REVIEW

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CD21$^{-/low}$ B cells: A Snapshot of a Unique B Cell Subset in Health and Disease

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Abstract

B cells represent one of the cellular components of the immune system that protects the individual from invading pathogens. In response to the invader, these cells differentiate into plasma cells and produce large amounts of antibodies that bind to and eliminate the pathogen. A hallmark of autoimmune diseases is the production of autoantibodies i.e. antibodies that recognize self. Those that are considered pathogenic can damage tissues and organs, either by direct binding or when deposited as immune complexes. For decades, B cells have been considered to play a major role in autoimmune diseases by antibody production. However, as pathogenic autoantibodies appear to derive mainly from T cell dependent responses, T cells have been the focus for many years. The successful treatment of patients with autoimmune diseases with either B cell depletion therapy (rituximab) or inhibition of B cell survival (belimumab), suggested that not only the autoantibodies but also other B cell features are important. This has caused a surge of interest in B cells and their biology resulting in the identification of various subsets e.g. regulatory B cells, several memory B cell subsets etc. Also, in other conditions such as chronic viral infections and primary immunodeficiency, several B cell subsets with unique characteristics have been identified. In this review, we will discuss one of these subsets, a subset that is expanded in conditions characterized by chronic immune stimulation. This B cell subset lacks, or expresses low, surface levels of the complement receptor 2 (CD21) and has therefore been termed CD21$^{-/low}$ B cells.

Introduction

Naïve B cells

In adults, B cells develop in the bone marrow where the immunoglobulin (Ig) genes, encoding antibody heavy and light chains, undergo recombination, giving rise to a vast antibody repertoire. Mature naïve B cells are found in blood and peripheral lymphoid organs, and express antibodies in a membrane bound form, i.e. B cell receptor (BCR), on their cell surface (Fig. 1). Before reaching the mature naïve B cell stage, those that express an autoreactive BCR undergo selection. To this end, various tolerance mechanisms are employed e.g. clonal deletion, receptor editing and anergy as well as regulation of survival factor levels [1, 2]. This ensures that the vast majority of naïve B cells express an innocuous surface BCR, and the cells are thus ready to respond to invading pathogens [3].

In autoimmune diseases, e.g. systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), mechanisms of tolerance induction appear to be defective allowing the development of naïve B cells that are enriched for autoreactivity [4, 5]. Although the significance of this is unknown, in a mouse model, the development of naïve B cells enriched for autoreactivity is sufficient to initiate autoimmune responses and autoantibody production [6, 7]. Furthermore, it has been proposed that autoreactive naïve B cells predispose as well as contribute to disease [5]. It is possible that a defect in B cell development might explain the presence of autoantibodies long (decades) before the disease onset both in RA and SLE [8, 9].

Memory B cells

Activation of naïve B cells under either T cell dependent or independent conditions can result in memory B cell formation (Fig. 1), which in general ensures that subsequent encounter with the same pathogen results in a faster antibody response. Formation of memory B cells is one of the keys to acquired immunity and the ultimate goal of vaccination, but at the same time it potentially maintains autoimmunity. Over the last decades, the complexity of the

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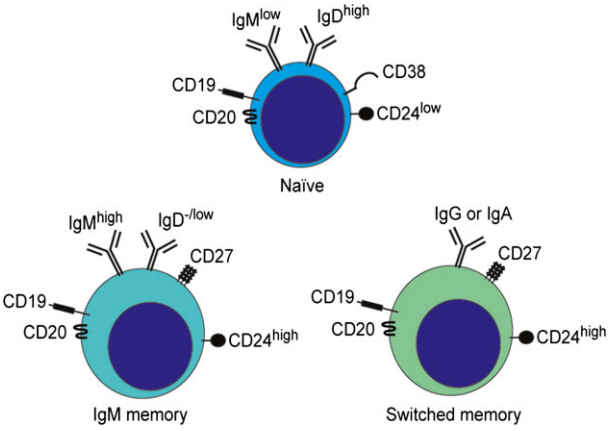

Figure 1 Human B cell subsets. Markers typical of naïve, IgM memory and switched memory B cells. The presence and surface levels of the depicted and additional markers are further described in table 1.

memory B cell compartment has grown, and previously unknown subsets have been uncovered [10–12]. Because of the difficulties to perform mechanistic studies on immunological memory in humans, most of the current knowledge derives from studies in mouse models [13–16]. These studies have defined memory B cells in spleen, lymph nodes and gut-associated lymphoid tissues but rarely in blood because of the limited volume. By contrast, most studies in humans investigate B cells in peripheral blood, as it is more difficult to obtain other tissue samples e.g. lymphoid tissue. Because the B cells in peripheral blood represent only a small fraction (<5%) of the total B cell population in the body, the information it provides may not be complete [17].

In humans, CD27 has long been used as a marker for memory B cells, although it is not a prerequisite. Moreover, class switch recombination (CSR) and somatic hypermutation (SHM) were also considered a clear sign of memory B cells, although this is not an absolute requirement as there are IgM memory B cells without SHM in their BCRs [10, 18]. Today, most studies identify memory B cells based on the presence or absence of a combination of markers including CD27/IgD/CD24/CD38 [12, 19], as well as BCR modification, i.e. SHM and CSR (Fig. 1) (Table 1). In this review, B cells that have undergone CSR are hereafter referred to as switched memory B cells.

Complement receptor type 2, CD21

Complement receptor type 2 (CR2, CD21) is a 145-kD glycosylated single polypeptide chain consisting of a long extracellular domain of 15–16 short consensus repeat sequences [20–22], a transmembrane region and a short cytoplasmic domain [21]. The receptor is expressed on B cells [23, 24], follicular dendritic cells, thymocytes and a subset of peripheral T cells [25, 26]. CD21 binds complement fragments C3d, C3dg and iC3b that are covalently bound to target antigens [27, 28]. In addition, CD21 has been proposed to bind ligands such as the Epstein–Barr virus (EBV) envelope glycoprotein gp350/220 [29], the low affinity Fc-receptor for IgE (FcεRII or CD23) [30], the cytokine interferon-alpha (IFN-α) [31].

On B cells, CD21 forms a complex together with CD19 and CD81 (TAPA-1) [32, 33], which functions as a co-receptor to the BCR. Upon simultaneous binding of complement-tagged antigens by CD21 and the BCR the threshold for B cell activation is reduced [27, 34]. Under these circumstances, CD21 acts as a bridge between the co-receptor complex and the BCR (Fig. 2). This co-ligation induces the phosphorylation of the cytoplasmic tail of CD19 by BCR-associated tyrosine kinases and amplifies downstream signalling [28, 35].

CD21 exists also in a soluble form as the receptor can be shed from the plasma membrane by proteolytic cleavage in the short consensus repeat 16 [36–38]. It has been shown that CD21 is constantly shed from peripheral blood B cells [39, 40], and that the levels in blood are increased after B cell activation [41]. The amounts of soluble CD21 in blood are also increased in diseases such as common variable immunodeficiency (CVID), B cell chronic lymphocytic leukaemia, and malignancies associated with EBV [42],

Table 1 Cell surface markers on different B cell subsets.

	Surface markers and expression levels								Surface BCR		
B cell subset	CD21	CD19	CD20	CD10	CD27	CD24	CD38	CD138	IgM	IgD	IgG/A
Transitional 1	−/low	+	+++	+	−	+++	+++	−	+++	+	−
Transitional 2	+	+	+++	Low	−	++	++	−	++	++	−
Transitional 3	+	+	+	−/+	−	+	+	−	+	++	−
Naïve	+	+	+	−	−	+	+	−	+	++	−
IgM memory	+	+	+	−	+	++	−/+	−	+++	+	−
Switched memory	+	+	+	−	+	++	−/+	−	−	−	+
Plasmablast	−/low	Low	−	−	+++	−	+++	+	−	−	−
Plasma cell	−	−	−	−	+++	−	+++	+++	−	−	−

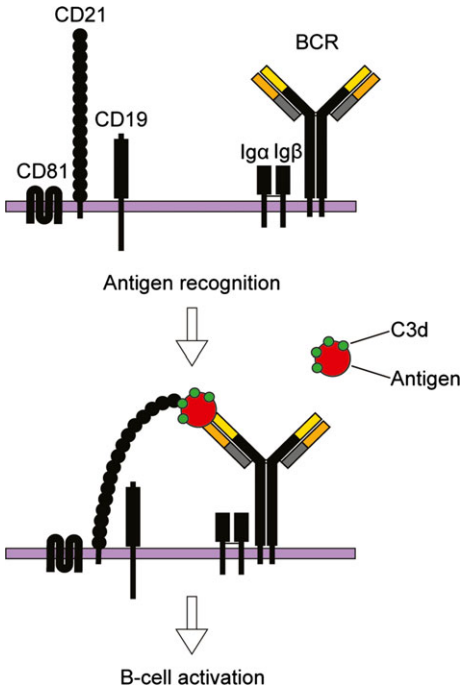

Figure 2 The BCR and its co-receptor. (Upper) The co-receptor complex, CD81, CD21 and CD19, and the membrane bound BCR together with its signal transduction molecules Igα/Igβ. (Lower) Upon simultaneous binding of complement-tagged antigens by CD21 and the BCR, the threshold for B cell activation is reduced. Under these circumstances, CD21 acts as a bridge between the co-receptor complex and the BCR. The opsonizing complement fragments are C3d, C3dg or iC3b.

whereas the levels are decreased in autoimmune diseases [43, 44].

CD21 expression on B cell subsets in peripheral blood

The surface levels of CD21 on B cells vary depending on the maturation stage of the cell (Table 1). Early transitional (T1) B cells express low levels [45, 46], the majority of circulating B cells including naïve and memory cells express CD21, whereas plasmablasts and plasma cells lack or express low levels [45, 47]. Moreover, the activation of B cells leads to reduced surface levels of CD21 [41]. In addition, a CD21$^{-/low}$ B cell subset has been described that is distinct from transitional B cells and plasmablasts/cells, as will be discussed below (Table 2).

CD21$^{-/low}$ B cells in health and disease

In health

Tonsils are constantly exposed to different microbes that we inhale or ingest. The immune cells of the tonsils therefore differ from those in peripheral blood as the tonsillar lymphocytes are constantly exposed to antigens and become activated. A decade ago a CD21$^{-/low}$ B cell subset was described as a novel CD27^{-} memory B cell subset that expressed the inhibitory receptor, Fc receptor-like 4, FcRL4 (FcRH4), and it was found in tonsils [48, 49]. These cells were defined as memory B cells because most were switched and their BCRs contained SHM (Table 2). The cells were large in size but were not plasmablasts/cells supported by the lack of Blimp-1 and other factors typical of terminal differentiation. However, they expressed surface activation markers such as CD69, CD80 and CD86, and the mRNA levels encoding the homing receptors CCR1, CCR5, CCR6 were elevated, whereas those encoding CXCR4 and CXCR5 were reduced. *In vitro* the cells showed poor proliferative capacity to BCR stimulation but responded to a combination of IL-2, IL-10 and CD40L. The latter also resulted in antibody secretion. These results suggested that the FcRL4^{+}CD21$^{-/low}$ B cells in tonsils are memory B cells homing to inflammatory sites, and that these cells are primed to respond to T cell help by mainly differentiating into antibody-secreting cells.

Although CD21$^{-/low}$ B cells have been extensively studied in several diseases there is relatively sparse data on CD21$^{-/low}$ B cells in peripheral blood from healthy individuals. Nevertheless, when CD21$^{-/low}$CD27^{-} B cells in peripheral blood from patients with autoimmune disease were compared to those from healthy individuals the results suggested that the CD21$^{-/low}$CD27^{-} B cells in healthy individuals consist of a mixture of naïve and memory B cells [50].

Human immunodeficiency virus infection

The human immunodeficiency virus (HIV) infects and depletes CD4^{+} immune cells, which, in the absence of treatment, leads to immunodeficiency and uncontrolled viral replication. Although HIV infects CD4^{+} cells, B cells are also severely hampered by the infection. This is evident in changed frequencies of different B cell subsets in peripheral blood. In addition, there is an increase in a CD21$^{-/low}$ B cell subset that correlates to a certain extent with the viral load [51, 52]. The CD21$^{-/low}$ subset contained both CD27^{+} and CD27^{-} memory B cells that also expressed FcRL4. The CD21$^{-/low}$CD27^{-} B cells were enriched for HIV specificity and were either IgM or switched with their BCRs showing signs of SHM. They also expressed similar homing receptors as the CD21$^{-/low}$ FcRL4^{+} memory B cell subset in tonsillar tissue and were therefore called tissue-like memory B cells [53]. The CD21$^{-/low}$ B cells in HIV viremia showed a reduced proliferation capacity in response to stimulation with different combinations of BCR agonist, T cell help (CD40L), toll-like receptor (TLR) 9 agonist, IL-2 and IL-10. However, they secreted antibodies in response to polyclonal activation. In HIV viremic individuals, the T cells upregulate inhibitory receptors and show reduced

Table 2 Definition and characterisation of CD21$^{-/low}$ B cell subsets in different conditions.

Tissue/Disease	Definition of the CD21$^{-/low}$ subset	Additional markers[a]	BCR	SHM	Reactivity	BCR stimulation[b]	Other stimulation[c]
Tonsils from healthy [48]	FcRL4^{+}	CD27^{-} CD20^{hi} CD138^{-} CD69^{+} CD80^{+} CD86^{+}*CCR1*↑ *CCR5*↑	IgG	+	N/A	–	+
HIV [53]	CD20^{hi}CD27^{-}	FCRL4^{+} CD11c^{hi} CXCR3^{hi} CXCR4^{low} CXCR5^{low} CCR7^{low}	IgM/IgG	+	HIV	–	+
HCV-MC [55–58]	IgM^{+}CD27^{+}	FcRL4^{hi} CD11c^{+} CD10^{-} CD20^{hi} CD22^{+} CD38^{-} CD86^{+} CD95^{+} CXCR3^{hi} CCR7^{low} CXCR5^{low}	IgM	+	HCV/Rheumatoid factor	–	+
Malaria [63,64,66]	CD27^{-}	FcRL4$^{?}$ CD11c↑ CD24↓ CD86↑ CD138^{-} CXCR3↑ CXCR4↓ CXCR5↓ CCR7↓	IgG	+	*P. falciparum*/ Polyreactive	–	–
CVID [72]		CD27^{low} FcRL4^{+} CD11c^{+} CD24↓ CD38↓ CD86↑ CD95^{hi} CXCR3↑ CXCR5↓ CXCR6↑ CCR7↓	IgM	–	Auto/polyreactive	–	+
CVID and RA [50]	CD27^{-}	CD11c^{+} CD10^{-} CD38^{-} CD72^{hi} CD86^{+} CD95^{+} CXCR4^{low} CCR7$^{-/low}$	IgM	–	Auto/polyreactive	–	–
RA [75]	CD11c^{+}	CD27^{hi} CD20^{hi} CD38^{low} CD80^{hi} CD86^{hi}	IgG	N/A	N/A	N/A	N/A
SLE [79]	CD19^{hi}	CD27^{int} CD10^{-} CD38^{-} CD86↑ CD95↑ CXCR4^{-} CXCR5↓ CXCR6↑	IgM/IgG	N/A	N/A	N/A	N/A
Sjögren's syndrome [80]	CD27^{-}	CD11c↑ CD10^{-} CD20↑ CD22↑ CD38^{low} CD69↑ CD72↑ CD86↑ CD95↑	IgM	+	Auto/polyreactive	–	+

[a]↑ upregulated ↓ downregulated; protein or *mRNA* expression.
[b]Response (proliferation and/or differentiation).
[c]Response (proliferation and/or differentiation) to e.g. different combinations of TLRs, CD40L (T cell help) and cytokines.
N/A, not assessed.

proliferative capacity, and are therefore referred to as exhausted [54]. By analogy, the CD21$^{-/low}$ B cells in HIV viremia are referred to as an exhausted cell population [53].

Hepatitis C virus-associated mixed cryoglobulinemia

Hepatitis C virus (HCV) is a leading cause of chronic liver disease and cirrhosis in the world. In a subgroup of patients it gives rise to autoantibody production and mixed cryoglobulinemia (HCV-MC), which is a non-malignant lymphoproliferative disorder. In patients with HCV-MC type II there is an expansion of CD21$^{-/low}$ B cells characterized as IgM^{+}IgD^{low}CD27^{+} that also express FcRL4 and CD11c [55], and a similar pattern of homing receptors to the tonsillar FcRL4+ memory B cells. In HCV-MC, the CD21$^{-/low}$ B cells showed an impaired proliferation capacity in response to BCR triggering [56], which could be overcome by stimulation with combinations of BCR, CD40L (T cell help), TLR9, IL-2 and IL-10 [57, 58]. These cells represented a clonally expanded population and used a particular combination of Ig heavy and light chain genes, V_H1–69 and V_κ3, which encode an autoantibody typically found in RA patients, rheumatoid factor that recognizes IgG. This autoantibody also reacts with the HCV [59–61]. By analogy to the HIV viremia the CD21$^{-/low}$ B cells in HCV-MC have been referred to as an exhausted B cell population [56, 62].

Malaria

Malaria is caused by mosquito-borne parasites, *Plasmodium* species, which first infect the liver and then enter the red blood cells. The parasite replicates inside the red blood cells, which rupture and can cause kidney as well as liver and brain damage. Antibodies are important in controlling the blood-stage of the infection; however, it takes many years to acquire antibody-mediated immunity and, in addition, it is often short-lived [63, 64]. This may be due to B cell functions being altered and to the genetic diversity of the parasite that allows it to escape humoral immunity. In individuals exposed to malaria an increased frequency of a CD21$^{-/low}$ B cell subset has been described [63, 65, 66]. These cells have been characterized as CD27^{-}, whereas it is unclear whether they express FcRL4 [64, 66–68]. The CD21$^{-/low}$ B cells also expressed increased levels of CD11c and a pattern of homing receptors indicating migration to inflammatory sites [63]. The CD21$^{-/low}$ B cells were mainly IgG^{+} and these BCRs contained SHM. Cloning and expression of the IgGs demonstrated that they recognized *Plasmodium* antigens and many were also polyreactive [63, 64, 66, 69]. The proliferative capacity of the CD21$^{-/low}$ B cells was poor when stimulated with anti-Ig or a combination of anti-Ig, anti-CD40, cytokines and TLR9 agonist, and they did not differentiate into antibody-secreting cells upon stimulation with a

combination of mitogens and cytokines [66]. In malaria the CD21$^{-/low}$ B cells have been referred to as an atypical memory subset.

Common variable immunodeficiency

Common variable immunodeficiency is a primary immunodeficiency and comprises a very heterogeneous group of patients where the underlying genetic defect is known in 10–20% of cases. Most patients are characterized by B cell dysfunctions such as hypogammaglobulinemia and a reduced response to vaccinations. The frequency of CD21$^{-/low}$ B cells is often increased in CVID patients, and in a subgroup of these the CD21$^{-/low}$ B cells constitute more than 20% of the total B cell compartment in peripheral blood. In the Freiburg classification this particular group of CVID patients are classified as type Ia [70]. Patients with CVID are prone to develop autoimmunity in particular haemolytic anaemia and autoimmune thrombocytopenic purpura [71]. In CVID type Ia the CD21$^{-/low}$ B cells expressed FcRL4 and low levels or lacked CD27 [72]. The cells were IgM^{+} that lacked SHM. The CD21$^{-/low}$ B cells exhibited a reduced proliferation capacity after BCR and CD40L (T cell help) stimulation but secreted antibodies upon activation [50, 72]. Moreover the CD21$^{-/low}$ B cell subset was enriched for autoreactivity. The CD21$^{-/low}$ B cells have been described as anergic naïve B cells as well as resembling exhausted tissue-like memory B cells [50, 72–74]. These two conclusions differ markedly as to the origin of the CD21$^{-/low}$ B cells; defective central tolerance would lead to anergic naïve B cells, whereas exhausted memory-like B cells would be a consequence of activation-driven peripheral exhaustion. This will be briefly discussed in the conclusions.

Rheumatoid arthritis

Rheumatoid arthritis is a heterogeneous chronic autoimmune joint disease, which is characterized by synovial inflammation as well as cartilage and bone destruction. The serological hallmark of the disease is production of autoantibodies to IgG (rheumatoid factor) and to citrullinated proteins (ACPAs), although about a third of RA patients do not produce any of these autoantibodies. The discovery of ACPA and the beneficial treatment effects of B cell deletion have lead to a renewed interest in B cells as disease mediators. Two CD21$^{-/low}$ B cell subsets have been found expanded in peripheral blood of patients with RA, one that expresses CD27 and another that lacks CD27. The CD27^{+} cells expressed CD11c and activation markers, and were defined as memory B cells as they were switched [75]. The CD27^{-} cells also displayed CD11c and activation markers. However, as these cells expressed unmutated IgM, responded poorly to BCR triggering and were enriched for autoreactivity they were described as anergic naïve B cells [50]. Moreover, in synovial fluid in patients with active disease a CD21$^{-/low}$ B cell subset seems to be a major B cell population [72, 76]. A subset of the synovial fluid B cells expresses FcRL4 and produces the cytokine RANKL that stimulates the differentiation and activation of osteoclasts [77]. The FcRL4^{+} cells expressed lower levels of CD21 than the FcRL4^{-} and were considered memory B cells based on combinations of CD27, IgD and CD38.

Systemic lupus erythematosus

Systemic lupus erythematosus is a heterogeneous systemic autoimmune disease characterized by a variety of abnormalities in the immune system, including immune complexes, leukopenia and autoantibodies recognizing nuclear antigens. The pathogenesis in SLE is complex and involves a multitude of cells and molecules both in the innate and adaptive immune responses. The patients display a reduced number of peripheral blood B cells, especially IgM memory B cells [78]. A CD21$^{-/low}$CD19^{high} B cell subset is expanded and it contains a mixture of switched and IgM^{+} B cells with low levels of CD27 [43]. The CD21$^{-/low}$ B cells expressed increased surface levels of activation markers and a pattern of markers indicating homing to inflammatory sites [79]. To our knowledge, the expression of FcRL4 on the CD21$^{-/low}$ B cells or their response to BCR stimulation in SLE have not been addressed, hence it is unclear whether this subset shares additional characteristics with the CD21$^{-/low}$ B cells described in other conditions.

Sjögren's syndrome

Sjögren's syndrome is a systemic autoimmune disease that targets primarily the exocrine salivary and lacrimal glands with a pronounced infiltration of lymphocytes, particularly B cells and plasma cells. In addition, extraglandular manifestations can occur in almost any organ, and there is also an increased risk (5%) for lymphoproliferative disease that is connected with disease activity. Patients with Sjögren's syndrome with lymphoproliferative disease have a significantly higher frequency of CD21$^{-/low}$ B cells in peripheral blood than those without lymphoproliferative disease and healthy controls [80]. The CD21$^{-/low}$ B cells, defined as CD27^{-}, expressed CD11c and activation markers. They were mainly IgM^{+} and based on SHM in their BCRs defined as memory B cells. This subset showed decreased proliferative capacity upon BCR triggering with and without TLR9 stimulations, and was enriched for autoreactivity.

Conclusions

The diseases discussed herein all have in common chronic immune stimulation where a CD21$^{-/low}$ subset is enriched

in relation to the total B cell population. Some of these diseases are associated with lymphocytopenia, e.g. SLE, whereas others are not, e.g. HCV-MC, suggesting that lymphocytopenia is not a requirement for the enrichment. It is, however, unclear whether the enrichment is due to an increase in absolute CD21$^{-/low}$ B cell numbers.

It is thus evident that a CD21$^{-/low}$ B cell subset is enriched in peripheral blood under many different conditions but are they the same cells? At a first glance they would appear not to be the same as they express different BCR isotypes, and these can be either mutated or unmutated. They also differ in their expression of CD27 though in some disorders they were selected based on being CD27^{+} or CD27^{-} (Table 2). Despite these differences there are several similarities e.g. expression of FcRL4, CD11c, activation and homing markers, the latter indicating migration to inflammatory sites e.g. low levels of CXCR4, CXCR5 and CCR7 but high levels of CXCR3 and CXCR6. This homing pattern is consistent with the presence of CD21$^{-/low}$ B cells in tonsils in healthy individuals, joints in RA patients and lungs in CVID patients but absence in non-inflamed peripheral lymphoid tissues e.g. spleen. Further, the proliferative capacity and antibody production by the CD21$^{-/low}$ B cells is poor in response to BCR stimulation, whereas in most disorders the cells respond to combinations of e.g. TLRs, CD40L (T cell help) and cytokines. Although it is not possible to determine whether the CD21$^{-/low}$ B cell subset are the same in these diseases, in most this subset seems to consist of memory B cells as they express either mutated and/or switched BCRs.

In CVID an unmutated IgM^{+}CD21$^{-/low}$ B cell subset has been described as an exhausted memory B cell population, and as a result of chronic activation. However, a similar unmutated IgM^{+}CD27^{-}CD21$^{-/low}$ B cell subset has also been described in CVID (and RA) but defined as a naïve anergic population. Here, the anergic state is a result of altered receptor editing and defective central B cell tolerance. Both notions are experimentally supported. One possible explanation for this discrepancy could be that the CD21$^{-/low}$ B cells consist of more than one subset, and another could be variation among patients.

In HIV, HCV-MC and malaria infections, the antibodies expressed by the CD21$^{-/low}$ B cells recognize viral/parasite antigens and hence, the infecting agent might drive the expansion of this B cell subset. In CVID and Sjögren's the antibodies produced by the CD21$^{-/low}$ B cells recognize e.g. nuclear antigens, autoantibodies known to be present in the serum of some of these patients. This could, therefore, indicate that in these disorders autoantigen drives expansion of this B cell subset. In for instance HIV and CVID, the CD21$^{-/low}$ B cells were shown to have undergone cell division supporting that chronic activation by disease-specific antigens may lead to an expanded CD21$^{-/low}$ B cell memory subset.

The CD21$^{-/low}$ B cells appear to be in an activated state but at the same time express several inhibitory receptors. They respond poorly to and are prone to undergo apoptosis upon BCR stimulation. However, the cells are able to respond to other stimuli and then mainly by differentiating into plasma cells. This could be an indication that the CD21$^{-/low}$ B cells interact with other cells e.g. other lymphocyte subsets and/or innate immune cells, either through cell–cell contact or in response to cytokines. Such interactions could potentially also lead to cytokine production by the CD21$^{-/low}$ B cells. However, whether the response of the CD21$^{-/low}$ B cells to such stimuli is beneficial or detrimental to the individual is currently unclear. In order to better understand the role and nature of this unique CD21$^{-/low}$ B cell subset in health and disease further investigations are needed.

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