV blocked strongly and dose dependently the CpG-induced up-regulation of the costimulatory molecules CD80, CD86 and CD40 (Figure 19A). Since CpG-stimulated B cells are known to produce several cytokines and chemokines, next we investigated whether inhibition of Syk also influences the production of prominent B cell-derived cytokines. We found, that the secretion of the regulatory IL-10, as well as the pro-inflammatory IL-6 and TNF-α was diminished in the presence of the kinase inhibitor (Figure 19B).

These results suggest a critical role for Syk in the CpG-induced proliferation, activation and cytokine production in human B lymphocytes.
Figure 18. Syk regulates CpG-driven B cell proliferation

$2 \times 10^5$ resting tonsillar B cells/well were activated with suboptimal concentration of CpG (0.5 µg/ml) in the presence or absence of Syk or Src inhibitors (iSykIV, iSykII or src inhibitor).

(A) Cells were harvested after pulsing with 1 µCi/well H$^3$-thymidine for the last 16 hours of culture. Results are expressed as normalized mean cpm ± SD of triplicate samples of one representative experiment out of five.

(B) Histograms show proliferation of CFSE-labeled B cells. Results are representative of two independent experiments and show the percentage of proliferated cells.
Figure 19. Syk regulates CpG-driven B cell activation and cytokine production

$2 \times 10^5$ resting tonsillar B cells/well were activated with 0.5 μg/ml CpG in the presence or absence of different concentrations of Syk inhibitor IV, as indicated.

(A) After 48 hours, changes in the expression of CD80, CD86 and CD40 were investigated by flow cytometry. Results are illustrated as flow cytometric histograms (i) and by diagrams showing normalized mean R_{MFI} ± SD of duplicate samples (ii). One representative experiment of three is shown.

(B) Secretion of IL-10, IL-6 and TNF-α after the stimulation of resting tonsillar B cells with CpG for 48 hours. Results of the Flow Cytomix assay show pg/ml secreted cytokines of pooled duplicate samples. One representative experiment of three is shown.
4.3.3. Syk regulates CpG-driven B cell differentiation

Upon activation by CpG ODN2006, B cells undergo isotype switching, differentiate into plasmablasts and secrete Igs. In view of our findings described above, we aimed to reveal whether plasmablast formation and antibody production are also influenced by the inhibition of Syk. As shown in Figure 20, Syk inhibitor IV caused a dose-dependent reduction in CpG ODN-induced differentiation of high-density B cells into CD20^low^CD27^{high/low}^CD38^{high/low} plasmablasts. In consequence, the number of IgM and IgG secreting cells also decreased (Figure 21). These data suggest that Syk is strongly involved in the regulation of CpG-driven B cell differentiation and Ab production.

**Figure 20. Effect of Syk inhibition on CpG-induced plasmablast differentiation**

2×10^5 resting tonsillar B cells/sample were activated with 0.5 µg/ml CpG in the presence or absence of different concentrations of iSyk IV. The percentage of CD20^low^CD27^{high/low}^CD38^{high/low} plasmablasts was determined at day 7 by flow cytometry.

(A) Data show mean frequency of CD20^low^CD27^{high/low}^CD38^{high/low} cells ± SD of duplicate samples. One representative experiment of three is shown.

(B) Dot plots of the same samples.
Figure 21. Syk regulates CpG-induced differentiation of IgM- and IgG-secreting B cells

2×10^5 resting tonsillar B cells/sample were activated with 0.5 µg/ml CpG in the presence or absence of different concentrations of iSyk IV. The number of IgM and IgG secreting cells was measured by ELISPot assay at day 7. Graph shows ASCs ± SD of duplicate samples. One representative experiment of three is shown.
4.3.4. Inhibition of Syk prevents the synergistic activation of MAPKs induced by the BCR and CpG

Stimulation of B cells via the BCR and CpG is known to initiate the synergistic activation of MAPKs and NF-κB, resulting in enhanced proliferation as well as up-regulation of costimulatory molecules and isotype switching.\(^{183,184}\) In order to find out if Syk might coordinate the conjunction of these two signaling pathways, we monitored how the upstream events, namely the phosphorylation of Syk and the membrane proximal Lyn are affected by the simultaneous stimuli. As shown in Figure 22A, the phosphorylation of both Lyn and Syk was significantly enhanced when resting tonsillar cells were activated via the BCR in the presence of CpG, highlighting that these signaling pathways already converge at the level of Lyn and Syk. Next we investigated whether inhibition of Syk has any effect on the phosphorylation of ERK, the first MAPK where the BCR- and CpG-induced signaling pathways might merge. Using Syk inhibitor IV we found that blocking the kinase prevents not only the BCR-, but also the CpG-initiated phosphorylation of ERK, underlining the indispensable role of this tyrosine kinase in the synergistic stimulatory effect of BCR and CpG.
Figure 22. Inhibition of Syk prevents the BCR- and CpG-induced synergistic activation of MAPKs

(A) Phosphorylation level of Syk and Lyn of $2 \times 10^6$ high density B cells/lane that were left untreated (Lane 1), activated with 0.1 µg/ml F(ab')$_2$ fragment of goat anti-human IgG/A/M (Lane 2), 1 µg/ml CpG (Lane 3) or by both stimuli simultaneously (Lane 4).

(B) Phosphorylation level of ERK of $2 \times 10^6$ high density B cells/lane that were left untreated (Lanes 1 and 5), activated with 0.1 µg/ml F(ab')$_2$ fragment of goat anti-human IgG/A/M (Lanes 2 and 6), 1 µg/ml CpG (Lanes 3 and 7) or by both stimuli simultaneously (Lanes 4 and 8). Lanes 1-4 show pERK levels in the absence, while Lanes 5-8 in the presence of 10nM iSyk IV.

(Ai and Bi) Immunoblots show results of one representative experiment out of three.

(Aii and Bii) Graphs show results of summarized densitometric analysis as mean relative intensity ± SEM of three independent experiments (Permutation-test. nsP>0.05; *P<0.05).
4.3.5. Extracellular CpG ODN induces Syk activation in B cells

Human B cells bind CpG ODNs by a receptor-mediated mechanism that is necessary but not sufficient for polyclonal activation. On the other hand, CpG-induced phosphorylation of Lyn and Syk was proven to occur both in a TLR9-dependent and -independent manner. Thus we wanted to test whether CpG might induce Syk phosphorylation upon extracellular triggering.

To test whether CpG might induce Syk phosphorylation through cell surface receptor(s), B cells were activated by CpG ODN2006 immobilized on the culture plate. We found that even under these conditions, i.e. when endocytosis of the ligand is prevented, Syk, Lyn and p38 are phosphorylated in a dose-dependent manner (Figure 23A). We ruled out the possibility that CpG was released from the surface and taken up by the cells, since no intracellular ODN was found after activation by the immobilized ligand (data not shown). As illustrated in Figure 23B, CpG immobilized on the culture plate does not induce B cell proliferation versus CpG offered to the cells in soluble form. This suggests that CpG binding to the cell surface is only sufficient to trigger the signaling pathway, but for the initiation of cellular functions its internalization is necessary.

To analyze the involvement of TLR9 in the CpG-induced phosphorylation of Syk, first we analyzed the expression of TLR9 in resting human tonsillar B cells by flow cytometry. We found that high-density B cells express TLR9 mainly intracellularly (mean relative MFI=11.45 ± 1.25, n=5) and 5-15% of them bear surface bound TLR9 as well (mean relative MFI=2.03 ± 0.23, n=5) (Figure 24A). Next we moved on to test whether inhibition of MyD88, a critical adaptor protein of TLR9-induced signaling events affects CpG-driven activation of the kinase. As shown in Figure 24B, we found that the blockade of MyD88-mediated signaling pathways in human B cells prevents the CpG-induced phosphorylation of Syk, similar to the control, src kinase inhibitor, confirming the role of TLR9 in the kinase activation.

These data clearly show that engagement of the CpG binding sites on the cell surface leads to the activation of the membrane proximal Lyn followed by Syk and p38 in human B cells. Since we found that TLR9 is expressed on the surface of resting tonsillar B cells, we suppose that Syk phosphorylation may occur at least partially through surface bound TLR9 in a MyD88-dependent manner.
Figure 23. Extracellular CpG ODN induces Syk activation in B cells

(A) Phosphorylation level of Syk, Lyn and p38 of 2×10^6 high-density B cells/lane that were left untreated (Lanes 1 and 5) or activated by different concentrations of biotinylated CpG (Lanes 2, 3, 4 and 6). For extracellular activation (Lanes 1-4), biotinylated CpG was coupled to neutravidin coated plates, while for extra- + intracellular activation (Lanes 5 and 6) the soluble complex of biotinylated CpG and neutravidin was added to the samples. (i) One representative experiment of three is shown. (ii) Graph shows summarized densitometric analysis as mean relative intensity ± SEM of three independent experiments (Permutation-test, "P>0.05; *P<0.05).

(B) 2×10^5 resting tonsillar B cells/well were activated with different concentrations of soluble (filled bars) or surface (neutravidin) -bound biotinylated CpG (opened bars) for 48 hours. Cells were harvested after pulsing with 1 μCi/well H^3-thymidine for the last 16 hours of culture. Results are expressed as normalized mean cpm ± SD of triplicate samples of one representative experiment of three.
Figure 24. Extracellular CpG ODN induces Syk activation without proliferation in a MyD88-dependent manner

(A) Flow cytometric histograms of high-density tonsillar B cells showing cell surface and intracellular TLR9 expression. TLR9 was detected by APC-labeled rat anti-human TLR9 (black line). As isotype control, rat IgG2a-APC was used (dashed line) and the grey line represents autofluorescence. Data shown are one representative of five independent experiments.

(B) Activation of Syk was measured after cells were pre-treated or not with Syk-, Src- or MyD88 inhibitors as described in Materials and Methods. Graph shows summarized densitometric analysis of Syk phosphorylation as % of activation ± SEM of five independent experiments (Permutation-test, **P>0.05; *P<0.05).
4.3.6. Inhibition of Syk does not influence the uptake of CpG oligonucleotides

To explore whether the reduced activation, proliferation, cytokine and Ig production are caused by the decreased endocytosis of CpG ODN due to the lack of Syk activity, we studied the uptake of fluorescently labeled CpG ODN by human B cells at different time intervals in the presence or absence of Syk inhibitor IV. As shown in Figure 25A, uptake of CpG ODN begins already after 5 minutes of exposure and reaches saturation within 30 minutes. Investigating either by flow cytometry (Figure 25) or confocal microscopy (Figure 26), we found that B cells bind and uptake the ODN even in the presence of Syk inhibitor IV. These data suggest that Syk is not involved in the uptake of CpG ODN by human B cells.

Interestingly however, treatment of the cells with Syk inhibitor IV clearly reduced the co-localization of CpG and its receptor, TLR9. Similarly, the appearance of CpG in TLR9-containing lysosomes was reduced however, it did not reach statistical significance (Figure 26). These results suggest that Syk is a prerequisite for optimal delivery of CpG into TLR9-containing endolysosomes.

**Figure 25. Syk inhibition does not influence the uptake of CpG ODN**

(A) High-density tonsillar B cells were stained with Alexa Fluor 488-labeled CpG ODN on ice (dashed lines) or at 37°C (solid lines) for the indicated times in the presence (red lines) or absence (black lines) of 10nM iSykIV. Binding (i) and uptake (ii) of the fluorescent ligand was measured by flow cytometry as described in Materials and Methods. Results shown are mean ± SD of one representative experiment out of three.

(B) Uptake of CpG-Alexa Flour 488 after 30 minutes in the presence or absence of 10nM iSykIV. Results shown are mean ± SEM of three independent tests (Permutation-test, nsP>0.05; *P<0.05).
Figure 26. Syk inhibition reduces co-localization of CpG and TLR9

B cells were stained with Alexa Fluor 488-labeled CpG ODN at 37°C for 30 minutes in the presence or absence of 10nM iSykIV. Binding of anti-TLR9 and anti-LAMP-1 was visualized using anti-mouse Alexa Flour 647 and anti-rabbit Alexa Flour 555, respectively. Shown are merged DIC and confocal images of CpG-Alexa Flour 488 (green), LAMP-1-Alexa Flour 555 (blue) and TLR9-Alexa Flour 647 (red). Cells were analyzed with Olympus FLUOView 500 confocal laser scanning microscope.

(A and Bi) One representative experiment of three is shown. Scale bars represent 10µM.

(Bii) Graphs show summarized mean ± SEM of Pearson coefficients of 100 cells for each sample from three independent experiments (Student’s t-test, NS P>0.05; *P<0.05).
4.3.7. Syk regulates CpG-driven up-regulation of TLR9 expression in B cells

It has been proposed that signaling through the BCR, CD40 and TLR9 leads to an increased TLR9 expression in B cells.\textsuperscript{185,186} Since we found that Syk blockade strongly inhibits CpG-induced proliferation, cytokine production and differentiation, we assumed that Syk is fundamentally involved in transducing signals which are required to maintain and up-regulate TLR9 expression in human B lymphocytes. Testing this hypothesis by flow cytometric analysis we found that in the presence of 5nM Syk inhibitor IV, B cells stimulated by CpG for 48 hours are unable to up-regulate intracellular TLR9 expression (Figures 27Ai and Aii). These results were strengthened by Western Blot analysis (Figure 27B).

Thus we can conclude that the kinase activity of Syk is a prerequisite for the CpG-induced up-regulation of TLR9 expression allowing efficient propagation of TLR9-mediated signaling and B cell functions.
Figure 27. Syk regulates CpG-driven up-regulation of TLR9 expression in B cells

(A) Expression of TLR9 after stimulation of B cells with 2 µg/ml CpG in the presence or absence of 5nM iSyk IV for 48 hours. (Ai) Histograms showing intracellular TLR9 expression are as follows: untreated cells (grey line), 2 µg/ml CpG treated cells (green line), 2 µg/ml CpG and 5nM iSyk IV treated cells (red dashed line). Results are representative of three independent experiments. (Aii) Graph shows summarized mean ± SEM of MFIs or R_MFIs of TLR9 expression of three independent experiments (Permutation-test, nsP>0.05; *P<0.05).

(B) Expression of TLR9 after stimulation of B cells with 2 µg/ml CpG in the presence or absence of 5nM iSyk IV for 48 hours. Extracts of 10^6 high-density tonsillar B cells were separated by SDS–PAGE and immunoblotted with anti-TLR9 Ab. Arrows indicate TLR9 of approximately 110 kDa and its activation fragments. Blot shown is representative of three independent experiments.
5. Discussion

A major role of B cells in the development and perpetuation of autoimmune diseases has been supported by the success of B cell depleting therapies and their efficacy in reducing symptoms and preventing disease progression. ICs containing autoantigens and complement proteins may alter effector B cell functions, making plausible that changes in the expression and function of complement- and Toll-like receptors may cause imbalance in the regulation of B cell functions breaking self-tolerance and inducing autoreactivity.

As shown in Figure 28, depending on the actual composition of ICs, the function of B lymphocytes may be influenced either positively or negatively. Co-crosslinking of FcγRIIb or CR1 with the BCR was reported to induce negative regulatory signals, leading to the inhibition of several B cell functions. At the same time, CpG motif-containing bacterial DNA or the C3d fragment of complement have been described as molecular adjuvants, which - after binding to TLR9 and CR2, respectively - lower the threshold for B cell activation.

![Diagram](image.png)

**Figure 28. Functional consequences of the engagement of immune complex-binding receptors in human B cells**
The expression pattern of the inhibitory CR1 is different from that of the enhancing co-receptor CR2 in human B cells

Several studies suggest that IC-binding receptors play an important role at checkpoints controlling the development of autoimmunity and maintaining B cell tolerance. Expression of CR1 and CR2 on human B cells has been studied in a number of autoimmune diseases. One important observation is that patients with SLE and RA display abnormalities in the expression of both CR1 and CR2 on B lymphocytes, compared to control subjects.\textsuperscript{35,36,113-115} As we know our studies are the first which addressed the expression and function of complement receptors on distinct B cell subsets in RA patients.

Analysing first B cells of healthy donors we found that the expression of CR1 is up-regulated during the differentiation of B lymphocytes into memory cells in contrast to CR2, which decreases in the same time (Figure 8). These data clearly prove that these two receptors are differentially regulated in human B cells – in contrast to the mouse system. Confirming earlier results,\textsuperscript{100,120} we also detected a significantly reduced level of complement receptors on human plasmablasts (Figure 8). To get a better understanding of the interplay between B cells and the complement system in RA, we analyzed the expression of CR1 and CR2 on the naive, memory and plasmablast pools of B cells isolated from these patients. Although in RA patients the expression level of both CR1 and CR2 was found lower than in healthy controls on all B cell subsets, during differentiation to memory cells a similar change could be observed. The maintained expression pattern of CR1 and CR2 further emphasizes their important role in the regulation of humoral immunity.

During recurrent infections, activated memory cells rapidly proliferate and a large number of PCs is generated. The signals required for differentiation toward PCs are relatively well-established however, much less is known about the factors which affect the development and survival of memory B lymphocytes. CR1 and CR2 have been proven to be indispensable for the maintenance of memory B cells by capturing Ags in the form of ICs on FDCs.\textsuperscript{71,97,98,189} Thus, up-regulation of inhibitory receptors, such as FcγRIIb and CR1 during differentiation toward Ab-secreting cells may increase the threshold for self-reactive memory B cell activation, thereby could prevent production of autoantibodies. Our hypothesis is supported by the fact that lower expression of FcγRIIb has been shown to correlate positively with autoantibody secretion of memory B cells.\textsuperscript{170} Furthermore, we have demonstrated that CR1 clustering reduces both Ab production and the frequency of CD27\textsuperscript{high}CD20\textsuperscript{CD19\textsuperscript{low}} plasmablasts in B cell cultures (Figures 10 and 11). Likewise, the lower expression of CR2 on
Peripheral memory B cells may have a similar effect by elevating the activation threshold of effector memory B cells.

In the case of RA patients, the expression of complement receptors was reduced on all tested B cell subpopulations, but the change in the expression pattern during the differentiation of B lymphocytes was maintained. This finding points to a strict regulation of complement receptors in RA, which seems to be missing in other B cell mediated autoimmune diseases, such as SLE. We have clearly demonstrated that the lower levels of CR1 and CR2 in RA patients were not caused by different frequencies of B cell subpopulations (Figure 7). This is not the situation in SLE, where higher frequency of plasmablasts with low level of inhibitory receptors may exacerbate the ongoing humoral immune response against self-Ags. Moreover, we could not associate CR1 and CR2 expression on B cells with the inflammatory status of the RA patients. These data further strengthen the notion that down-regulation of IC-binding receptors on B cells may contribute to the induction of autoimmunity rather than affecting disease severity. Indeed, CR1 and CR2 were shown to decrease progressively before any major clinical manifestation of autoimmunity in MRL/lpr mice. Furthermore an age-associated decline in CR1 and FcγRIIb levels of female RA patients has also been described. The fact that B cells of elderly women tend to lose their CR1 and FcγRIIb may be one of the causes why women are more susceptible to RA after menopause.

Taken together, our results highlight the complex regulation of B cell tolerance, which can be broken down by the simultaneous alteration of complement receptors and changes in the actual composition of the immune complexes. However, further investigations are needed to fully understand how this altered receptor expression may contribute to the dysregulation of B cell functions.

Possible mechanisms responsible for the loss of up-regulation of CR1 and CR2 on RA B cells

Mechanisms, which are responsible for the down-regulation of IC-binding receptors on B cells of RA patients may be based on either inherited or acquired elements.

One of the possible explanations is that complement-containing ICs act as ligands and reduce receptor levels through a negative feedback circle. Indeed, IC-induced down-regulation of complement receptors has been suggested in several studies; however, most of these had been restricted to erythrocyte CR1 due to its role in immune complex clearance. In
SLE, large loads of ICs were shown to acutely and chronically induce the loss of CR1 from erythrocytes when ICs are transferred to macrophages.\textsuperscript{190} Similarly, CR1 appears to be consumed in patients with Factor I deficiency - characterized by a continuous generation of C3b.\textsuperscript{191} Despite the relatively well-established causes of the decreased erythrocyte CR1 level in autoimmune patients, only few studies attempted to reveal the relationship between leukocyte CR1/CR2 and circulating IC level. These results show that transcription and protein levels of both CR1 and CR2 correlate inversely with disease activity and presence of circulating ICs in RA and SLE patients.\textsuperscript{35,113,192} When we analyzed the ligand-induced modulation of CR1 and CR2 expression on isolated B cells, we found that the natural ligands, C3b and C3d resulted in a 20-30% decrease in receptor levels (data not shown). Furthermore, model ICs - generated by incubation of heat aggregated IgG with serum - caused a similar reduction in CR1 and CR2 expression, strengthening the hypothesis of the ligand-induced regulation of complement receptors. Since IC overload is a general phenomenon in RA, one can assume that \textit{in vivo} high levels of complement-containing ICs may result in a similar drop in receptor densities. Despite the fact that higher levels of ICs in RA patients were proven to correlate positively with disease activity and negatively with leukocyte CR1 transcript, we could not detect any positive correlation between down-regulation of the B cells’ complement receptors and disease activity. Therefore we propose that the persistence of circulating ICs above a critical concentration generates long-lasting negative feed-back signals with subsequent down-modulation of receptors, and further increase in IC level has no influence on receptor densities.

IC-mediated down-regulation of CR1, CR2 and FcγRIIb may also be caused by the proteolitic cleavage of the receptors’ extracellular domains, a phenomenon called ectodomain shedding. Several reports have proven that CR1 and CR2 as well as the inhibitory FcγRIIb can be released from B cells upon activation and might be involved in regulatory processes.\textsuperscript{116,117} Shedding of receptors results in abrogation of receptor-mediated signaling, meanwhile the released fragments may act as potential soluble modulators by interacting with their ligands. However when we examined the ligand-induced down-modulation of CR1 employing immobilized or soluble C3b, we could not detect the release of soluble CR1 into the culture supernatant (data not shown). Moreover, at least for CR2, it has been shown that the B cells of RA patients shed the same amounts of CR2 as healthy donors and display decreased levels of soluble CR2.\textsuperscript{114,193} Based on these results we suggest that the partial loss
of IC-binding receptors from the cell surface may be caused by endocytosis and/or reduced transcription rather than ectodomain shedding.

**CR1 clustering inhibits BCR-induced B cell functions in healthy donors and RA patients**

Our group has previously shown that CR1 has a negative regulatory effect on human B cell functions. Treatment of B cells with aggregated C3, which mimics multimeric C3b and binds to CR1, strongly and dose-dependently inhibits the anti-IgM-induced proliferation as well as Ca\(^{2+}\) mobilization of resting tonsillar B cells.\(^{89}\) Despite the relatively well-characterized expression pattern of CR1, it is not known how reduced receptor density affects certain B cell functions in autoimmune diseases. We demonstrate here that clustering CR1 by aggregated C3 or by the CR1-specific monoclonal To5 antibody inhibits B cell proliferation as well as antibody production dose-dependently (Figures 9, 10 and 11). These functional results together with the expression data give further evidence that CR1 and CR2 have opposite roles in human B lymphocytes. Consequently these complement receptors are important players in the IC-mediated fine tuning of various B cell functions under both physiological and autoimmune conditions. In spite of the significant decrease of CR1 expression in RA patients, its inhibitory capacity is still preserved, and ligand binding results in a significantly reduced B cell proliferation and antibody secretion. Similar results were obtained assessing the function of CR2 in the case of SLE patients, where despite the fact that B cells were found to express half as many surface CR2 as normal B lymphocytes, the Ca\(^{2+}\) response and the percentage of responding cells were significantly increased after co-ligation of surface Ig and CR2.\(^{121}\) It is possible that at suboptimal Ag concentration - as employed in our experiments - the B cells of RA patients still express enough CR1 to inhibit BCR-induced responses. However, persistent stimulation by abundantly generated immune complexes, as seen in chronic autoimmune diseases, might overcome this inhibitory capacity and the B cells would become inappropriately activated. Therefore we propose that the reduced expression of CR1 and CR2 affects receptor functions only when their level fall below a critical density and only the combined alteration in receptor expression may be sufficient to cause dysregulation of B cell functions.

The intracellular signaling mechanisms which are involved in the inhibition caused by CR1 ligation have not been clarified yet. In contrast to CR2, which contains possible tyrosine phosphorylation sites in its intracellular tail,\(^{194}\) the short cytoplasmic domain of human CR1 comprises only one potential threonine phosphorylation motif which does not seem to have the ability of initiating signals. CR2 alone or in complex with CD19 lowers the threshold for
B cell activation induced by suboptimal cross-linking of surface Igs.\textsuperscript{195,196} CR1 molecules in the cell membrane of human B cells appear not only in free form, but also in association with CR2, though this CR1/CR2 complex does not contain CD19.\textsuperscript{102} The inability of CR1 to associate with CD19 may explain the lack of its activatory effect and may account for its inhibitory role in several B cell functions.

Our results clearly show that CR1 clustering on B cells reduces the phosphorylation of key signaling molecules, such as the membrane proximal Syk and Lyn kinases, and the p38 MAPK (Figure 16), resulting in the observed inhibitory effect of CR1. This dephosphorylation might be induced by active recruitment of certain phosphatases (like SHP-1, SHP-2 or SHIP) or by the association of CR1 with inhibitory receptors, such as CD22 or CD72. Alternatively, CR1 clustering may affect the membrane localization of the BCR, which transduces signals from cholesterol- and sphingolipid-rich membrane microdomains. In contrast to the CR2/CD19/CD81 complex, which prolongs the retention of the BCR in lipid rafts, clustering of CR1 may sequester BCR from other important signaling molecules and thereby results in reduced tyrosine phosphorylation of proteins. Molecular mechanisms responsible for the CR1-induced inhibition in human B cells are under investigation in our laboratory.

In conclusion, our results demonstrate that despite the significant decrease of CR1 expression in RA patients, the inhibitory capacity of this complement receptor is still maintained, and its ligation results in inhibition of B cell proliferation and antibody production – similarly to that found in the case of healthy individuals. Therefore the altered expression of complement receptors on B cells alone does not seem to be sufficient to break B cell tolerance. However, concurrent changes in the expression and/or function of IC-binding receptors on B lymphocytes and other cell types may result in aberrant complement activation as well as Ag retention on B cells and FDCs, leading indirectly to an altered memory response, autoantibody secretion and induction of autoimmunity.
**CR1 clustering inhibits TLR9-induced proliferation and cytokine production of human B cells, but it does not affect their plasmablast differentiation**

TLRs and the complement system are two well-characterized elements of innate immunity and both are known to play a key role in priming the adaptive immune response. In addition to clonally rearranged Ag-specific receptors, B lymphocytes also express germline-encoded pattern recognition receptors on their surface. Thus, it is likely that during infection B cells will receive signals through additional receptors, including complement- and Toll-like receptors in addition to the BCR. In response to CpG DNA human B cells undergo proliferation, secrete pro-inflammatory cytokines and IgM.\(^{123-125}\) The role of complement and C3 opsonized antigens in B cell activation and Ab production has also been thoroughly studied.\(^{71,89,91,105}\) Recognising the indispensable role of both TLR9 and CR1 in B cell biology, we investigated how the interaction between these two elements of innate immunity affects B cell responses.

Our results provide evidence of a strong interaction between CRs and TLRs in B cells. We have shown that CR1 clustering inhibits CpG-induced B cell activation, proliferation as well as cytokine production even in the presence of additional BCR stimulus (Figures 12, 13 and 14). These results may be explained by the CpG-induced Lyn and Syk phosphorylation (Figure 17) and the inhibitory effect of CR1 on the activation of these kinases (Figure 16). In contrast to this, ligand binding to CR1 did not inhibit TLR9-induced differentiation of B cells, moreover, their clustering resulted in elevation of plasmablasts’ number after stimulation via both BCR and TLR9 (Figure 15).

Despite the relatively well-established role of TLR9 in the maintenance of serological memory and generation of high affinity Ab-secreting plasma cells, much less is known about the exact role of B cell-produced cytokines and other factors that regulate CpG-induced differentiation of B cells. Considering the inhibitory function of CR1 on both TLR9 and BCR+TLR9-induced activation and cytokine production, it was unexpected that CR1 did not influence the TLR9-driven plasmablast formation and Ab production of B lymphocytes. Furthermore, in contrast to other B cell functions, concurrent engagement of BCR and TLR9 resulted in equal or even lower number of Ab-producing plasmablasts than individual TLR9 stimulus. The lack of CR1-mediated inhibition and decreased generation of Ab-producing cells under simultaneous triggering of BCR and TLR9 may be due to several reasons. Firstly, BCR-driven activation may result in the production of various factors, which inhibits TLR9 expression and/or function in B lymphocytes. Indeed, several B cell-produced cytokines and
growth factors (IL-4, IL-10, IFN-γ, BAFF, etc.) were proven to control B cell responses to CpG DNA by up- or down-regulation of TLR9 expression. For example, IL-4 was shown to decrease TLR9 transcription and this down-regulation was found to be more pronounced after 48 hours. Similarly, IL-10 was reported to inhibit CpG-induced proliferation as well as antibody production when added without IL-2. In this manner, stimulation of B cells through the BCR may induce secretion of cytokines or other factors which diminish the response of B cells to CpG. This effect however may become evident only under long-term incubation but not in proliferation assays. This could also explain the lack of CR1-mediated inhibition on TLR9-induced plasmablast formation in contrast to other B cell functions. Long-term stimulation of B cells by CpG-motif containing DNA with simultaneous triggering through the BCR may induce enhanced expression of TLR9 and/or increased production of growth factors. This may result in a more robust B cell activation which can’t be overridden by the CR1-induced inhibitory signals. Our hypothesis is supported by the fact that both BCR and CpG stimuli were proven to induce up-regulation of TLR9 and IL-10 secretion, thereby may contribute to the augmented B cell response under simultaneous activation. Otherwise, CR1 itself may hamper the production of cytokines (like IL-10 (Figure 14)) or expression of transcriptional factors (like bcl-6) responsible for negative regulation of CpG-induced plasmablast formation, thereby give rise to enhanced differentiation and subsequent Ab production of B cells.

An alternative explanation for the observed lack of CR1-mediated inhibition on TLR9-driven plasmablast formation may be the selective triggering of individual B cell subpopulations. While CpG DNA alone activates mainly memory B cells, the anti-Ig stimulus is able to potentiate both naïve and memory B cells in the presence of T cell help. Memory B cells form Ig-secreting effector cells more rapidly than do naïve B lymphocytes. Thus in the presence of CpG, more memory B cells could differentiate into Ab-secreting plasmablasts that proliferate at a rate exceeding that of memory as well as naïve B cells. Moreover, IL-2 - which was applied to induce plasmablast formation - was proven to selectively enhance proliferation of memory B cells activated with CD40L and IL-10, but had no effect on naive B cells. Considering the selective induction of memory B cells after TLR9 engagement and the enhanced generation of CD38+ Ig-secreting blasts we hypothesize, that the CpG stimulus induces an accelerated and efficient Ab response selectively by memory B cells which can’t be overwritten by CR1. In contrast to this, anti-Ig stimulus results in the formation of both CD38+ memory B cells and CD38+ plasmablasts, thereby decreases the
number of rapidly proliferating Ig-secreting cells, and creates the possibility for CR1 to exert its inhibitory function.

Taken together, our results give evidence of a negative control of TLR-induced signaling in B cells by CR1 and provide new insights into the interaction between the complement system and TLRs to control humoral immunity. The CR1-mediated inhibition of TLR9-induced B cell proliferation and cytokine production may become clinically relevant in a number of pathogenic processes, where RNA- or DNA-containing autoantigens can directly induce aberrant activation of B cells through autoreactive BCRs and TLR9.

**Syk is integrally involved in CpG-induced activation of human B cells**

It is known for long that binding of CpG-containing ODNs to endosomal TLR9 triggers B cell proliferation, inflammatory cytokine and Ab production by a mechanism that is dependent of the adaptor protein MyD88. Our results gave evidence that CR1 is a potent inhibitor of both BCR- and CpG-induced activation of human B cells. Investigating the mechanisms responsible for the CR1-mediated inhibition we found that both anti-Ig and CpG stimuli induce phosphorylation of key membrane proximal signaling molecules, such as the src kinase Lyn and Syk. Although the important role of Syk in immunoreceptor-mediated signaling pathways has been established earlier and its role in TLR-associated innate immunity has raised attention, their possible interaction has not been studied yet. Regarding the CpG-induced phosphorylation of Syk, data published earlier are contradictory. It has been shown that the activation of this kinase can be induced by CpG type A DNA in human monocyte-derived dendritic cells independent of TLR9 and by CpG type B ODN2006 in peripheral mononuclear cells in a TLR9-dependent manner.\(^{174,175}\) Furthermore, in two other studies CpG alone did not affect Syk phosphorylation.\(^{176,201}\) Discrepancy between these results is most probably caused by differences in the applied ODNs, target cells and antibodies reacting with Syk.

Our major finding is that activation of Syk is indispensable for the most important CpG-driven B cell functions such as Ab and cytokine production (Figures 18, 19, 20 and 21). Iwata et al. reported that concurrent activation of B cells through the BCR, CD40 and TLR9 induces robust activation, proliferation, cytokine and Ab production, and all these functions are abrogated by the inhibition of Syk. In this study the decrease of the B cells’ response in the presence of Syk inhibitors was attributed to the lower BCR-induced NFκB phosphorylation and the subsequently reduced up-regulation of TLR9.\(^ {176}\) Our results exceed these observations, since we found that CpG alone can induce efficient propagation of B cell
activation without the involvement of BCR and CD40. Data shown here point to the importance of CpG-driven direct phosphorylation of Syk, because inhibition of the kinase blocks all kinds of B cell functions. Jabara et al. found that the CpG-induced Syk activation occurs through DOCK8 in peripheral mononuclear cells, resulting in robust proliferation and IgG secretion. In these experiments DOCK8 induced Syk phosphorylation did not seem to be necessary to the up-regulation of CD86 and cytokine (such as IFN-α) production. These data strengthen our results showing that CpG-induced Syk activation can be initiated not only by intracellular signals in a DOCK8-MyD88-TLR9-dependent manner, but also via so far unidentified cell surface receptors which function upstream of DOCK8 and are activated by src-family kinases at a membrane proximal site. Based on this scheme it becomes conceivable why DOCK8 deficient but not Syk inhibited cells are able to maintain certain B cell functions such as activation of p38 and secretion of IFNα.

Studying the mechanism of the CpG-induced Syk activation we found that inhibition of CpG internalization did not prevent the phosphorylation of Syk or Lyn, indicating that the activation is initiated by cell surface bound CpG (Figure 23). Since we found that TLR9 is expressed on the surface of resting tonsillar B cells and that blockade of MyD88-mediated signaling pathways prevents the CpG-induced phosphorylation of Syk (Figure 24), we suppose that Syk phosphorylation may occur through surface expressed TLR9 in a MyD88-dependent manner. Nevertheless it has to be emphasized that our results do not rule out the presence of other CpG-binding cell-membrane structures/receptors, which may cause initiation of the Syk-driven signaling platform in response to CpG stimulation. Based on our data and those published by Jabara et al., we hypothesize that the CpG-induced Syk activation is initiated from the cell surface, intersects the TLR9-MyD88-cascade through Bruton’s tyrosine kinase and results in a subsequent, most probably DOCK8-dependent recruitment of Lyn and Syk.

*Syk mediates TLR9 up-regulation as well as transport of CpG into TLR9-containing endosomes*

The actin cytoskeleton plays an important role in the process of B cell activation by reorganization of the microtubular-actin network following Ag stimulation. Syk was proven to be essential for optimal actin reorganization in human B cells allowing BCR-induced spreading, Ag internalization and assembly of signalosomes. Moreover, Syk-dependent BCR uptake was shown to govern the subcellular location of TLR9, however CpG-induced B cell activation was observed without BCR stimulation as well. Here we show that hindrance
of CpG-induced B cell functions in the presence of Syk inhibitor is not due to the impaired uptake of the ODN (Figure 25) but is most probably caused by insufficient delivery of CpG into the TLR9-containing endolysosomes (Figure 26). Indeed, CpG and TLR9 co-localization decreases in the presence of Syk inhibitor, suggesting an important role of Syk in the transport of ODNs in human B cells.

We provide evidence here that a further important consequence of Syk inhibition is the suppression of CpG-induced up-regulation of TLR9 in B cells (Figure 27). The BCR-, CD40- and CpG-driven up-regulation of TLR9 has already been reported earlier, however its expression has been studied mainly at the mRNA level.\textsuperscript{173,185,186} Investigating the expression of TLR9 at the protein level we show here for the first time that binding of CpG to human B cells results in up-regulation of its own receptor. This finding points to a positive feed-back loop in which CpG can facilitate its own effects. Thus the blockade of Syk and its downstream signaling molecules leading to enhanced TLR9 expression could inhibit the positive feed-back loop, thereby block the efficient propagation of CpG-induced B cell functions.

In conclusion, we suggest the following model for CpG-induced B cell stimulation (Figure 29). Binding of CpG ODN to TLR9 or to other surface receptor(s) (such as DEC-205\textsuperscript{207}, SR-B1\textsuperscript{201} or CR2\textsuperscript{208}) results in CpG uptake and initiates Syk phosphorylation commencing from the cell surface. Thereafter activation of Syk induces actin reorganization, which is indispensable for the effective endosomal maturation and trafficking of CpG into TLR9-containing endolysosomes. Here, binding of CpG to its specific receptor induces a MyD88-dependent second wave phosphorylation of Syk, which causes B cell proliferation, differentiation and TLR9 up-regulation in synergism with the BCR and other cell surface co-receptors.
Syk regulates intracellular cross-talk between BCR, TLR9 and CR1

At the end of my PhD dissertation, I would like to emphasize the significance of Syk in the interaction between BCR, TLR9 and CR1 in human B cells (Figure 29). Our results give evidence that CR1 is a potent inhibitor of the humoral immune response. This inhibition is achieved by a decreased phosphorylation of BCR- and TLR9-induced membrane proximal kinases, such as Lyn and Syk, reducing B cell activation at the initial phase of humoral immunity. Our finding that the inhibitory capacity of CR1 is still maintained despite its reduced expression in RA patients suggests that this receptor might serve as a new target for therapeutical interventions to reduce the activity of autoreactive B cells. This inhibitory function of CR1 could be especially important in IC-mediated autoimmune diseases, where high overload of self-RNA and DNA-containing ICs may lead to aberrant B cell activation by the simultaneous engagement of both BCR and TLR9.

Considering the dual requirement of both autoantigen-triggered BCR signals and bacterial products in activation of self-reactive B cells, our results further emphasize the clinical usefulness of small molecule Syk inhibitors or CR1 targeting in B cell-mediated autoimmune diseases assumed to be driven by the inappropriate activation of the autoreactive B cells by their BCR and/or TLR9.

Figure 29. Cross-talk between BCR, TLR9 and CR1
References


110. Luxembourg AT, Cooper NR. Modulation of signaling via the B cell antigen receptor by CD21, the receptor for C3dg and EBV. *J Immunol.* 1994;153(10):4448-4457.


93


Summary

The importance of antigen- and IC-binding complement- and Toll-like receptors in regulating B cell responses has been demonstrated in physiological conditions as well as in several autoimmune animal models. Considering the complex role of B cells in pathological processes, it is reasonable to assume that simultaneous alteration in the expression and/or function of these IC-binding receptors may contribute to breaking of B cell tolerance and induction of autoimmunity.

To get a better understanding of the interplay between B cells and the complement system under physiological and autoimmune conditions, we analyzed the expression of CR1 (CD35) and CR2 (CD21) on the naive, memory and plasmablast pools of B cells. We found that memory B cells of healthy individuals and active RA patients express significantly higher levels of CR1 than naive B cells. In contrast to this, the appearance of CR2 decreases during differentiation to memory cells, proving that these two receptors are regulated in a different manner in human B cells. Confirming earlier results we also detected a significant reduction in the level of CR1 and CR2 on human plasmablasts. We have clearly demonstrated that the differences in receptor expression between healthy individuals and RA patients are not caused by the different frequencies of the various B cell subpopulations and it does not correlate with disease activity.

We have shown that clustering of CR1 inhibits BCR-induced B cell proliferation as well as antibody production dose-dependently. Despite the significant decrease of CR1 expression in active RA patients, the inhibitory capacity of this complement receptor is preserved and its ligand-induced clustering results in a significant inhibition of B cell functions – similarly to that found in the case of healthy individuals. These functional results together with the expression data suggest that CR1 and CR2 have opposite effects on human B lymphocytes and they play an important role in the IC-mediated fine tuning of B cells’ function both under physiological and pathological conditions.
We have revealed that CR1 inhibits not only the BCR-induced activation of human B lymphocytes, but also has a striking regulatory effect on TLR9-initiated B cell functions. Ligation of CR1 exerts a dose-dependent inhibition on the CpG-induced B cell proliferation as well as on cytokine production. We have demonstrated for the first time that clustering of CR1 results in dephosphorylation of key signaling molecules, such as Syk and MAPKs, thus it is able to interfere with the BCR- and TLR9-mediated signals at an early stage of B cell activation.

We have shown that - beside the "classical", MyD88-mediated signaling pathway - CpG induces dose- and time-dependent phosphorylation of Syk in resting human tonsillar B cells. We have demonstrated that this phosphorylation is initiated from the cell surface and does not occur via the cell surface BCR. Our data prove that activation of Syk is indispensable for the most important CpG-driven B cell functions, since treatment of the cultured cells with Syk inhibitors results in a strong and dose-dependent decrease of the CpG-induced proliferation as well as cytokine and antibody production. We have shown for the first time that the BCR- and CpG-mediated signaling pathways already converge as early as the level of Lyn and Syk in human B cells. We have given evidence that the CpG-induced Syk activation is a prerequisite for the delivery of CpG oligonucleotides into TLR9-containing endolysosomes and for the induction of TLR9 expression, allowing efficient propagation of TLR9-mediated activation of human B cells.

In conclusion, our results suggest that under physiological conditions CR1 clustering has a negative regulatory effect on both BCR- and TLR9-dependent functions of B cells by the induction of dephosphorylation of membrane proximal kinases at an early stage of B cell activation. Since the reduced expression of CR1 on the B cells of RA patients does not affect its inhibitory function, this receptor might serve as a new target for therapeutical interventions in rheumatoid arthritis.
Összefoglalás

Autoimmun állatmodelleken végzett kísérletek igazolják, hogy az antigént-, illetve immunkomplexeket-kötő komplement- és Toll-szerű receptorok kiemelkedően fontos szerepet játszanak a B-sejtek működésének szabályozásában. Figyelembe véve a B-sejtek összetett szerepét a patológiás folyamatok kialakulásában kézenfekvő, hogy ezen immunkomplex-kötő receptorok megjelenésében és/vagy funkcióiban bekövetkező egyidejű változások nagymértékben hozzájárulhatnak a B-sejtes tolerancia áttöréséhez és az autoimmun folyamatok kialakulásához.


Eredményeink alapján a CR1 ligandum általi keresztkötése dózis-függően gátolja a B-sejtek BCR által kiváltott proliferációját, valamint ellenanyagtermelését. Ez a gátló hatás - a csökkent CR1 expresszió ellenére - az RA-ban szenvedő betegek esetében is kimutatható, vagyis a receptor aggregációja szignifikánsan csökkenti a BCR-indukált B-sejt funkciókat, hasonlóan az egészséges kontrollokhoz. A funkcionális vizsgálatok eredményei az expressziós adatokkal összhangban egyértelműen igazolják, hogy a CR1 és a CR2 ellentétes hatással rendelkezik emberi B-sejtek esetében, és a receptorok kiemelten fontos szerepet játszanak a B-sejtek funkcióinak immunkomplexek általi finom szabályozásában fiziológiai és patológiás folyamatok során egyaránt.
Kimutattuk, hogy a CR1 képes szabályozni a Toll-szerű receptorok funkciját és jelátviteli folyamatát is. A CR1 receptorok keresztkötése dózis-függő módon gátolja a B-sejtek CpG-indukált osztódását, valamint citokin- és ellenanyagtermelését. Elsőként igazoltuk, hogy a CR1 keresztkötése olyan "kulcsszerepet betöltő" jelátviteli molekulák defoszforilációját váltja ki, mint a Syk, valamint a különböző MAPK-ok, és így hatékonyan gátolja a BCR- és a CpG-indukált B-sejt aktiváció kezdeti lépéseit.


Összességében elmondhatjuk, hogy fiziológiás körülmények között a CR1 hatékonyan gátolja a B-sejtek BCR és TLR9-indukált aktivációját egyaránt. Ez a gátlás már a jelátviteli folyamatok kezdeti lépéseibe is megfigyelhető a membrán-közeli kinázok defoszforilációján keresztül. Mivel a csökkent CR1 expresszió nincs hatással a receptor gátló szerepére, ezért ez a komplementreceptor új terápiás célpontként tekinthető a reumatoid artritiszben szenvedő betegek B-sejtjeinek szelektív gátlása során.
Acknowledgements

First of all I would like to thank my supervisor Prof. Anna Erdei for the possibility to work in a really friendly environment at the Department of Immunology. I am very grateful for her continuous support during my doctoral work. Special thanks to her for introducing me into the field of complement system.

I warmly thank my closest colleagues, Andrea Isaák, Noémi Sándor, Katalin Török, Melinda Herbáth, Zoltán Szittner, Krisztián Papp, Andrea Papp-Balogh, Daniel Szili and Mihály Józsi, for their friendly help and interesting discussions.

I am especially grateful to Bernadett Mácsik-Valent, Zoltán Prohászka and Zsolt Vági, who always encouraged me both mentally and emotionally.

I also wish to acknowledge Dr. Anna Polgár, Dr. Emese Kiss and Prof. Gyula Poór (National Institute of Rheumatology and Physiotherapy, Budapest, Hungary) for their collaboration during the investigation of RA patients.

I would also like to thank all of my friends and colleagues at the Department of Immunology for their kindness and the excellent working environment.
List of publications

Publications connected to the thesis

Kremlitzka M, Polgár A, Fülöp L, Kiss E, Poór Gy, Erdei A
Complement receptor type 1 (CR1, CD35) is a potent inhibitor of B-cell functions in rheumatoid arthritis patients

Kremlitzka M, Mácsik-Valent B, Erdei A
Syk is indispensable for CpG-induced activation and differentiation of human B cells
Manuscript under revision

Expression and role of CR1 and CR2 on B and T lymphocytes under physiological and autoimmune conditions
Molecular immunology 46:(14 Special Issue) pp. 2767-2773. (2009)

Other publications

Török K, Kremlitzka M, Sándor N, Tóth EA, Bajtay Z, Erdei A
Human T cell derived, cell-bound complement iC3b is integrally involved in T cell activation
Immunology letters 143:(1) pp. 131-136. (2012)

Published abstracts

Kremlitzka M, Valent B, Erdei A
New insights into the inhibitory function of CR1 on human B lymphocytes; the functional consequences of the crosstalk between BCR, TLR9 and CR1
Kremlitzka M, Polgár A, Kiss E, Poór Gy, Bajtay Z, Erdei A
Expression and function of CR1 and CR2 on B cells of rheumatoid arthritis patients
Molecular immunology 48:(14) pp. 1715-1716. (2011)

Erdei A, Kremlitzka M, Isaák A, Poór Gy, Bajtay Z
Complement-mediated regulation of B-cell function - physiological upregulation of CR1 and Fc gamma RII on memory B cells is lacking in SLE
Clinical and experimental rheumatology 29:(1) p. 177. 1 p. (2011)

Erdei A, Kremlitzka M, Isaák A, Prechl J, Bajtay Z
Differential expression and function of complement receptor type 1 (CD35) and 2 (CD21) on human B lymphocytes
Molecular immunology 47:(13) p. 2223. 1 p. (2010)

Erdei A, Isaák A, Kremlitzka M, Poór G
Physiological upregulation of CR1 and Fc gamma RII on memory B cells is lacking in SLE patients, but is not related to the cells' activation state
Molecular immunology 46:(14) p. 2829. 1 p. (2009)