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# The versatile functions of complement C3-derived ligands

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**Running title:**

**Functions of complement C3-derived ligands**

**Summary**

The complement system is a major component of immune defence. Activation of the complement cascade by foreign substances and altered self-structures may lead to the elimination of the activating agent, and during the enzymatic cascade several biologically active fragments are generated. Most immune regulatory effects of complement are mediated by the activation products of C3, the central component. The indispensable role of C3 in opsonic phagocytosis as well as in the regulation of humoral immune response is known for long, while the involvement of complement in T cell biology have been revealed in the past few years. In this review we discuss the immune modulatory functions of C3-derived fragments focusing on their role in processes which have not been summarized so far. The importance of locally synthesized complement will receive special emphasis, since several immunological processes take place in tissues, where hepatocyte-derived complement components might not be available at high concentrations. We also aim to call the attention to important differences between human and mouse systems regarding C3-mediated processes.

**Keywords:**

Complement, B cell, monocyte/macrophage, dendritic cell, cell activation

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## Introduction

The complement system is one of the most versatile constituents of the immune system. This network of more than 30 soluble and cell surface proteins plays an important role in innate defence as well as instructing and regulating adaptive responses. Complement activation can be triggered by a wide variety of substances, including immune complexes, foreign structures, altered host cells and apoptotic bodies. The cascade involves sequential activation of complement components, leading to inflammation, clearance of pathogenic microorganisms and apoptotic cells, thus contributes to maintaining host homeostasis. In the course of activation several biologically active fragments are generated, which exert immune regulatory functions via binding to various complement receptors and regulators expressed by a wide variety of cell types.

Activation of the complement cascade can occur via three pathways: the classical, lectin-dependent and alternative route (*Fig.1*). The classical pathway is set off by activation of the C1-complex, which is initiated by binding of its C1q subunit to the Ig Fc-region in antigen-antibody complexes, to apoptotic cells and to C reactive protein. The lectin pathway is triggered by Mannan Binding Lectin and ficolins, the pattern recognition molecules, which recognize carbohydrates on microbial surfaces. The alternative route is initiated by C3($H_2O$), which is deposited on activator surfaces as a result of spontaneous and slow hydrolysis of C3 ("tick over mechanism") and serves as an amplification loop for the other two activation routes. Independent of the initial trigger however, all these pathways lead to the formation of an enzymatic complex which cleaves C3, the central complement component.

In this review we focus on the immune modulatory role of C3-derived fragments, with particular attention to functions which have not been summarized so far. We emphasize

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studies in human systems and aim to highlight similarities and important differences between the human and mouse systems.

## Activation of component C3, generation of biologically active C3-fragments

Component C3 is the most abundant complement protein in serum and body fluids. It is present in the circulation in a concentration of 1.2–1.5 mg/ml, which is comparable to the amount of IgG3 in human serum. The primary source of serum C3 is the liver, however its local production by several cell types – including macrophages, dendritic cells, lymphocytes and epithelial cells – in various tissues is also known to play an important role in the initiation and regulation of adaptive immune responses (1-3).

The first cleavage products of the central component C3 are C3a and C3b (*Fig.1*). The small soluble C3a peptide binds to guanine nucleotide binding protein (G-protein) coupled C3a receptors, which are widely distributed in peripheral tissues and the central nervous system, suggesting that this peptide plays a diverse and more prominent role in various biological functions than earlier thought. The anaphylatoxic effect of C3a is known for long, however its role in adaptive immunity has been revealed only in the past few years. These results have been extensively reviewed recently elsewhere (4, 5).

Upon activation into fragment C3b the molecule undergoes marked conformational changes exposing binding sites for interaction with other proteins (6). C3b in its nascent state has the capacity to bind covalently to C3b-acceptor (C3bA) sites via its newly exposed, short-lived thioester group (7). C3bAs are hydroxyl and amine groups of proteins or carbohydrates, which are present on the activator surface, including cell membranes and various foreign structures. It is known for long that covalently fixed C3b is not only a subunit of additional

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C3-convertases, but serves as a regulator of the complement cascade and is the major ligand to complement receptor type 1 (CR1, CD35). Activator-bound C3b can be further processed by factor I to generate inactivated C3b (iC3b) and C3d(g), which remain covalently attached to C3bAs and are ready to interact with complement receptors CR3, CR4, CRIg and CR2, respectively (*Fig.1*). The phagocytic complement receptor of the immunoglobulin superfamily (CRIg), which binds C3b and iC3b has also been described on tissue resident macrophages (8).

**Covalent binding of nascent C3b to C3bAs**

As alluded above, when C3 is activated by C3-convertases or trypsin-like serine proteases, the internal thioester is exposed and approximately 10% of the generated nascent C3b molecules form ester or amide bonds with proteins and carbohydrates during their short half-life. The covalent binding reaction has been thoroughly studied employing red blood cells (RBCs) (7), Sepharose-trypsin (9) and glycine and glycerol as representative small molecules with amino and hydroxyl groups (10).

The phenomenon of immune adherence has been described long time ago by Nelson, who demonstrated that complement-opsonized microorganisms adhere to human erythrocytes (11). Later Fearon demonstrated that binding and clearance of C3-opsonized particulate antigens and immune complexes (ICs) is mediated by CR1 present on human red blood cells, and identified this complement receptor on other human cell types, including monocytes, B cells and neutrophil granulocytes (12).

It is known for long, that the covalent fixation of C3 is necessary for opsonisation of pathogens and foreign structures, which enhance their uptake by phagocytes via CR1, CR3

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and CR4 receptors. Several molecules have been identified as C3bAs in various pathogens, such as major outer membrane protein from Legionella pneumophila (13), lipophosphoglycan from Leishmania major (14), glycolipid-1 from Mycobacterium leprae (15), and heparin-binding hemagglutinin from Mycobacterium tuberculosis (16). In the membrane of RBCs glycophorin was identified as the main C3bA molecule (17). As will be detailed in the next section, the covalent attachment of C3b to nucleated cells has been demonstrated in several studies, but so far only CR2 on human B lymphocytes has been identified as a C3bA site (18).

**C3b deposition on nucleated cells**

Autologous cells normally are protected by the regulatory molecules from C3b-fixation which may lead to complement dependent lysis. Nevertheless, deposition of C3b onto the surface of nucleated cells can occur not only in pathologic conditions, like in the case of malignantly transformed cells (19, 20) and virus infected cells (21, 22), but also in physiological conditions (23, 24) and in the case of the monocytic human cell line U937 (25). Furthermore Matsumoto and Seya demonstrated C3 deposition and homotypic cell adhesion in the case of P39, a human myeloid cell line (26). In mouse studies di Renzo et al. found that Lewis lung carcinoma cells - which do not express complement receptors - fix C3 covalently to the cell membrane, and after internalization of the acceptor-bound C3b the growth of these cells is elevated (27).

Despite the fact that hydroxyl or amine groups of proteins and carbohydrates are available on the cell membrane for the covalent interaction, nascent C3b does not react randomly or non-specifically (28). This is confirmed by the study of Marquart et al. and our findings, which demonstrate that normal human peripheral blood mononuclear cells (PBMCs) are heterogeneous from this aspect and mainly B cells, macrophages and dendritic cells (DCs)

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– i.e. cells which also may serve as antigen presenting cells (APCs) bind C3b covalently (23, 24, 29-36). T cells seem to acquire this capability only after activation (37, 38). In mice macrophages and B cells, but not T lymphocytes were found to fix C3b covalently (34). These data suggest that both the availability of C3bA sites and the limited proteolysis of deposited C3-fragments on the cell membranes are regulated by so far unravelled factors and mechanisms.

In the case of human B cells CR2 has been identified as the main C3bA, and it has been proven that CR2 is involved in the formation of the alternative pathway convertase that generates further nascent C3b fragments for covalent fixation (18, 30). However, CR2 negative human monocytes and granulocytes (24), human monocyte derived macrophages (MDMs) (31), human monocyte derived dendritic cells (MDCs) (36), carcinoma cells (27) and B cells derived from CR1/2 knock out (KO) mice (34) also have the capacity to fix C3b covalently. Therefore additional, so far unidentified cell membrane molecules may also serve as C3bAs. In the extract of serum or C3-treated cells high molecular weight complexes containing C3-fragments had been detected by several groups, but the C3b-acceptor molecules could not be identified (26, 27, 29, 39). Therefore the intriguing question still needs to be answered: which cell membrane molecules serve as C3bAs on nucleated cells which do not express CR2?

Nevertheless, as will be discussed in the next sections, various immunomodulatory functions of C3bA-bound C3-fragments have been described in the case of several cell types.

## Immunomodulatory function of C3bA bound fragments

C3-fragments as bivalent ligands enhance cytotoxic reactions

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Potentiation of antibody dependent cellular cytotoxicity (ADCC) by target cell-bound C3b has been shown using human peripheral lymphocytes and C3b-coated chicken erythrocytes by Ghebrehiwet et al. (40), who demonstrated that at low anti-target antibody concentrations erythrocyte bound C3b significantly increased target cell lysis. The inverse situation has also been described; namely, effector cell-bound C3b was found to elevate ADCC, too. In these experiments concanavalin A stimulated human lymphocytes were demonstrated to cleave C3 by their cell membrane proteases and fix C3-fragments covalently, which enhanced the lysis of antibody-sensitized CR1-positive erythrocytes (37). These results clearly show that C3b, as a bivalent ligand, amplifies the cytotoxic reaction by improving the contact between the effector and target cells, regardless of the orientation of the complement fragment. This was strengthened by the studies of Ramos et al. using cells of Epstein-Barr virus (EBV)-positive Burkitt lymphoma lines which activate and bind C3 (22). They demonstrated that these lymphoblasts possess increased sensitivity to lymphocyte mediated lysis during complement dependent cellular cytotoxicity (CDCC).

## C3-fragments fixed to APCs and exosomes enhance T cell responses

C3-derived fragments C3a, C3b and iC3b have been shown to affect T cell functions directly or via APCs. Modulation of T cell proliferation, differentiation and survival by the anaphylatoxic peptides C3a and C5a and their receptors has been extensively studied and reviewed recently (41-44).

Here we focus on the T cell modulatory effect of the larger C3-derived fragments; C3b and iC3b. The role of cell bound C3b in the process of antigen presentation was revealed by demonstrating that A20 murine cells of the B lymphoblastoid cell line and concanavalin A-



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elicited peritoneal macrophages, which serve as APCs, are able to fix C3-fragments covalently when incubated in autologous serum. These C3b-bearing cells were shown to promote the proliferation of antigen-specific T cells, particularly at suboptimal antigen doses. It has been proven that CR1/CR2 is expressed on the responding, activated T cells, which interacts with C3b deposited to C3bAs on the surface of APCs, thus strengthening the contact between the two cell types (32) (*Fig.2*). The possible *in vivo* significance of this bridge-forming capacity of C3b was demonstrated by using APCs derived from mice injected with gamma-inulin, a known adjuvant which activates the alternative pathway. It was found that C3 split-products are deposited on the surface of macrophages *in vivo,* which elevate the response of antigen specific T cells 2.5-fold compared with APCs of untreated animals (33). The mechanism proposed might apply in all conditions when complement activating antigens/adjuvants are used.

In addition to C3b fixed to APCs, exosomes released by these cells were also shown to influence T cell responses. Earlier Raposo et al. demonstrated that activated APCs release exosomes containing major histocompatibility complex (MHC), co-stimulatory and adhesion molecules, which mediate antigen presentation to T cells (45). These studies were extended and exosomes secreted by C3-fragment bearing murine B lymphocytes and macrophages were investigated (34). It was found that these vesicles contain C3-fragments and significantly enhance specific T cell responses at suboptimal antigen doses (*Fig.2*). This finding might have an important relevance *in vivo*, since both virus-infected and tumor cells are known to fix C3b (19-22). Therefore, C3b-coated exosomes released by these cells might contribute to the immune response in pathologic conditions, particularly when the antigen stimulus is low.

Dendritic cells (DCs) – similarly to macrophages - are known to produce most of the complement components, including C3, furthermore several functions of DCs were shown to be influenced by complement receptors CR3 and CR4 (43, 46, 47). Using C3 KO animals

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Zhou et al. proved the importance of this protein in antigen presentation (48). Importantly, the role of C3 in the differentiation of DCs has been clearly demonstrated in a C3-deficient case by Ghannam et al. (49).

It is known from the studies of several groups that ligation of CR3 and CR4 inhibits DC maturation (50-53). To obtain a deeper insight into the role of complement in DC functions, we focused our attention on the phenotypical and functional changes induced by acceptor-bound C3b. Consistent with previous studies carried out with murine cells, human MDCs were able to fix C3b covalently (36). When native C3 or fresh human serum was added to MDCs allowing the covalent binding of C3b, a markedly increased expression of MHCII, CD83 and CD86 molecules, and a significantly enhanced secretion of TNF-α, IL-6 and IL-8 cytokines were detected, while C3 inactivated by the nucleophilic methylamine had no effect. These results are in agreement with data of Ghannam et al. who reported that in a primary human C3-deficient patient DC maturation was lacking (49). They also found that the total number of MDCs was significantly lower in this patient than in healthy individuals. It has to be noted that the phenotype of MDCs stimulated by covalently bound C3-fragments was markedly different from LPS matured MDCs, proving that a “danger signal” and a physiological stimulus direct DC maturation into different ways (36). A further important functional consequence of C3b-fixation was the elevated capacity of MDCs to stimulate allogeneic T cells. The possible involvement of CR3 in fixing C3-fragments was excluded by using CD11b RNA silenced cells. Modelling an *in vivo* occurring situation in secondary lymphoid organs, activated macrophages and MDCs were cocultured, which resulted in fixation of sufficient amounts of C3-fragments on the APCs to elevate T cell activation. Regarding the mechanism of this process we propose that DC-bound C3b interacts with CR1 expressed on T lymphocytes and functions as an adhesion molecule between DCs and T cells (*Fig.3*). In these experiments C3 production by MDCs could not be detected, therefore these

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APCs were most probably "opsonized" by macrophage-derived C3-fragments. These results broaden the concept of "local opsonisation" of microbes occurring in the close vicinity of activated, C3 producing macrophages (54), and reveal a novel way to enhance the interplay between macrophages, DCs and T lymphocytes (36). It should be noted, that a substantial portion of the covalently fixed C3-fragments were internalized by MDCs in 30 minutes in our experiments (36). Although the possible involvement of this intracellular C3 in promoting T cell response was not investigated, it might have a role described recently by Botto et al. (55). According to their studies C3 bound to dying cells can direct the cargo in APCs and influence T cell response to antigens.

Considering that DCs and macrophages are present in tissues at strategically critical sites where hepatocyte-derived complement is not available, C3 produced locally by these cells might strongly influence the acquired immune response.

Human T cell derived iC3b deposited on the cell membrane promotes autologous T cell proliferation

Production of the anaphylatoxic peptides C3a and C5a during cognate interactions between T cells and APCs has been thoroughly investigated, and their role in T cell biology was demonstrated over the past years in excellent papers (44, 56-58). Regarding complement synthesis by human T cells and the effect of the larger C3-fragments on T cell functions however, data are much sparse and controversial. Earlier Pantazis et al. showed that virus infected T cell lines produce C3 (59) and recently Cardone et al. demonstrated C3b deposition on activated CD4+ T cells (60). Studying human T cells derived from the blood and tonsil it was demonstrated recently that after stimulation with anti-CD3 these cells produce C3, and

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iC3b fragments become deposited on the cell surface (38). T cell bound iC3b was shown to act as a costimulatory molecule by interacting with CR3 and probably CR4 receptors expressed by MDCs, and significantly enhanced autologous T cell proliferation.

**CR1 (CD35) and CR2 (CD21)**

As mentioned earlier, covalently fixed C3b and its proteolytic cleavage fragments iC3b and C3d(g) serve as ligands to complement receptors CR1 (CD35), CR2 (CD21), CRIg and members of the β2-integrin family: CR3 (CD11b/CD18) and CR4 (CD11c/CD18), respectively (*Fig.1*). These receptors are widely distributed on different cell types and exert a great variety of biological functions. Although the overall structure of these receptors is identical or very similar in men and mice, it has to be emphasized that regarding their tissue distribution and function there are significant differences between these two species (*Table 1*).

In mice both CR1 and CR2 are encoded by the same gene (*Cr2*), and alternative splicing gives rise to the two protein products which have identical functions (61). Mouse CR1 comprises 21 Short Consensus Repeats (SCRs) while mouse CR2 is identical to this protein, except that it lacks the first 6 SCRs. In humans two separate genes (*Cr1 and Cr2*) encode for CR1 and CR2, producing two different cell membrane proteins with distinct tissue distribution and functions (62) (*Table 1, Fig.4*).

In humans CR1 is expressed by polymorphonuclear (PMN) leukocytes, monocytes, macrophages, B and T cells, follicular dendritic cells (FDCs) and erythrocytes, while in mice CR1/2 expression is restricted to B cells and FDCs (63, 64) and appears on activated T cells (32). Human CR1 is an approximately 200 kDa single chain transmembrane glycoprotein with a short cytoplasmic tail. In the most common human allotype the extracellular portion of the

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molecule is composed of 30 SCRs. CR1 binds activated fragments of C3 and C4, such as C3b and C4b and with lower affinity, iC3b. It belongs to the complement regulators and possesses decay accelerating activity for the C3/C5-convertases of the classical and alternative pathways. Additionally, it serves as a cofactor for Factor I-mediated cleavage of C3b, thus blocks further activation of the complement cascade. Its further important activities include phagocytosis by macrophages and granulocytes, transport of C3-opsonized immune complexes by erythrocytes and maintenance of immunological memory by FDCs (reviewed in: (65). Recently CR1 has been demonstrated to bind EBV on human B cells (66). On T cells inhibition of CR1-mediated functions were demonstrated by Wagner et al. (67) and CR1 was found to be involved in the generation of Treg cells (68). As will be discussed later, CR1 expressed by human B lymphocytes exerts a strong inhibitory effect (69, 70) (*Table 1*).

CR2 is a surface glycoprotein expressed on B cells, FDCs, epithelial cells and a subset of T cells (63, 71). In both species, CR2 appears either as a subunit of the CD19/CD21/CD81 complex, or associated with CR1 (26, 72). In the trimolecular complex CR2 functions as the ligand binding unit whereas CD19 mediates signal transduction (73). A further functional link between human CR1 and CR2 might be provided by the co-factor activity of CR1, which in the presence of Factor I facilitates the production of C3d, the ligand for CR2. Although CR1 and CR2 can be found associated on normal human B cells, each receptor can cap independently (74).

Besides promoting antigen (Ag)-induced B cell activation, CR2 was also proven to rescue peripheral B cells from apoptosis and to enhance Ag processing and presentation of C3d-bound particles (75). In addition, FDC-expressed CR2 is essential for trapping C3 fragment-tagged Ags – similarly to CR1 – thereby contributes to the maintenance of immunological memory and development of long-term B cell responses. CR2 is the receptor

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for EBV on human B cells (76), and has been demonstrated to serve also as the receptor for CD23 (77).

Murine CR1and CR2 enhances humoral immune response

The immunomodulatory role of CR1 and CR2 on B lymphocytes was mainly studied using animal systems. In 1974 Pepys found an impaired humoral immune response to both T cell dependent and T cell independent antigens in mice deficient in C3 (78). Later it has been shown that mice lacking CR1 and CR2, the receptors which interact with the C3-derived fragments, do not develop antibody response (79, 80). In the same year the adjuvant effect of C3d was proven by Dempsey et al. Employing the recombinant model antigen, hen egg lysozyme (HEL) coupled to two or three copies of C3d was 1000–10.000-fold more immunogenic than HEL alone (81). It is important to note however, that the construct containing only one copy of C3d was tolerogenic *in vivo*. This might be explained by earlier findings which demonstrate that soluble C3d controls the growth of activated B cells (82) in contrast to polyvalent C3-fragments (83).

As a B cell receptor (BCR) independent function, targeting antigen to murine CR1 and CR2 either by single chain antibody fragments or by chemically engineered whole antibodies was shown to enhance the efficiency of antigen presentation (84, 85). Targeting of antigen-bound C3b and C4b and its role in antigen presentation were proven in human systems as well, using EBV-transformed lymphoblastoid cells (86, 87). The role of CR1 and CR2 in murine B cell differentiation and function has been extensively reviewed elsewhere (88-90).

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Human CR1 and CR2 exert opposing functions

As mentioned earlier there are significant differences between the expression and function of human and murine CR1 and CR2, therefore results obtained in mouse systems should be translated to humans with great caution. In the following section the immunomodulatory role of complement receptors expressed by human B cells will be discussed with particular attention to CR1, which down-modulates B cell responses in contrast to the activatory CR2.

In contrast to mouse B cells, where ligation of BCR to CR1 and CR2 or clustering complement receptors by multimeric ligands strongly elevates antibody production, the response of human B cells to ligation of CR1 and CR2 is much more diverse (*Fig.4*). Regarding the role of CR2 on human B cells results are in line with data on mouse B cells. Namely, depending on whether C3d, the natural ligand is presented in soluble or crosslinked form, CR2 can mediate either negative or positive signals (91-93). Antibody to CR2 was shown to induce proliferation of human B cells in the presence of T cells (94, 95) and ligand binding to CD21 was found to augment BCR induced activation via a selective, c-fos dependent signaling pathway (96). Nevertheless, it has to be pointed out that a remarkable CR2-dependent enhancement of the antibody response which was found in mouse systems, has not been demonstrated in any human systems studied so far.

Concerning the function of CR1 on human B cells, results of studies carried out between 1980 and 1990 are much more controversial. In experiments using $F(ab')_2$ fragments of antibodies reacting with CR1, Daha et al. showed enhancement of non-specific Ig-production when the cells were stimulated with a suboptimal dose of pokeweed mitogen (PWM) in the presence of T cells (97). Under these conditions monomeric C3b was ineffective. Later Weiss et al. found that CR1-specific monoclonal antibodies did not affect nonspecific B cell proliferation but elevated specific antibody production in the presence of T

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cell-derived factors and suboptimal antigen dose (98). Tedder et al. however found no effect of CR1-specific antibodies in a similar experimental system (99). In contrast to these data, employing the natural ligand Berger and Fleisher had shown that C3b inhibited PWM-induced Ig-production of human B cells (100). Similarly, Tsokos et al. also demonstrated the negative modulatory effect of C3b in conditions where the participation of other immunoregulatory cells could be excluded (101).

Assuming that these controversial results are most probably due to the mixed cell populations and the different experimental conditions used by the authors, we decided to reinvestigate the role of CR1 in human B cell functions. We used highly purified B cells which were activated suboptimally via the BCR crosslinking in serum-free culture conditions, and for CR1 ligation C3b, the natural ligand was applied in a multimeric form (69). We demonstrated that clustering CR1 inhibited proliferation and $Ca^{2+}$-response of human B cells, and proved that this inhibitory activity also occurs in the presence of the costimulatory cytokines IL-2 and IL-15. These results give evidence that human CR1 exerts an opposite effect to CR2, thus provides an additional level of regulation of humoral immunity (*Fig.4*). The therapeutic potential of CR1 inhibition was proven in a humanized SCID model, where selective co-cross-linking native DNA-specific BCR with the inhibitory CR1 suppressed B cell proliferation and autoantibody production (102). The inhibitory effect of CR1 in autoimmune patients (70) will be detailed in the next section.

Human CR1 and CR2 are expressed differentially in various B cell subsets

Based on the opposing functions of human CR1 and CR2 we assumed that their expression is different in various B cell subsets. Indeed, employing tonsil-derived B cells, where



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complement receptor expression on germinal center (GC) B cells can be assessed in addition to the subpopulations present in blood, we have found that CR1 and CR2 expression changes oppositely during B cell differentiation. While $CD19^+$, $CD27^-$ naïve B cells upregulate their inhibitory CR1 during differentiation to $CD19^+$, $CD27^+$ memory cells, the expression of the activatory CR2 receptor is downregulated (70). Similarly to plasmablasts, both CR1 and CR2 expression are markedly reduced on $CD27^+$, $CD10^+$, $CD38^+$ GC B cells (our unpublished observation).

We presume that the contrasting expression of CR1 and CR2 during human B cell differentiation have an essential role in the maintenance of peripheral B cell tolerance. The significantly decreased expression of CR1 - as well as the other main inhibitory receptor, FcγRIIb - on GC B cells may support the intensive proliferation of centroblasts and the differentiation of centrocytes into memory cells or plasmablasts. Alternatively, decreased appearance of both CR1 and CR2 on these B cell subsets can be the consequence of receptor internalization due to the continuous Ag screening and uptake of complement-tagged ligands. Later, however, the elevated expression of CR1 on memory B cells with parallel down-regulation of the activatory CR2 may prevent the activation and differentiation of low-affinity, non-specific B cells into antibody secreting plasma cells and restrict the immune response only to Ag-specific B cell clones, preventing autoimmunity (*Fig.5*). The important question still needs to be answered: how the ligands – namely C3b and C3dg - are generated and offered to B cells at the different stages? We think that it is a complex process, where antigen-bound C3-fragments, local C3-production by different cells, C3-fragments present in ICs bound to FDCs, the presence or lack of regulatory proteins on the membrane of various cells, etc., might all play a role.

Expression and function of CR1 and CR2 on human B cells in autoimmune conditions

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In autoimmune conditions - including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) - ICs containing the autoantigen, autoantibody and C3-fragments are not properly cleared from circulation. Hence their excessive binding to B cells together with the observed abnormal expression of FcγRIIb, CR1 and CR2 on the surface of human B cells may cause imbalance in the regulation of B cell functions, breaking self-tolerance and inducing autoreactivity.

Although the reduced expression of CRs on erythrocytes as well as on leukocytes of autoimmune patients is known for long (103-105), the distribution of CR1 and CR2 on various B cell subsets in autoimmune conditions has been studied only recently (70, 106). Our group assessing CR1 and CR2 expression on different B lymphocyte subpopulations of healthy donors and SLE patients revealed that under physiological conditions, $CD27^{+}CD19^{+}$ memory B cells express higher level of CR1 than $CD27^{-}CD19^{+}$ naïve cells. Its expression however is markedly decreased in both $IgM^{+}$ and switched memory B cells of SLE patients. In contrast to CR1 however, no difference was found in the expression of CR2 between naïve and memory B cells of healthy individuals, and the reduced receptor expression affected all B cell populations of the patients (106). In the case of RA patients, although the expression of CR1 and CR2 was reduced on all tested B cell subpopulations, the change in the expression pattern during the differentiation of B lymphocytes was maintained. This finding points to a strict regulation of complement receptors in RA, which seems to be missing in other B cell-mediated autoimmune diseases, such as SLE.

Despite the reduced expression of CR1 in autoimmune patients, we have found that its inhibitory capacity is maintained and reduces both B cell proliferation and antibody secretion similarly to healthy individuals (70). Similar results were obtained investigating the function of CR2 on B cells of SLE patients, where despite the fact that B cells were found to express half as many surface CR2 as normal B lymphocytes, the $Ca^{2+}$ response and the percentage of

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responding cells were significantly increased after co-ligation of BCR and CR2 (107). These observations suggest that down-regulation of IC-binding receptors on B cells may contribute to the induction of autoimmunity rather than affecting disease severity. Indeed, CR1 and CR2 have been proven to play an essential role in the development of tolerance by presentation of self-antigens to autoreactive B cells at the immature stage (108). At this stage encounter of B cells with autoantigens leads to negative selection, thus defect in retention of self-antigens due to abnormal expression of CR1 and CR2 may cause impaired deletion and escape of anergic, self-reactive B cell clones to the periphery. This hypothesis is supported by the fact that $CD21^{low/-}$ B lymphocytes have been found enriched in patients with autoimmune diseases (109) and these cells also fail to express CD35 (our unpublished results). $CD21^{low/-}$ B cells are refractory to most stimulation and – instead of being eliminated – they remain in the blood of autoimmune patients where infections or abnormal clearance of ICs may create a favourable environment for their activation, leading to break of B cell tolerance and development of autoimmunity.

Although the exact mechanism is still unresolved, these data suggest that concurrent changes in the expression and/or function of IC-binding receptors on B lymphocytes and other cell types may lead to aberrant B cell activation as well as Ag retention on B cells and FDCs, resulting in autoantibody secretion and induction of autoimmunity.

**CR3 (CD11b/CD18) and CR4 (CD11c/CD18)**

Complement receptor CR3 (CD11b/CD18, also known as Mac-1; αMβ2) and CR4 (CD11c/CD18, also known as p150, 95; αXβ2) belong to the family of β2 integrins. These molecules are heterodimeric transmembrane glycoproteins consisting of a non-covalently coupled alpha and beta polypeptide chain, which have fundamental role in cell-cell, cell-



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extracellular matrix connections and locomotion. The 18 different α and 8 β-chains combine into 24 integrins that are classified based on their β chains (110, 111). In immunology, special attention is drawn to the leukocyte specific β2 family, which has four members; the well-known LFA-1 (CD11a/CD18), the complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) and CD11d/CD18.

Human CR3 and CR4 are closely related integrins; their α-chains share 87% identity in amino acid sequence in the extracellular part, and 56% in the short intracellular tail (112, 113). The functional regions in human CD11b and CD11c are also shared with mouse CD11b (114).

The common ligands of CR3 and CR4 include iC3b, the inactivated form of C3, fibrinogen and ICAM-1 (115-117). In both receptors the I-domain of the α-chain is responsible for ligand binding, although the β chain also plays an important role in maintaining the proper conformation for ligand binding (111, 118-123). By mapping the binding sites for various ligands Zhang et al. revealed that the fibrinogen binding site is different from the sequences which interact with iC3b (124). CR3 has an additional lectin type ligand binding site that can interact with the yeast product β-glucan and has an important role in effective tumour killing (125). Both CR3 and CR4 can bind LPS and several pathogens directly (117, 126-130).

The tissue distribution of CR3 and CR4 is different in men and mice (*Table 1*). In humans CR3 and CR4 are mainly expressed on monocytes, macrophages, dendritic cells, PMNs and natural killer (NK) cells, and recently they have been detected on certain B lymphocyte populations as well (114, 131-133). In mice, expression of CD11c is far more limited, therefore it is used as a marker of mouse DCs.

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Dissecting the functions of CR3 and CR4

The similarities between CR3 and CR4 in structure and ligand specificity makes the study of their individual role extremely challenging. Furthermore, the competition between the ligands for CR3 and CR4, which exists in humans is lacking in mice due to the above mentioned differences in the receptors' tissue distribution. Considering these facts it can be easily accepted that results obtained in mouse experiments cannot be simply translated to humans. Nevertheless, studies in human systems are also hindered by technical obstacles. Namely, when the function of CR3 and CR4 is investigated on phagocytes, both receptors are targeted at the same time, therefore the outcome of the experiment cannot be clearly associated only with one of them. These difficulties are further increased by the high intracellular pool and rapid recycling of integrins (134, 135). Due to these features, blocking one receptor with monoclonal antibodies to study the function of the other is challenging, because antibody covered integrins may translocate and reside inside the cell, while the unoccupied ones come to the surface, thereby lower the efficiency of receptor blocking. Finally, the possible proximity of CR3 and CR4 in the cell membrane may also be a source of false results caused by steric hindrance between adjacent receptors having bound antibodies or ligands.

The best-known and most important function of CR3 is the phagocytosis of iC3b opsonized pathogens and apoptotic cells (136-139). The latter process is an immunologically silent mechanism, which is important in the induction of tolerance (51-53, 140). Based on the similarities between human CR3 and CR4 in structure, ligand specificity and expression, earlier it was hypothesized that CR4 is involved in similar processes as CR3 – most importantly in the phagocytosis of iC3b opsonized particles. The first indication of distinct roles for CR3 and CR4 came from the studies of Georgakopoulos et al., who showed that CD11c and CD11b are differentially involved in adhesion of human monocytes to fibrinogen

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(141). Later Pliyev et al. proved that phagocytosis of iC3b opsonized particles by PMNs is dependent on CR1 and CR3, but not on CR4 (142).

The function of CR4 has not been studied intensively so far most probably due to lack of specific monoclonal antibodies. Moreover, expression of CR4 is limited in mice, and in humans its expression level in some cell type is lower compared to CR3. Nevertheless, interesting studies performed in mice draw the attention to the importance of discriminating between CR3 and CR4. While the anti-inflammatory effect of CR3 is widely accepted, CR4 seems to have rather a pro-inflammatory role (143, 144).

CR3 and CR4 are accepted to play important roles in pathological conditions and it is known for long that inflammation can alter the expression level of these receptors. Due to mutations in the common β-chain, CR3 and CR4 are associated with leukocyte adhesion deficiency (LAD), an autosomal recessive disorder characterised by recurrent bacterial infections. Much attention is drawn to the role of CR3 in the pathogenesis of autoimmune diseases such as SLE and RA, and recently a single nucleotide polymorphism in the CD11b chain (rs1143679) has been identified as a risk factor in SLE (145). Although its function has not been described so far, CD11c was found to appear on the B cells of lupus prone mice and in human leukemic patients (146, 147).

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CR3 is the dominant receptor in opsonic phagocytosis by human DCs

Our strong hypotheses is that due to an evolutionary thrift, CR3 and CR4 play different roles in human cell types. Therefore we set out to dissect their functions using human monocyte-derived dendritic cells (MDCs), which express these two integrins in approximately equal amounts. Studying the interaction of MDCs with HIV-1 it was found that C3b-opsonized

virus particles caused up to ten-fold higher productive infection than the non-opsonized ones. This significantly higher infection rate could be reduced to background level by blocking the ligand binding site of CD11b (138). These results demonstrate that CR3 is the dominant receptor to mediate the uptake of iC3b opsonized HIV by MDCs, and this receptor directs opsonized HIV to cellular compartments which are favourable for productive infection. The prominent role of CR3 over CR4 was also demonstrated in the phagocytosis of iC3b opsonized yeast and bacterial particles by MDCs (148) (*Fig.6*). These results were confirmed by using siRNA; when downregulation of CD11b blocked the uptake of microbes by MDCs, while silencing CD11c had no effect at all. Although it has not been proven so far, one possible explanation for the differential function of CR3 and CR4 in these experiments is that the cytoskeletal association and mobility of the two receptors is different (113).

CR3 and CR4 are differentially involved in adhesion of human DCs

Mouse models revealed an important role for CR3 expressed by DCs in adhesion and cytokine production upon binding to fibrinogen (149, 150). These data, however, cannot be simply applied to the human system, where both CR3 and CR4 are expressed on several cell types which bind fibrinogen (151). Furthermore, integrin expression has been shown to be regulated differentially in the two species (152). In the study of Thacker et al. human MDCs were found to bind fibrinogen and secrete cytokines in a CD18-dependent manner, but the role of CR3 and CR4 could not be separated in these experiments (153). Georgakopoulos et al. showed that the contribution of these integrins to fibrinogen binding is dependent on the experimental conditions (141). We set out to dissect the role of CR3 and CR4 in adhesion, employing human monocyte derived macrophages (MDMs) and DCs and antibodies specific to the ligand binding site of the receptors. We found that CR4 ligation significantly decreases



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the number of adhered cells and the force of adhesion in the case of both cell types, and inhibits macrophage spreading. These data show that strong adhesion to fibrinogen is mainly mediated by CR4, while the role of CR3 is negligible in this process (accepted in PLOS ONE, 2016) (*Fig.6*).

**Conclusions, perspectives**

We have reviewed new aspects of complement mediated functions and exposed the importance of C3-derived fragments not only in bridging innate and adaptive immunity, but also in regulating immune responses.

We are convinced that in addition to valuable data obtained in animal studies it is important to investigate human systems and understand the differences of complement functioning between men and mice. With the help of new methods to isolate and characterize various subpopulations of human T and B lymphocytes as well as macrophages and dendritic cells we shall be able to reveal the expression and role of various complement receptors at different developmental stages of these cells. Due to the complexity of signals integrated when B cells engage immune complexes bearing diverse complement fragments, the opposing role of CR1 and CR2 on human B cells needs further investigations, both in physiological and pathological conditions.

Since several immunological processes take place in tissues where hepatocyte-derived complement proteins might not be available, locally produced complement components and their receptors are most likely involved in modulating cellular responses *in situ*. Recognition of further details of these processes will help better understand physiological and pathological processes.

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## Legend to Figures

***Fig.1*. Generation of ligands for C3-receptors**

The complement cascade can be activated via three pathways: the classical, lectin-dependent and alternative route, and all these pathways lead to the activation of the central complement component, C3. The first cleavage products are C3a and C3b. The soluble C3a peptide may bind to G-protein coupled C3a receptors, while the larger activation fragment C3b has the capacity to bind covalently to C3b-acceptor sites (C3bA) present on the activating surface. Covalently fixed C3b is the main ligand of CR1 (CD35) and CRIg. C3b can be further processed to generate iC3b and C3d(g), which remain covalently attached to C3bA sites and are ready to interact with complement receptors CR3 (CD11b/CD18), CR4 (CD11c/CD18), CRIg and CR2 (CD21) expressed on several cell types.

***Fig.2.* Enhancement of mouse T cell response by APC-bound and exosome deposited C3-fragments**

Antigen presenting cells (APCs) - mouse macrophages and B cells bearing C3bA-bound C3b and iC3b are able to interact with CR1/2 expressing mouse T cells, resulting in augmented T cell response. Exosomes released by C3-fragment bearing APCs can exert a similar effect.

***Fig.3.* Enhancement of human T cell response by locally generated C3-fragments fixed to DC**

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Activated human macrophages produce and activate C3, which results in covalent deposition of C3-fragments onto DCs present in their close vicinity („local opsonization"). DC-bound C3b and iC3b may then interact with CR1 and CR3 expressing T cells and increase the adherence between the APC and the responding T cell, leading to enhanced proliferation.

***Fig.4.*** **Role of CR1 and CR2 in the regulation of B cell functions in men and mice**

Complement receptors CR1 (CD35) and CR2 (CD21) in human B cells are produced by two separate genes, *Cr1* and *Cr2*. Occupancy of CR1 inhibits BCR induced proliferation, antibody and cytokine production both in physiological and autoimmune condition. In contrast to this, ligation of CR2 (CD21) which is in complex with CD19 and CD81, rather promotes B cell functions under certain experimental conditions. In mice CR1 and CR2 are encoded by a single gene, *Cr2,* and the cell membrane proteins CR1 and CR2 are generated by alternative splicing. On mouse B cells both CR1 and CR2 appear as a subunit of the co-receptor complex containing CD19 and CD81, and their ligation strongly enhance BCR dependent activation of murine B cells. The co-stimulatory effect of CR2 ligation on human B cells is considerable less pronounced than in mice.

***Fig.5.*** **Differential expression of the inhibitory CR1 (CD35) and the activatory CR2 (CD21) during human B cell differentiation**

CR1 expression on $CD27^{+}$, $CD10^{-}$, $CD38^{+}$ human germinal center (GC) B cells is significantly diminished compared to $CD27^{-}$ naive B cells, which may support the intensive proliferation of centroblasts and the differentiation of centrocytes into memory cells or plasmablasts. The elevated expression of the inhibitory CR1 on memory B cells parallel with



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the down-regulation of the activatory CR2 may prevent the activation of low-affinity, non-specific B cells and restrict the immune response to the Ag-specific B cell clones, preventing autoimmunity.

***Fig.6.*** **CR3 and CR4 mediate different functions on human DCs**

Although iC3b and fibrinogen are common ligands for CR3 and CR4, these two integrins have different functions on human MDMs. The uptake of iC3b-opsonised particles is mediated mainly by CR3 (CD11b/CD18), in contrast to the adherence to fibrinogen, where CR4 (CD11c/CD18) plays a dominant role.

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*Table 1* **Tissue distribution and function of C3-binding structures in men and mice**

| *Binding structure* | *Cell type* | *Function in humans* | *Function in mice* | | |
|---|---|---|---|---|---|
| **C3b Acceptor** | **B cells** | serves as a site for formation of alternative pathway convertases on CR2 | enhancement of antigen presentation | | |
| | **T cells**[a] | enhancement of cytotoxicity | *not expressed* | | |
| | **NK cells** | enhancement of cytotoxicity | *no data* | | |
| | **Mf** | enhancement of T cell stimulation, inhibition of FcγR-mediated phagocytosis and ADCC | enhancement of antigen presentation | | |
| | **DC** | enhancement of T cell stimulation | *no data* | | |
| **CR1** (CD35) | **B cells** | inhibition of BCR dependent proliferation, plasmablast formation, Ab production and cytokine production, EBV infection, complement regulator | **CR1/2** (CD21/ CD35) | | |
| | **T cells**[a] | inhibition of proliferation and cytokine synthesis, induction of Treg cells, complement regulator | | **B cells** | BCR-dependent enhancement of antibody response<br>BCR-independent enhancement of Ag-presentation |
| | **Mo/mph** | phagocytosis, complement regulator | | **T cells**[a] | enhancement of cell-cell interactions |
| | **FDC** | maintenance of memory, complement regulator | | **Mo/mph** | *not expressed* |
| | **PMN** | phagocytosis, complement regulator | | **FDC** | maintenance of memory |
| | **RBC** | IC transport, complement regulator | | **PMN** | *not expressed* |
| **CR2** (CD21, + CD19 + CD81) | **B cells** | slight enhancement of proliferation and intracellular $Ca^{2+}$ release binds EBV, CD23 and interferon | | | |
| | **T[a] cells** | enhancement of cell-cell interactions, EBV infection | | | |
| | **Mo/mf** | *not expressed* | | | |
| | **FDC** | maintenance of memory | | | |
| | **PMN** | *not expressed* | | | |
| | **RBC** | *not expressed* | | | |
| **CR3** (CD11b/ CD18) | **B cells** | expressed on B-1 cell subpopulation | regulation of T-cell activity in autoimmunity | | |
| | **T cells** | regulation of cytotoxicity and proliferation | upregulated upon virus infection on cytotoxic subpopulations | | |
| | **Mo/mf** | phagocytosis, adhesion and migration | phagocytosis, adhesion and migration | | |
| | **DC** | phagocytosis, adhesion and migration | phagocytosis, adhesion and migration | | |
| | **PMN** | phagocytosis, degranulation, adhesion and migration | phagocytosis, degranulation, adhesion and migration | | |
| | **NK cells** | enhancement of cytotoxicity (β-glucan and iC3b dependent) | enhancement of cytotoxicity (β-glucan and iC3b dependent) | | |
| **CR4** (CD11c/ CD18) | **B cells**[a] | adherence, proliferation– | *not expressed* | | |
| | **T cells** | cell-mediated cytolysis by subset of $CD8^+$ T-cells - | *not expressed* | | |
| | **Mo/mf** | adhesion, phagocytosis | *not expressed* | | |
| | **DC** | adhesion, phagocytosis | cell specific marker (CD11c), phagocytosis | | |
| | **PMN** | adhesion, enhancement of bactericidal activity | *not expressed* | | |
| | **NK cells** | *no data* | *not expressed* | | |

[a] expressed on activated cells

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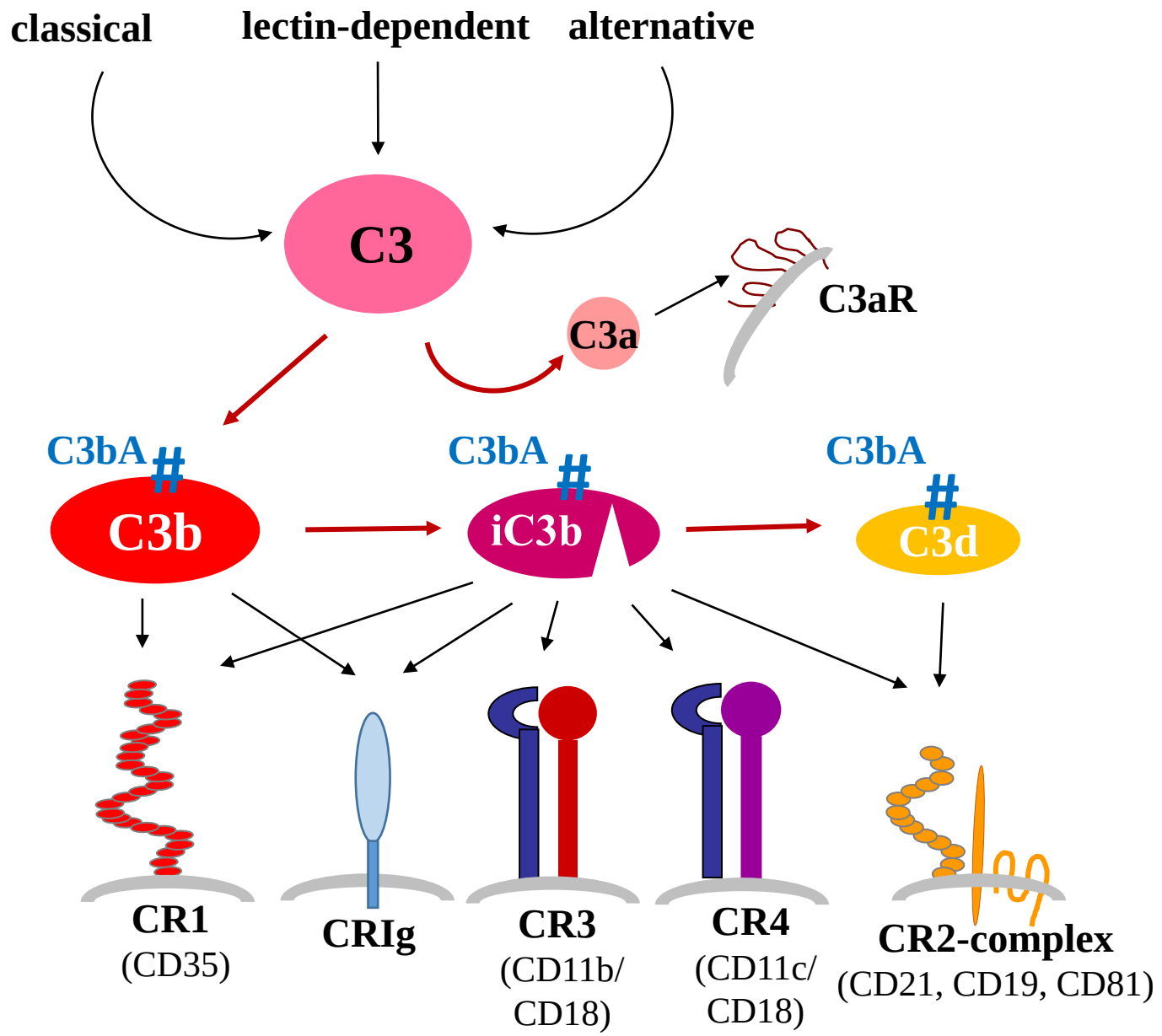
# Figure 1

*Complement activation*

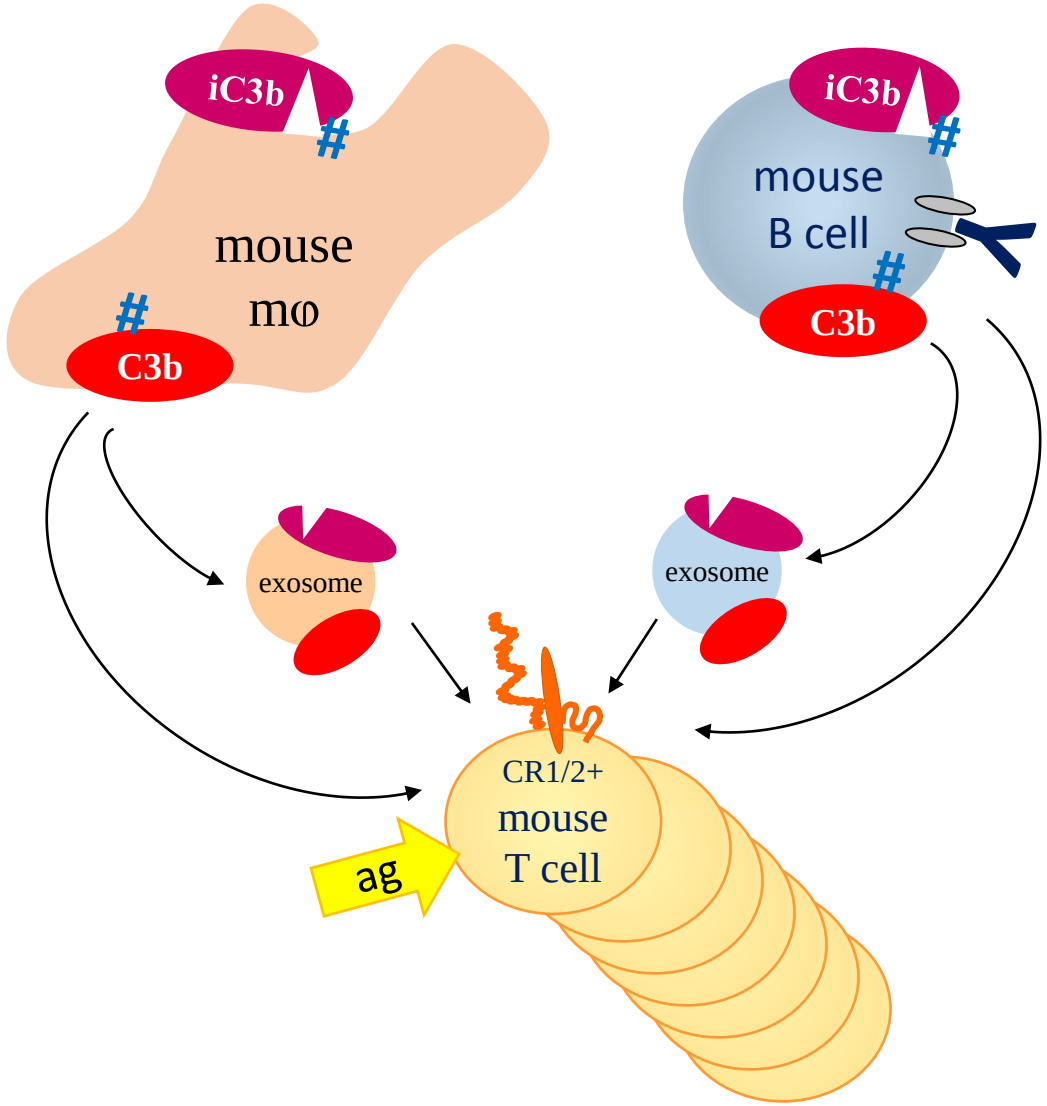
*Cleavage of C3*

*Covalently bound C3-fragments, ligands for C3-receptors*

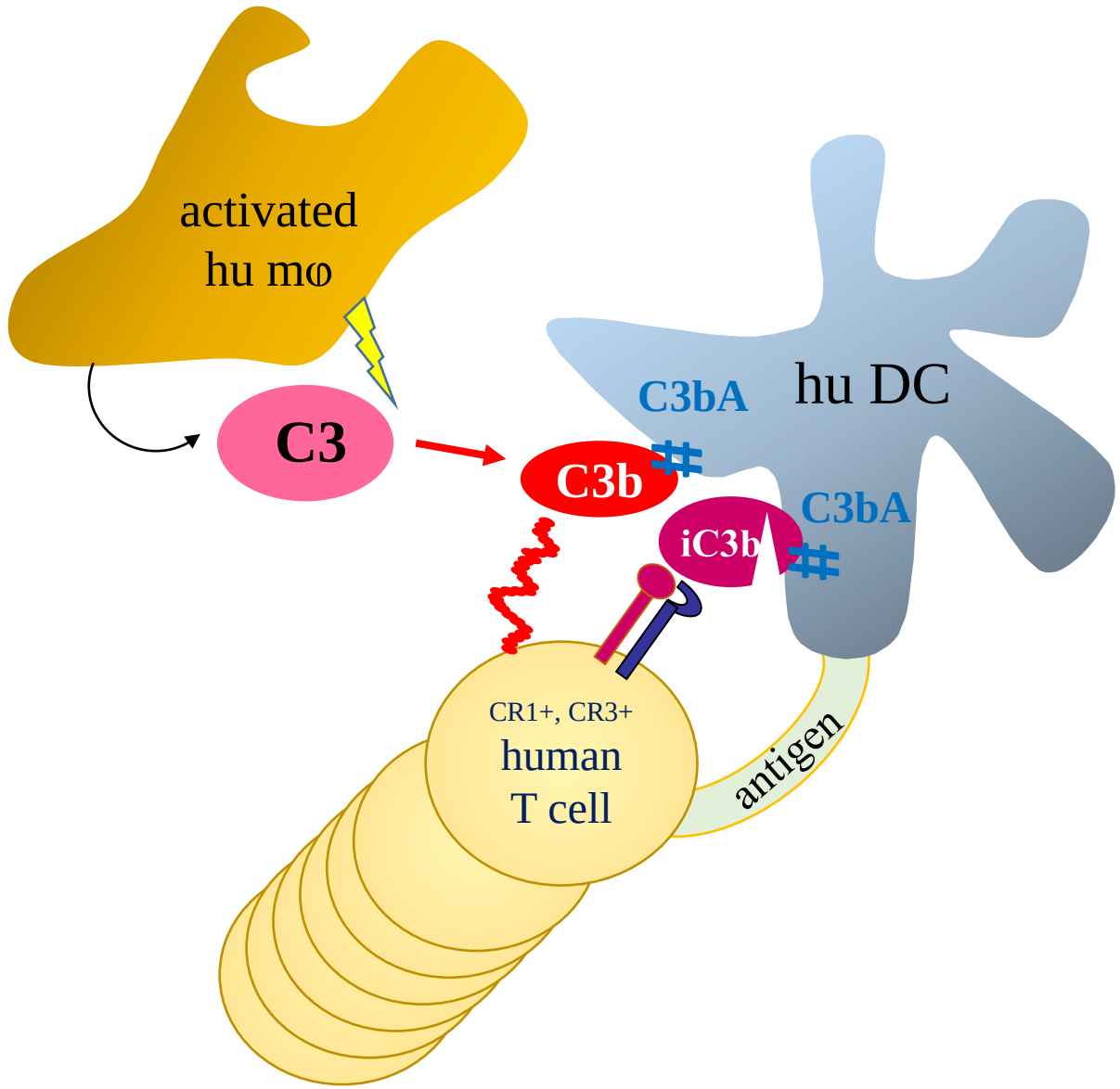
*Receptors for covalently bound C3-fragments*



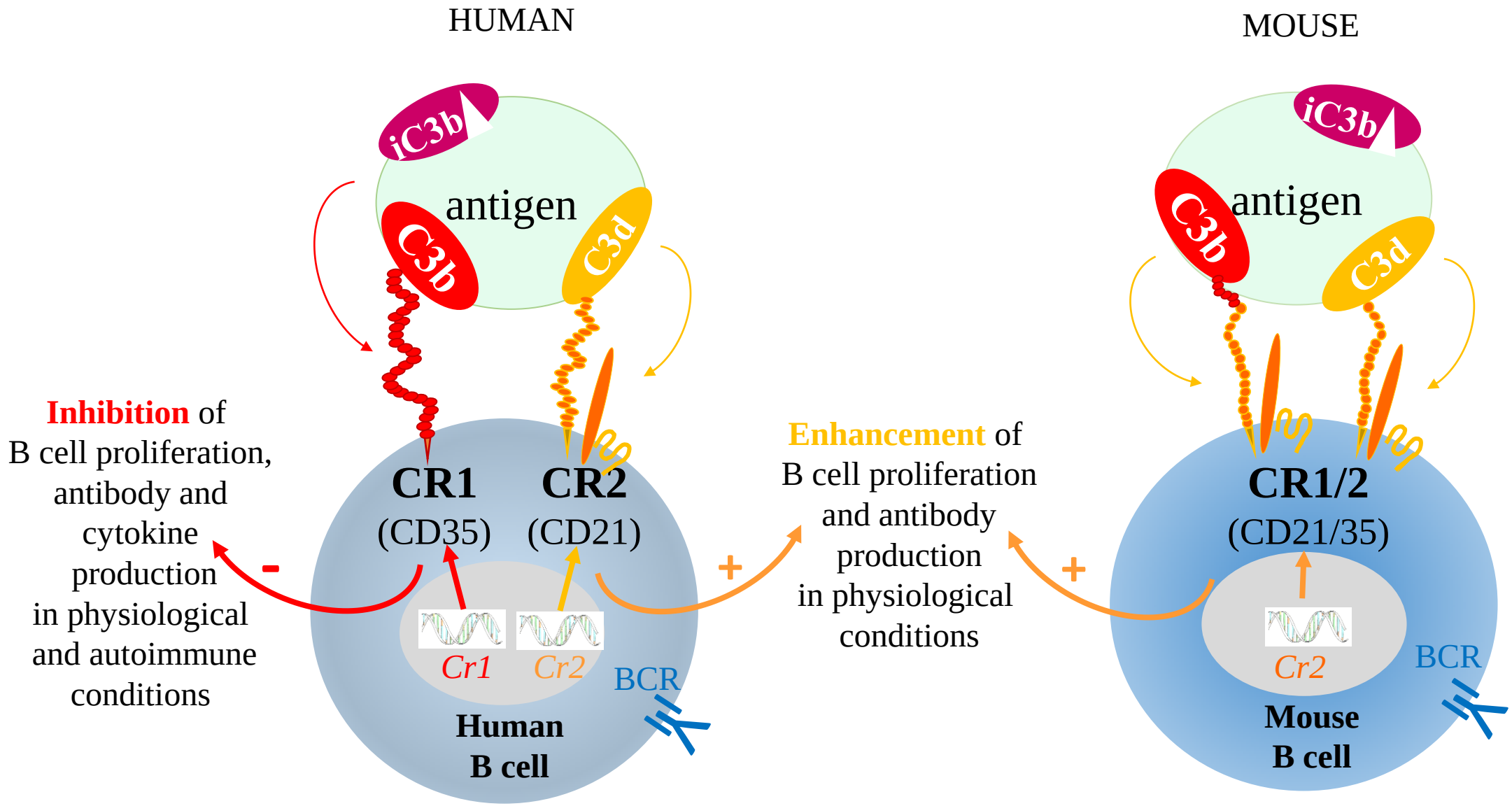
# Figure 2


iC3b
#
mouse
mφ
#
C3b
iC3b
#
mouse
B cell
#
C3b
exosome
exosome
CR1/2+
mouse
T cell
ag

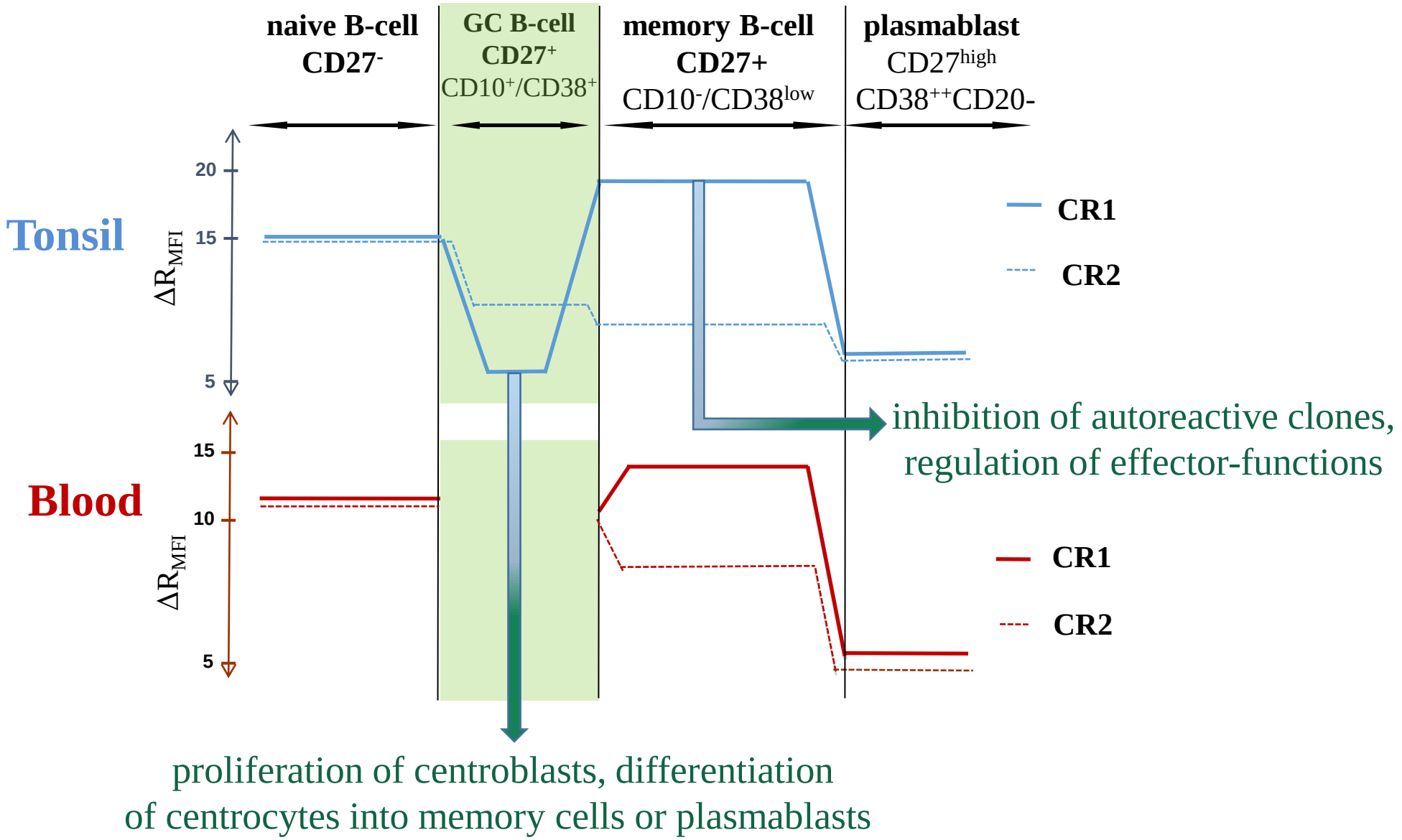
# Figure 3


activated
hu mφ
C3
C3b
C3bA
hu DC
C3bA
iC3b
CR1+, CR3+
human
T cell
antigen

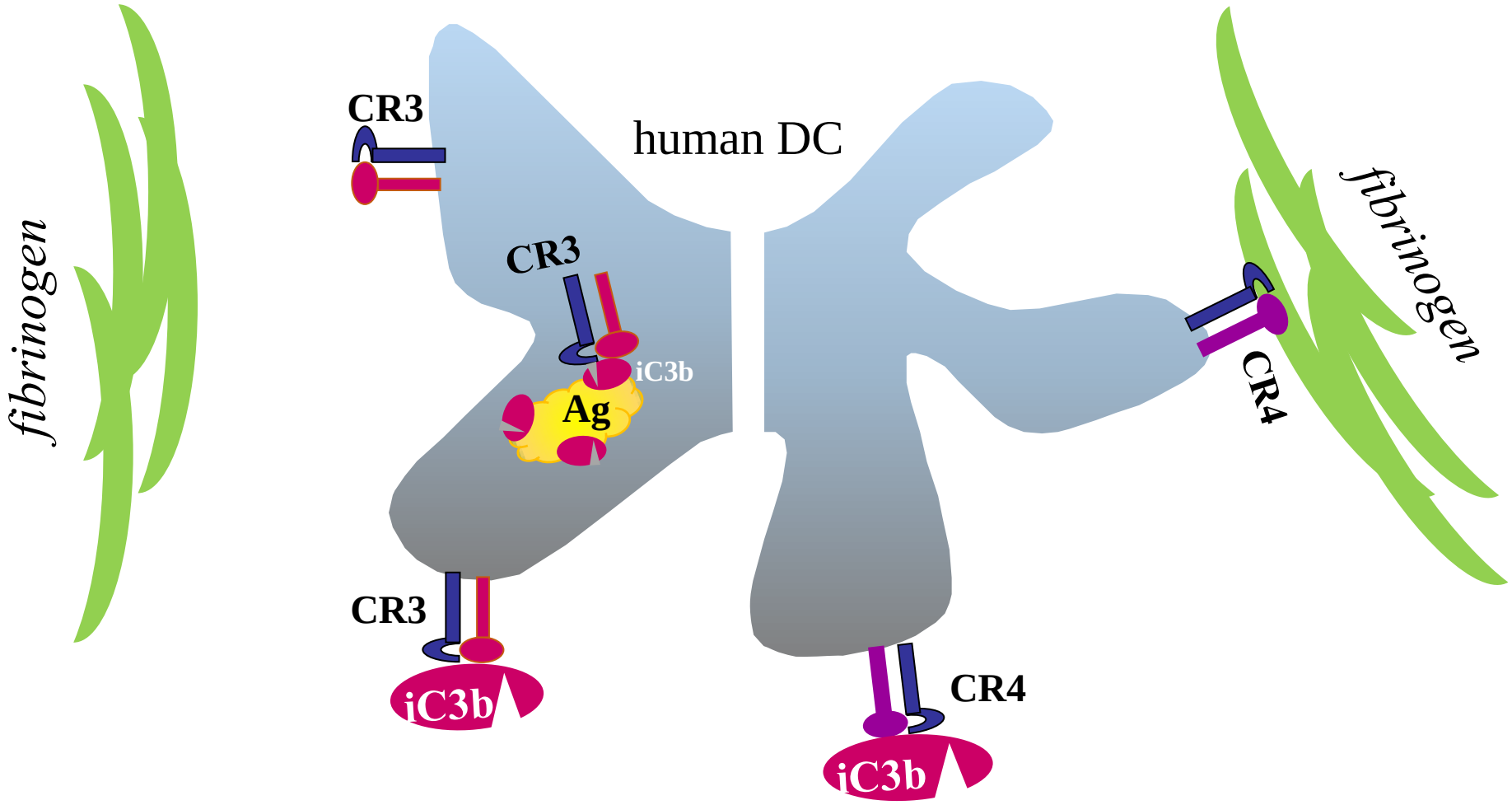
# Figure 4


HUMAN
MOUSE
iC3b
antigen
C3b
C3d
CR1
(CD35)
CR2
(CD21)
CR1/2
(CD21/35)
Cr1
Cr2
BCR
Human
B cell
Mouse
B cell
Inhibition of
B cell proliferation,
antibody and
cytokine
production
in physiological
and autoimmune
conditions
Enhancement of
B cell proliferation
and antibody
production
in physiological
conditions
-
+
+

# Figure 5


naive B-cell
CD27-
GC B-cell
CD27+
CD10+/CD38+
memory B-cell
CD27+
CD10-/CD38low
plasmablast
CD27high
CD38++CD20-
Tonsil
Blood
ΔRMFI
20
15
5
15
10
5
CR1
CR2
CR1
CR2
inhibition of autoreactive clones,
regulation of effector-functions
proliferation of centroblasts, differentiation
of centrocytes into memory cells or plasmablasts

# Figure 6




- phagocytosis
- no adherence to fibrinogen

- no phagocytosis
- adherence to fibrinogen