Alterations in the expression and role of immunecomplex-binding receptors on B lymphocytes under physiological and autoimmune conditions

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List of abbreviations

ANA	antinuclear antibody
BCR	B cell receptor
C3	complement component 3
CIA	collagen-induced arthritis
CIC	circulating immune complex
CR1	complement receptor type1
CR2	complement receptor type 2
DC	dendritic cell (s)
dsDNA	double straind DNA
FcγR	IgG receptor
FDC	follicular dendritic cell (s)
FITC	fluorescein isothiocyanate
FSC	forward scatter
GC	germinal centre
HEL	hen egg lysozyme
IC	immune complex (es)
IFN	interferon
IL	interleukin
ISC	Ig-secreting cells
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
LHR	long homologous repeat(s)
lpr	generalized lymphoadenopathy
LPS	lipopolysaccharide
MAC	membrane attack complex
MACS	magnetic cell sorting
MAPK	mitogen-activated protein kinase
MFI	mean fluorescence intensity

mRNA	messenger ribonucleic acid
MZ	marginal zone
NK	natural killer
NOD	non obese diabetic
ODN	oligodeoxynucleotide
PBMC	peripheral blood mononuclear cell (s)
PCs	plasma cell(s)
PE	phycoerythrin
RAG	Recombinase Activating Genes
RFI	relative fluorescence intensity
SCR	short concensus repeat(s)
SHIP	Src-homology-2-domain-containing inositol 5'-phosphatase
sIg	surface immunoglobulin
SLE	systemic lupus erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SRBC	sheep red blood cell
SSC	side scatter
ssDNA	single-stranded DNA
TLR	toll-like receptor
TNF	tumor necrosis factor

1. Introduction

B cells are central components of the humoral immune response, being responsible for the production of serum immunoglobulins. Membrane immunoglobulins displayed on their surface, termed the B-cell antigen receptor, allow the clonal expansion of B cells with the appropriate specificity during the response to infections. Several checkpoints during B-cell development ensure that self-reactivity is kept at a minimum, while a highly diverse (10^9) population is continuously released from the bone marrow. The secondary lymphoid organs provide a microenvironment suitable for the efficient presentation of antigens to B-cells. If additional secondary signals are present in the form of cytokines, FDC and T-cell contact, B-cells give rise to a memory population and the antibody producing plasma cells.

Autoimmune pathogenesis in SLE involves complex intercellular-dependent pathogenetic processes with considerable heterogeneity in clinical manifestations and disease course. Although multiple immunologic abnormalities are important for the development and clinical expression of SLE, a growing body of evidence, most recently including the efficacy of B cell depletion therapy, supports the key role of B lymphocytes in disease pathogenesis. Both antibody-dependent and antibody-independent mechanisms of B cells are important in SLE. The pathogenic roles of B cells in autoimmune diseases occur through several mechanistic pathways that include autoantibodies, immune complex (IC) formation, dendritic cell (DC) and T cell activation, cytokine synthesis, chemokine-mediated functions, and ectopic neolymphogenesis. Each of these pathways participates to different degrees in autoimmune diseases. B lineage depletion and the inhibition of B cell activation and survival are beneficial in multiple autoimmune diseases and provide a basis to further explore the role of B cell subsets and their function in physiological and pathological conditions.

1.1. The role of B cells in Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a rheumatic disease characterized by autoantibodies directed against self-antigens, IC formation, and immune dysregulation, resulting in damage to essentially any organ, including the kidney, skin, blood cells, and the nervous system. The natural history of this illness is unpredictable; patients may present with many years of symptoms or with acute life-threatening disease. A large body of experimental and observational studies in both mice and humans indicate that B cells play complex and important roles in SLE. The presence of autoantibodies is the feature common to all SLE

patients. Increased production of autoantibodies leads to IC formation and tissue damage due to direct binding and/or deposition in tissues. Whether these antibodies are produced in reaction to exposure of normally nonexposed self-antigens or because of a broad spectrum of immune dysregulation resulting in excessive production of many antibodies without regard to prior stimulation, is unclear; both mechanisms may play a role. Patients with SLE make antibodies against DNA and other nuclear antigens, ribosomes, platelets, erythrocytes, leukocytes, as well as other tissue-specific antigens. The generated IC result in widespread tissue damage. In addition, cell-mediated autoimmune responses also play a pathophysiologic role (1-3). Within the healthy population, little measurable evidence exists of antibody to self. A subset of individuals has small amounts of autoantibody as manifested by a low-titer ANA or other autoantibody (4;5). Autoantibody production, by relatively few B lymphocytes, may be a by-product of polyclonal B-cell activation in which many more B lymphocytes are activated, perhaps not in response to specific antigenic stimuli (6-8). Viral stimulation of the immune system coupled with genetic defects in the innate and adaptive immune responses could lead to loss of tolerance and increase autoantibody formation (9-12).

Autoreactive B lymphocytes are abundant in the mature peripheral B-cell repertoire and need to be censored to avoid autoimmunity (13). This censoring is accomplished in diverse ways and may be broken down by multiple mechanisms both intrinsic and extrinsic to the B cells. Although undoubtedly multiple immunologic abnormalities are important for the development and clinical expression of SLE, a growing body of evidence, more recently including the effectiveness of therapeutic B-cell depletion (14-19), strongly points to the B cell as a central player in the pathogenesis of this disease. Although self-reactive B lymphocytes produce the autoantibodies essential to the diagnosis of disease, B cells have proven in recent years to be active participants in the development of disease irrespective of autoantibody production (20).

Autoantibody-independent functions that may contribute to SLE include the ability of B cells to regulate T cells and DCs and their ability to produce cytokines such as interleukin-10, interleukin-6, and interferon (IFN)-γ (21;22). Immunologic tolerance for self-antigens is enforced at several checkpoints throughout B-cell development. Although central mechanisms such as receptor editing and clonal deletion initiate control of autoreactivity during early bone marrow development, accumulating evidence supports the assumption that despite early tolerance checkpoints a significant fraction of autoreactive B cells proceed unchecked into the peripheral repertoire (23). To prevent autoimmunity additional censoring mechanisms are needed at later stages (23-25). Although the frequency of autoreactive B-cells significantly

declines in the transition from the immature B-cell stage into the peripheral mature compartment, as much as 20% of peripheral naive B cells are still reactive with nuclear antigens (26).

1.2. Immune complex formation and interaction with IC-binding receptors

The formation of IC due to the interaction of foreign substances with specific antibodies is a physiological process, which constitutes an essential part of normal immune defence mechanisms. Under physiological conditions IC are rapidly removed from the bloodstream, and provide important feedback signals for the immune system via Fcγ– and complement receptors (CR) (27-29). In some circumstances, however, potentially pathogenic IC might form and not be cleared properly. Large quantities of precipitating IC continue to circulate and become trapped and deposited in the tissues of the kidney, lung, skin, joints or blood vessels. These IC can fix complement and cause local damage at their sites of deposition (30;31). The balance between rapid and safe clearance versus tissue localization is fundamentally influenced by the biophysical and immunochemical properties of IC.

It is well accepted that for the generation and control of an appropriate humoral immune response, in addition to signaling through the B cell receptor (BCR), a balanced signaling through IC-binding complement (CR1, CR2) and Fcγ (FcγRI, FcγRII, FcγRIII) receptors is necessary. The importance of these receptors has been demonstrated in several autoimmune animal models (32-34). Complement containing IC may influence effector cell functions as well as the fate of autoreactive B cells. Alterations in the expression and/or function of these receptors may contribute to the initiation and/or maintenance of IC-mediated autoimmune disorders. Signaling pathways initiated upon the engagement of Fcγ- and complement receptors on various cell types by IC regulate autoimmune responses at different levels. Control of cognate interactions and inflammatory responses, IC-clearance, B cell selection and activation may all be affected by altered expression or function of these receptors.

1.3. Modulation of B cell antigen-receptor signalling by cross-talk between IC-binding receptors

During the development of the humoral immune response, several mechanisms regulate the functions of B cells. In addition to the determining antigen-specific interaction

via the BCR, the fate of a B lymphocyte is strongly influenced by the integration of signals transduced via several other cell membrane molecules. Among those are the IC-binding cell membrane structures such as CR1, CR2 and FcγRII (Fig.1.). The composition, amount and ratio of various elements – such as Ig-isotype, complement activation fragments - in the IC play an important role in determining the response of B cells via their interactions with these receptors. The cross-talk between these cell membrane structures interacting with certain constituents of the complex significantly alters the quality and magnitude of the responses induced by the BCR.

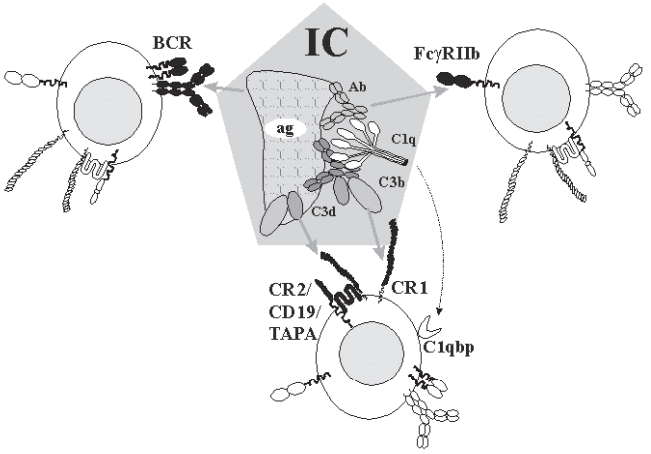


Figure 1. *Composition of IC determines possible interactions with B lymphocytes (Erdei et al., 2003)*

1.4. The role of complement in autoimmune disease

The complement system is comprised of a number of serum and cell membrane proteins that play an important role in the elimination of foreign structures and pathogens while protecting the host organism from complement-related damage. Complement has also been shown to participate in the generation of normal humoral immune responses to foreign antigens. Recent studies suggest that the function of the complement system may be extended

to include the maintenance of B cell tolerance, too. Complement activation occurs via three distinct pathways, known as the classical, alternative, and lectin pathways. Each of these routes culminates in the cleavage of major complement protein C3 by specific multi-protein convertase enzymes (Fig.2.) and the subsequent generation of the membrane attack complex (MAC). A certain portion of C3b fragments generated from C3 by cleavage becomes covalently attached to the activating substrate and serves as ligands, - along with C4bbound to antigens -, for complement receptor type 1 (CR1/CD35). Inactivation of C3b to iC3b and C3d generates the specific ligands for complement receptor type 2 (CR2/CD21), and serves as a means of targeting antigen or IC to cells expressing CR2. All these processes might link complement activation to B cell biology and tolerance.

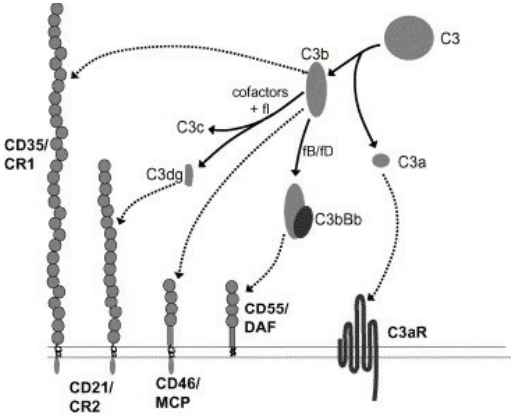


Figure 2. *Cleavage of native C3 and generation of its biologically active fragments; CR1 preferentially binds C3b while the main ligand of CR2 is C3d*

1.4.1. Complement receptor type 1 (CR1/CD35)

CR1 is an approximately 200 kDa single-chain glycoprotein. The extra membranous portion of its most common size allotype is composed of 30 short consensus repeats (SCR). The 28 N-terminal SCR can be organized, based on a degree of homology, into four long homologous repeats (LHR) A–D, each composed of seven SCR (Fig.3.) (35). There are two

distinct sites on CR1 that interact with C3b and C4b. Site 1 spans SCR 1–3 in LHR A, and binds C4b and, weakly, C3b. Site 1 also carries decay-accelerating activity for the C3 convertases. Site 2 spans SCR 8–10 in LHR B and the nearly identical SCR 15–17 of LHR C, and binds both C3b and C4b efficiently, although it has a higher affinity for C3b. It is also the major site of CR1 cofactor activity and is indispensable for the decay-accelerating activity of the C5 convertases (36-38).

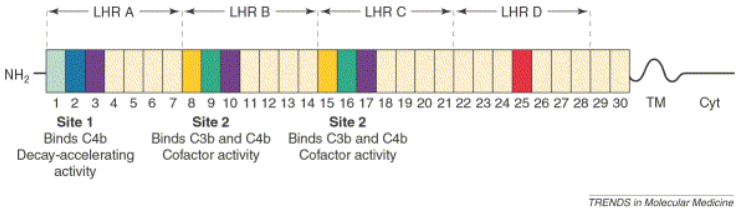


Figure 3.*The structure of human complement receptor type 1***,** *showing 30 SCR organized in four LHR and their functions (Atkinson et al., 2002)*

CR1 is expressed by all peripheral blood cells except platelets, natural killer cells and most T lymphocytes (39). In tissues, it is expressed by follicular dendritic cells (FDC), glomerular podocytes and some astrocytes. In the circulation, CR1 on erythrocytes is an immune adherence receptor that binds C3b/C4b-opsonized immune complexes and ferries them to the liver and spleen for removal (40). Another function of CR1, facilitated by its ability to bind C3b and C4b, is the retention of opsonized antigens necessary to maintain immunological memory on FDC in germinal centers (GC) (41;42). In addition to its ligand-binding capability, CR1 has decay-accelerating activity for C3- and C5-convertases. By limiting the deposition of C3b and C4b, this decay-accelerating activity might regulate the size of immune complexes, and prevent excessive complement activation. CR1 also possesses cofactor activity for the limited cleavage of C3b and C4b by the plasma serine protease, factor I (43). Cleavage of C3b by factor I, with CR1 as cofactor, generates C3dg. This fragment and C3d, the final cleavage product of C3, interact with complement receptor type 2 on B cells, thus lowering the threshold for B-cell activation by the antigen receptor (44-46). Recently, CR1 was also shown to bind C1q and mannan-binding lectin, raising the possibility that they too, along with C3b and C4b, contribute to CR1-mediated immune adherence (47;48).

Whereas the role of human CR2 in B cell activation is relatively well-established, much less is known about the exact function of CR1 (CD35). Data presented by our group indicate that CR1 expressed by human B lymphocytes mediates inhibitory signals, thus plays an opposite role to CR2 in the regulation of B cell activation (49). C3b-like C3, which mimics multimeric C3b and binds to CR1, strongly and dose-dependently inhibits the anti-IgM-induced proliferation of human B cells. Parallel to this, the anti-IgM-induced transient increase of intracellular free Ca^{2+} level and phosphorylation of tyrosine residues of several cytoplasmic proteins are also inhibited by multimeric C3 (49).

1.4.2. Complement receptor type 2 (CD21)

CR2 is a surface glycoprotein located almost exclusively on B cells and FDC in mice, with a somewhat broader tissue distribution in humans. In mice, both CR1 and CR2 are transcribed from a single gene by alternative mRNA splicing, while in humans these proteins are derived from two distinct but closely linked genes on chromosome 1 (50). Both human and mouse CR2 bind the C3 activation products iC3b, C3dg, and C3d; human CR2 also binds Epstein–Barr virus, CD23, and interferon-γ (51;52). CR2 is composed of 15 repeating 60–70 amino acid extracellular subunits termed short consensus repeats (SCR), a transmembrane domain, and a short cytoplasmic tail. In humans, a 16SCR form of CR2 has been identified that is generated by alternative splicing of a single exon (53).

Expression of CR2 on B cells is regulated in a cell-type specific manner and depends on the developmental stage. CR2 is first expressed at the T1-T2 transitional stage on $B220^{low}/IgM^{high}$ B cells and is terminated when they finish their maturation and differentiate into plasma cells (54). While terminally differentiated plasma cells lack CR2, memory B lymphocytes express this complement receptor. FDC, which capture antigen complexes in GC,constitutively express CD21. In the splenic compartment the highest levels of receptor expression are found on marginal zone B lymphocytes followed by follicular B cells expressing intermediate CR2 level (55). Non-conventional B1 cells have the lowest CR2 density on their surface.

In humans, during B cell development in the bone marrow, CR2 is primarily expressed on IgM^{+}/IgD^{-} cells, although expression on a small subpopulation of $CD19^{+}/IgM^{-}$ pre-B cells has also been described. CR2 is expressed almost on all mature peripheral human B lymphocytes. Similar to mice, expression of human CR2 on splenic B cells is highest in the

marginal zone subpopulation and lost from plasma cells along with other surface molecules characteristic for B cells (56).

CR2 has been demonstrated to amplify antigen-induced B cell activation through surface IgM (sIgM), to rescue peripheral B cells from sIgM-mediated apoptosis, to promote antigen processing and presentation of C3d-bound targets, to modulate the expression of costimulatory molecules, to stabilize the B cell receptor in lipid rafts, and to target IC to GC in secondary lymphoid organs (57-61). Many of these functions may occur via interaction of CR2 with CD19 and CD81 on the B cell surface, where these receptors form a multi-molecular signal transduction complex. Mice made deficient in CR2 by homologous recombination have defects in T-dependent and T-independent antibody responses, GC formation, generation of memory B cells, and the development of a normal natural antibody repertoire. The defect in T-dependent antibody responses results from a lack of CR2 on both B cells and FDC.

1.4.3. Possible relationship between CR1/CR2 and the development of autoimmunity in mice

In the B6/*lpr* mouse model of lupus, CR1/CR2 deficiency results in the earlier onset of a more aggressive disease. On a mixed B6/129 background, CR1/CR2-deficient *lpr* mice develop marked splenomegaly and lymphadenopathy, increased anti-nuclear and anti-dsDNA antibodies, and increased glomerular deposits of IC (62). In mice expressing transgenes for hen egg lysozyme (HEL) and HEL-specific membrane Ig, the lack of CR1/CR2 resulted in impaired induction of tolerance. In the absence of CR1/CR2, HEL-specific B cells were not anergized and were found to be accumulated in secondary lymphoid organs with an increased life-span. Since both CR1 and CR2 are deficient in these animals, a specific effect of CR2 deficiency cannot be assessed (63). This double-transgenic model supports a role for CR1/CR2 in negative selection, suggesting that complement may be involved in the maintenance of tolerance. In one report, the effect of C4 deficiency in these models mimicked those of CR1/CR2 deficiency, suggesting that the primary effect on B cell tolerance was mediated by CR1 in its interactions with C4 activation fragments. The *Cr2 gene* is demonstrated to be a strong candidate for lupus susceptibility in NZM2410 mice, which is widely used as a model of lupus (64). Biochemical analyses of CR1 and CR2 in these mice revealed that both proteins are increased in molecular weight because of a single nucleotide polymorphism in the ligand-binding domain. Molecular modelling of the mouse CR2

sequence revealed that the altered amino acid (asparagine) is located at a site in the human receptor that is critical for receptor dimerization. Glycosylation of this asparagine residue would alter receptor dimerization. Subsequent functional analyses demonstrated defects in C3d-ligand binding as well as signaling mediated by CR2. Because the functions of CR2 were affected in this model, these data support a role for CR2 in the development of the autoimmune phenotypes observed.

The exact mechanisms by which CR2 plays a role in B cell tolerance have also not yet been delineated. Since coligation of CR2 with sIg is known to lower the threshold for B cell activation, one hypothesis is that coligation of CR2 with sIg also lowers the threshold for B cell tolerance to autoantigens. If CR2 expression or function is impaired, then autoreactive B cells may be able to escape from tolerance. Altered CR2 expression or function may also diminish the levels of complement-coated autoantigens that are targeted to FDC in secondary lymphoid organs, and remove a reservoir of self-antigens that may be critical in maintenance of self-tolerance. Expression of CR2 on both B cells and FDC is important in the generation of normal immune responses, and ligand binding to both cell types may also be important in the regulation of B cell tolerance.

CR2 may indirectly regulate B cell tolerance via effects on T cell tolerance, too. CR2 has been shown to bind and internalize antigen for presentation to T cells (65). Since CR2-mediated antigen presentation in the absence of sIg coligation does not result in upregulation of costimulatory molecules, this mechanism may be important in the regulation of T cell tolerance (66). In addition, coligation of CR2 with sIg may alter the levels or types of costimulatory molecules upregulated, and thus skew the T cell cytokine profile to create an environment that favors loss of tolerance to self-antigen. In the cardiac myosin-induced model of experimental autoimmune myocarditis, which is believed to be mediated by autoreactive T cells, complement depletion, CR1/CR2 blockade or deficiency markedly decreased the incidence and severity of myocarditis. In addition, reduced levels of proinflammatory cytokines (TNF-α, IL-1 and IFN-γ) and increased levels of an anti-inflammatory cytokine (IL-10) were noted in these animals, as well as decreased autoantibody titers of total IgG. These data suggest that CR1/CR2 regulates the production of proinflammatory cytokines by autoreactive T cells, with an associated increase in autoantibody production resulting in the induction of autoimmune myocarditis. Although these results contrast with those observed in the B6/*lpr* and HEL double transgenic models, it is possible that in certain autoimmune disorders CR2 regulates tolerance to autoantigen, while in others it induces an autoimmune response.

1.4.4. Alterations of CR1/CR2 receptor expression in human autoimmune diseases

Contribution of complement receptors CR1 (CD35) and CR2 (CD21) to the initiation and sustenance of tissue damage was studied in various animal models. The findings support the notion that malfunctioning of CR1/CR2 might be involved in the breakdown of tolerance and excessive autoantibody production by autoreactive B cell clones. Results obtained from mouse experiments however should be extended to human system with great care, since there are basic differences between the structure and function of human and murine CR1 and CR2. As mentioned earlier in the mouse CR1 and CR2 are coded by one gene and the receptors are generated by alternative splicing. In contrast to this, different genes encode human CR1 and CR2. While murine CR2 shows structural and functional homology to human CR2 and has a similar expression pattern, human CR1 is functionally distinct from murine CR1; moreover its tissue distribution greatly differs in these two species.

While CR1 expressed on mouse B cells have a similar role as CR2, in humans they have distinct functions. Regarding their role in B cell activation probably the most important function of CR2 is its capacity to promote B cell activation by lowering the signaling threshold of antigen-specific BCR (46). In contrast, human CR1 clustered on anti-μ–activated human B lymphocytes have been shown to induce negative regulatory signals (49), a phenomenon also demonstrated recently on human T cells (67). Thus, in humans, depending on the actual composition of IC and the degradation stage of C3 and C4 in the complex, CR1 and CR2 may influence the fate of autoreactive B cells differently.

Expression of CR2 and CRl on human B cells has been studied in a number of human autoimmune diseases. One important observation is that patients with SLE demonstrate abnormalities in the expression of both CR2 and CRl on B lymphocytes (68;69). Peripheral B cells show a marked decrease in both CR2 and CRl density as compared to control subjects. It has also been reported that CR2 expression is strongly reduced on synovial B cells of arthritis patients as compared to their peripheral B cells (70).

The functional consequences of reduced complement receptor expression in human diseases have been quite rarely studied so far. In one of these investigations B cells from SLE patients were stimulated with an anti-IgD antibody conjugated to the Epstein-Barr virus gp350 protein, which binds to CR2. Despite the fact that these B cells were found to express half as many surface CR2 as normal B cells, the Ca^{2+} response and the percentage of

responding cells were significantly increased (71). These observations suggest that CR2 may be involved in the exaggerated autoreactive B cell activity as a regulator, independent of its density on the cell surface.

1.5. The role of FcγR on B cells

FcγRs are widely expressed throughout the immune system (72). By binding the antibody Fc-portion, they provide a link between the specificity of the adaptive immune system and the powerful effector functions triggered by innate immune effector cells. In all mammalian species studied to date, four different classes of Fcγ receptors have been defined: FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and FcγRIV (72). Whereas FcγRI displays high affinity for the antibody-constant region and restricted isotype specificity, FcγRII and FcγRIII have low affinity for the Fc region of IgG but a broader isotype-binding pattern. FcγRIV, a recently identified receptor conserved in all mammalian species reacts with Ig with intermediate affinity and restricted subclass specificity. Functionally, there are two different types of FcγR: the activating and the inhibitory receptors, which transmit their signals via immunoreceptor tyrosine-based activation (ITAM) or inhibitory motifs (ITIM), respectively. The paired expression of activating and inhibitory molecules on the same cell is the key for the generation of a balanced immune response. Additionally, it has only recently been appreciated that the IgG Fc receptors show significant differences in their affinity for individual antibody isotypes, rendering certain isotypes more strictly regulated than others.

Murine models of autoimmune diseases indicate the roles of the inhibitory Fcγ receptor in the suppression of such disorders, whereas activating-type FcγR are crucial for the onset and exacerbation of the disease (73-76). Activating and inhibitory FcγR that are co-expressed on the same cell set a threshold for immune cell activation by IC. Besides their involvement in the efferent phase of an immune response, they are also important for modulating adaptive responses by regulating B cell activation. Uptake of IC by FcγR on DC and the concomitant triggering of activating and inhibitory signaling pathways will determine the strength of the initiated T-cell response. Loss of this balanced signaling results in uncontrolled responses that can lead to the damage of healthy tissues and ultimately to the initiation of autoimmune processes. There are several factors that can influence the balance between activation and inhibition, such as the expression levels of activating versus inhibitory receptors and the modulation of their ratio by cytokines (77-79). The pathogenicity of an

autoimmune response will be determined by both cytokine-mediated regulation of class switching and the changes of expression levels of the responsible activating versus inhibitory Fcγ receptors.

1.5.1. The inhibitory FcγRIIB

FcγRIIB belongs to the family of immune inhibitory receptors. These proteins can be found on a wide variety of immune effector cells, share similar properties, and are important regulators of their activating counterparts (80). The loss of these negative regulators leads to imbalanced immune responses resulting in autoimmunity and overt autoimmune disease (81;82). FcγRIIB is a single-chain receptor that carries an ITIM motif in its cytoplasmic domain, a hallmark of this inhibitory protein family. It functions through the recruitment of the inositol phosphatase SHIP through binding to an SH2 site generated on the FcγRIIB ITIM motif as a consequence of the transphosphorylation that is initiated upon its coligation to an ITAM-bearing receptor. It is the only "classical" Fcγ receptor on B cells where it regulates activating signals delivered to the BCR by IC retained on the FDC (83).

1.5.2. FcγRIIB as a regulator of B cell activation

Because of its role in regulating BCR signals, which ultimately will decide whether a B cell proliferates, undergoes class switching, and matures into an antibody-secreting plasma cell, FcγRIIB has been suggested to play an important role in maintaining peripheral tolerance. When coligated with the BCR, it triggers two ITIM-dependent signaling pathways that inhibit cell activation and proliferation. While regulation of cell activation is dependent on the recruitment of SHIP to the ITIM motif which ultimately leads to the inhibition of calcium-dependent signaling pathways, control of proliferation seems to involve SHIP-independent signaling pathways, including the adaptor molecule Dok and MAP kinases (84). Besides these ITIM-dependent signaling events, the crosslinking of FcγRIIB on B cells independent of BCR is known to lead to B cell apoptosis (85;86). The capacity of FcγRIIB to trigger B cell apoptosis has been proposed to be another mechanism for controlling B cell responses and maintaining self tolerance by deletion of low-affinity or self-reactive B cells (86). This hypothesis was supported by the generation of FcγRIIB-deficient mice that spontaneously develop a lupus-like disease characterized by the production of autoantibodies. This autoimmune phenotype is strain dependent, indicating that other epistatic modifiers are

involved in disease susceptibility and severity. Autoimmune-prone mouse strains such as NZB, NOD, BXSB, and MRL express reduced levels of FcγRII receptor on activated and germinal-center B cells, which has been attributed to a polymorphism in the promoter of this gene (87-90). More recently, a similar polymorphism in the human FcγRIIB promoter that is linked to lupus has been identified (91). Additionally, a polymorphism in the transmembrane domain of FcγRIIB has been identified, which was associated to human SLE. This allelic variant of the inhibitory receptor loses its function due to the inability to associate with lipid rafts (92;93). It has been described that transfer of FcγRIIB-deficient bone marrow into irradiated RAG or IgH knockout recipients leads to the development of autoimmunity. In these animals, the monocytic compartment still expressed FcγRIIB while it was absent from peripheral B cells (94). Moreover, induced up-regulation of FcγRIIB expression on B cells to wild-type levels by retroviral transduction restore tolerance and thus prevent the development of the fatal autoimmune disease. Restoration of FcγRIIB expression to wild-type levels on approximately 40% of peripheral B cells was sufficient to prevent the development of autoantibodies and autoimmune glomerulonephritis (95). To protect against autoimmunity it was not necessary to restore FcγRIIB function on all B cells. This finding has important implications for the design of therapeutic approaches, as it suggests that despite the complex nature of autoimmune diseases, therapeutic effects are achievable by targeting specific cell populations.

In another experiment overexpression of FcγRIIb on B cells reduced IgG production and the development of spontaneous SLE in mice (96). These results clearly demonstrate the contrasting roles played by FcγRIIb on B cells and macrophages in the control of autoimmunity, and emphasize the therapeutic potential for modulation of FcγRIIb expression on B cells in autoimmune disease.

The stages in B cell development at which FcγRIIB exerts its function in the maintenance of tolerance has recently been defined. Autoreactive B cells can be generated at several stages during B cell ontogeny. There is accumulating evidence that FcγRIIB mediates its function during late stages of B cell maturation, thus representing a distal checkpoint. FcγRIIB deficiency did not influence early events in the bone marrow like receptor editing, nor did it prevent the development of IgM-positive autoreactive B cells. After class switching to IgG, however, FcγRIIB was essential to prevent the expansion of autoreactive B cells and their maturation into plasma cells (97). As IgG has higher pathogenic potential than IgM, a relatively late stage of FcγRIIB-mediated negative regulation might be sufficient to prevent

the initiation of severe autoreactive processes. Altered regulation of FcγRIIb expression, caused by either genetic or disease activity related factors, could contribute significantly to the hyperactivity of B cells in SLE.

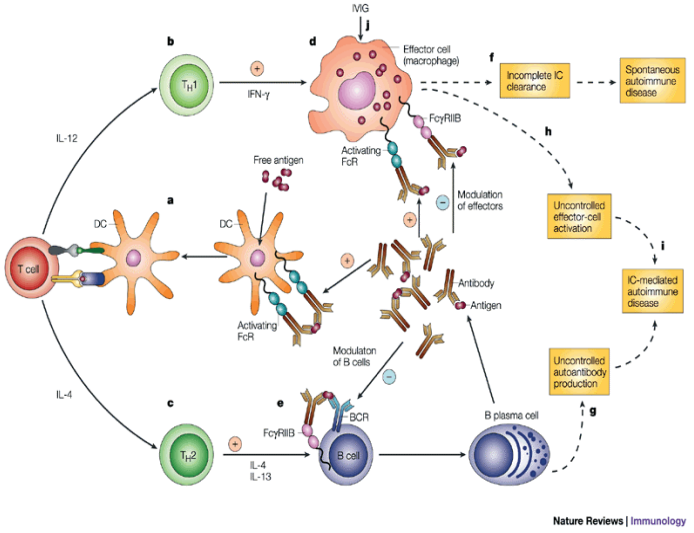


Figure 4. *FcγR can initiate or augment an immune response at the initial antigen-presentation step (a) by facilitating immune complex (IC) uptake, and can drive cellular (b) and humoral (c) immune responses. (Takai et al., 2002)*

1.6. SLE and memory B cells

1.6.1. The role of IC-binding receptors on memory B cells

Humoral memory for foreign antigens is essential for long-term protection against invading pathogens. However, autoreactive memory cells may have life-threatening consequences in autoimmune diseases such as SLE, a disease associated with a breakdown in B cell tolerance and elevated serum levels of high-affinity IgG autoantibodies. In addition to altered tolerance of IgG-producing B cells, individuals with SLE show abnormalities in early

B cell tolerance checkpoints, leading to increased numbers of autoreactive mature naïve B cells independent of disease activity (98). Naïve B cells do not secrete antibodies, but antigen-mediated activation induces their differentiation into antibody-secreting short-lived plasmablasts and long-lived plasma cells or memory B cells. The finding that high frequencies of autoreactive naïve B cells are present in SLE patients suggests that these cells might be the precursors of high-affinity IgG^+ B cells contributing to humoral autoimmunity in SLE. Alternatively, defects that lead to abnormalities in memory B cell tolerance in SLE might be independent of the earlier tolerance defects.

The relative contribution of IgG^+ memory B cells to the production of serum autoantibodies in SLE is not known, but memory B cells readily respond to activation by differentiation into antibody-secreting B cells. Indirect evidence for a role of memory B cells in human autoimmune diseases comes from clinical studies with rituximab, a monoclonal anti-CD20 antibody that efficiently depletes all circulating B cells. Recent evidence suggests that relapses after anti-CD20 therapy are strongly associated with the reappearance of high numbers of circulating memory B cells (99). However, it is not yet clear whether this reflects early regeneration of such cells or efflux of non-depleted memory B cells from secondary lymphoid organs.

1.6.2. Development of antigen-specific memory B cells

Memory B lymphocytes are mainly generated in the GC of secondary lymphoid organs. Within these structures proliferating B cell blasts can increase the affinity of their surface Ig through somatic hypermutation of their Ig variable region genes and positive selection of high affinity mutants. Isotype switch can also take place during GC reaction. After leaving the GC, memory B cells either join the recirculating pool of lymphocytes, or home to antigen draining sites such as the marginal zone of the spleen. Memory B cells display several intrinsic differences compared with naive B cells, i.e.: (*a*) lower threshold for activation, (*b*) ability to directly present antigen to helper T cells, and (*c*) longer life span.

Helper T (Th) cell–regulated B cell immunity progresses in an ordered cascade of cellular development that culminates in the production of antigen-specific memory B cells. The recognition of peptide MHC class II complexes on activated antigen-presenting cells is critical for effective Th cell selection, clonal expansion, and effector Th cell function development. Cognate effector Th cell–B cell interactions then promote either development of

short-lived plasma cells (PC) or GC. These GC expand, diversify, and select high-affinity variants of antigen-specific B cells for entry into the long-lived memory B cell compartment. Upon antigen re-challenge, memory B cells rapidly expand and differentiate into PC under the cognate control of memory Th cells (100).

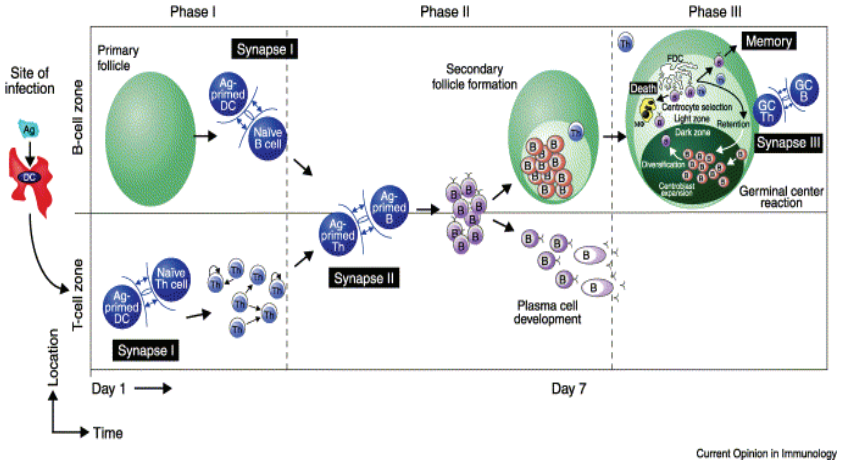


Figure 5. *B cells can either differentiate into long-lived plasma cells, or remain as non-secreting precursors for antigen recall. (Louise et al., 2005)*

1.6.3. Maintenance of serological memory by polyclonal stimuli

Whether persisting antigen is required to maintain serological memory remains debated. Antigen-driven proliferation and differentiation of memory B cell to short-lived plasma cells induce high levels of protective antibodies. Yet, if persistence of antigen were the only mechanism available to maintain antibody production, immunological memory would be limited to persisting antigens. All these suggest that there should be a so far unrevealed alternative mechanism that may ensure sustained proliferation and differentiation of memory B cells, independently of persisting antigen (101).

Under *in vivo* conditions there are two types of polyclonal stimuli that can trigger B lymphocyte proliferation and differentiation in the absence of antigen. Microbial structures

such as LPS or unmethylated DNA motifs (CpG oligonucleotides), which stimulate B cells via TLR4 and TLR9, respectively or stimuli by activated bystander T cells, which stimulate B cells in a non-cognate fashion (102-104).

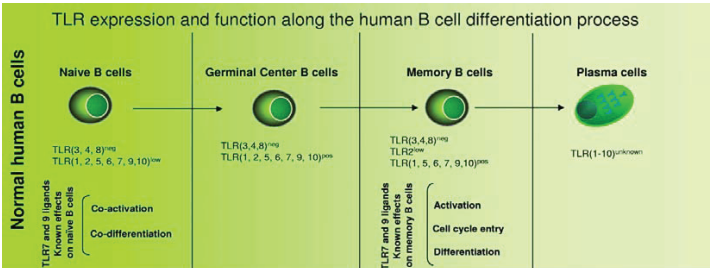


Figure 5. *TLR expression and function on human B cell subpopulations*

Detailed studies on human B-cell populations have shown that blood–derived naive and memory B cells express distinct levels of TLR6, 7, and 9. Indeed, TLR6, 7, 9, and 10 are barely expressed by circulating naive B cells whereas memory B cells display a higher sensitivity to TLR activation with a concomitant higher capacity for differentiation into plasma cells.

In the antigen-dependent response mode, memory B cells undergo a massive expansion and differentiation to become short-lived plasma cells. This response is transient, because of the negative feedback exerted by the high level of antibody present. However, some plasma cells generated in this way become long-lived if rescued in available niches such as the bone marrow. These cells sustain serum antibody levels, but can do so only for a few months, because of their limited life span. In contrast, in the polyclonal response mode, all memory B cells respond to environmental stimuli by undergoing continuous proliferation and differentiation. In this way, a constant level of plasma cells and serum antibodies could be maintained throughout a human life span. Because this mechanism is non-specific, it would act indiscriminately to maintain the broad spectrum of antibody specificities generated during the antigen-driven immune response (101).

B cells were the first human immune cell subset known to recognize CpG motifs within microbial DNA. Based on B-cell activation and proliferation, the human CpG motif was identified and an oligo-nucleotide was developed (CpG ODN 2006) that triggered the MAP kinase pathway and NFκB translocation in purified B cells. This sequence turned out to be a potent adjuvant to support humoral immune responses in primates (105-107). Foreign CpG-DNA from viruses and bacteria can activate memory B cells through binding to TLR9, and this pathway has been hypothesized to be involved in the continuous activation of memory B cells ensuring life-long humoral immunity (101).

The discrimination between human and bacterial/viral DNA is based on the fact that CpG dinucleotides are underrepresented and generally methylated in vertebrate DNA, while they are present at expected frequency and are unmethylated in bacteria and viruses. Bacterial infection has been suspected as a triggering factor for lupus and TLR9 has been implicated in the activation of autoreactive B cells in vitro. Its role in promoting autoantibody production and disease in vivo, however, has not been determined (108-110).

1.6.4. IgM memory B cells

In mice, memory B cells have mostly switched from the initial expression of IgM to that of other Ig classes and the frequency of memory B cells is about 5% of all peripheral B lymphocytes. In the human this ratio is much higher;i.e. 25%. The difference might largely be explained by the longer life span of humans, leading to the accumulation of a much larger fraction of memory B cells.

In humans, B lymphocytes expressing cell surface IgM and CD27 comprise a significant proportion of the B-cell population in the peripheral circulation. These cells have been termed IgM memory B cells because, like conventional class-switched memory cells, they possess somatically hypermutated variable region genes and express CD27 (111).

The splenic marginal zone (MZ) has been implicated as a source of circulating IgM memory B cells, as their representation in the peripheral circulation is reduced in asplenic subjects, and their progressive appearance in the peripheral circulation during the first and second year of life correlates with the development of the splenic MZ (112). Although it presently cannot be ruled out that $IgM^{+}/CD27^{+}$ B lymphocytes are generated in a GC-independent pathway, these observations collectively suggest that these cells, besides class-switched memory cells, represent a second, phenotypically defined memory B cell subset present in human blood..

In vitro-activated IgM memory B cells differentiate into plasma cells, but do not change isotype even under conditions that promote switching. This indicates that IgM memory B cells are committed to secrete IgM. The role of IgM- expressing memory cells in T cell-dependent immune responses remains elusive. Pentameric IgM enables efficient cross-linking of antigen and permits a strong activation of the complement system. These features are advantageous in the defence against bacteria. Since IgM memory B cells require the spleen for their generation and/or survival and are responsible for the protection against encapsulated bacteria, splenectomized and asplenic patients have a high incidence of infections by encapsulated bacteria and do not respond to polysaccharide vaccines (113;114).

2. Aims

Studies in animal models have suggested that genetically determined abnormalities in IC-binding Fcγ– and complement receptors may have a causative role in autoimmune disordersThe pathogenic potential of these receptors in human autoimmune disorders is still controversial. Since B cells play complex and important roles in SLE, alterations in the expression of IC-binding receptors expressed on the surface of their various subsets may contribute to the dysregulation of several B cell functions. An appropriate cross-talk between these receptors is necessary to control immune responses by B cells.

Our aims were

1. To describe the expression pattern of FcγRII (CD32), complement receptors type 1(CD35) and 2 (CD21) as well as membrane Ig on naïve, IgM^+ and IgG^+ memory B cells of healthy individuals.

2. To compare the expression pattern of these IC-binding receptors on distinct B cell subpopulations in SLE patients and healthy individuals.

3. To reveal the effect of CR1 clustering on CpG and- BCR –induced proliferation of naïve, IgM^+ and IgG^+ memory B cells from healthy donors.

4. To describe how ligand-induced clustering of CR1 modulates CpG and BCR-induced antibody-producing plasmablast differentiation of IgM^+ and IgG^+ memory B cells.

3. Materials and methods

3.1. Materials

3.1.1. Buffers,media

RPMI medium 1640+10%FCS

RPMI powder	Dissolved in 1000ml distilled water
$NaHCO_3$	2 mg/ml
Na-piruvate	0.22 mg/ml
L-glutamine	2mM
Streptomcyin	0.1 mg/ml
Penicillin	100 U/ml

GKN

NaCl	8 g/l
KCl	0.4 g/l
NaH_2PO_4	1.77 g/l
Na_2HPO_4	0.77 g/l
D-glucose	2 g/l
Phenol-red	10 mg/l

TMB buffer (pH5.5) for ELISA

Na-acetate	0,1 M

PBS buffer (pH 7.4)

NaCl	8 g/l
KCl	0,2 g/l
$Na_2HPO_4*H_2O$	1,4 g/l
KH_2PO_4	0,2 g/l

FACS buffer

PBS	
FCS	1%
Na azide	0.1%

MACS buffer

PBS	
EDTA	2mM
BSA	0,5%

Sample buffer (2X) for SDS-PAGE

Tris/HCI (pH 6.8)	125 mM
SDS (10%)	2%
Glycerol	20%
BromphenolBlue (4%)	0,001%
2-ME	2%

Running buffer for SDS-PAGE (pH 8.3)

Tris	3g/l
SDS (10%)	10ml/l
Glycin	14.4 g/l
Distilled water	Up to 1000ml

Transfer buffer for Western blot

Tris	3g/l
Glycin	14.4g/l
Distilled water	Up to 1000ml

AEC solution for ELISPOT

AEC(-amino-9-ethyl carbazole)	100mg
N,N-dimethyl formamide	Up to 10ml

AEC buffer for ELISPOT (pH 5.0)

Acetic acid (0,2M)	148ml
Sodium acetate (0,2M)	352ml
Distilled water	Up to 1L

3.1.2. Antibodies

Name	Host	Origin
a-hCD27-PE	mouse	Caltag
a-hCD32-FITC	mouse	BD Pharmingen
a-hCD35-PE	mouse	BD Pharmingen
a-hCD35-FITC	mouse	Southern Biotech
a-hCD21-FITC	mouse	Dako
a-hIgG-AlexaCy5	goat	Jackson ImmunoResearch
a-hIgM-AlexaCy5	goat	Jackson ImmunoResearch
a-hCD19-biotin	mouse	BD Pharmingen
a-hIgG-HRPO	rabbit	Dako
a-hIgM-HRPO	rabbit	Dako
a-hIgG $F(ab')_2$	goat	Jackson ImmunoResearch
a-hIgM $F(ab')_2$	goat	Jackson ImmunoResearch
StreptavidinPerCpCy5.5		BD Pharmingen

3.2. Methods

3.2.1. Patients and controls

Twenty-three patients with SLE (20 females, age 39 ± 5 years; 3 males age 44 ± 10 years) were involved in the present study. As controls, 30 age- and sex-matched subjects with no clinical or laboratory signs of an inflammatory rheumatic disease were included. All patients satisfied the criteria for a definitive diagnosis of SLE (115). Of the 23 SLE patients, 15 were receiving corticosteroids (equivalent to 5–25 prednisone mg/day) and 8 were treated with immunosuppressive drugs, including hydroxychloroquine (n=2, 200 mg/day) and azathioprine (n=5, 50 mg/day) or methotrexate (n=3, 12.5-20 mg/wk). Disease activity was assessed by the SLE disease activity index (SLEDAI) score (115;116). 15 patients had a SLEDAI ≤10 and were considered relatively inactive. The remaining 8 patients with SLEDAI >10 were considered active. 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 each participant.

3.2.2. Flow cytometry

Immunofluorescence measurements were performed using a FACSCalibur flow cytometer and the CellQuest software (BD Biosciences, Mountain View, CA). Peripheral blood mononuclear cells (PBMCs) from individual patients and controls were isolated from heparinized blood by Ficoll Hypaque (Amersham Biosience) density gradient centrifugation and washed twice in PBS. On the basis of forward (FSC) and side scattering (SSC) properties lymphocytes were differentially counted. A total of $3*10^5$ PBMC were washed in PBS, and were stained using the appropriate antibodies according to the manufacture's instruction. $CD19^+$ B cells were generally stained with FITC, PE and AlexaCy5 conjugated monoclonal antibodies to CR1, CR2, sIgG/IgM, FcγRII and CD27. After incubation on ice for 30 min., cells were washed and were resuspended in 200µl PBS containing 1% FCS and 0.15 NaN_3. Data of 100,000 cells were collected. Relative fluorescence intensity (RFI) was obtained by dividing the mean fluorescence intensity (MFI) of the sample with isotype-matched control.

3.2.3. B-cell preparation

3.2.3.1. B cell preparation from blood

Heparinized blood was mixed with an equal volume of PBS at room temperature and slowly layered over the Ficoll-Hypaque solution. After centrifugation at 2000 rpm for 30 min at 18 °C with no brake, mononuclear cells were isolated and washed three times with PBS. Cells were finally resuspended in complete RPMI 1640 medium. Monocytes were depleted by plastic adherence, while T cells were depleted by rosette formation using 2-aminoethylisothiouronium bromide (AET) treated sheep red blood cells (SRBC) followed by separation of rosette-forming cells on Ficoll-Hypaque gradient. The purity of the $CD19^+$ B cells was usually over 80% (< 2% $CD14^+$, < 8% $CD3^+$and < 10% $CD56^+$) as measured by flow cytometry.

B cells were alternatively isolated from PBMCs by negative selection using magnetic separation (Miltenyi Biotec Inc.). Fc-receptor-blocking Ab and non-B-cell hapten antibody coctail was added to freshly isolated mononuclear cells followed by an anti-hapten Ab coupled to magnetic microbeads. Cells were then passed through a magnetic column and B cells were collected in the flowthrough fraction. Over 95% of the cells obtained were $CD19^+$ with no detectable $CD3^+$ and $CD14^+$ cells in the suspension.

3.2.3.3. Isolation of $CD27^+$ memory B cells

$CD27^+$ memory B cells and $CD27^-$ naïve cells were separated by magnetic cell sorting. Isolated B cells were passed through 30μm nylon mesh to remove cell clumps. $5x10^7$ cells were mixed with CD27 microbeads and incubated for 30 minutes at 4 °C. Cells were washed three times by adding 1-2 ml PBS containing 0,5% BSA. The cell suspension was loaded onto a column, which was placed in the magnetic field of a MACS Separator. The magnetically labeled $CD27^+$ memory B cells were retained on the column, while the unlabelled naïve B cells run through. After removal of the column from the magnetic field, the retained memory cells were eluted. The purity of the cells obtained after the procedure was assessed by flow cytometry.

3.2.4. PCR

3.2.4.1. RNA extraction

Total RNA was extracted from magnetic separated B cells using TRI reagent (Sigma) according to manufacturer's instructions (Chomczynski and Sacchi, 1987). 400µl of TRI reagent (Sigma) was added to $5x10^6$ cells. The cell extract was pipetted up and down 20 times until homogeneity. Samples were allowed to stand for 7 minutes at room temperature for complete dissociation of nucleoprotein complexes. 80µl of chloroform was added to the samples, vortexed for 10 sec. and allowed to stand for 10 min. at room temperature. The resulting mixture was centrifuged at 12,000g for 15 min. at 4°C. At this step the mixture was separated into 3 phases: the protein containing red organic phase in the bottom, an interphase pellet containing DNA in the middle, and a colourless aqueous phase containing RNA at the top of the tube. 200µl of isopropanol was added to the separated aqueous phase. Samples were mixed well and allowed to stand for 10 min. at room temperature. RNA was pelleted by centrifugation at 12,000g for 10 min. at 4°C and washed with 80% ethanol (~1.5 ml). After vortexing, the samples were centrifuged at 7,500g for 5 min. at 4°C. Ethanol was poured out and the RNA pellet was resuspended in 10µl RNase free H_2O. RNA concentration and purity of the preparation was determined by measuring absorbance at 260 and 280 nm. The optimal ratio was greater than 1.7.

3.2.4.2. RT-PCR

Reverse transcription was performed according to the method of Gerard and Alessio (1993). The reaction was carried out with 1µg of total RNA, 0.5µg oligo dT, 2µl of 10 mM dNTPs (1mM final concentration), 20 U RNase Inhibitor, 4µl 5X reaction buffer containing 250mM Tris-HCl, 250mM KCl, 20mM $MgCl_2$, 50mM ditiothreitol, 200U Reverse Aid^{TM}M-MuLV reverse transcriptase (Fermentas). The final volume was made 20 µl and incubated at 42^{o}C for 60 min. The reaction was stopped by heating to 70^{o}C for 10 minutes.

3.2.4.3. PCR

Polymerase chain reaction was carried out with 0.2 µl cDNA, 1µl 10X reaction buffer (250mM Tris-HCl, 250mM KCL), dNTP and $MgCl_2$ with 0.2mM and 1.5mM final concentration, respectively, 5 pmol of each oligonucleotide and 1U Taq DNA Polymerase in a 10µl final reaction volume. Reactions were performed in a Perkin Elmer Thermal Cycler. The amplification protocol comprised 30 cycles of 1 min at 94°C (denaturation), 30 s at 50°C (annealing) and 30 s at 72°C (extension). The first cycle was preceded by an extra 5 min at 94°C and the final extension step was continued for 10 min.
Primers pairs used for human β-actin, human FcγRIIa and FcγRIIb1/b2 were as follows:

Sense β-actin : 5'-GGCTACAGCTTCACCACCAC-3'
Antisense β-actin : 5'-GCGCTCAGGAGGAGCAATG-3'
Sense FcγRIIa: 5'-AGTGGCCTTGATCTACTGCA-3'
Antisense FcγRIIa: 5'-GAGTCATGTAGCCGCCGTCA-3'
Sense FcγRIIb1/2: 5'-AGTGGCCTTGATCTACTGCA-3'
Antisense FcγRIIb 1/2: 5'-ATGTGGAACGGAAGAGCCTT-3'
The expected size of PCR products are 400 bp for β-actin, 157bp for FcγRIIa and 457/400 bp for FcγRIIb1/b2. PCR products were visualized with ethidium bromide in agarose gel electrophoresis.

3.2.5. Level of circulating immunecomplexes (CIC)

To measure IC present in plasma we used the CIC-C1q Enzyme Immunoassay, which is based on the principle that complement-fixing CIC will bind to immobilized human C1q protein. Plasma samples (diluted 1:50) and heat aggregated human gamma globulin, as standard were added to the C1q-coated microtiter wells and incubated for 60 minutes at room temperature. After washing (6x) with PBS containing 0.05% Tween-20^{TM}, HRP-conjugated goat anti-human IgG was added to each test well and incubated for 30 minutes at 25^{o}C. After washing, 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was used as a chromogen. OD values were measured at 405 nm.

3.2.6. Detection of antibody production by ELISA

Supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) for IgM and IgG production. Duplicate wells of ninety-six–well plates (Costar) were coated with 3 µg/ml capture antibody reacting with the human immunoglobulin of interest, i.e.: polyclonal $F(ab')_2$ goat anti–human IgG and polyclonal $F(ab)_2$ goat anti–human IgM at 4°C overnight. Plates were washed 3 times with PBS containing 0.1% Tween-20 and blocked with PBS containing 2% BSA. 50µl of B cell culture supernatants and - as negative control – medium RPMI alone were added to the wells and incubated for 1 hour at 37°C. Plates were again washed with PBS-Tween and HRP-conjugated detection antibody specific for human IgM and IgG (Dako) was added (1:2000 dilution) and incubated again for 1 hour at room temperature. Plates were again washed 6 times in PBS-Tween buffer, and developed with TMB substrate. Optical densities were read at 450nm.

3.2.7. Enzyme-linked immunospot (ELISPOT) for detecting antibody secreting cells

Ninety-six-well PVDF membrane plates (Millipore) were coated with 50µl 3 µg/ml human IgM and IgG specific antibodies at 4°C overnight. Plates were washed 3x with PBS, blocked with 200µl medium RPMI containing 10% FCS and incubated for two hours at room temperature. After washes, cells were added to the wells in triplicate at $3x10^5$ cells per well in RPMI medium containing 10%FCS for 20 hours at 37°C in 5% CO_2. Than cells were washed and the second HRPO-conjugated anti-IgM and anti-IgG antibodies were added to the plate for 90 minutes at 37°C. After removing the cells, the plates were washed 3x with PBS-Tween (0,5%). AEC (3-amino-9-ethyl carbazole) as chromogen substrate was added to the wells. Colour development was stopped by rinsing the plate with distilled water. After drying at room temperature, spots were counted using an ImmunoScan (CTL) reader.

3.2.8. Proliferation assay

Isolated B lymphocytes were cultured at 3×10^5 cells/well in 200 µl medium RPMI containing 10% FCS (Gibco) in flat-bottom, 96-well microtiter plates (Costar), at 37°C in a humidified atmosphere containing 5% CO_2. Cells were stimulated with 3 µg/ml $F(ab')_2$ of goat anti-human IgM (Fc5µ) or anti-human IgG (Jackson) and cultured in the presence of various amounts of heat-aggregated C3 for 72h. Cells were pulsed with 1 µCi/well [^{3}H]thymidine

(NEN, Boston, MA) for the last 16 h of culture. Incorporated radioactivity was measured using a Wallac 1409 liquid scintillation beta counter (Wallac, Allerod, Denmark). The results are expressed as mean cpm ± SEM of triplicate samples.

3.2.9. Isolation of human C3, generation of C3b-like C3

Human C3 was isolated from freshly drawn serum by fast protein liquid chromatography (FPLC) as described by Basta and Hammer. Purified C3 was concentrated, dialysed against PBS, followed by incubation with protein G beads (Pharmacia Biotech) to minimize the amount of contaminating IgG. The remaining IgG content was <1%, as assessed by ELISA. The purity of the C3 preparation was assessed by SDS-PAGE and Coomassie bluestaining. Aliquots were stored at -20°C and aggregated at 63°C for 20 min before use. Any possible toxic effect of various C3 preparations had been excluded by assessing the viability of treated cells, using trypan blue staining for microscopical and propidium iodide staining in FACS analysis.

3.2.10. Plasmablast differentiation from memory B cells in vitro

3×10^5 purified naive or memory B cells were cultured for 6 days in 200µl RPMI medium complemented with 10% FCS. Cells were activated with anti-IgM, anti-IgG antibodies (at 8µg/ml final concentration) and CpG (at 1µg/ml) in the presence of IL-2 (50ng/ml) and IL-10 (50ng/ml) (Immunotools), respectively. The frequency of plasmablasts was measured by flow cytometry.

3.2.11. Statistical analysis

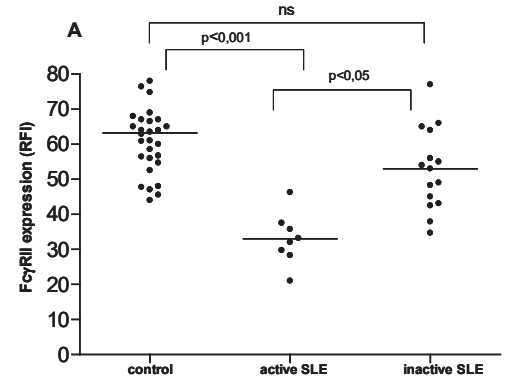
Data are expressed as mean ± SEM. Statistical significance of the difference between disease group and controls was determined using Mann-Whitney *U* test. Multiple group analyses were performed using one-way ANOVA with Kruskall–Wallis test. Correlation coefficients were calculated using Spearman's rank correlation. Data were analysed by Prism software, version 4.0 (GraphPad Software). Results were considered statistically significant if $P<0.05$.

4. Results

4.1. Flow-cytometric analysis of IC-binding receptors expressed on human B cell of healthy controls and SLE patients

4.1.1. Surface expression of Fcγ- and complement receptors on peripheral B cells of healthy controls and SLE patients

Cells isolated from 15 patients diagnosed with inactive, 8 with active SLE according to the SLEDAI and 30 healthy controls were investigated by flow cytometry. Peripheral B cells were identified by gating forward and side scatter profiles and CD19-positivity. Relative fluorescence intensity (RFI) of the samples was calculated as described in the section of Materials and Methods. As shown in Figure 5, despite individual variations there was a significant decrease in the expression of IC-binding receptors on B cells of SLE patients compared to the normal controls. CR1 expression on B cells from SLE patients with active disease (SLEDAI>10) was significantly lower (median 16,4; 25th to 75th percentile, 14,8 to 21,5) compared to healthy controls (median 37; 25th to 75th percentile, 32,5 to 43; P<0,001) or patients with inactive SLE (median 32; 25th to 75th percentile, 25,8 to 36; P<0,05). Reduction in FcγRII expression was also more pronounced in patients with active SLE (median 32,6; 25th to 75th percentile, 29 to 36,7) compared with age-matched controls (median 63,5; 25th to 75th percentile, 55,3 to 67,5; P<0,001) or patients with inactive SLE (median 53; 25th to 75th percentile, 43,1 to 64; P<0,05). CR2 expression was also reduced in patients with SLE (median 5,7; 25th to 75th percentile, 3,7 to 6,5) compared to controls (median 6,5; 25th to 75th percentile, 5,8 to 7,8; P<0,01), however, there was no statistically significant difference between patients with active or inactive disease (data not shown). Patients with SLE segregated into groups expressing CR2 at lower and higher level, irrespective of the disease activity. We next analyzed the possible correlation of changes with receptor expression, and found positive correlation between the expression of FcγRII and CR1 ($p<0.05$).


A
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p<0,001
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FcγRII expression (RFI)
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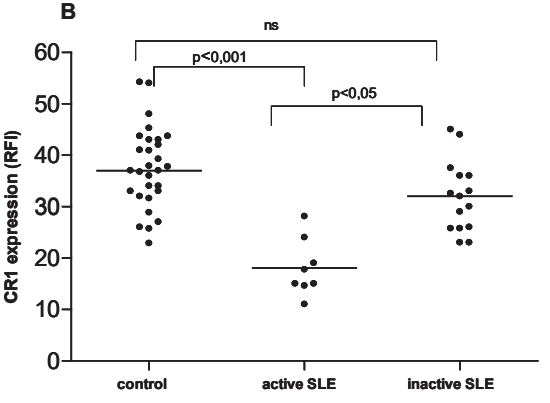
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CR1 expression (RFI)
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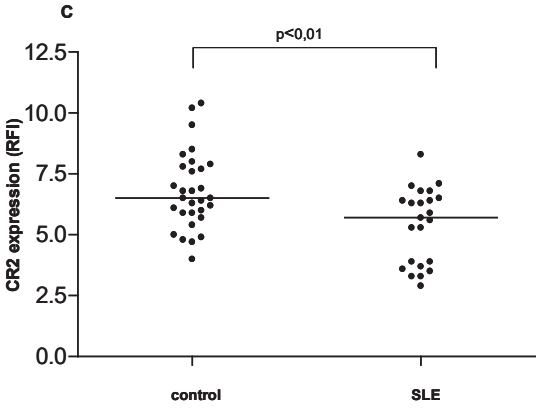

Figure 5. Surface expression of Fcγ- and complement receptors on peripheral B cells of SLE patients and healthy controls

Blood mononuclear cells (BMC) isolated from individual patients and healthy volunteers were stained with specific antibodies against CR1, CR2 and FcγRII. B cells were analyzed selectively by gating forward and side scatter profiles and CD19-positivity. Relative fluorescence intensity (RFI) was obtained by dividing the mean fluorescence intensity (MFI) of the sample with that of the isotype–matched control. RFIs are given for FcγRII (A) CR1 (B) and CR2(C) expression. Differences between donor groups were compared using the nonparametric Kruskall–Wallis test. Horizontal lines show the median values for each group.

4.1.2. Patients with active SLE display disturbed ratio of various B cell subpopulations

Lymphocyte counts are known to be significantly decreased in SLE, and lymphopenia is the most prevalent initial laboratory abnormality in this disease. The first report of disturbed peripheral B lymphocyte homeostasis in SLE demonstrated that the frequencies of $CD27^+$ B cells were significantly enhanced, mainly in patients with active SLE. As shown in Figure 6, we found that patients with SLE have a disturbed ratio of $CD27^-$ naïve and $CD27^+$ memory B cells (53% - 47%) compared to healthy individuals (75% - 25%). Circulating memory B cells express either IgM or secondary antibody isotypes and are distinguished from naive B cells by the appearance of cell surface CD27. As Figure 6 illustrates we observed a striking reduction in the ratio of IgM memory compartment, and in the same time a large expansion of $CD27^{high}$ plasmablast/early plasma cells in patients with active SLE shown (Figure 7).

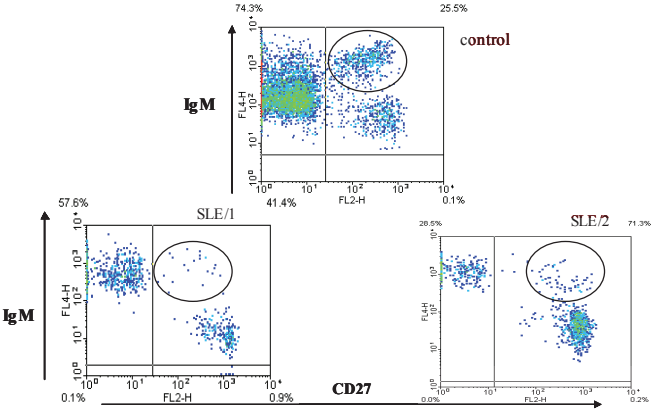

Figure 6. The ratio of $IgM^+/CD27^+$ unswitched memory B cells are greatly reduced in active SLE

Blood mononuclear cells (BMC) isolated from individual patients and controls were stained with specific antibody and analyzed. Density plot of gated $CD19^+$ B cells is shown, double-stained with the following antibodies: PE-labelled anti-CD27, Cy5-labelled anti-IgM $F(ab')_2$ as indicated. Circles emphasize major differences in the ratio of $CD27^+/sIgM^+$ memory B cells. Data shown are representative of six independent experiments.

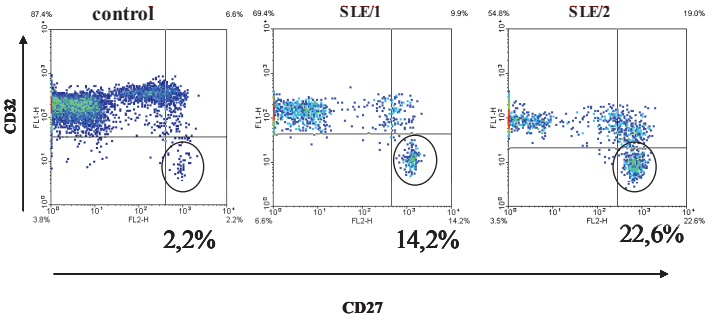

Figure 7. Increased frequency of the CD27^{high}/sIg^{low} plasmablasts population in active SLE

Density plot of gated CD19^{+} B cells is shown, triple-stained with the following antibodies: PE-labelled anti-CD27, Cy5-labelled anti-IgM F(ab')$_2$ and FITC-labelled anti-CD32 as indicated. Ig-isotype matched FITC- and PE-conjugated mouse antibodies were used as negative controls for non-specific staining. Circles emphasize major differences in the ratio of CD27^{high}/sIg^{low} plasmablasts. Data shown are representative of six independent experiments.

4.1.3. IgM^{+}/CD27^{+} and IgM^{-}/CD27^{+} memory cells from SLE patients fail to up-regulate the inhibitory FcγRII

Investigating FcγRII expression on CD19^{+} B cells isolated from patients with SLE a striking decrease was found compared to the controls. This reduction was more pronounced in the group of patients with active disease. Since it has been shown that FcγRII expression on B cells is changing during maturation, in further studies we aimed to clarify which B cell subpopulations are affected. CD19^{+} peripheral B cells were divided into four groups based on their expression of CD27: IgM^{+}/CD27^{-} naïve, IgM^{+}/CD27^{+} and IgM^{-}/CD27^{+} memory cells and CD27^{high} plasmablasts. While non-autoimmune individuals displayed an increased level of FcγRII expression on memory cells compared to naïve cells, memory B cells from SLE failed to up-regulate this receptor (Figure 8.). Comparing IgM^{+} and switched memory cells from healthy individuals, the former ones showed higher FcγRII expression (Figure 8/A.). The reduced FcγRII expression found in SLE affected both IgM^{+} and switched memory cells, to

somewhat higher extent the latter (Figure 8/C). Interestingly, the pattern of FcγRII expression on IgM^+ memory cells was different from that on switched memory cells from healthy donors; namely, the expression range was much wider in the latter case. In SLE both IgM^- and IgM^+ cells displayed a wide FcγRII expression range.

As seen in Figure 8/C the expression level of FcγRII was almost the same on the $CD27^-$ naive B cell population in both SLE patients and healthy individuals. In contrast to this, $CD27^+$ memory B cells from the autoimmune patients had significantly lower surface levels of FcγRII than the healthy controls (Figure 8/C), most probably due to a failure to up-regulate FcγRII expression as B cells become memory cells.

Although the FcγRII monoclonal antibody (clone FL18.26) we used can not distinguish activating FcγRIIa and inhibitory FcγRIIb isoforms, we found that the mRNA level of FcγRIIa in peripheral B cells was below the limit of detection (see detailes in 4.1.8.), hence the inhibitory IIb isoform dominates in these samples.

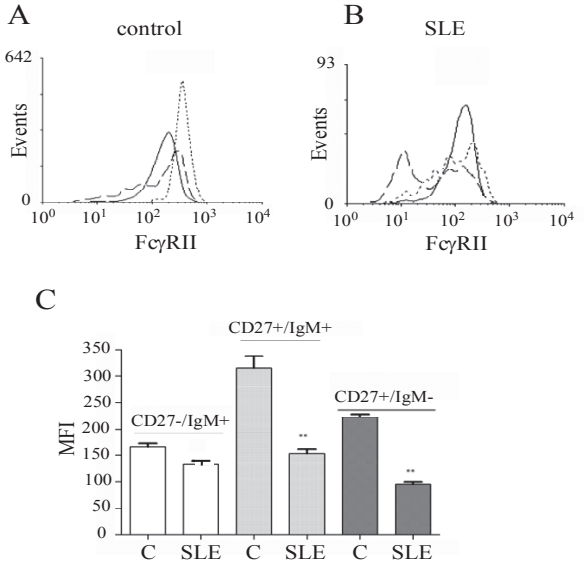

Figure 8. FcγRII expression on naïve and memory B cells of healthy individuals and SLE patients

Flow cytometric analysis of gated $CD19^+$ B cells, triple-stained with the following antibodies: FITC-labelled anti-CD32, PE-labelled anti-CD27 and Cy5-labelled anti-IgM. Ig-isotype matched FITC- and PE conjugated mouse antibodies were used as negative controls for non-specific staining.

(A) The expression of FcγRII on IgM^+ (dotted line) and IgM^- (dashed line) $CD27^+$ memory B cells of healthy controls compared to naïve cells (solid line). Histogram of one representative experiment is shown. (B) The expression of FcγRII on IgM^+ (dotted line) and IgM^- (dashed line) memory B cells of SLE patients compared to naïve cells (solid line). Histogram of one representative experiment is shown. (C) Average MFI-values of 8 samples treated as described above. Open columns: $CD27^-$ IgM^+ cells, grey columns: $CD27^+$ IgM^+ cells, black comuns $CD27^+$ IgM^- cells of healthy control individuals and SLE patients, as indicated. Data are expressed as mean ± SE. (Mann –Whitney U test $P < 0.01$)

4.1.4. Distinct pattern of CR1 and CR2 expression on $CD27^-$ naive and $CD27^+$ memory B cells of healthy donors and SLE patients

The reduced level of CR1 on erythrocytes is considered a key feature contributing to IC overload and exaggerated complement activation in SLE. Comparatively fewer studies documented lower levels of CR1 on leukocytes in this disease. The decline in erythrocyte CR1 is considered an acquired phenomenon caused by the proteolytic cleavage of CR1 from the membrane of erythrocytes. The mechanism underlying reduced CR1 expression on nucleated cells is not yet known and is under constant investigation.

In studies using lupus prone MRL/lpr and female (NZB X NZW) F1mice it had been shown that the level of complement receptors CR1/2 is strongly reduced on B lymphocytes. In the former model, CRl/CR2 levels were found to be decreased from an early time point, before nephritis and elevated levels of autoantibodies appear, indicating the relevance of these receptors in autoimmunity.

In contrast to mice in humans, where CR1 and CR2 are encoded by distinct genes and thus have different functions, the expression level of these receptors on distinct B cell subpopulations has not been defined so far, not even in healthy individuals.

We found that the level of CR1 was higher on $CD27^+$ memory B cells compared to $CD27^-$ naïve cells in healthy donors (Figure 9/A) - similarly to the expression of FcγRII. In contrast to FcγRII levels, there was not, however, any difference between IgM^+ and switched memory compartment (Figure 9/A). In contrast to FcγRII and CR1, the expression of CR2 was at the same level both on $CD27^-$ naïve and $CD27^+$ memory cells and no up-regulation could be seen in the control group, reflecting again differences in the regulation and function of human CR1 and CR2 (Figure 10/A).

Investigating CR1 and CR2 expression on $CD19^+$ B cells isolated from patients with SLE, a significant decrease of both receptors was found, however different subpopulations were affected. While a reduced CR1 level could be measured only on $CD27^+$ memory cells (Figure 9/C), a lower CR2 expression was characteristic for both the $CD27^-$ naïve and $CD27^+$ memory cells (Figure 10/C). In the case of CR1-expression both IgM^+ and switched memory cells were affected (Figure 9/C), similarly to our finding with FcγRII.

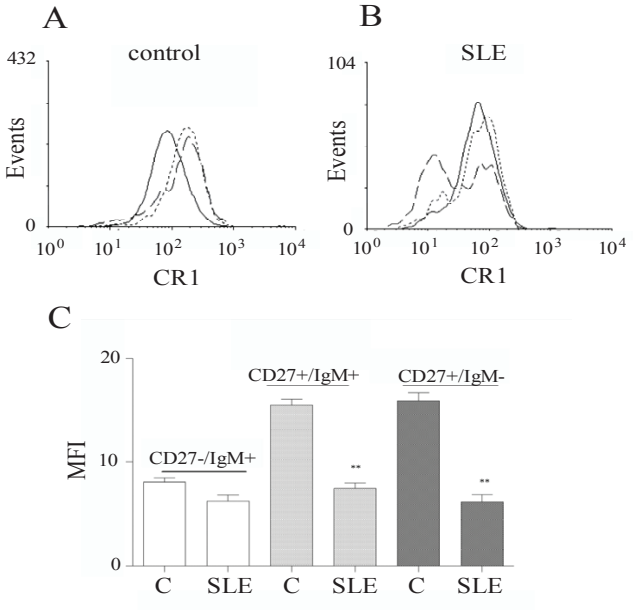

Figure 9. CR1 expression on naïve and memory B cells of healthy individuals and SLE patients

Flow cytometric analysis of gated $CD19^+$ B cells, triple-stained with the following antibodies: FITC-labelled anti-CD35, PE-labelled anti-CD27 and Cy5-labelled anti-IgM. Ig-isotype matched FITC- and PE conjugated mouse antibodies were used as negative controls for non-specific staining.

(A) The expression of CR1 on IgM^+ (dotted line) and IgM^- (dashed line) $CD27^+$ memory B cells of healthy controls compared to naïve cells (solid line). Histogram of one representative experiment is shown. (B) The expression of CR1 on IgM^+ (dotted line) and IgM^- (dashed line) memory B cells of SLE patients compared to naïve cells (solid line). Histogram of one representative experiment is shown. (C) Average MFI-values of 8 samples treated as described above. Open columns: $CD27^-$ IgM^+ cells, grey columns: $CD27^+$ IgM^+ cells, black comuns $CD27^+$ IgM^- cells of healthy control individuals and SLE patients, as indicated. Data are expressed as mean ± SE. (Mann –Whitney U test $P < 0.01$)

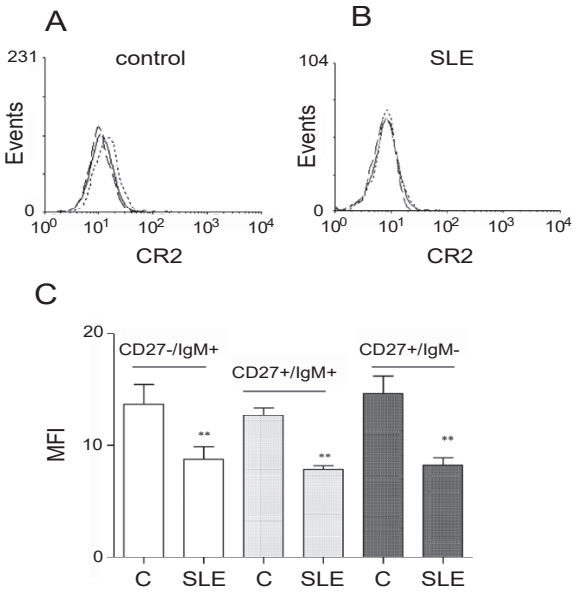

Figure 10. CR2 expression on naïve and memory B cells of healthy individuals and SLE patients

Flow cytometric analysis of gated $CD19^+$ B cells, triple-stained with the following antibodies: FITC-labelled anti-CD21, PE-labelled anti-CD27 and Cy5-labelled anti-IgM. Ig-isotype matched FITC- and PE conjugated mouse antibodies were used as negative controls for non-specific staining.

(A) The expression of CR2 on IgM^+ (dotted line) and IgM^- (dashed line) $CD27^+$ memory B cells of healthy controls compared to naïve cells (solid line). Histogram of one representative experiment is shown. (B) The expression of CR2 on IgM^+ (dotted line) and IgM^- (dashed line) memory B cells of SLE patients compared to naïve cells (solid line). Histogram of one representative experiment is shown. (C) Average MFI-values of 6 samples treated as described above. Open columns: $CD27^-$ IgM^+ cells, grey columns: $CD27^+$ IgM^+ cells, black comuns $CD27^+$ IgM^- cells of healthy control individuals and SLE patients, as indicated. Data are expressed as mean ± SE. (Mann –Whitney U test $P < 0.01$)

4.1.5. $IgM^+/CD27^+$ and $IgG^+/CD27^+$ memory cells of SLE patients express lower sIg levels than B cells of healthy donors

Comparing sIg expression on $CD27^-/IgM^+$ naïve B cells and $CD27^+/IgM^+$ memory cells from healthy individuals, a significantly higher level could be detected on the latter (Table I.). The difference between $CD27^-$ naïve and $CD27^+$ memory B cells was not observed in the SLE group. $CD27^-/IgM^+$ naïve B cells of SLE patients expressed higher sIgM levels than that of the healthy donors. Since naïve and transitional B cells present in the $CD27^-$ compartment were not investigated separately, a higher frequency of transitional B cells expressing higher sIgM-levels in SLE may lie behind this phenomenon. Investigating sIg expression we found a decrease on both $IgM^+/CD27^+$ and $IgG^+/CD27^+$ memory B cells of SLE patients compared to healthy controls (Table I.).

	$CD27^-$	$CD27^+$
sIgM expression (MFI)		
Control (n=8)	338 ± 59 [a b]	1404 ± 139 [a c]
SLE (n=8)	767 ± 118 [b]	746 ±71 [c]
sIgG expression (MFI)		
Control (n=8)	-	1173 ± 99 [d]
SLE (n=8)	-	689 ± 113 [d]

Table I.
Surface Ig expression on $CD27^-$ naïve and $CD27^+$ memory B cells of healthy individuals and SLE patients

MFI: mean fluorescence intensity. Data are presented as mean ± SE.
[a,b,c]: $p < 0.01$; [d]: $p < 0.05$

4.1.6. CD19^{+}/CD27^{high}/sIg^{low} plasmablasts of healthy donors and SLE patients express minimal levels of FcγRII and CR1

The terminally differentiated CD19^{+}/CD27^{high}/sIg^{low} B cell population displays very low levels of FcγRII and CR1. All of the control individuals showed also minimal levels of these receptors on their plasmablasts. A typical FACS profile of FcγRII and CR1 expression on plasmablasts from a healthy donor and an SLE patient is shown in Figure 11.

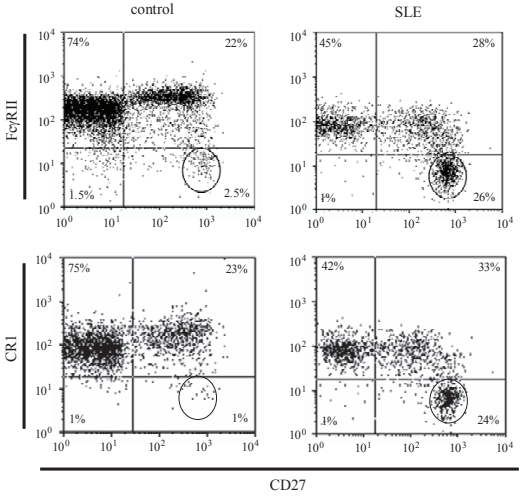

Figure 11. FcγRII and CR1 expression on CD19^{+}/CD27^{high}/sIg^{low} plasmablasts of healthy donors and SLE patients

Dot plot of gated CD19^{+} B cells, triple-stained with the following antibodies: PE-labelled anti-CD27, Cy5-labelled anti-IgM F(ab')$_2$ and FITC-labelled anti-CD32 or FITC-labelled anti-CD35, as indicated. Ig-isotype matched FITC- and PE-conjugated mouse antibodies were used as negative controls for non-specific staining. The upper two dot-plots show data obtained after staining for FcγRII on cells of a healthy, control individual and an SLE patient. The lower two dot-plots show data obtained after staining for CR1 on cells of a healthy, control individual and an SLE patient. Major differences in the ratio of CD27^{high}/sIg^{low} plasmablasts are emphasized by circles. Data shown are representative of 6 independent experiments.

4.1.7. Longitudinal study of the expression of IC-binding receptors on B cells of SLE patients and healthy donors

Genetically determined alterations in receptor expression are expected to be relatively stable over time. To test whether the reduced expression of IC-binding receptors on B cells from SLE patients changes over a two-year period, we repeated flow cytometric measurements 1 and 2 years after the first study in 3 patients with SLEDAI > 10. As shown in figure 12, the inhibitory receptors FcγRII and CR1 showed less stability during the tested period in contrast to the enhancing receptor CR2, which proved to be relatively stable.

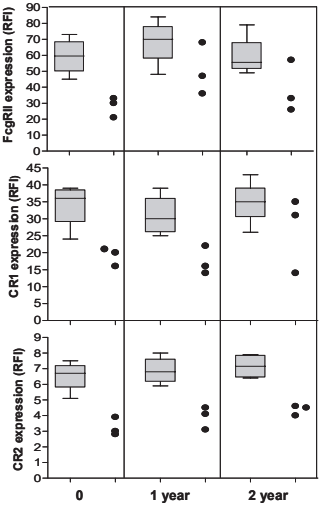

Figure 12. Follow –up analysis of FcγRII, CR1 and CR2 expression on peripheral B cells of three patient with SLE

Blood mononuclear cells (BMC) isolated from three patients with SLE (dots) and four healthy controls (boxes) were monitored repeatedly. Cells were stained with specific antibody against CR1, CR2 and FcγRII. B cells were analyzed selectively by gating forward and side scatter profiles and CD19-positivity. Relative fluorescence intensity (RFI) was obtained by dividing the mean fluorescence intensity (MFI) of the sample with that for the isotype–matched control. The upper and lower limits of the boxes represent the 75th and 25th percentile, respectively. The line inside the boxes indicates the median.

4.1.8. Expression profile of FcγRII in B cells at mRNA level

To confirm the expression profile of FcγRII in B cells from healthy controls and SLE patients, we performed semiquantitative RT-PCR to detect the message for the inhibitory FcγRIIb1/b2 and activating FcγRIIa isoform, using MACS-purified $CD19^+$ B cells. The inhibitory FcγRIIb has two major splice variants, IIb1 and IIb2. An important functional difference is that FcγRIIb2 lacks the 19 aminoacids of the first exon encoding the cytoplasmic domain and is more capable of endocytosis. PCR primers for FcγRIIb were designed to amplify both IIb1 and IIb2 messages with amplicons distinguishable on the basis of size. The specificity of the primers and PCR were confirmed by PCR using FcγRIIb1, FcγRIIb2-containing plasmids as the template (data not shown). In agreement with previous data, $CD19^+$ B cells express more IIb1 message than IIb2 (13/A) and in contrast to myeloid cells, do not express detectable IIa isoform (13/C). In the B cells of SLE patients we could only detect the IIb1 isoform (13/B).

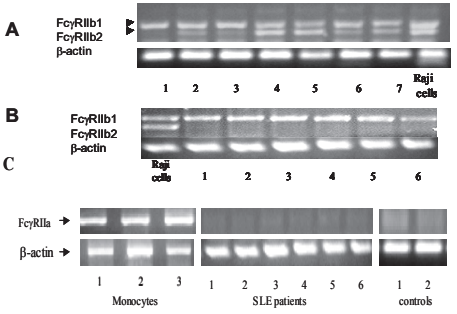

Figure 13. Comparison of FcγRIIb1/2 and FcγRIIa mRNA expression pattern in B cells from healthy controls and patients with SLE

Total RNA from MACS-purified $CD19^+$ peripheral B cells from healthy donors and SLE patients was isolated and used for RT-PCR. Amplification of β-actin served to check RNA amounts loaded on the agarose gel. PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. 7 samples of healthy donors (A) and 6 samples of SLE patients (B) for FcγRII are shown. 2 samples of healthy and 6 samples of SLE patients for FcγRIIa (C) are shown. Raji cells and monocytes were used as positive control.

4.1.9. There is no correlation between the reduced expression of IC-binding receptors and serum IC concentrations in SLE patients

Each SLE patient may present different combinations of pathological manifestations and serum factors. However, IC overload is a universal feature of this disease. Measurement of the concentration of IC provides a more sensitive index of disease activity than either serum C3 or C4 concentrations, though C1q containing IC represents only a limited species of complexes. Since ligand-induced down-regulation of Fcγ- and complement receptors is a feasible scenario that may happen in SLE, we tested whether complement containing immune complexes (CIC), as ligands, may lie behind the reduction in receptor density found in this case. We set out to investigate if there is a correlation between C1q binding circulating IC levels and receptor expression. As expected, we found significantly (P<0.01) higher levels of CIC in the sera of SLE patients as compared to that of healthy controls (Figure14). However, we could not observe consistent correlations between CIC concentration and surface density of any of the IC receptors on B cells (data not shown).

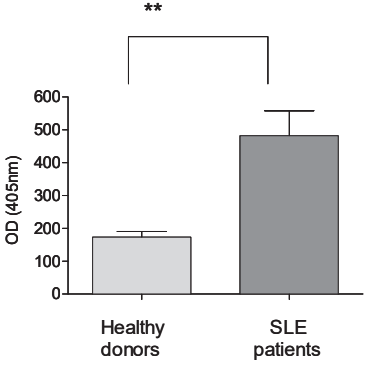

Figure 14. Circulating immune complex levels in healthy donors (30) and SLE (20) patients

4.1.10. Culturing on immobilized C3b prevents CR1 up-regulation on memory B cells

Next, we wanted to reveal whether CR1 clustering has any modulatory effect on CR1 expression. To this end we incubated the B cells on surfaces coated with purified C3b and assessed CR1 expression in different B cell subpopulations 16 hours later. We found, that the immobilized natural ligand of CR1, offered in a cross-linked form, had no effect on CR1 expression in the naïve B cell population, but induced the loss of up-regulation of the receptor on memory B cells. This effect is not associated with a general down-modulation of membrane antigens, as we could not observe any changes in the expression of other receptors such as FcγRII and CD19. As illustrated in Figure 15/C the decrease of CR1 is selectively induced by immobilized C3b, since treating the cells with soluble C3 had no such effect. The fact that CR1 clustering is able to induce receptor down-modulation selectively on memory B cells in a similar manner what we observed in SLE patients, suggests that CR1 expression might be regulated by ligand-induced crosslinkig on these cells.

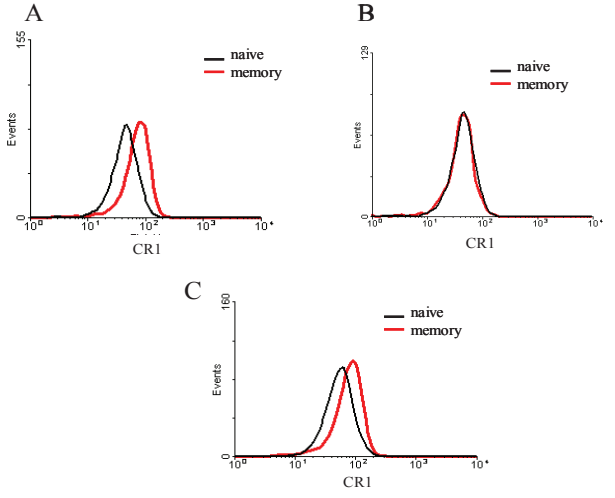

Figure 15. The effect of immobilized and soluble C3b on CR1 expression

Purified mononuclear cells from healthy donors were cultured in the absence (A) or in the presence (B) of immobilized C3b (Calbiochem) overnight. Histogram C shows the effect of soluble C3b on CR1 expression. After incubation, CR1 expression on naïve and memory B

cells was measured by flow cytometry. Analysis of gated $CD19^+$ B cells was performed after staining the cells with the following antibodies: FITC-labelled anti-CD35, PE-labelled anti-CD27. Ig-isotype matched FITC- and PE conjugated mouse antibodies were used as negative controls for non-specific staining. One representative experiment of three is shown.

4.2. Functional consequences of CR1 clustering on antigen-dependent and antigen-independent activation of B cells

4.2.1. Naïve B cells are more sensitive to CR1 induced inhibition of BCR triggered proliferation than memory B cells

It has been earlier shown by our group that ligand-binding to CR1 - in contrast to CR2 - inhibits BCR-induced proliferation of human B cells. In our present experiment purified $CD19^+$ B cells were divided into two groups based on their expression-levels of CD27 as a typical marker for mutated memory B cells. $CD27^-$ naïve B cells were activated with 10 μg/ml of goat anti-human IgM $F(ab')_2$, while $CD27^+$ memory B cells with 10 μg/ml of goat anti-human IgM $F(ab')_2$ and IgG $F(ab')_2$, respectively. As it has been shown that human C3 in aggregated form behaves as C3b-like C3 and reacts preferentially with CR1 on B lymphocytes, first we tested which concentration of aggregated C3 would be optimal in the case of our present culture conditions (Fig. 16, panel A). Based on these results, separated naïve and memory B cells were cultured in the presence of 20 μg/ml aggregated C3 for 72 h. As shown in panel B of Figure 16 the multimeric CR1 ligand exerts an inhibition on the BCR-induced proliferation in the case of both subpopulation, the degree of inhibition, however, was different between the naïve and memory cell-compartment. While the inhibitory capacity of aggregated C3 was quite similar on IgM^+ and IgG^+ memory cells, on naïve cells higher inhibition could be observed.

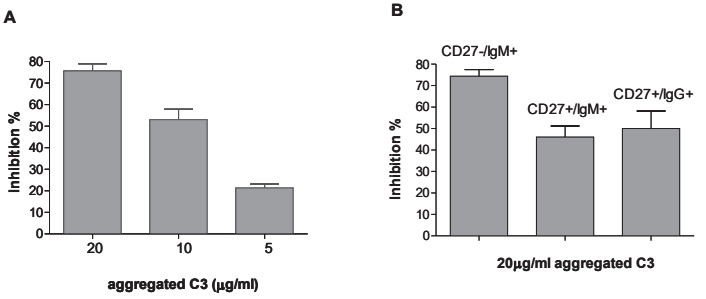

Figure 16. Inhibition of naïve and memory B cell proliferation by aggregated C3

Purified $CD19^+$ B cells were separated into $CD27^-$ and $CD27^+$ cells. **(A)** $CD27^-$ naïve cells were activated with 10 μg/ml goat anti-human IgM $F(ab')_2$ and cultured in the absence or

presence of various amounts of aggregated C3 (ranging between 5-20μg/ml.) **(B)** Naïve and memory B cells were activated with10 μg/ml of goat anti-human IgM $F(ab')_2$ and anti-human IgG $F(ab')_2$ respectively, and cultured in the presence of aggregated C3 at the concentration of 20μg/ml. ^{3}H-thymidin incorporation was measured after 72h. Data are presented as inhibition (%) of activation. Columns represent the mean±SEM of triplicate samples. One representative experiment of three is shown.

4.2.2. Lower levels of CR1 on B cells from SLE patients do not influence their BCR-induced proliferation

As we found that up to 50% of CR1 and FcγRII is lost from the surface of memory B cells of active SLE patients, we investigated whether this reduction results any in change in the inhibitory capacity of CR1 on the BCR-induced B cell proliferation. Because only very low amounts of blood could be obtaiend from SLE patients (25-30 ml), not enough B cells could be isolated to investigate naïve and memory cells separately. This is why we used unseparated B cells to test whether the decreased CR1 density on B cells has any impact on BCR-induced proliferation. B cells were activated with 10 μg/ml of goat anti-human IgM/IgG $F(ab')_2$ and cultured in the presence of various amounts of aggregated C3 for 72 h. As illustrated by Figure 12 the highest concentration of the ligand (20μg/ml) strongly inhibits BCR-induced proliferation. The degree of this inhibition is similar to that found using naïve B cell population of healthy donors (~80%, Fig.17.).

These data suggest that although there is a significant reduction in CR1 density on B cells of SLE patients, this does not affect their inhibitory capacity - the cells respond to the inhibitory signal with the same sensitivity as the naïve cells of healthy donors do.

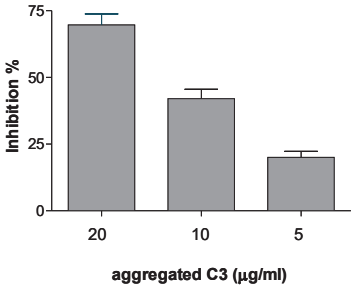

Figure 17. The effect of CR1 clustering on BCR- induced proliferation of lymphocytes from SLE patients

Purified $CD19^+$ peripheral B cells were activated with 10 µg/ml goat anti-human IgM $F(ab')_2$ and cultured in the absence or presence of various amounts of aggregated C3 (ranging between 5-20µg/ml.) ^{3}H-thymidin incorporation was measured after 72h. Data are presented as inhibition (%) of activation. Columns represent the mean±SEM of triplicate samples. One representative experiments of three is shown.

4.2.3. CR1 ligation does not affect the BCR-induced plasmablast formation of memory B cells

Because IL-2 and IL-10 have been reported to induce peripheral memory B cells to differentiate into antibody secreting cells (ASC) in vitro, we were interested in determining the rate of differentiation in distinct subsets of human B cells. Purified B lymphocytes were separated into $CD27^-$ and $CD27^+$ fractions. Following stimulation for 6 days with IL-10 and IL-2, $CD27^-$ naive B cells yielded <1% $CD27^{high}/sIg^{low}$ ASC, irrespective of whether anti-IgM $F(ab')_2$ was present or not. In contrast, we could detect approximately 6% of $CD27^{high}/sIg^{low}$ cells in cultures of memory B cells stimulated with IL-2/IL-10 alone. When anti-IgM $F(ab')_2$ and anti-IgG $F(ab')_2$ antibodies were also present in the culture, the frequency of $CD27^{high}/sIg^{low}$ plasmablasts increased up to 15-18%. We next questioned whether adding aggregated C3 to the culture could influence plasmablast formation from memory B cells induced by BCR/IL-2/IL-10 stimulus. As illustrated in Figure 18, we could not detect any

effect of CR1 clustering on differentiation. In vitro activated naïve and memory B cells were harvested after 6 days of culture and then were incubated with PE-anti-CD27 and FITC-anti-IgM/IgG antibodies for cytometric analysis. The generation of functional ASC was assessed using ELISPOT by determining the frequency of cells secreting IgM, and IgG (data not shown).

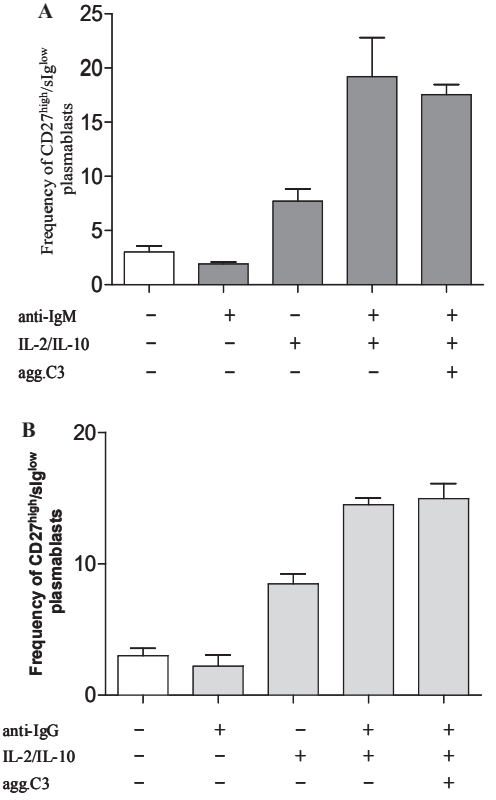

Figure 18. Generation of sIg^{low}/CD27^{high} plasmablasts from memory B cells upon BCR stimulus in healthy donors

Separated memory B cells were cultured in medium alone; anti-IgM (10μg/ml) (**A**) or anti-IgG (**B**); IL-10 (50ng/ml)/IL-2(50ng/ml) and anti-IgM or IgG (10μg/ml) /IL-10 (50ng/ml)/IL-2(50ng/ml) in the presence or absence of aggregated C3 (20μg/ml) at a final cell

density of 3×10^5per well for 6 days. The percentage of $CD27^{high}/sIg^{low}$ plasmablasts was measured by flow cytometry. Analysis of gated $CD19^+$ B cells was performed after staining the cells with the following antibodies: FITC-labelled anti-IgG/IgM, PE-labelled anti-CD27. Ig-isotype matched FITC- and PE conjugated mouse antibodies were used as negative controls for non-specific staining. One representative experiment of three is shown.

4.2.4. CR1 aggregation has no effect on CpG induced proliferation of $CD27^-$ naïve and $CD27^+$ memory B cells from healthy donors

Naïve and memory B cells were isolated from blood by sorting for expression of CD27. First we tested the capacity of isolated subpopulations to proliferate in response to CpG. Naïve and memory B cells were activated with 1 μg/ml of CpG oligodeoxynucleotides and cultured in the presence of various amounts of aggregated C3 (ranging between 5-20μg/ml) for 72 h. As illustrated in Figure 19 CpG-induced proliferation in both the naïve and memory B cell population. Cluestering CR1, by aggregated C3 did not influence the CpG-induced proliferation even at the highest concentration of 20μg/ml.

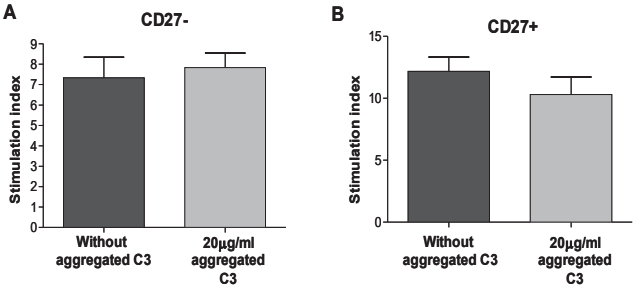

Figure 19. CR1 clustering has no effect on CpG induced proliferation of $CD27^-$ naïve and $CD27^+$ memory B cells from healthy donors

Purified $CD19^+$ peripheral B cells were separated into $CD27^-$ and $CD27^+$ cells. $CD27^-$naïve **(A)** and $CD27^-$ memory cells **(B)** were activated with 1 μg/ml CpG oligodeoxynucleotides and cultured in the absence or presence of various amounts of aggregated C3 (ranging between 5-20μg/ml). ^{3}H-thymidin incorporation was measured after 72h. Data are presented

as stimulation index. Columns represent the mean±SEM of triplicate samples. Onrepresentative experiments of three is shown.

4.2.5. CR1 clustering has no influence on CpG induced plasma-cell differentiation of memory B cells in vitro

Antigen-independent activation of B cells is thought to be one of the mechanisms responsible for antibody-mediated autoimmunity, as seen in SLE and other autoimmune diseases. Some investigators have hypothesized that an absolute requirement to activate naive B cells via BCR would allow proper self/non–self-discrimination at this developmental stage and prevent the emergence of autoreactive plasma cells via polyclonal antigen-independent B-cell activation. In contrast, TLR-9 activation of $CD27^{+}$ memory B cells by unmethylated CpG DNA may be a homeostatic mechanism that maintains adaptive long-term B-cell memory in the absence of antigen. In agreement with others we observed only a small extent of plasmablast induction in the $CD27^{-}$ naïve population upon CpG stimulus. However, predominant differentiation into plasma cells occurred in the $CD27^{+}$ B fraction, as illustrated in Figure 20 The relative frequency of $CD27^{high}/sIg^{low}$ plasmablasts increased up to 30% after CpG stimulus. Plasmablasts were defined by the $CD27^{high}/sIg^{low}$ phenotype.

Next we investigated whether aggregated C3, applied for CR1 clustering on the cell surface, influences the plasmablast differentiation from memory cells. As shown in Figure 20 the presence of the CR1 ligand had no effect on the differentiation. It has been reported that IL-10 rapidly up-regulates TLR-9 in memory B cells. In order to test whether IL-10 has a synergistic effect on CpG-induced plasmablast formation, we added IL-10 to the culture. Under these conditions, we found a slight increase in the frequency of plasmablasts compared to the culture when we used CpG as stimulus alone.

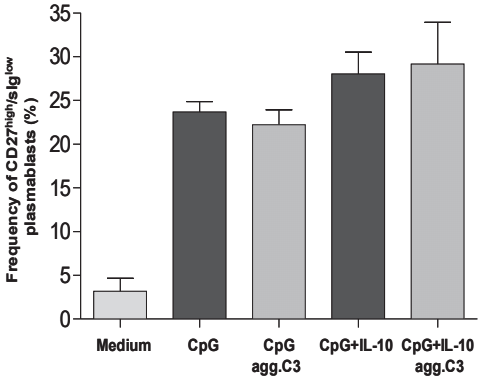

Figure 20. Generation of $sIg^{low}/CD27^{high}$ plasma cells from $CD27^{+}$ memory B cells of healthy donors upon CpG stimulus

Separated $CD27^{+}$ memory B cells were cultured in medium, CpG (1μg/ml), CpG + IL-10 (50ng/ml) in the presence or absence of aggregated C3 (20μg/ml) at a final cell density of 3×10^{5} per well for 6 days. The cells were then stained with anti-IgM/IgG-FITC and anti-CD27-PE. The relative percentages of $CD27^{high}$-positive cells are shown. The error bars represent the mean ± SEM of duplicate samples.

4.2.6. CpG-mediated plasmablast formation of $CD27^{+}$ memory B cells is accompanied by an increased number of IgM but not IgG secreting cells; B cells from SLE patients fail to differentiate into IgM secreting plasmablasts upon induction by CpG

We found that $CD27^{+}$ memory B cells from healthy donors are able to differentiate to $CD27^{high}/sIg^{low}$ plasmablasts upon CpG stimulation, as measured by flow cytometry. We used ELISPOT to assess the generation of functional antibody secreting cells (ASC) by determining the frequency of cells secreting IgM, and IgGs. After removing monocytes by plastic adherence isolated mononuclear cells were incubated for 6 days in the presence of CpG. As shown in Figure 21, CpG-mediated plasmablast formation of $CD27^{+}$ memory B cells was accompanied by an increased number of IgM but not IgG secreting cells, indicative of plasma-cell formation in the IgM memory compartment. Similarly to our flow cytometric

result, aggregated C3 had no impact on the differentiation of functionally active antibody forming cells.

Isolated mononuclear cells from SLE patients were also incubated for 6 days in the presence of CpG and were sorted directly into ELISPOT plates previously coated with anti-human IgM and IgG antibodies. The number of cells secreting IgM and IgG was determined. While in healthy individuals, we could detect IgM producing cells after CpG stimulus, B cells from SLE patients failed to form IgM secreting plasmablasts under the same conditions, as shown in Figure 21. It also has to be mentioned that BCR stimulation could not overcome the proliferative defect (data not shown).

Next, we investigated the effect of CpG on the spontaneous IgG production by B cells of SLE patients. As seen in Figure 21 spontaneous IgG production was not influenced by CpG and most probably not any IgG-producing plasmablasts were generated during culture. Aggregated C3 had any effect neither on spontaneous IgG secretion nor on formation of IgG–producing plasmablats by B cells from SLE patients upon CpG stimulus. As SLE patients have high frequency of autoreactive, ssDNA specific B cells, CpG is able to engage surface IgG and TLR-9 simultaneously. Co-engagement of these receptors however, did not result in enhanced antibody production.

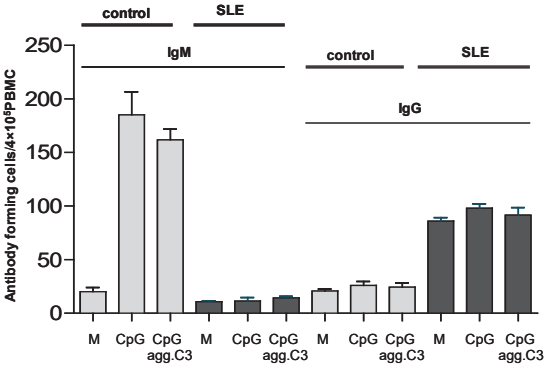

Figure 21. CpG-mediated formation of antibody secreting cells in healthy donors and patients with SLE

Isolated peripheral mononuclear cells from healthy donors and SLE patients with active disease were cultured with CpG ODN for 6 days in the presence or absence of aggregated C3

(20μg/ml). Cells were then transferred into wells previously coated with either polyclonal anti-human IgM or anti-human IgG antibody and cultured overnight. Columns show the total number of IgM and IgG producing B cells/4x10^5 BMC. The error bars represent the mean ± SEM of triplicate samples. One representative experiment of three is shown.

Discussion

It is widely accepted that dysfunctions of Fcγ–, complement- and B-cell antigen receptors contribute to the initiation and/or maintenance of IC-mediated autoimmune disorders (34;39;117-119). Control of cognate interactions and inflammatory responses, IC-clearance, B cell selection and activation may all be affected by altered expression or function of these receptors. Various censoring mechanisms, including anergy and sequestration prevent the participation of mature autoreactive B cells in germinal center reactions, thereby avoiding their expansion into memory and plasma cells. Several studies suggest that IC-binding receptors play a crucial role at checkpoints responsible for avoiding autoimmunity (120-123).

As shown in Figure 22, depending on the composition of IC and the degradation stage of complement components C3 and C4 in the complex, IC-binding receptors may influence the fate of autoreactive B cells in distinct ways. FcγRIIb co-crosslinked with BCR regulates B cell activation negatively. Similarly, human CR1 clustered by multimeric C3b on BCR-activated human B lymphocytes has also been shown to induce negative regulatory signals, a phenomenon demonstrated recently on human T cells, as well (67). On the other hand CR2, which interacts with C3d, the final cleavage product of C3 has been described as a molecular adjuvant, which lowers the threshold of B cell activation by cross-linking CR2 to surface Ig both on human and murine cells (124).

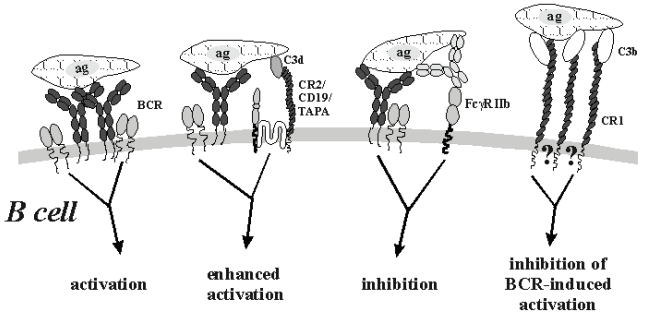

Figure 22. Regulation of B cell activation by IC binding receptors (Erdei et al., 2003)

Failed up-regulation of inhibitory FcγRIIb and CR1 on memory B cells from SLE patients

Human B cells express various FcγRII isoforms at different ratios in resting state and during their activation. Their appearance is highly regulated by various stimulatory signals (125-127). Activation of human B cells results in enhanced FcγRII expression on the cell surface accompanied by the up-regulation of the b1 and the concomitant down-modulation of b2 isoform (128). Expression of FcγRIIB is typically upregulated on activated B cells in mice, too. Autoimmune mouse strains lack this upregulation through polymorphisms in the promoter of *Fcgr2b* (129-131). Restoring upregulation of FcγRIIB on activated B cells in autoimmune strains is sufficient to block IgG anti-DNA accumulation and the development of autoimmune disease in these strains (132). Regarding a possible role of modulating CR1 expression on human B cells similar to this, there is no documented evidence in the literature so far.

We carried out a phenotypic analysis of the expression of IC-binding receptors on B cells taken from healthy individuals and SLE patients. Despite of individual variations there was a significant decrease in the expression of these receptors on B cells of SLE patients compared to the normal controls. CR1 expression on B cells from SLE patients with active disease (SLEDAI>10) was significantly lower compared to healthy controls or patients with inactive SLE. Reduction in FcγRII expression was also more pronounced in patients with active SLE compared with age-matched controls or patients with inactive SLE. We observed a positive correlation between the expression of FcγRII and CR1, indicating that these inhibitory receptors are simultaneously down-modulated. We found that the reduced expression of FcγRII and CR1 on B cells from SLE patients was unstable over a two-year period. This finding indicates that the reduced expression of these receptors associated with down-regulating mechanisms specifically related to the disease process rather than to primary defects predisposing the individual to the disease.

Distribution of these receptors on distinct B cell subpopulations in the blood has not been documented in detail so far, not even in healthy individuals. In our studies SLE patients with reduced number of IC-binding receptors on $CD19^+$ B cells were selected and further examined in detail to reveal whether the previously found reduction affects certain B cell subpopulations. We are convinced that investigation of the naïve, IgM^+ and switched memory B cell compartment might provide further insights into knowledge of the relevance of IC-binding receptors at the late stage of B cell maturation in both physiological and pathological

conditions. We found that the expression of the inhibitory receptors FcγRII and CR1 is significantly up-regulated on the memory B cells of healthy controls; however their expression is considerably decreased on memory B cells of SLE patients, reaching the levels of receptor density found on naive cells.

Immunological memory protects host organisms from recurrent infections. Memory responses are faster, larger, and qualitatively different from primary responses. It is however, unclear what the exact requirements are for maintaining the ability to make such a response (100;133). Many factors can affect the development and survival of memory lymphocytes. It has long been proposed that Ag in the form of IC deposited on the surface of follicular dendritic cells via complement receptors and FcγRs is necessary for the maintenance of memory B cells and secondary responses (42;134;135). In this manner, up-regulation of the inhibitory CR1 and FcγRII on memory cells may have a role in the sensitivity of their response to antigen recall under physiological conditions. The observed loss of this up-regulation in SLE patients may have a strong influence on the memory response and differentiation into antibody secreting plasma cells.

The expression pattern of the enhancing co-receptor CR2 is different from that of the inhibitory receptors

While CR1 expressed on mouse B cells have a similar role as CR2, in humans, where CR1 and CR2 are encoded by different genes, they have been shown recently to exert distinct functions. Regarding the role of CR2 in B cell activation probably its most important function is to promote B cell activation by lowering the signaling threshold of BCR. In our studies, regarding the expression of CR2, the co-receptor for BCR, no difference was found between naive and memory cells of healthy individuals – in contrast to FcγRII and CR1. In SLE however, a reduced expression of CR2 was characteristic for both the naive and memory cells. These findings further strengthen the notion that CR1 and CR2 expression is differentially regulated on human B lymphocytes under physiological conditions as well as in SLE. Taking into consideration that these two complement receptors have opposite effects on human B cells, their differential expression can be considered as an important regulatory component of B cell function.

The loss of up-regulation of FcγRII and CR1 observed in SLE affects both IgM$^+$ and switched memory B cells

To get a further insight into which memory B-cell population is affected in SLE, we examined IgM$^+$ and class-switched memory B cells separately. We found that the reduced expression of Fcγ- and complement receptors as well as sIg observed in SLE affected both IgM$^+$ and switched memory B cells.

In humans, B lymphocytes expressing cell surface IgM and CD27 comprise a significant proportion of the B-cell population in the peripheral circulation (136). These cells have been termed IgM memory B cells because, like conventional class-switched memory cells, they possess somatically hypermutated variable region genes and express CD27 (137;138). Despite the prevalence of IgM-expressing over "classical" class-switched memory B cells, the role of IgM- expressing memory cells in T cell-dependent immune responses remains elusive. It has been documented recently that in healthy individuals the IgM$^+$ memory B cell compartment is depleted of self-reactive and polyreactive antibodies relative to the naive B cell pool (139). Thus, transition from naïve B cells into circulating IgM$^+$ memory B cells is accompanied by efficient counter-selection against self-reactive naïve B cells before the onset of somatic hypermutation. Based on this finding and along with the fact that IgM pathogenic autoantibodies are less characteristic for SLE, we suppose that reduced levels of IC-binding receptors on IgM$^+$ memory cells may contribute to autoimmunity in a way that is different from switched memory cells. Although little is known about the role of IgM$^+$/CD27$^+$ B cells in autoimmunity, disturbances in the memory cell compartment may result not only in higher susceptibility to infection by encapsulated bacteria, but may also have other consequences.

It has been shown, however, that self-reactive antibodies including antinuclear and polyreactive immunglobulins were frequently expressed by IgG$^+$ memory B cells in healthy donors.

Memory B cells can be induced in vitro to differentiate into Ig-secreting plasmablast upon stimulation (140-142). The checkpoints controlling the differentiation of self-reactive memory B cells into antibody secreting plasma cells has yet to be determined, but it is feasible to suppose that abnormal expression of IC-binding receptors on anergic memory B cells may provide a signal sufficient to reactivate them. Under normal conditions these cells remain in anergic state, but in situations where IC concentrations reach pathological levels, failed up-

regulation of the inhibitory FcγRII and CR1 on isotype-switched memory B cells may lead to activation and autoantibody production. Thus the inhibitory receptors FcγRII and CR1 might serve as a final barrier to prevent these B cells from maturing into plasma cells, a function lacking in SLE patient.

In mice, there is accumulating evidence that the inhibitory FcγRII mediates its function during late stages of B cell maturation(143). Absence of this receptor resulted in the expansion of IgG^+ plasma cells secreting autoreactive antibodies, but did not affect early events in the bone marrow such as receptor editing, nor did it prevent the development of IgM^+ autoreactive B cells. In humans, however, there is no documented evidence about the role of FcγRII and CR1 in the process of plasmacell differentiation from memory B cells. Taken together, we can say that although both IgM^+ and switched memory B cells from SLE patients expressed lower levels of FcγRII and CR1 on their surface, the functional consequences of this reduction may be different in the two memory populations.

Possible mechanisms responsible for the loss of up-regulation of IC-bindig receptor expression on memory B cells in SLE

Mechanisms, which are responsible for the reduced expression of IC-binding receptors in SLE may be due to both inherited and acquired elements. Whether the complement containing immune complexes acting as ligands, or antibodies directed against these receptors are responsible for the reduction, is unclear (144). We examined the ligand-induced modulation of CR1 expression after overnight exposure to immobilized C3b in different B cell subpopulations. The natural ligand of CR1 had no effect on CR1 expression in the naïve population, but induced the loss of up-regulation of this receptor on memory B cells. This effect is not associated with a general down-modulation of membrane antigens, and is selectively induced by immobilized C3b which clusters cell surface CR1. The fact that CR1 aggregation is able to induce receptor down-modulation in a similar manner we observed in SLE patients, suggest the ligand-induced regulation of this complement receptor. We found that CR1 down-regulation was not readily reversible, since its appearance could not be observed after an additional 48 h incubation in the absence of C3b. Since immune complex overload is a universal phenomenon in SLE, high levels of complement containing complexes may cause reduction in receptor density. Although we found significantly higher levels of

CIC in the SLE patients, we could not detect any correlation between CIC levels and receptor expression.

We propose that the observed receptor up-regulation on memory B cells disappears once a critical concentration of CIC is exceeded, and further increase in CIC levels does not affect receptor levels.

An alternative way leading to reduced receptor density could be the proteolytic cleavage of extracellular domains of membrane proteins (ectodomain shedding). Several reports have proved that complement receptors CR1, CR2 as well as the inhibitory FcγRIIb from B cells can be released upon activation and might be involved in regulatory processes (145;146). Extracellular proteolysis of receptors irreversibly inactivates receptor-mediated signalling. In the same time soluble fragments are released into the plasma where they act as potential modulators by interacting with their ligands. This provides additional levels of regulation (proteinase and receptor), and a higher order of control of cellular function.

As we could only detect a partial loss of IC-binding receptors from the cell surface, we assume that both FcγRII and CR1 segregate into two types of membrane domains, one that is susceptible to receptor loss and another that is resistant to this event. Thus, their localization determines their accessibility for serine proteases resulting in the shedding of these receptors.

Terminal differentiation toward plasma cells may also account for the reduced receptor densities observed on B cells. Similarly to earlier reports (147;148) we also found a significantly higher percentage of $CD19^{+}/CD27^{high}$ plasmablast/early plasma cells and a disturbed ratio of naïve and memory B cells in SLE patients. These cells are known to proliferate intensively and secrete antibody, thereby being good targets for therapeutic interventions. Investigating IC-binding receptors on these terminally differentiated $CD27^{high}$ plasmablasts we detected minimal levels of FcγRII, CR1 and sIg both in healthy individuals and SLE patients. Minimal levels of inhibitory FcγRII and CR1 on $CD27^{high}/sIg^{low}$ plasmablasts suggest that these receptors are probably not involved in the regulation of B cells at this differentiation stage and have no influence on antibody production.

CR1 clustering and BCR-mediated B cell proliferation in healthy donors and SLE patients

Our laboratory has previously shown that heat-aggregated C3 has an inhibitory effect on BCR induced B cell proliferation. Aggregated C3 behaves like C3b-like C3 in respect to its receptor-binding activity. Because aggregation results in the reduced accessibility of the

C3d regions of C3 molecules, it primarily binds to human CR1 and mimics multimeric C3b. Ag-bound C3d is known to promote B cell activation by lowering the threshold of Ag-specific BCR for activation, however, clustering of CR1 by its natural ligand C3b, inhibits the proliferation of B cells activated via the BCR. Now we have shown that clustering of CR1 by aggregated C3 on the B cell surface inhibits proliferation dose-dependently both in the case of naïve and memory cells, in healthy donors. We have found no difference between the IgM and IgG memory compartment, however, CR1 ligation exerted a stronger inhibition on the proliferation of naïve cells as compared to memory B lymphocytes. Since we measured higher expression levels of CR1 on memory B cells, we assume that the up-regulation of this receptor may influence the inhibitory capacity of CR1. It is still an open question, what molecular mechanisms are responsible for the inhibition casused by CR1 clustering.

Human CR1 has no activating and/or inhibitory motifs in its cytoplasmic domains. In contrast to CR2, which includes potential tyrosine phosphorylation sites in its cytoplasmic tail, the short intracytoplasmic domain of human CR1 does not seem to have the ability of transducing signals. The intracellular part of CR1 contains only one potential threonine phosphorylation site and two PDZ-motifs (149). A general function of the PDZ domain seems to be directing cellular proteins to multiprotein complexes allowing CR1 to associate with other membrane proteins or actin cytoskeleton. Actin cytoskeleton is known as a regulator of the distribution of membrane regions and proteins upon receptor ligation in lymphocytes (150). CR1 molecules in the cell membrane of human B cells appear not only in free form, but also in association with CR2, but not within the trimolecular complex of CR2/CD19/CD81 (151). The role of this CR1/CR2 complex however is yet not clear, but a recent report described a co-operation between the two receptors in the internalisation of ligands (152). So far no other receptors have been described, which could be associated with CR1 in the cell membrane.

Recent evidence indicates that following antigen binding the BCR rapidly translocates into cholesterol- and sphingolipid-rich membrane microdomains termed lipid rafts (153). Lipid rafts appear to function as platforms for the induction and regulation of the BCR signaling cascades. It has been reported that BCR co-ligated with the CD19/CD21 complex is retained within the lipid rafts for considerably longer time than the BCR that is simply cross-linked to itself (154). Prolonged retention of the BCR in rafts resulted in sustained tyrosine phosphorylation of proteins within the lipid microdomains indicating the formation of a long-lived signaling complex. Independent cross-linking of the BCR and the CD19/CD21 complex resulted also in increased residency and signalling, but the duration of the effect was not as

prolonged as with co-ligation (154). There is no evidence regarding the membrane localization of human CR1. Its strong immunomodulatory potential on BCR-mediated proliferation may be explained by its capacity to induce sequestering the BCR receptor from other important signalling molecules present in the lipid rafts, thereby destabilize the BCR signalling complex. On the other hand CR1 clustering may cause down-regulation of signalling by enhancing BCR internalization.

Investigating the inhibitory effect of CR1 clustering on the BCR-induced proliferation of naïve and memory B cells from healthy individuals we could observe stronger inhibition in the naïve population. One possible explanation for this observation may be that during culturing ligand-induced down-regulation of CR1 happens on memory B cells by shedding large amounts of receptors. Soluble CR1 may then cover C3b epitopes in aggregated C3, thereby resulting in partial blocking of its inhibitory capacity. One may speculate that under physiological conditions the ligand–induced receptor shedding may have similar regulatory functions. Our finding that no change could be observed in the inhibitory capacity of CR1 in SLE patients, where up-regulation of CR1 on memory B cells is lost, supports this suggestion as well.

CR1 ligation does not block the BCR-induced plasmablast formation of memory B cells in healthy individuals

Little is known about the formation and maturation of plasmacells (PC) in humans. Following immunization, specific Ab-forming cells (AFC) are generated and can be transiently detected in the circulation. The nature of these circulating AFC has not been fully clarified, and it is possible that they are the main precursors of terminally differentiated PC in the bone marrow. Experimental data accumulated in recent years which demonstrate that PC are a diverse cell compartment comprising cell subsets differing in several significant respects (155). As illustrated in Figure 18, human PC differentiation requires transitions through successive maturational steps under the control of several factors (155;156). Memory B cells are shown to differentiate into Ig secreting plasmablasts in the presence of antigen and the T cell–derived cytokines IL-2 and IL-10, in vitro. Molecules that regulate and modulate this transition positively or negatively are under investigation. Since several rounds of divisions are necessary to the differentiation, CR1 is a potential receptor that may be able to inhibit the BCR-induced plasmablast formation. We determined the relative rates of BCR-mediated

differentiation of distinct subsets of human B cells into antibody producing cells in the presence of IL-2 and IL-10. Naive B cells yielded <1% of $CD27^{high}/sIg^{low}$ plasmablasts following stimulation. In contrast, under the same conditions the frequency of $CD27^{high}/sIg^{low}$ B cells increased up to 15-18% in the memory population. We could not observe any plasmablast differentiation in the absence of cytokines, therefore it is likely that co-signals from cytokine receptors are necessary to differentiation. Although we found that CR1 ligation strongly inhibits the BCR-induced proliferation, it had no effect on plasmablast differentiation of memory B cells. It is likely that events mediated by cytokines override the inhibitory signaling from CR1.

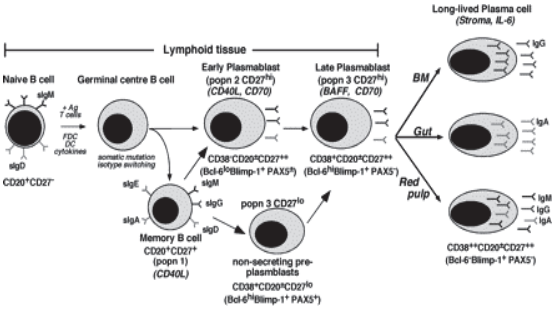

Figure 23. The model of terminal B cell differentiation

CR1 clustering and CpG-mediated B cell proliferation and differentiation in healthy donors and SLE patients

Unmetilated bacterial CpG oligonucleotids are potent activators of human B cells (157-159). In contrast to the results of Lanzavecchia's group, who reported that only memory but not naive B cells proliferate in response to CpG ODN 2006 (101) we observed a strong proliferating capacity in the naïve populations, too. The same group have published that naive B cells start to proliferate upon CpG stimulus only if they receive simultaneous antigen-specific stimulation via the BCR. Here we demonstrate that naive B cells may also be

activated by polyclonal stimuli in the absence of cognate interaction, without BCR triggering. Bernasconi and colleagues have reported that the density of TLR9, the receptor required for recognition of CpG motifs, was low on naive B cells but high on memory B cells (160). Moreover TLR9 mRNA was rapidly upregulated in naive B cells upon ligation of the BCR. Several reports have described that CpG DNA synergizes with BCR mediated signals and amplifies B cell proliferation (160). Moreover CpG and BCR-mediated signals were shown to integrate at the level of the MAPK, NF-kB activation pathways (161). In contrast to the BCR-induced proliferation that was strongly inhibited by aggregated C3, our results clearly show that CR1 clustering does not interfere with the signalling pathways activated by CpG. Altogether these findings suggest that aggregated C3 interferes with the BCR coupling network at an earlier stage, i.e. before it becomes integrated with the CpG-pathway.

A recent study suggests that CpG DNA potentiates B-cell response by enhancing terminal differentiation (162). We found that plasmablast formation could be detected only to a little extent in the $CD27^-$ naïve B cell population upon CpG stimulus. However, in $CD27^+$ B cells, a predominant differentiation into plasma cells occurred.

The relative frequency of $CD27^{high}/sIg^{low}$ plasmablasts increased up to 30% after CpG stimulus. Aggregated C3, applied for CR1 clustering on the cell surface, had no influence on the plasmablast differentiation of memory cells, indicating that the inhibitory signals mediated by CR1 has an effect only at the early stages of differentiation.

We used ELISPOT assay to assess the generation rate of antibody secreting cells by determining the frequency of cells secreting IgM and IgG under the same culture conditions. CpG-mediated plasmablast formation of $CD27^+$ memory B cells was accompanied by an increased number of IgM but not IgG secreting cells indicative of plasma-cell formation in the IgM memory compartment. The differential response to polyclonal stimuli may reflect distinct roles of switched and IgM memor B cell subsets. Thus, although the discriminating sensitivity of switched memory B cells to help bystander cells may be instrumental in maintaining systemic IgG antibody levels, the capacity of IgM memory B cells to respond to CpG in the absence of cytokines could be instrumental in maintaining levels of natural antibodies to bacterial antigens.

After stimulation of memory B cells with CpG oligodinucleotid in the presence of IL-10 cytokine we found a slight increase in the frequency of plasmablast population compared to the culture when we used CpG as the only stimulus. The response of memory B cells was not significantly affected by the addition of anti-Ig, indicating that the stimulatory conditions provided by polyclonal activators were already optimal. Summing up, these results indicate

that, in the absence of specific antigen, only IgM memory B cells proliferate and differentiate into antibody-secreting cells in response to polyclonal stimuli derived from microbes, and this process could not be inhibited by CR1 clustering.

In contrast to memory B cells of healthy individuals, B cells from SLE patients failed to form IgM secreting plasmablasts upon stimulation with CpG. The failure of CpG to induce proliferation of memory B cells in SLE may be due to the fact that TLR9 is either expressed at too low levels or is inefficiently coupled to signal transduction pathways. Regarding TLR-9 expression in B cells from SLE patients there are inconsistent data in the literature. Verthelyi et al. have described that the response of PBMC from SLE patients to CpG ODN is abnormal (163).The activation of monocytes and dendritic cells was impared, as shown by their decreased cytokine secretion. In contrast, the production of IgM and IL-10 by B cells was comparable with that of in samples of healthy controls. These authors have also shown that reduction in cellular responsiveness to CpG ODN stimulation was not due to a reduction in TLR-9 expression. Nakano et al. have demonstrated that mRNA levels of TLR-9 in B cells was up-regulated in SLE patients and B cell activation by CpG resulted in enhanced production of anti-dsDNA antibodies and IL-10 (164;165). Our results, however, demonstrate a decreased ratio of IgM memory B cell population in SLE patients indicating that the low number of these cells may be the cause of the defective response to bacterial stimuli. Our finding further supports this assumption, because if BCR ligation was applied parallel to the CpG stimulus, which may trigger TLR9–up-regulation in B cells, the responsiveness was also abolished.

In conlusion, the reduction in the expression level of IC-binding receptors on $CD19^+$ B cells is not only due to a disturbed memory compartment, but the higher frequency of $CD19^+/CD27^{high}$ plasmablasts expressing very low levels of these receptors. Our functional studies further suggest that under physiological conditions the CR1-mediated inhibition might have a role in the antigen-dependent homeostasis of memory B cells, but this does not affect later differentiation.

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Summary

Investigations in autoimmune animal models suggest that alterations in the expression or function of IC-binding Fcγ– and complement receptors may contribute to the initiation and maintenance of IC-mediated autoimmune disorders. As B cells have a crucial role in the pathological processes by producing autoreactive antibodies, examination of the expression and role of these IC-binding receptors in the maintenance of B cell tolerance has elemental role.

We carried out a phenotypic analysis of the expression of IC-binding receptors on B cells taken from healthy individuals and SLE patients. In spite of individual variations there was a significant decrease in the expression of these receptors on B cells of SLE patients compared to the age-matched healthy controls. CR1 expression on B cells from SLE patients with active disease (SLEDAI>10) was significantly lower compared to healthy controls or patients with inactive SLE. Reduction in FcγRII expression was also more pronounced in patients with active SLE compared with age-matched controls or patients with inactive SLE. We observed a positive correlation between the expression of FcγRII and CR1, indicating that these inhibitory receptors are simultaneously down-modulated. We found that the reduced expression of FcγRII and CR1 on B cells from SLE patients was unstable over a two-year period.

Distribution of these receptors on distinct B cell subpopulations in the blood has not been documented in detail so far, not even in healthy individuals. We found that the expression of the inhibitory receptors FcγRII and CR1 is significantly up-regulated on the memory B cells of healthy controls; however their expression is considerably decreased on memory B cells of SLE patients, reaching the levels of receptor density found on naive cells. Regarding the expression of CR2, the co-receptor for BCR, no difference was found between naive and memory cells of healthy individuals – in contrast to FcγRII and CR1. In SLE however, a reduced expression of CR2 was characteristic for both the naive and memory B cells. We found that the reduced expression of Fcγ- and complement receptors as well as sIg observed in SLE affected both IgM^+ and switched memory B cells.

We have shown that clustering of CR1 by aggregated C3 on the B cell surface inhibits proliferation dose-dependently both in the case of naïve and memory cells, in healthy donors. We have found no difference between the IgM and IgG memory compartment, however, CR1 ligation exerted a stronger inhibition on the proliferation of naïve cells as compared to

memory B lymphocytes. Although we found that CR1 ligation strongly inhibits the BCR-induced proliferation, it had no effect on plasmablast differentiation of memory B cells. In contrast to the BCR-induced proliferation that was strongly inhibited by aggregated C3, our results clearly show that CR1 clustering does not interfere with the signalling pathways activated by CpG. In the absence of specific antigen, only IgM memory B cells proliferate and differentiate into antibody-secreting cells in response to polyclonal stimuli derived from microbes, and this process could not be inhibited by CR1 clustering.

In conlusion, the reduction in the expression level of IC-binding receptors on $CD19^+$ B cells is not only due to a disturbed memory compartment, but the higher frequency of $CD19^+/CD27^{high}$ plasmablasts expressing very low levels of these receptors. Our functional studies further suggest that under physiological conditions the CR1-mediated inhibition might have a role in the antigen-dependent homeostasis of memory B cells, but this does not affect later differentiation.

Összefoglalás

Állatmodellekkel végzett kísérletek igazolják, hogy az IC-kötő Fcγ- és CR1/CR2 receptorok expressziójában és funkciójában bekövetkező zavarok hajlamosító tényezők autoimmun betegségek kialakulásában, fenntartásában. Mivel autoreaktív ellenanyagok termelése révén a B-sejteknek kitüntetett szerepük van a patológiás folyamatok létrejöttében, az IC-kötő receptorok megjelenésének, szerepének vizsgálata a B-sejt tolerancia fenntartásában alapvető fontosságú.

Munkánk során egészséges donorokból és SLE-ben szenvedő betegekből származó B-sejteken vizsgáltuk az IC-kötő receptorok sejtfelszíni megjelenését. Szignifikáns csökkenést találtunk a betegek B-sejtjein mind az FcγRII mind a komplementreceptorok expresszióját tekintve. A CR1 és az FcγRII receptorok esetében a betegség aktivitása nagymértékben befolyásolta a sejtfelszíni megjelenést, a CR2 receptor esetében azonban nem volt szignifikáns eltérés az aktív és az inaktív csoport között. Kimutattuk, hogy a CR2 esetében a betegség aktivitásától függetlenül elkülöníthető egy a receptort kisebb mértékben kifejező csoport. A továbbiakban megvizsgáltuk, hogy az aktív SLE-s csoportban található receptorcsökkenés mely B-sejt alpopulációkat érinti. Ezen receptorok expressziós mintázatának eltéréseit különböző B-sejt alpopulációkon eddig egészséges donorokban sem vizsgálták. Eredményeink azt mutatják, hogy a gátló CR1 és az FcγRII megjelenése fokozott az IgM^+ és az izotípusváltáson átesett IgM^- memória B-sejteken, szemben a naív B-sejtekkel. Míg a CR1 esetébe nem találtunk különbséget a két memóriasejt populáció között, addig az FcγRII megjelenése nagyobb mértékű volt az IgM^+ memória sejteken. A CR2 esetében nem találtunk fokozott expressziót a memória-populáción. A CR1 és a CR2 eltérő megjelenése különböző fiziológiás szerepükre is utal, szemben az egérben kifejeződő CR1/CR2-vel.

Aktív SLE-ben szenvedők B-sejtjeit vizsgálva nem találtuk az CR1 és az FcγRII receptorok memória sejtekre jellemző fokozott megjelenését. A CR2 csökkenés azonban a naív és a memória populációt egyaránt érintette. Az irodalommal megegyezően a $CD27^{high}/sIg^{low}$ plazmablaszt populáció nagymértékű növekedését tapasztaltuk SLE-ben. Ezen sejteken az IC-kötő receptorok expressziója a kontroll csoportban tapasztaltakhoz hasonlóan csekély volt. Longitudinális vizsgálataink arra utalnak, hogy a betegekben található receptorcsökkenés a CR1 és az FcγRII esetében átmeneti jellegű.

Funkcionális vizsgálatainkban megállapítottuk, hogy egészséges donorokból izolált memória B-sejtek BCR által kiváltott proliferációja a naív sejtekhez képest kevésbé gátolható a CR1 receptor keresztkötése által. SLE-s betegekből izolált csökkent CR1 expressziót mutató

B-sejteket vizsgálva a kontrollhoz képest nem találtunk különbséget a CR1 által kiváltott gátlás mértékében. Metilálatlan CpG motívumokat tartalmazó oligonukleotidok által indukált B-sejt proliferációt a CR1 receptor aggregációja nem befolyásolta.

Megállapítottuk, hogy míg a naív és a memória B-sejtek BCR-indukált proliferációját a CR1 receptor keresztkötés gátolja, addig a receptor aggregációja nem befolyásolja a memória sejtek BCR ill. CpG által indukált plazmablaszt differenciációját.

Összességében elmondhatjuk, hogy aktív SLE-ben a gátló CR1 és FcγRII receptorok csökkenése egyrészt a memória populáció sejtjein az up-reguláció hiányából, másrészt az IC-kötő receptorokat minimálisan kifejező plazmablaszt populáció felszaporodásából adódik. Funkcionális vizsgálataink arra utalnak, hogy fiziológiás körülmények között a CR1 által közvetített gátlásnak a memória B sejtek antigén függő homeosztázisában lehet szerepe, a késői differenciációs folyamatokat azonban nem befolyásolja.

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List of publications

Publications connected to the thesis

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Regulation of B-cell activation by complement receptors CD21 and CD35
Curr Pharm Des. 2003; 9 (23): 1849-60. Review (IF: 5,385)

Isaák A, Prechl J, Gergely J, Erdei A.
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Peripheral and tonsillar B cells differ in their CR1 (CD35) expression and function
Manuscript in preparation

Other publications

Molnár, E., J. Prechl , **A. Isaak**, A.Erdei
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Murine CR1/2 targeted antigenized single-chain antibody fragments induce transient low affinity antibodies and negatively influence an ongoing immune response.
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On-chip complement activation adds an extra dimension to antigen microarrays.
Mol Cell Proteomics. 2007 Jan;6(1):133-40. Epub 2006 Oct 27

Published abstracts

Eszter Molnár, József Prechl, Andrea **Isaák**, Eszter Csomor, Krisztián Papp, Anna Erdei
The Role of C3-Binding Receptors (CD21/CD35) in the Survival of Murine B Lymphocytes
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Erdei,A., J. Prechl, **A. Isaák**, M. Józsi, Zs. Bajtay, E. Molnár, P. Gergely, Jr., Gy. Poór and J.Gergely
Regulation of B-cell activation by complement receptors CR1 (CD35) and CR2 (CD21)—possible involvement in the pathogenesis of autoimmune diseases • ABSTRACT
Autoimmunity Reviews, Volume 3, Issues 7-8, November 2004, Pages 624-625

Zs. Szekeres , **A. Isaák**, J. Prechl and A. Erdei
Generation of a fusion protein containing DNA-like peptide and a single chain antibody
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Targeting Antigen to CR1/CR2 by Single Chain Antibody Fragments Results in Low Affinity IgM Response Without the Induction of Memory
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