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Therapeutic Targeting of B Cells for Rheumatic Autoimmune Diseases

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Abstract——**Autoreactive B cells are characterized by their ability to secrete autoantibodies directed against self-peptides. During the last decade, it has become increasingly apparent that B lymphocytes not only produce autoantibodies but also exert important regulatory roles independent of their function as antibody-producing cells. This is especially relevant in the context of autoimmunity, because autoreactive B cells have been shown to possess the ability to activate pathogenic T cells, to produce pro-inflammatory cytokines, and to promote the formation of tertiary lymphoid tissue in target organs. The production of monoclonal antibodies against B-cell-surface molecules has facilitated the characterization of several distinct B lymphocyte subsets. These cell-surface molecules have not only served as useful cell differentiation markers but have also helped to unravel the important biological functions of these cells. Some of these molecules, all of which are expressed on the cell surface, have proven to be effective therapeutic targets. In both animal models and in clinical assays, the efficient elimination of B lymphocytes has been shown to be useful in the treatment of rheumatoid arthritis and other autoimmune diseases. The treatment of most rheumatic autoimmune diseases relies mainly on the use of cytotoxic immunosuppressants and corticosteroids. Although this has resulted in improved disease survival, patients may nonetheless suffer severe adverse events and, in some cases, their relapse rate remains high. The increasing need for safer and more effective drugs along with burgeoning new insights into the pathogenesis of these disorders has fueled interest in biological agents; clinical trials involving the B-cell depletion agent rituximab have been especially promising. This article reviews the current knowledge of B-cell biology and pathogenesis as well as the modern therapeutic approaches for rheumatic autoimmune diseases focusing in particular on the targeting of B-cell-specific surface molecules and on the blocking of B-cell activation and survival.**

I. Introduction

B lymphocytes are cells that express cell-surface immunoglobulin (Ig) receptors capable of recognizing antigens (Ags). One of the major roles of these cells is to generate antibody-secreting plasma cells and memory B cells in response to specific Ags. Secreted antibodies are the principal molecules involved in humoral immunity, with the capacity not only to neutralize pathogens and pathogen toxins, but also to facilitate their elimination by activating several effector mechanisms, such as phagocytosis or complement system proteins.

The discovery of B lymphocytes during the 1960s and 1970s, based on experimental animal models and clinical evaluation of patients with immune deficiency diseases, demonstrated that a subset of cells derived from the bone marrow was responsible for mediating antibody production. It is well known that alterations in B-lymphocyte development can give rise to certain types of primary immunodeficiencies, leukemias, lymphomas,

[1]Abbreviations: AAV, anti-neutrophil cytoplasmic antibody-associated vasculitis; Ab, antibody; ACR, American College of Rheumatology; AE, adverse event; Ag, antigen; ANA, antinuclear antibody; ANCA, anti-neutrophil cytoplasmic antibody; APRIL, a proliferation-inducing ligand; BAFF, B-cell activating factor; BCR, B-cell receptor; BILAG, British Isles Lupus Assessment Group; BLyS, B-lymphocyte stimulator; Breg, B cells with suppressor or regulatory function; CCP, cyclic citrullinated peptide; 95% CI, 95% confidence interval; CNS, central nervous system; CSF, colony-stimulating factor; DAS, disease activity score; DMARD, disease-modifying antirheumatic drug; dsDNA, double-stranded DNA; ESR, erythrocyte sedimentation rate; EULAR, European League Against Rheumatism; FNGN, focal necrotizing glomerulonephritis; FO, follicular; GC, germinal center; GPI, 6-phosphate isomerase; HACA, human anti-chimeric antibody; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; IRR, infusion-related reaction; JCV, polyomavirus JC; LAMP-2, lysosomal membrane protein 2; LPS, lipopolysaccharide; mAb, monoclonal antibody; MC, mixed cryoglobulinemia; MHC, major histocompatibility complex; MPO, myeloperoxidase; mRSS, modified Rodnan skin score; MTX, methotrexate; MZ, marginal zone; NOD, nonobese diabetic; NZB, New Zealand black; PBO, placebo; PML, progressive multifocal leukoencephalopathy; PR3, proteinase 3; RA, rheumatoid arthritis; RAG, recombination-activating gene; RCT, randomized controlled trial; RF, rheumatoid factor; RTX, rituximab; SF-36, Short Form-36; SLE, systemic lupus erythematosus; SRI, SLE responder index; SS, Sjögren's syndrome; SSc, systemic sclerosis; TFH, follicular helper T cell; TGF, tumor growth factor; Th, T helper; TI, T-independent; TNF, tumor necrosis factor; VAS, visual analog scale; WG, Wegener's granulomatosis.

and autoimmune diseases (Küppers et al., 1999). During the last decade it has become increasingly apparent that B lymphocytes exert important regulatory roles independent of their function as antibody-producing cells. These roles include efficiently presenting Ag to the T lymphocytes, secreting cytokines and chemokines, and inducing lymphoneogenesis (Jacob and Stohl, 2010). Thus, B lymphocytes have emerged as cells that, in addition to producing antibodies, carry out important immunoregulatory functions (Dörner et al., 2009b).

B lymphocytes are phenotypic and functionally heterogeneous (Jackson et al., 2008). The production of monoclonal antibodies (mAbs) against B-cell surface molecules, together with the advent of multicolor flow cytometric analysis and functional studies, has facilitated the characterization of several distinct B-lymphocyte subsets (Table 1, Fig. 1). These cell-surface molecules have not only served as useful markers for identifying B-cell differentiation subsets but have also been helpful in unraveling the important biological functions of these cells. Most of these molecules regulate B-cell development and function, facilitate communication with the extracellular environment, or modulate signals triggered by Ag-specific binding to cell-surface Ig (BCR). Other molecules that populate the cell surface of B cells, and that are not B-cell restricted, can also play key roles in their functionality [e.g., MHC class II molecules, CD40, and B-cell activating factor (BAFF) receptors or Toll-like receptors]. CD27 and CD38 molecules have also proven essential in defining several B-cell subsets (Figs. 1 and 2).

Moreover, some of these molecules, all of which are expressed on the cell surface, have proven to be effective therapeutic targets for the treatment of B-cell malignancies (Carter, 2006). In both animal models and clinical essays, the efficient elimination of B lymphocytes has been shown to be useful in the treatment of rheumatoid arthritis (RA) and other autoimmune diseases (Dörner et al., 2009a).

To date, the treatment of most rheumatic autoimmune diseases including RA, systemic lupus erythematosus (SLE), progressive systemic sclerosis (SSc), anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, primary Sjögren's syndrome (SS), and inflammatory myopathies has largely relied on cytotoxic immunosuppressants and corticosteroids. Although this has resulted in improved disease survival, these patients may still suffer severe adverse events and, in some instances, the disease relapse rate is high, whereas other cases remain refractory to conventional immunosuppressive therapies. The increasing need for safer and more effective drugs along with burgeoning new insights into the pathogenesis of these disorders has prompted interest in biological agents; clinical trials involving the chimeric monoclonal antibody rituximab (RTX) have raised high expectations. In fact, this anti-CD20 agent has shown efficacy in patients with RA who fail to respond to anti-tumor necrosis factor α (anti-TNFα) agents. Furthermore, RTX has proven useful in open trials in those patients with SLE who are refractory to conventional immunosuppressants. However, recent methodologically controversial randomized controlled trials have failed to demonstrate its superiority against conventional treatments. B-cell depleting therapy with RTX offers a similarly promising treatment for the other rheumatic autoimmune diseases mentioned above.

This article reviews the current knowledge of B-cell biology and pathogenesis as well as the modern therapeutic approaches for rheumatic autoimmune diseases, focusing in particular on the targeting of B-cell-specific surface molecules and on the blocking of B-cell activation and survival.

II. B-Cell Biology

A. B-Cell Development

From the very onset of life, B cells continuously develop from hematopoietic stem cells (CD34+CD19−) in the bone marrow through a series of precursor stages during which they randomly rearrange their Ig genes to generate Ag-specific B-cell receptors (BCRs) capable of recognizing a wide variety of Ags (Tonegawa, 1983) (Fig. 1). During their maturation B cells undergo two key processes: the generation of functional Ag-specific receptors, and the selection of lymphocytes that express useful Ag receptors. B-lymphocyte development, which takes 2 to 3 days, requires the concerted action of a network of cytokines and transcription factors that positively and negatively regulate gene expression (Melchers, 2005). Bone marrow stromal cells produce IL-7, which both determines Ig gene rearrangements and promotes the proliferation and survival of B precursor cells. Although IL-7 is absolutely essential for mouse

TABLE 1
Cell-surface CD molecules preferentially expressed on B cells

CD Name	Alternative Name	Gene Family	Expression	Function
CD19	B4	Ig superfamily	All B cells	Regulates B-cell signaling
CD20	B1	Tetraspan family	Mature B cells	Membrane-embedded Ca^{2+} channel
CD21	CR2	Complement receptor family	Mature B cells and FDC	Acts as a complement receptor (C3d)
CD22	BL-CAM	Ig superfamily	Mature B cells	Lectin-like adhesion molecule
CD23	FcεR	C-type lectin	Activated B cells and FDC	Low-affinity IgE receptor
CD79a,b	Ig α/β	Ig superfamily	Ig + B cells	Mediates Ig signaling
CD267	TACI	TNFR superfamily	Subset of mature B cells	Cell-survival receptor
CD269	BCMA	TNFR superfamily	Mature B cells and plasma cells	Cell-survival receptor
CD307	FCRL5	Ig superfamily	Mature B cells	Regulates B-cell activation

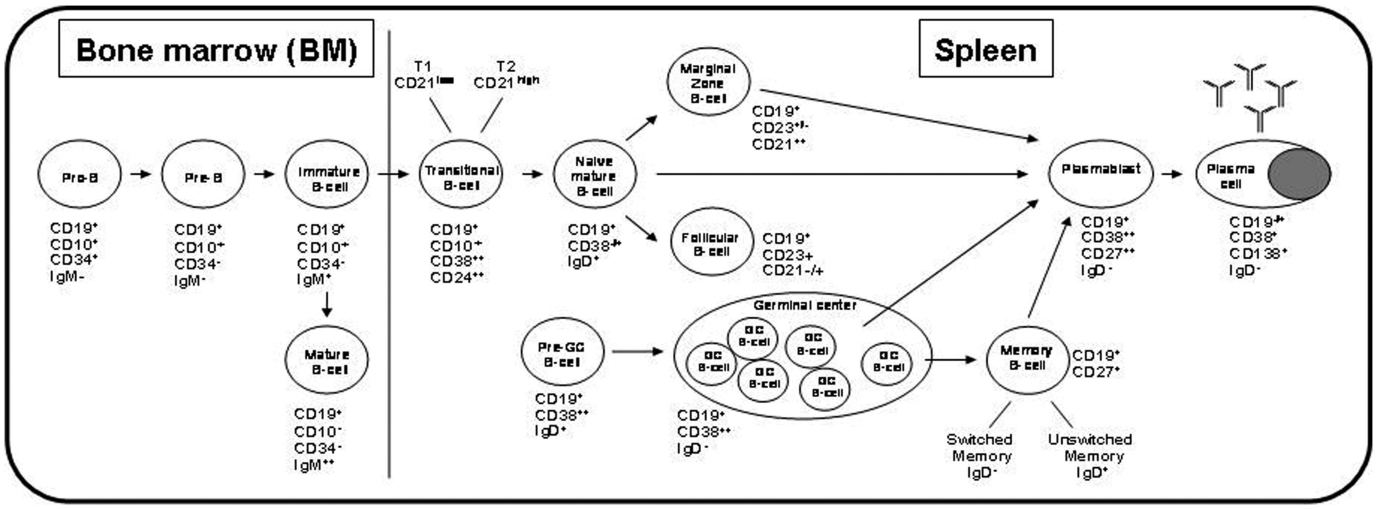

FIG. 1. Cell-surface expression during B-cell development. The differential expression of several cell-surface markers allows for a precise dissection of B-cell differentiation and subsets from the pro-B cells to the plasma cells.

B-cell development, this cytokine in not required by these cells in humans (Bertrand et al., 2000). Several transcription factors regulate the early stages of B-cell development, EA2, EBF, and Pax5 being particularly important in promoting B-cell lineage commitment and differentiation (Bartholdy and Matthias, 2004).

The wide variety of Ag receptors results from a combination of different variable (V) region gene segments with diversity (D) and/or joining (J) gene segments and by the addition or removal of nucleotides at the junctions of the V, D, and J segments (Jung et al., 2006). B-cell maturation occurs in stages characterized by successive rearrangements and expressions of IgH and IgL gene segments. The human B-cell developmental program includes the expression of CD19, the regulated expression of recombination-activating genes (RAG-1, RAG-2), and terminal deoxynucleotidyl transferase. In the earliest B-cell lineage committed precursors (pro-B cells) Ig gene genes exist in a germline configuration. During the differentiation of pro-B (CD34+CD19+) cells into pre-B (CD34−CD19+) cells, a rearrangement in the heavy chain locus starts via a somatic recombination of the D_H to J_H (IgH diversity segment to IgH joining segment), then of the V_H to DJ_H. Once these rearrangements are successfully completed, transcription begins and a mature mRNA encoding the μ heavy chain is produced, which accumulates in the cytoplasm. A part of this μ chain associates with the invariable surrogate light chains (λ5 and Vpre-B9) to form the pre-B B-cell receptors (Mårtensson et al., 2007). The expression of this receptor on the cell surface delivers survival and proliferation signals, thereby inducing the rearrangements in the light chains. In immature B cells, the heavy and light chains are thus assembled and IgM molecules are expressed on the cell surface. Allelic exclusion guarantees that all Igs molecules expressed by a single B-cell will recognize a specific Ag (Mostoslavsky et al., 2004).

The immune system has to discriminate between B lymphocytes capable of producing protective antibodies directed against pathogens versus those that produce harmful autoantibodies (Burnet, 1957). Therefore, immature B lymphocytes are selected against the high-affinity recognition of self-Ags to prevent the development of autoimmune diseases. This process is known as negative selection and involves either 1) the elimination of developing lymphocytes in which Ag receptors bind to self-Ags or 2) the editing of their receptor genes (von Boehmer and Melchers, 2010). Immature B cells exit the bone marrow and enter into the blood to complete their maturation program in secondary lymphoid tissues, preferentially in the spleen. These immature B cells are known as transitional T1 B cells (IgM+CD10+) (Fig. 1). Next, cells enter the follicles and acquire cell-surface IgD and CD23, as well as the ability to recirculate. Nevertheless, they still carry markers of immaturity and are thus known as transitional T2 cells (IgM+, IgD+, CD10+) (Sims et al., 2005). Transitional T2 cells then finalize their maturation process and give rise to mature naive B cells (IgM+IgD+CD10−) (Fig. 1).

In this way, bone marrow precursors that were initially Ig-negative develop into mature B cells that can now be induced to proliferate and differentiate in antibody-producing plasma cells and memory B cells after encounters with Ag.

B. B-Cell Activation and Maturation of Conventional B Cells

As described above, B cells originate in the adult bone marrow, where the Ag receptor repertoire is generated randomly and independently of the pathogens. Once the immature B cells leave the bone marrow for the periphery and undergo the aforementioned transitional stages, they develop either into marginal zone B cells or into follicular B cells (Figs. 1 and 2) (Pillai and Cariappa, 2009). Most mature B cells coexpress IgM and IgD, as

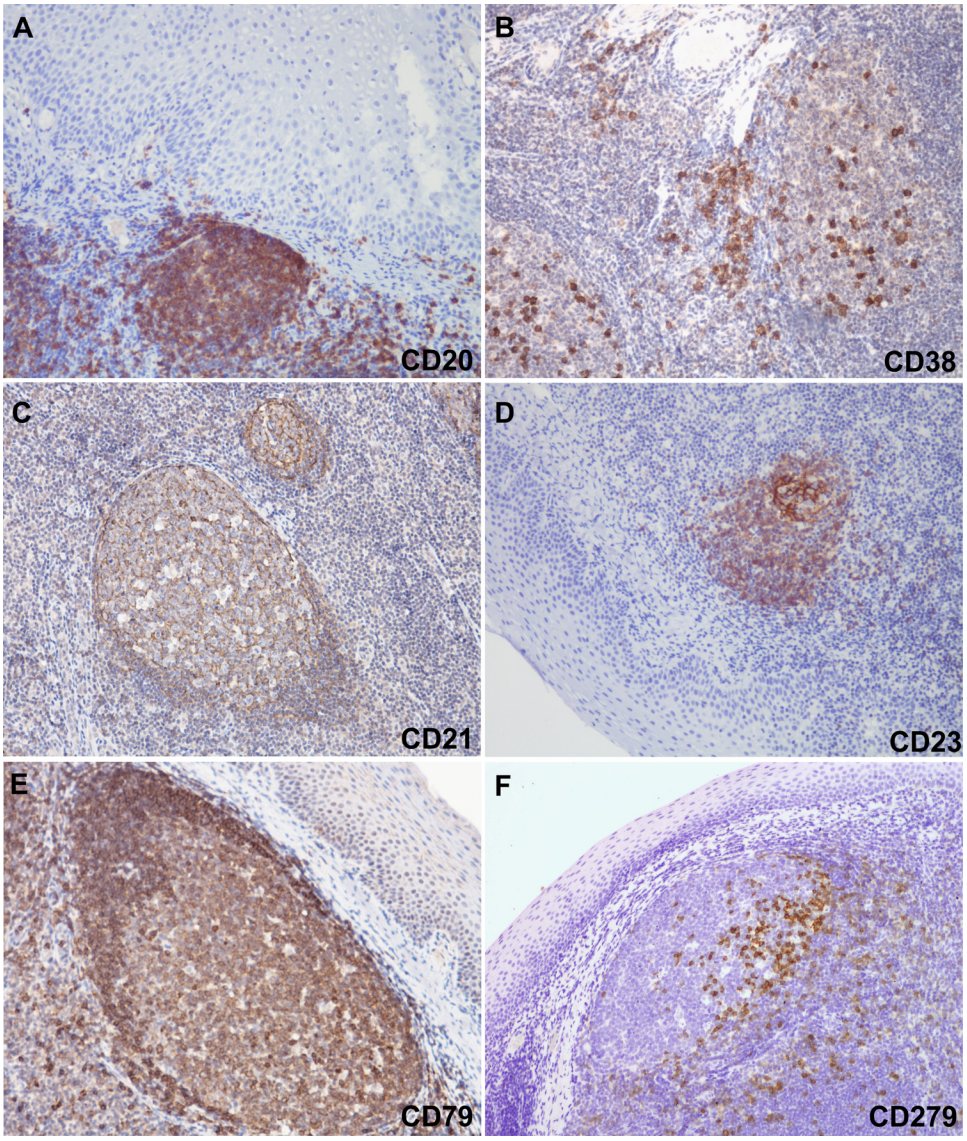

FIG. 2. Tissue expression of cell-surface molecules. Paraffin-embedded tonsil sections stained with mAbs against cell-surface molecules. A, pan B-cell marker, CD20; B, B-cell activation marker, CD38; C, resting B-cell and follicular dendritic cell marker, CD21; D, B-cell activation marker, CD23; E, pan B-cell marker, CD79b; and F, follicular helper T-cell marker CD279 (PD-1). Magnification, 25×.

well as the B-cell-specific markers CD19 and CD20 on the cell surface. These mature B cells can now be selected by specific Ags for activation and clonal expansion. Ag-induced B-cell activation and differentiation takes place in secondary lymphoid tissues. Both protein and nonprotein Ags (e.g., lipids, sugars, or DNA) can induce B-cell activation. B-cell responses to proteins require the participation of T helper cells specific for the Ag. T-dependent B-cell responses are initiated at the edges of the follicles where B cells present Ag to T cells. Helper T cells that specifically recognize the Ag peptides associated with MHC class II molecules are then activated. The combination of signals produced by the interaction of CD40 expressed on the B cells with CD40L expressed on activated T cells, together with the action of T-cell-secreted cytokines, induces B-cell proliferation and differentiation into short-lived extrafollicular antibody-secreting cells (MacLennan et al., 2003). Some of the activated B cells enter inside the follicles and generate a germinal center (GC) reaction (Fig. 3). The participation of a distinct subset of T cells, known as follicular helper T (TFH) cells, is required for germinal center generation through cognate interaction with GC B cells (McHeyzer-Williams et al., 2009) (Fig. 2). These cells are characterized by the expression of CXCR5, PD-1, and by the secretion of IL-21 (King et al., 2008). Essential to this interaction of T_{FH} cells with GC B cells are a series of ligand-receptor interactions, including those mediated by CD40L/CD40, by the inducible costimulator ligand/inducible costimulator, and by the SLAM family molecules (Vinuesa et al., 2009; Schwartzberg et al., 2009). GC B cells are induced to proliferate and clonally expand and are located in the dark zone and are known as centroblasts (Fig. 3). This clonal expansion process is

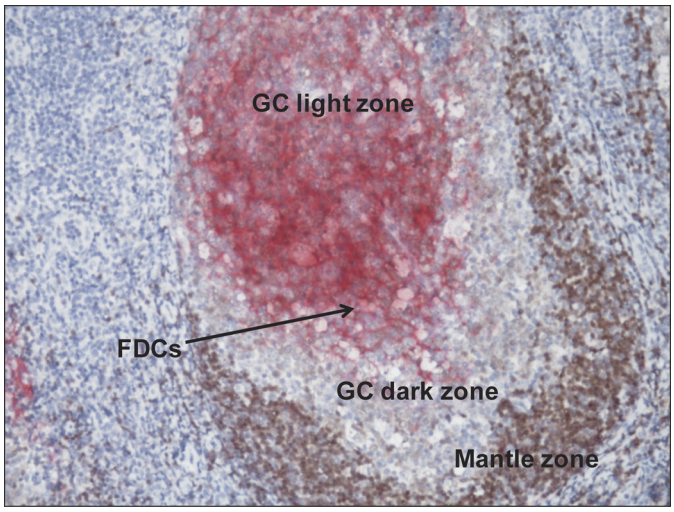

FIG. 3. Germinal center reaction. Double staining of a tonsil secondary follicle; centrocytes and follicular dendritic cells in the germinal center light zone positive for CD23 (red); mantle zone B cells positive for TCL1 (brown); centroblasts in the germinal center dark zone are negative for both markers.

needed to generate a sufficient number of specific cells to mount an efficient immune response, because the number of B lymphocytes specific for a certain pathogen is very low. During the course of a T-dependent response, an increase in the affinity of the produced antibodies can be observed; this is especially true after repeated interactions with the same Ag. This increase in antibody affinity stems from the somatic hypermutation and selection of high avidity cells capable of recognizing the Ag (Neuberger, 2008). This selection process is mediated by contact with the Ag retained on the surface of follicular dendritic cells (Fig. 3). Those follicular dendritic cells that express the complement receptor CD21 (Fig. 2) trap antibody- and complement protein-bound AG, displaying them on their cell surface for recognition by B lymphocytes located in the light zone (Fig. 3). B-cell clones bearing amino acid changes, which lead to increased Ig Ag affinity, will then be selected for differentiation into the plasma cells of memory B cells (Kosco-Vilbois and Scheidegger, 1995).

During the GC reaction, naive Ag-specific B cells mature into either memory B cells or into long-lived Ig-secreting CD138+ plasma cells (Fig. 1). These plasma cells migrate to the extrafollicular regions of lymphoid organs, as well as to the bone marrow, where most of the antibodies found in the blood are produced (Shapiro-Shelef and Calame, 2005). After Ag encounter, some B cells begin to produce antibodies with different Ig heavy chains, which mediate various effector functions, a process known as Ig class switching. Because these two Igs use the same VDJ exons for both the heavy and light chains they exhibit the same antibody specificity (Chaudhuri and Alt, 2004). Upon activation, these cells can produce and secrete different classes of antibodies (IgM, IgG, IgA, IgE, and IgD). The interactions of the various constant regions with specific Ig Fc receptors, or with their complements, confer upon them distinct effector functions (Ravetch and Bolland, 2001). However, the secreted antibodies have the same specificity as those mature cell-surface receptors that recognized the Ag, which first triggered the activation. Both somatic hypermutation and Ig class switching are initiated by a single enzyme: activation-induced cytidine deaminase (Longerich et al., 2006).

Memory B cells (CD19+CD27+) are also capable of living for long periods of time. They recirculate between the peripheral lymphoid tissues, via the blood and lymphoid vessels, to efficiently respond to Ags (Tangye and Tarlinton, 2009).

T-independent Ags, including polysaccharides, glycolipids, and nuclear acids are characterized by their polyvalent nature. Moreover, they can induce B-cell activation by cross-linking multiple surface Ig molecules without the participation of T helper cells. However, these activated B cells are unable to undergo somatic hypermutation, class switch recombination, or memory cell generation, although some exceptions to this rule have recently been reported (Vinuesa et al., 2003).

C. Memory B Cells

Two distinct B-cell populations, long-lived plasma cells and memory cells, which are generated during encounters with Ag, contribute to humoral immunological memory (Sanz et al., 2008; Tangye and Tarlinton, 2009). In humans, memory B cells recirculate through the blood, although their main reservoir is located in lymphoid tissues, such as the spleen (Mamani-Matsuda et al., 2008). Memory B cells can generally persist throughout the life of the host (Crotty et al., 2003). Although the mechanisms responsible for their longevity remain unknown, there is evidence that under steady-state conditions human memory B cells are slowly dividing. This suggests that the memory B-cell pool is maintained by homeostatic proliferation (Lanzavecchia and Sallusto, 2009). Memory B cells are characterized by the expression of the cell-surface marker CD27 (Fig. 1) and somatically mutated Ig V region genes. They also present significantly higher levels of several costimulatory molecules and activation Ags (e.g., CD80, CD86, and CD95) compared with naive B cells (Good et al., 2009). The main functional property of memory B cells is their capacity to rapidly proliferate and differentiate into plasma cells after re-exposure to Ag, which is important for eliminating the pathogens before the onset of clinical disease. This feature, combined with the increased number of these cells after Ag encounter, is key for the enhanced production of those specific antibodies found during the secondary immune responses. Multiple subsets of memory B cells have been identified. Whereas most of the memory B cells express surface IgG or IgA, a significant number of CD27+ memory cells express IgM (Tangye and Good, 2007). In contrast, not all memory B cells express CD27. Some CD27−IgG+ B cells

have been shown to display all the characteristics of classic B memory cells such as mutated Ig V genes and high expression of costimulatory molecules (Fecteau et al., 2006). It is noteworthy that the frequency of this memory subset rises significantly in patients with SLE and correlates with disease activity and titers of anti-DNA antibodies (Wei et al., 2007). Moreover, another CD27− memory B-cell subset has been found that is exclusively present both underneath and within the epithelium of mucosal-associated lymphoid tissues. These memory B cells express the cell-surface inhibitory receptor FCRL4, have distinct protein profiles, and have an impaired capacity to generate plasma cells in culture (Ehrhardt et al., 2003).

The concept that memory B cells are derived only from a T-cell-dependent response has been challenged by the finding that in mice T-cell-independent type II responses to bacterial polysaccharides lead to the expansion of memory B cells with a distinct phenotype (Obukhanych and Nussenzweig, 2006).

D. B-Cell Activation and Maturation of Other B Cells

Although follicular B cells, also known as conventional B cells or B2, represent the vast majority of B cells, other B-cell types have been identified. Among these are MZ cells and B-1 (B1a and B1b) cells. It is generally thought that the B-cell compartment is a representative example of the adaptive branch of immune defense, with mature recirculating B cells having evolved to a point where they are capable of generating a vast receptor repertoire, one that can mount T-dependent B-cell responses with high-affinity and long-term memory cells. Whereas follicular (FO) B cells are involved in responses to T-dependent Ag, B-1a, B-1b, and MZ, B cells are more specialized and respond to T-independent Ag.

T-independent Ags are divided into types I and II. The former include mitogenic stimuli such as lipopolysaccharide (LPS), CpG, or poly-IC that elicit polyclonal B-cell activation via Toll-like receptors, whereas the latter are polysaccharides that engage the B-cell receptor, thereby inducing Ag-specific B-cell responses. TI-2 Ags typically induce splenic MZ B cells to grow as plasmablasts in the extrafollicular foci of the spleen (Zandvoort and Timens, 2002; Viau and Zouali, 2005). T-independent-2 Ag responses are especially important in the defense mechanisms mounted against the capsulated and cell-wall polysaccharides expressed by a number of bacterial pathogens, such as *Streptococcus pneumonia*, *Neisseria meningitis*, and *Haemophilus influenzae* (Martin and Kearney, 2002; Lopes-Carvalho et al., 2005). Because of their anatomical location, B-1 and MZ B cells are the first cell populations to encounter Ags acquired through the gut/peritoneum and blood stream. These two B-cell subsets have evolved to provide a first line of defense against pathogens by responding rapidly and vigorously to Ag stimulation. These data suggest not only that B-1 and MZ B cells are poised to respond to plasma cell differentiation in the microbial environment, but also that TLR agonists are instrumental in stimulating Ab-mediated innate immune protection during microbial infections (Genestier et al., 2007).

1. Marginal Zone B Lymphocytes. Although MZ B cells, which represent 5 to 10% of spleen cells, seem capable of responding to all three major classes of Ags (T-dependent, T-independent-1, and T independent-2), their rapid response to TI-2 Ags is unique. They do this by generating short-lived antibody responses to the antigenic determinants expressed on invading viral and encapsulated bacterial species. These cells express canonical Ig receptors that are important for the responses mounted against bacterial Ags. Moreover, they may also contribute to autoimmunity and malignancy (Martin and Kearney, 2000; Mandik-Nayak et al., 2006). MZ and follicular B cells are distinguished by the differential expression of several cell-surface markers; specifically, MZ B cells are $IgD^{low}CD21^{high}CD23^{low/-}$, whereas FO B cells are $IgD^{high}CD21^{inter}CD23^{high}$ (Oliver et al., 1999) (Fig. 1). In addition, MZ B cells express various activation markers, such as high basal levels of CD80, CD86, CD40, and CD44, but low levels of CD62L. MZ B cells exhibit rapid and robust proliferation and execute Ig secretory responses when stimulated with LPS, anti-IgM, and/or CD40 ligands (Snapper et al., 1993; Oliver et al., 1997, 1999). The hyper-reactivity and polyreactivity of MZ B cells, as well as their unique anatomic localization at the red pulp junction, strongly suggest that these B cells mediate rapid Ab responses to blood-borne Ag. Intact responses to TI-2 Ags are critical for the generation of protective immunity against encapsulated extracellular bacteria such as *Streptococcus pneumoniae* (a Gram-positive bacterium and the predominant cause of otitis media), community-acquired pneumonia, septicemia, and meningitis in humans (Wuorimaa and Käyhty, 2002). Although numerous reports have demonstrated the necessity of MZ B cells, the molecular mechanisms regulating TI-2 Ag responses remain only partially understood.

2. B-1 Lymphocytes. In contrast to conventional B-2 cells, B-1 cells are derived from the fetal-liver hematopoietic stem cells that persist after birth, particularly in the peritoneal cavity and in gut-associated lymphoid tissues. B-1 cells are further subdivided into B-1a (CD5+) and B-1b (CD5−) subsets (Hardy, 2006). However, it should be pointed out that most of the observations about the B1 cell subsets have been obtained in mice, and very few data are available on human B1 B cells. B-1a cells and their natural antibody products provide innate protection against bacterial infections in naive hosts, whereas B-1B cells function independently as the primary source for long-term adaptive antibody responses to polysaccharides and other T-cell-independent type 2 Ags during infection (Alugupalli et al., 2004; Haas et al., 2005). The origins of B-1 cells, and whether

B-1a and B-1b cells are derived from the same or from distinct progenitor cell populations, remain unknown. Remarkably, B-1 cells can interact with the innate immunity via their association with natural killer T cells (Askenase et al., 2004). B-1b cells in the peritoneal cavity carry out a function similar to that of MZ B cells and may be enriched in clonal specificities for T-independent Ab responses (Martin and Kearney, 2001). Thus, it is clear that MZ and B-1 B cells may share overlapping functional capabilities, although these subsets occupy unique topographical niches. This close relationship is further indicated by their partially shared phenotype, which includes the recently defined marker CD9 and the FcR homolog 3 (Won and Kearney, 2002; Won et al., 2006).

Two hallmarks of MZ B cells and B-1 B cells are their polyreactivity and their low affinity for various ligands, a possible consequence of positive selection by self-Ags. However, their low threshold of activation makes them highly reactive to high loads and/or altered self-Ags, which potentially exacerbates autoimmune disease. It is likely that sequestration of autoreactive B cells in the MZ and in the peritoneal cavity is essential for the maintenance of self-tolerance (Li et al., 2002). Their expansion in autoimmune models and their association with autoantibody secretion suggest that they contribute to tissue damage. The reduced antibody responses observed in CD1d(−/−) or Jα18(−/−) mice, compared with WT mice, during autoimmune disease are also consistent with natural killer T- and B-cell cooperation. Increasing success with cell-depletion therapies for autoimmune disorders suggests that the targeted elimination of B-1 and MZ B cells may represent an effective immuno-intervention strategy for systemic autoimmunity (Viau and Zouali, 2005).

E. Regulatory B Cells

The idea that B cells can negatively regulate cellular immune responses was suspected as early as the 1970s (Katz et al., 1974; Neta and Salvin, 1974). Since then, several experimental models of autoimmunity (Wolf et al., 1996; Mizoguchi et al., 1997; Mauri et al., 2003), infection (Jankovic et al., 1998), and cancer (Inoue et al., 2006) have supported the notion that, similar to regulatory T cells, B cells with suppressor or regulatory function (Breg) form an important component in the maintenance of peripheral tolerance. This fact underscores the caution that must be exercised when using B-cell depletion therapies for the long-term treatment of autoimmune disorders, because they might deplete not only pathogenic but also regulatory B cells. However, the existence of a human counterpart of Breg cells remains a matter of debate and continued study (Jamin et al., 2008). It is noteworthy that one recent report described a subset of human regulatory B cells that was enriched within a $CD19^{+}CD24^{hi}CD38^{hi}$ subset, capable of suppressing T helper 1 cell differentiation in vitro (Blair et al., 2010). Unlike healthy B cells, these regulatory B cells from patients with SLE display impaired IL-10 production when activated via CD40.

Multiple mouse B-cell subsets with varying phenotypes and origins have been described as possessing regulatory properties. This regulatory function seems to be directly mediated by their capacity to produce IL-10 and/or TGF-β and by their ability to directly interact with pathogenic T cells (Mauri and Ehrenstein, 2008). Among the IL-10-producing B-cell populations with reported Breg functionality, there are CD5-positive and CD5-negative ones. The former includes both the peritoneal B1a cells (O'Garra et al., 1992) and the recently reported spleen B10 cells, a rare subset characterized by the $CD1d^{high}CD5^{+}CD19^{high}$ phenotype (Yanaba et al., 2008a). The latter includes spleen MZ B cells ($CD21^{high}CD23^{-}IgM^{high}CD1d^{high}$) and transitional 2-marginal zone precursor B cells ($CD21^{high}CD23^{+}IgM^{high}CD1d^{+}IgD^{+}$) (DiLillo et al., 2010). Adoptive transfer of these B-cell populations (either resting or activated in vitro) has been shown to be beneficial in preventing or reversing the progression of chronic colitis, diabetes, arthritis, encephalitis, and contact hypersensitivity in experimental mouse models (Bouaziz et al., 2008). Therefore, understanding how to amplify Breg numbers or functionality may reveal a mechanism for ameliorating autoimmune diseases. In this regard, both innate (LPS) and adaptive (CD40L) signals seem to be required for optimal IL-10 production by B cells (Mizoguchi and Bhan, 2006; Yanaba et al., 2009). In vitro and in vivo expansion of IL-10-producing B cells has been reported for BAFF (Yang et al., 2010).

F. B-Cell Functions That Occur Independently of Antibody Production

B cells are able to perform other functions independently of their role to secrete antibodies. This is especially relevant in the context of autoimmunity, because autoreactive B cells have been shown to possess the capacity to activate pathogenic T cells, to produce proinflammatory cytokines, and to promote the formation of tertiary lymphoid tissues in target organs (Jacob and Stohl, 2010).

1. Antigen Presentation. B cells are very efficient Ag presenters to T cells because of their ability to specifically capture and internalize Ag through their BCRs (Lanzavecchia, 1987). BCR signaling triggers receptor internalization and targeting of the Ag to an endocytic compartment optimized for peptide loading onto class II MHC. The MHC-peptide complex is transported to the cell surface, where it is recognized by the T-cell receptor of the CD4 T helper.

B cells are not only capable of presenting to T cells but also can affect T-cell activation by mediating a number of costimulatory interactions. The costimulatory molecules CD80 and CD86, which are up-regulated on the B-cell surface after activation, engage T-cell surface CD28, thereby promoting T-cell activation, amplifying

proliferation, and inducing cytokine production. B cells are then induced to proliferate and differentiate as a result of the combined action of the T-cell cytokines and the signals generated by the interaction of B-cell-expressed CD40 with the T-cell activation molecule CD40L (Elgueta et al., 2009).

Another important function carried out by B cells independently of Ig production is the role they play in the formation of T follicular helper cells. Several studies have established that these cells do not form in B-cell-deficient mice (Haynes et al., 2007) and that expression of inducible costimulator ligand by B cells is necessary for the formation of T_{FH} cells (Nurieva et al., 2008).

2. Cytokine Production. In addition to their function as Ag-presenting cells, it has now been well established that activated B cells can be induced to produce a vast array of cytokines and chemokines including IL-1, IL-4, IL-6, IL-8, IL-7, granulocyte-colony stimulating factor (CSF), granulocyte macrophage-CSF, IL-10 IL-12, TNFα, lymphotoxin (LTα), TGFβ, bone morphogenic protein-6/7, vascular endothelial growth factor-A, macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, IL-16, and CXCL13 (Lund, 2008; Manjarrez-Orduño et al., 2009). Moreover, B cells can produce cytokines in a polarized fashion, mimicking Th1/Th2 cells. These cells are called B effector 1 and B effector 2 cells (Harris et al., 2000). Cytokines produced by these B-cell effector cells can regulate T-cell differentiation (Th1, Th2, and Th17). TNFα production by B cells can amplify Th1 differentiation and IFN-γ production by T cells (Menard et al., 2007). B-cell-derived IL-6, in combination with TGFβ, promotes T-cell differentiation into highly pathogenic Th17 cells, which secrete high levels of IL-17 and other proinflammatory cytokines (Bettelli et al., 2007). Secretion of type 2 cytokines by B cells is associated with the development of Th2 cells (Harris et al., 2005). B-cell-produced cytokines such as IL-12, IFNγ, IFNα, and IL-2 can act on natural killer cell activation (Haddad et al., 2009). In contrast, IL-8, granulocyte-CSF, and granulocyte macrophage-CSF have been shown to play a role in the recruitment of inflammatory cells (Noronha et al., 2009).

The LTα and TNF produced by B cells are required for the maturation of follicular dendritic cells and for the organization of GC (Gonzalez et al., 1998; Vinuesa and Cook, 2001; Tumanov et al., 2002). It has recently been shown that the formation of ectopic or tertiary lymphoid tissues induced by lymphotoxin could lead to the local amplification of autoimmune responses including those found in RA and SLE (Takemura et al., 2001; Lorenz et al., 2003).

As mentioned above, the capacity of a distinct B-cell regulatory subset to specifically produce IL-10 (B10 cells) may be of crucial importance to the control of autoimmunity (DiLillo et al., 2010).

G. Mechanisms of B-Cell Tolerance

As described above, B lymphocytes with a vast amount of specificities are generated by V(D) J recombination during B-cell development in the bone marrow and during somatic hypermutation of the variable Ig domains in the GC. However, the downside of producing this enormous random diversity of antibodies is the generation of autoantibodies. Tolerance to self-Ags is a fundamental property of the immune system, and the failure of self-tolerance leads to autoimmunity.

B-cell tolerance is induced when immature B cells recognize self-Ags in the bone marrow. If this involves a high-affinity recognition, it may lead to the death of the cell by apoptosis (cell deletion) or to the inactivation of the cell (cell anergy) (Goodnow et al., 2005). However, the most frequent result is the induction of a change in Ag receptor specificity by a mechanism known as receptor editing. Receptor editing consists of the reactivation of recombinase genes leading to a secondary rearrangement that induces the expression of a new light chain, thus facilitating the acquisition of a new specificity (Nemazee and Weigert, 2000). Twenty to fifty percent of developing B cells have been shown to undergo receptor editing (Wardemann et al., 2004). In fact, the majority ($\sim$75%) of early immature B cells not only express self-reactive antibodies including antinuclear antibodies (ANAs) but also exhibit a high degree of polyreactivity (Wardemann and Nussenzweig, 2007). This number drops dramatically during the transition from early immature to immature B cells, a phenomenon that is closely linked to receptor editing and that represents the first checkpoint in the establishment of self-tolerance (Wardemann and Nussenzweig, 2007).

Transitional B cells undergo a second self-tolerance checkpoint, leaving the number of autoreactive B mature cells at around 20% (Wardemann et al., 2003). If self-reactive transitional B cells are repeatedly stimulated by self-Ags they can undergo anergy and remain unresponsive to Ag stimulation (Ekland et al., 2004). Most of the mature B cells that retain the capacity to recognize self-Ags produce IgM autoantibodies against intracellular Ags, which are not pathogenic IgG high-affinity autoantibodies, such as are found in autoimmune diseases (Dighiero, 1997). An important regulator of the immature-to-mature B-lymphocyte transition in the spleen is the TNF-family member BAFF (Kahn et al., 2008). It is noteworthy that deregulation of BAFF expression results in autoimmunity and in an SLE-like syndrome in mice and humans (Mackay et al., 1999; Stohl et al., 2005).

Somatic hypermutation in the germinal center may also be a source of autoreactive B lymphocytes, because the random changes introduced in the Ig genes can also modify Ag specificity and produce self-reactive antibodies (Vinuesa et al., 2009). Thus, other mechanisms must operate during the late stages of B-cell maturation to

maintain tolerance of the remaining pool of potentially harmful self-reactive mature B lymphocytes (Goodnow et al., 2007). For example, autoreactive mature B lymphocytes in the peripheral tissues in the absence of specific helper T cells may be rendered functionally inactive or may die by apoptosis. Thus, it is clear that BCR-controlled checkpoints during B-cell development are essential for preventing autoimmune diseases (von Boehmer and Melchers, 2010).

Genetic predisposition and polymorphisms in a large number of genes that directly or indirectly regulate B-cell differentiation and activation, as well as environmental factors (e.g., viral infections), are likely to contribute to the appearance and outcome of autoimmune diseases. However, it has been observed that in some instances, the presence of autoantibody-producing cells is not sufficient to induce B-cell-mediated autoimmune disease. Therefore, a model has been put forward proposing that autoreactive B cells acquire other pathogenic properties, such as the ability to activate pathogenic T cells, to produce proinflammatory cytokines, and to induce ectopic tertiary lymphoid tissues in the target organs (Manjarrez-Orduño et al., 2009). In concordance with this hypothesis, it has been postulated that the efficiency of B-cell depletion therapies in several autoimmune diseases could stem in large part from their effect on the antibody-independent function of these cells (Wei et al., 2007; Sanz et al., 2008).

III. B-Cell Pathogenesis

A. Human Autoimmunity

Autoimmunity can appear when the mechanisms that control tolerance to self-Ags are unable to eliminate or inactivate all pathogenic autoreactive B cells. Gene perturbations that affect B-cell activation thresholds (e.g., CD19, CD22, lyn, SHP2) or that regulate apoptosis (e.g., bcl-2, bcl-x, Fas/FasL) increase the likelihood that an autoreactive B cell will escape, clonally expand, and then determine the induction of an autoimmune process (Dörner and Lipsky, 2006; Maniati et al., 2008).

Autoreactive B cells are characterized by their ability to secrete autoantibodies directed to self-peptides. Testing specific patterns of autoantibody reactivity in the sera of patients is essential for establishing the correct diagnosis and, in some cases, the prognosis of most autoimmune diseases, despite the lack of any direct proof that their primary pathogenic characteristics have an affect on such diseases (Joseph et al., 2010). For example, the presence of ANAs serves as a very sensitive diagnostic test for SLE. However, these autoantibodies are also found in other autoimmune diseases, and their serum levels do not correlate with the severity of clinical disease (Martin and Chan, 2004).

B cells can contribute to the pathogenesis of autoimmune diseases through the production of autoantibodies (Lipsky, 2001). Although not all autoantibodies are pathogenic, those that persist in the circulation or that change their isotype to IgG and increase their affinity for self-Ags are directly responsible for the pathology in both systemic and organ-specific autoimmune diseases, employing multiple mechanisms to cause tissue damage. In some cases, pathogenic autoantibodies are directed to a cell-surface receptor, such as the acetylcholine receptor in myasthenia gravis or the thyroid-stimulating hormone receptor in Graves' disease (Vincent, 2002; Jones et al., 1985). Autoimmune reactions initiated against a specific Ag often result in the release of other Ags and the subsequent production of autoantibodies to these Ags, a phenomenon called epitope spreading (Vanderlugt and Miller, 2002). In patients with SLE and RA, autoantibodies can also form immune complexes with circulating auto-Ags, which accumulate in the kidneys and joint synovium, activating the complement cascade and thereby inducing local inflammation and tissue destruction (Koffler et al., 1971).

Although some autoimmune diseases, such as RA, have been regarded as classic T-dependent diseases based on adoptive transfer experiments of T cells from diseased to healthy animals, the therapeutic success of B-cell depletion using a mAb against the B-cell surface molecule CD20 (RTX) in several autoimmune diseases has brought about a renewed focus on the role of B cells in the pathogenesis of these diseases (Martin and Chan, 2004; Yanaba et al., 2008b). CD20 is a B-cell-specific molecule highly expressed on mature B cells but absent from pro-B cells and plasma cells (Tedder and Engel, 1994). It is noteworthy that depletion of CD20 B cells, which causes dramatic clinical effects, does have a moderate effect on antibody levels and clinical responses often exceed any drops in antibody levels (DiLillo et al., 2008). This has been explained by the observation that such treatment does not affect the pool of long-lived plasma cells lacking CD20 expression. This realization, together with multiple experiments in mice, strongly supports the emerging view that those B-cell functions that occur independently of antibody production play important pathogenic roles. Specifically, their capacity to initiate and regulate T-cell activation, in tandem with the production of pro-inflammatory and regulatory cytokines or lymphoneogenesis, contributes to the development of autoimmune disease (Blank and Shoenfeld, 2007; Manjarrez-Orduño et al., 2009; Jacob and Stohl, 2010). For example, B cells form aggregates, in some cases presenting GC-like structures in the synovium of patients with RA, where they may function as Ag-presenting cells promoting T-cell activation and expansion (Weyand and Goronzy, 2003).

B. Animal Models

Some of the most relevant evidence for the role of B cells and autoantibodies in the pathogenesis of RA has come from an animal model of inflammatory arthritis, the K/BxN transgenic model of arthritis (Kotzin, 2005).

K/BxN is a hybrid mouse resulting from the cross between a K/B transgenic mouse and a nonobese diabetic (NOD) mouse. This K/BxN mouse developed very severe arthritis with a pathologic condition typical of RA (Kouskoff et al., 1996). It has been shown that the anti-glucose 6-phosphate isomerase (GPI) autoantibody produced in this model was pathogenic. When serum from the K/BxN mice was injected into normal mice, the recipient mice developed marked arthritis. It has also been shown that the GPI molecule itself seems to be present on the cartilage surface, and that anti-GPI autoantibodies can therefore bind to the articular surface and form immune complexes (Matsumoto et al., 2002).

Although anti-GPI antibodies are present in many patients, they are neither specific nor sensitive for RA. The ability of ubiquitous Ags such as GPI to induce synovial inflammation is probably related to their adherence to the cartilage surfaces. The presentation of immobilized Ag-antibody complexes on cartilage provides an exceptionally good substrate for complement fixation, similar to the rheumatoid factor (RF) embedded in rheumatoid cartilage. An analogous situation occurs in murine passive collagen-induced arthritis, where anti-type II collagen antibody binds to the collagen arrayed on the surface of articular cartilage (Firestein, 2003).

Another potential mechanism for B-cell involvement in RA has emerged from work conducted with another hybrid mouse model of the disease, one in which rheumatoid synovial tissue from patients with RA is implanted into NOD-severe combined immunodeficient (NOD-SCID) mice (Takemura et al., 2001). Tissues that lacked B cells did not support the infiltration and activation of adoptively transferred CD4+ T cell clones. The dependence of T-cell activation on B cells was demonstrated in studies in which mice were treated with RTX to deplete their B cells. Subsequent examination of the grafted synovial tissue showed that the antibody treatment reduced T-cell infiltration. In addition, the ability of the tissues to produce the inflammatory cytokines (INFγ and IL-1β) was inhibited in a dose-dependent manner in the B-cell-depleted mice. Thus, the ability of synovial T cells to drive a Th1- type inflammatory process seems to be governed by the presence of B cells.

In mouse models, the kinetics of B-cell depletion and the sensitivity of B cells to RTX varied among different tissues (Gong et al., 2005; Hamaguchi et al., 2005). In a human CD20–transgene mouse model, B cells in the peripheral blood and lymphoid tissues were highly sensitive to cell death in response to RTX treatment, whereas B cells located in the GCs in lymphoid tissues were far less sensitive to depletion (Gong et al., 2005). This could be explained by the protective effect of B-cell–activating factors such as B-lymphocyte stimulator (BLyS) and by the requirement for B cells to access the circulation for efficient depletion.

A transgenic mouse model of CD19-directed immunotherapies had been developed (Zhou et al., 1994; Engel et al., 1995b). These mice are able to express the human CD19 gene regulated by its endogenous promoter, which recapitulates the developmental pattern of human CD19 (hCD19) cell-surface expression (Zhou et al., 1994; Engel et al., 1995b). Because of CD19 overexpression, hCD19 transgenic (hCD19TG) mice also develop autoimmune disease (Sato et al., 2000).

Using a transgenic model, Yazawa et al. (2005) analyzed the depletion of B cells after an injection with anti-hCD19 mAbs. Two weeks after a single injection of CD19 mAb, serum IgM, IgG, and IgA Ab levels were significantly reduced, remaining so for at least 10 weeks. CD19 mAb treatment also reduced humoral immune responses and autoantibody production in hCD19TG mice.

Murine models, such as (NZBxNZW)F_1, MRL/Fas^{lpr} (MRL/*lpr*), and BXSB mice, spontaneously develop SLE-like syndromes with a comparable heterogeneity and have provided a powerful tool for studying human disease. The NZBxNZW model was the first murine model described for lupus nephritis. The F_1 hybrid between the NZB and New Zealand white mouse strains develops renal lesions that are remarkably similar to the pathologic lesions described in human SLE (Monneaux et al., 2001).

Intrinsic B-cell defects are also found in MRL/*lpr* mice, which develop a similar lupus-like disease. Therefore, aberrant B-cell function is now thought to be central to the development and/or progression of lupus-like diseases in mice and humans (Lipsky, 2001; Grammer et al., 2003).

Ahuja et al. (2007)) analyzed the efficacy and impact of B-cell depletion on clinical disease in a mouse model after crossing human CD20 transgenic onto the MRL/*lpr* strain. The authors found that treatment of transgenic MRL/*lpr* mice with high doses of depleting Ab for prolonged periods of time led to substantially reduced B-cell counts. Depletion of B cells led, in turn, to reductions in T-cell activation and amelioration of clinical disease, formally demonstrating that B-cell depletion affects multiple immunological parameters and aspects of clinical disease in a mouse model.

B-cell depletion had significant effects on NZB/W F1 mouse survival that were dependent upon the timing of treatment initiation. Prophylactic depletion of mature B cells in NZB/W F1 mice by CD20 mAb prolonged survival. Therapeutic low-dose CD20 mAb treatment successfully prolonged survival and delayed the appearance of proteinuria during spontaneous lupus in 12- to 20-week-old NZB/W F1 mice when administered during the onset of disease symptoms. In contrast, mature B-cell depletion in young NZB/W F1 mice before disease symptoms led to accelerated mortality, nephritis, and ANA production (Haas et al., 2010). Therefore, distinct B-cell

populations can have opposing protective and pathogenic roles during the progression of lupus.

The tight-skin (TSK/+) mouse is a model for human SSc and was originally identified as a spontaneous mutation that resulted in increased synthesis and accumulation of collagen and other extracellular matrix proteins in the skin. (Green et al., 1976). TSK/+ mice produce autoantibodies against SSc-specific target auto-Ags including topoisomerase I, fibrillin 1, and RNA polymerase I. Similar to human SSc, heightened CD19 signaling is present in TSK/+ mice, although CD19 overexpression has not been detected on TSK/+ B cells (Saito et al., 2002). Loss of the CD19 signaling pathway dramatically inhibits autoantibody production in TSK/+ mice. In addition, CD19 deficiency in TSK/+ mice results in an approximately 40% reduction in skin thickness. Therefore, B cells contribute to skin fibrosis in TSK/+ mice through a CD19-dependent pathway.

B-cell depletion using CD20 monoclonal antibody treatment prevents autoantibody production in TSK/+ mice and significantly reduces skin hyperplasia if administrated before disease onset. B-cell depletion did not affect skin fibrosis, hypergammaglobulinemia, or autoantibody levels in adult mice with established disease (Hasegawa et al., 2006).

Vasculitides associated with serum positivity for ANCAs that affect small- to medium-sized vessels are commonly known as ANCA-associated vasculitis (AAV) and include Wegener's granulomatosis (WG), microscopic polyangiitis, and Churg-Strauss syndrome. ANCAs have long been suspected of playing a pathogenic role. Since Falk et al. (1990) showed that ANCA could stimulate respiratory bursts in neutrophils to trigger the release of primary granule constituents, in vitro studies have revealed that ANCAs can not only cause vascular damage by inducing a wide range of neutrophil effector functions such as release of cytokines and chemokines but also can adhere to cultured endothelial cells, resulting in their lysis (Bosch et al., 2006). The pathogenic role of ANCAs in vivo was convincingly demonstrated by Xiao et al. (2002), who passively administered murine anti-myeloperoxidase (MPO) IgG to Rag2(−/−)mice, which lack functioning T and B lymphocytes, resulting in the development of focal necrotizing glomerulonephritis (FNGN) lacking immune deposits (indistinguishable from human ANCA-associated glomerulonephritis); approximately 15% of glomeruli suffer damage.

In contrast to accumulated evidence from MPO-ANCA animal models, the pathogenic potential of ANCAs against proteinase 3 (PR3), which are mainly found in patients with WG, remains unproven, and these antibodies have not reproduced the typical granulomata in any animal models (Gómez-Puerta and Bosch, 2009).

Recent experimental studies are now beginning to clarify some of these issues. WG is thought to begin with an aberrant cell-mediated immune response to an exogenous or endogenous Ag in the respiratory tract, which results in granuloma formation and the subsequent development of humoral autoimmunity to PR3 (Bosch et al., 2008). One theory on PR3-directed autoimmunity involves the complementary peptide of proteinase 3, which is encoded by the antisense strand of the *PR3* gene (Pendergraft et al., 2004). Exposure of the immune system to this peptide triggers the formation of antibodies that cross-react with PR3. DNA sequences complementary to the PR3 gene have been identified in microorganisms including *Staphylococcus aureus*, which supports the role of infectious agents as triggers of PR3 autoimmunity via molecular mimicry (Bosch et al., 2008). Cotrimoxazole treatment reduces the relapse frequency in patients with WG who have achieved remission, probably by eliminating or reducing *S. aureus* in the upper airways (Stegeman et al., 1996).

Kain et al. (2008) recently suggested that molecular mimicry is also the fundamental mechanism underlying the development of pauci-immune *focal* FNGN in patients with ANCA. However, the Ag involved is neither PR3 nor MPO, but rather lysosomal membrane protein 2 (LAMP-2). In neutrophils, LAMP-2 is located on the membranes of intracellular vesicles that contain MPO and PR3 and is also abundant on the surface of endothelial cells. LAMP-2 plays a role both in Ag presentation and in the adhesion of peripheral blood mononuclear cells to the vascular endothelium. In their study, Kain et al. (2008) provided a novel molecular explanation for the origin and development of pauci-immune FNGN, which could have profound clinical implication. These authors showed that infection by fimbriated bacteria can trigger cross-reactive autoimmunity to LAMP-2, resulting in the production of autoantibodies that activate neutrophils and damage human microvascular endothelium in vitro and that cause pauci-immune FNGN in rats.

IV. Targeting B Cells

A. Targeting B-Cell-Specific Surface Molecules

1. Anti-CD20 Therapy (rituximab). RTX, a chimeric monoclonal antibody that specifically depletes CD20 B cells, has demonstrated promising potential for use in the treatment of B-cell-mediated autoimmune diseases such as RA and other systemic autoimmune diseases. RTX, at least early in therapy, causes complete but transient depletion of B cells. Although RTX has some intrinsic cytotoxic activity toward B cells in vitro, the consensus is that its main B-cell-depleting activity involves antibody-dependent cellular cytotoxicity and, to a lesser extent, complement-dependent cytotoxicity. It should be noted that CD20 expression is retained on plasmablasts (unlike plasma cells), and thus these cells are still a target of RTX treatment. This most likely explains the aforementioned difference between the phenotypes of mice treated with anti-CD19 versus anti-CD20 monoclonal antibodies with respect to serum Ig

levels, because CD19 is also expressed on plasma cells, whereas CD20 is not.

a. Rheumatoid arthritis. RTX has been approved by both the Food and Drug Administration in the USA and by the Committee for Medicinal Products for Human Use of the European Agency for the Evaluation of Medicinal Products in Europe for the treatment of patients with RA who have had an inadequate response to anti-TNFα therapy.

With the growing availability of expensive therapeutic agents involving RA, it has become increasingly important to tailor treatment to individual patients to maximize the cost-benefit ratio and to minimize the duration of time in which suboptimal treatments are given. Patients in whom anti-TNFα therapies fail have various treatment options, including the combined use of conventional disease-modifying antirheumatic drugs (DMARDs), switching to an alternative anti-TNFα, or changing to an agent with a different mechanism of action [anti-CD20 (RTX), anti-CTLA-4 (abatacept), or anti-IL-6 (tocilizumab)].

The available evidence indicates that switching to a different anti-TNFα agent may be effective (Bombardieri et al., 2007); however, several cohort studies have demonstrated that its efficacy declines over time and that drug retention decreases in patients who have received one or more TNFα inhibitors previously (Olsen, 2007).

RTX has shown efficacy in the treatment of patients with RA who failed to respond to DMARD therapy, in patients previously exposed to anti-TNFα therapy, and more recently in early patients with RA naive for methotrexate (MTX) and anti-TNFα therapies. At present, RTX is indicated for the treatment of patients with active RA who have had an incomplete response or who are intolerant to at least one TNFα inhibitor and an adequately dosed MTX. Other potential indications include those patients with an inadequate response to DMARD therapy and a previous history of solid organ or hematological neoplasm.

i. Rituximab after Anti-Tumor Necrosis Factor α Failure. Various RTX randomized controlled trials (RCTs) in patients with RA are summarized in Table 2. In phase II trials, different dosing regimens of RTX (four weekly infusions of RTX 375 mg/m^2 versus 500 or 1000 mg of RTX administered 2 weeks apart) were tested in combination with MTX and were shown to achieve similar efficacy and to produce superior clinical responses compared with the use of MTX alone (Edwards et al., 2004; Emery et al., 2006).

In early studies, RTX has shown efficacy when used alone or in combination with other agents, including MTX. The efficacy and durability of monotherapy seems to be inferior to that of combined treatment with MTX (Edwards et al., 2004).

A second phase II study, the DANCER trial (Emery et al., 2006), was designed to determine the appropriate regimen of RTX combined with MTX, with or without corticosteroids. The trial involved 465 patients with active RA despite the use of MTX in one of the nine dosing groups: placebo (PBO) with no steroids, PBO with only intravenous steroids, PBO with intravenous and oral steroids, 500 mg of RTX $\times$ 2 with no steroids, 500 mg of RTX $\times$ 2 with intravenous steroids, 500 mg of RTX $\times$ 2 with intravenous and oral steroids, 1000 mg of RTX $\times$ 2 with no steroids, 1000 mg of RTX $\times$ 2 with intravenous steroids, or 1000 mg of RTX $\times$ 2 with intravenous and oral steroids. All patients were on an MTX background. RTX was given intravenously at 2-week intervals. The intravenous steroid regimen consisted of 100 mg of methylprednisolone administered on days 1 and 15. The oral steroid regimen consisted of 60 mg/day prednisone on days 2 to 7 and 30 mg/day prednisone on days 8 to 14. The primary endpoint targeted only RF-positive patients at 24 weeks.

There was a statistically significant benefit in the RTX arm of the study compared with the PBO arm in terms of American College of Rheumatology (ACR) responses. An ACR-20, ACR-50, or ACR-70 response requires a patient to have a 20, 50, or 70% reduction, respectively, in the number of swollen and tender joints, and a 20, 50, or 70% reduction, respectively, in three of the following five parameters: 1) physician global assessment of disease, 2) patient global assessment of disease, 3) patient assessment of pain, 4) C-reactive protein or erythrocyte sedimentation rate (ESR), and 5) degree of disability in the Health Assessment Questionnaire score. The ACR-20 responses were 54, 55, and 28% in the 1000-mg, 500-mg, and PBO RTX regimens, respectively. The steroid regimens did not seem to affect the efficacy results at 24 weeks, although the use of intravenous steroids did significantly reduce the number of infusion reactions associated with RTX. The use of intravenous steroids before RTX administration has now become routine in a concerted effort to limit infusion reactions. The ideal dose of RTX has become less certain, given the similar results observed in the 500- and 1000-mg regimens. Subsequent studies have proven that RTX in combination with MTX can markedly reduce inflammatory activity and increase functional ability and quality of life (Cohen et al., 2006).

In a large phase III trial, Cohen et al. (2006) examined patients who had inadequately responded to anti-TNFα agents (etanercept, adalimumab, and infliximab) and who had been randomly chosen to receive MTX therapy with either two PBO or 1000-mg RTX infusions 2 weeks apart. At week 24, the RTX-treated group (n = 311) showed significantly greater improvement than the PBO-treated group (n = 209), with higher ACR-20, ACR-50, and ACR-70 response rates compared with a PBO of 51 versus 18%, 27 versus 5%, and 12 versus 1%, respectively.

As with the first two studies, RTX treatment produced a rapid and sustained peripheral depletion of B cells. In addition, Igs levels slightly decreased, IgM levels in a few patients falling below the lower limits

TABLE 2
Randomized clinical trials with rituximab in patients with rheumatoid arthritis

Author	No. Patients	Arms	RA	Primary Endpoint/ Follow-up	Clinical Outcomes	Remarks
Edwards et al., 2004	161	MTX (n = 40) or RTX (n = 40) or RTX + MTX (n = 40), or RTX + CYC (n = 41)	Anti-TNFα naive	ACR50 response/24 weeks	ACR50: 13 vs. 33 vs. 43 vs. 41	Only RF patients were included. MTX arm used very low doses (10 mg/week)
Cohen et al., 2006 REFLEX trial	520	MTX + PBO (n = 209) vs. RTX + MTX (n = 308)	Anti-TNFα failure	ACR20 response/24 weeks and post-treatment 2 years	At week 24: ACR20: 18 vs. 51 ACR50: 5 vs. 27 ACR70: 1 vs. 12 EULAR moderate or good: 22 vs. 65	Most patients positive for RF (79%); nonsignificant differences at 6 months in radiographic progression. Significant reduction at 1 year[a]
Emery et al., 2006 DANCER trial	465	PBO + MTX (n = 149) or RTX 2× 500 mg + MTX (n = 124) or RTX 2× 1000 mg + MTX (n = 192)	DMARD or anti-TNFα failure	Proportion of RF-positive patients who met the ACR 20%/24 weeks	ACR20: 28, 55 and 55, respectively. ACR70: 5,13, 20[b] EULAR good response: 4, 14, 28[b]	No radiological outcomes measured. Better responses in patients not previously exposed to anti-TNFα
Finckh et al., 2007	116	RTX (n = 50) vs. alternative anti-TNFα (n = 66)	Anti-TNFα failure	Change from baseline of DAS-28 score/at least 6 months	Mean decrease in DAS-28: −1.61 in RTX vs. −0.98 in anti-TNFα	Treatment with RTX was more effective than a 2nd or 3rd anti-TNFα
Tak et al., 2009 IMAGE study	715	PBO + MTX (n = 232), RTX + MTX (2× 500 mg) (n = 239) or RTX + MTX (2× 1000 mg) (n = 244)	Early RA MTX naive	Change from screening in the mTSS[b] at week 52/ 52 weeks	Mean change in mTSS at 52 weeks 1.08 vs. 0.65 vs. 0.36[a] ACR50: 41 vs. 59 vs. 64	RTX (2× 1000 mg) + MTX significantly improved clinical outcomes and inhibited joint damage compared with MTX alone. Published only in abstract form
Mease et al., 2010 SUNRISE trial	475	One open-label course of RTX. From week 24 RTX (retreatment group) vs. PBO	Anti-TNFα failure	ACR20 response at 48 weeks/48 weeks	ACR20: RTX vs. PBO 54 vs. 45%, mean change in DAS 28: −1.9 vs. −1.5	Patients with very high disease activity at baseline (mean DAS-28, 6.7). No differences in ACR50, ACR70, and EULAR responses in both groups

CYC, cyclophosphamide; mTSS, modified total Sharp score.
[a] Determined by Genant-modified Sharp Score.
[b] $p > 0.001$.

of normal, although with no higher incidence of infectious episodes during the study. Overall, adverse events were noted almost equally in both treatment groups. Twenty-three percent of patients with RTX developed an acute infusion reaction during or after the first infusion compared with 18% of patients receiving PBO. Infusion-related reactions are thought to be related to cytokine release and include pruritus, fever, rash, pyrexia, rigors, sneezing, throat irritation, cough, bronchospasm, hypotension, and hypertension. There was also a slightly higher incidence of minor and serious infections in the RTX-treated group compared with the PBO group.

In summary, the REFLEX trial demonstrated that patients who had previously undergone anti-TNFα therapy experienced significant improvements in nearly all areas after treatment with RTX, including swollen and tender joint counts, patient's and physician's global assessments of disease activity, pain scores, Health Assessment Questionnaire scores, C-reactive protein and ESR levels, and mental and physical health scores [measured by Short Form-36 (SF-36)], fatigue scores, and a positive trend in the improvement of joint narrowing as assessed radiographically.

Current evidence on the efficacy of RTX has thus far been related only to RF-positive patients (Edwards et

al., 2004; Emery et al., 2006). In a phase III study on TNFα nonresponders, a marked response was seen in RF-negative patients, (Cohen et al., 2006), whereas in a separate study on RF-negative patients who were not part of the primary endpoint analysis, the response was no different from that observed in PBO-treated patients, although their response to PBO was unusually high (Emery et al., 2006).

Strand et al. (2006) randomized 161 patients with RA who had failed to respond to treatment with different DMARDs (from 1 to 5), as follows: 40 in each of the PBO/MTX, RTX monotherapy, and RTX plus MTX groups and 41 in the RTX/cyclophosphamide group. Median doses of MTX at study entry were 12.5 to 15 mg/week across the three groups. The primary endpoint of the study was the proportion of patients at week 24 with an ACR-50 response.

Compared with PBO/MTX group, the proportion of patients achieving ACR-20, ACR-50, and ACR-70 proved higher with RTX treatment. At the same time, improvements in physical function were greater in the RTX plus MTX group compared with other groups at weeks 48 and 72 of follow-up.

The preferred timing of repeated RTX treatment courses remains a matter of debate. Recent data have supported fixed retreatment every 24 weeks versus on-demand retreatment (Teng et al., 2009; Mease et al., 2010).

From a cohort of 559 patients with RA who had failed to respond to anti-TNFα therapy but who had received an open-label first course of RTX, 475 patients were randomized to a second course (RTX retreatment: n = 318 versus PBO retreatment: n = 157) (Mease et al., 2010). Compared with baseline, the administration of RTX during retreatment showed significantly improved efficacy in patients at week 48 compared with those who took a PBO during retreatment [greater ACR20 responses and mean change in Disease Activity Score using 28-joint-counts (DAS-28) score]. The DAS-28 is a composite index that includes variables such as the number of tender and swollen joints, ESR, and, as an option, the patient's assessment of disease activity.

There were no significant differences in ACR-50, ACR-70, and EULAR responses in RTX and PBO retreatment groups. The EULAR response criteria are based on the assessment of disease activity using the DAS-28 score. The EULAR response criteria include not only change in disease activity but also current disease activity. To be classified as responders, patients should have a significant change in the DAS-28 score and also low current disease activity. There were no differences in the proportion of patients with overall infections in either group. The authors concluded that two courses of RTX treatment approximately 6 months apart resulted in improved and sustained efficacy at 1 year, compared with 1 course, using a similar safety profile.

A prospective cohort study nestled within the Swiss Clinical Quality Management RA(SCQM-RA) cohort included all 116 patients who had inadequately responded to at least one TNFα inhibitor and who were subsequently treated with either RTX or a second TNFα inhibitor (Finckh et al., 2007). The primary outcome was the evolution of RA disease activity as measured by changes from baseline in the DAS-28. At six months, the mean decrease in the DAS-28 was -1.61 (95% CI, -1.97 to -1.25) among patients on RTX, compared with -0.98 (95% CI, -1.33 to -0.62) of those given a second TNFα inhibitor. Other significant predictors of the DAS-28 response included previous failures with more than one anti-TNFα agent [mean change in DAS-28, -0.44 (95% CI 0.004, 0.87); $P = 0.05$ versus previous failure with 1 anti-TNFα agent] and concomitant DMARD use [mean change in DAS-28, -0.30 (95% CI, 0.04, -0.56; $P = 0.03$ versus no DMARD use].

Further extension of the SCQM-RA registry and subgroup analysis of 318 patients (155 patients received RTX and 163 patients received a second or third anti-TNFα) revealed that for patients who had failed anti-TNFα because of inefficacy (either primary or secondary), switching to RTX was more effective than switching to another anti-TNFα agent (Finckh et al., 2010). When the motive for switching was not ineffectiveness, but rather was due to an adverse event or another reason, the longitudinal improvement in DAS-28 was similar for RTX and for alternative anti-TNFα s.

ii. Rituximab as a first-line biological therapy and early rheumatoid arthritis. RTX treatment has also been tried in patients with early RA, even in those previously unexposed to anti-TNFα treatment. Some brief reports (McGonagle et al., 2008; Renato, 2009) have demonstrated that RTX might be useful as first-line biological therapy for the treatment of severe seropositive RA. The efficacy of RTX in daily clinical practice as a first-line biological therapy was evaluated in 39 patients with RA. At baseline, the mean DAS-28 score was 6.35, and 81% of patients were positive for RF. After 12 months of follow-up, there was a significant improvement in the DAS-28, with a EULAR response in 23 of 30 patients (76%).

One study consisted of an open pilot trial using RTX in 20 consecutive patients suffering early seropositive [RF and anti-cyclic citrullinated peptide (CCP)] RA with highly active disease (DAS-28 $>$ 5.1 in all of them) (Renato, 2009). After 6 months of follow-up, a satisfactory response was achieved in most patients, with ACR-20, ACR-50, and ACR-70 responses of 62, 42, and 21%, respectively. No serious adverse events were reported.

The IMAGE study is an ongoing randomized, phase III trial (clinicaltrials.gov identifier NCT00299104) evaluating RTX in combination with MTX in MTX-naive patients with active RA. This study (Tak et al., 2009) included 755 patients with early RA ($<$4 years' duration)

who were randomized into three groups: PBO plus MTX (n = 232), RTX 2500 mg × 2 plus MTX (n = 239), and RTX 1000 mg x 2 plus MTX (244).

The primary endpoint was designated as the change from screening in the Genant-modified Sharp method at week 52. A secondary endpoint was major clinical response (ACR-70 maintained for at least 6 months). There was a significant reduction in radiological progression in the RTX plus MTX groups after 24 and 52 weeks of follow-up (see Table 2). At the same time, patients receiving RTX plus MTX experienced a significant clinical response in terms of ACR responses and reductions in DAS-28 scores. Despite some favorable data, RTX currently lacks U.S. Food and Drug Administration approval as a first-line therapy in patients with early RA.

iii. Synovial effects of anti-CD20 therapy. Analysis of the synovia of patients with RA receiving RTX has shown that a reduction in B cells and an inhibition of structural joint damage does occur (Kavanaugh et al., 2008; Thurlings et al., 2008; Keystone et al., 2009). Depletion of circulating B cells failed to reveal any direct relationship with clinical response. In a transgenic mouse model, susceptibility to RTX therapy was shown to vary by tissue, presumably as a result of factors in the local microenvironment (Gong et al., 2005).

Vos et al., (2007) demonstrated that early depletion of B cells in peripheral blood and synovial tissue occurs after 4 weeks of RTX treatment. However, the synovial depletion was incomplete and did not occur in all patients. In addition, the authors found no correlation between the changes in B-cell numbers and the change in DAS-28 scores at week 4.

In an open-label trial with 13 patients with RA who had undergone a synovial biopsy via arthroscopy (Kavanaugh et al., 2008) several serological and synovial biomarkers were tested both at baseline and at 8 weeks after RTX treatment. In peripheral blood, circulating $CD19^+$ B-cell counts, RF, CCP antibodies, and Igs were then determined. Infiltrating cell populations in the synovium were assessed by immunohistochemistry using digital image analysis, including B-cells (CD19 and CD20), T cells (CD3), macrophages (CD68), and plasma cells (CD138). As had been observed in other clinical trials, treatment with RTX resulted in a profound depletion of circulating B cells, with a 95% depletion of $CD19^+$ cells in the peripheral blood. As reported previously, there was no correlation between clinical response and B-cell depletion levels. At the same time, changes in RF and CCP levels did not correlate with clinical outcome. In the synovium, there was no significant change in the numbers of $CD3^+$ T cells, $CD68^+$ macrophages, or $CD138^+$ plasma cells. However, a significant decrease in the numbers of B cells in the synovium was observed, with a mean decrease of approximately 80%. Among patients who achieved an ACR-50 response, there was a significant depletion of synovial B cells, a finding that seemed more consistent than that observed among nonresponders. No differences in synovial B-cell survival factor expression [BAFF, APRIL (a proliferation-inducing ligand), and stromal cell-derived factor 1] or in synovial lymphoid aggregates were observed in responders and nonresponders. Although there was a trend toward greater reduction of synovial B-cell numbers among responders, these differences did not achieve statistical significance. A potential explanation for these unexpected results may be the small sample of the study, the open label design, and/or the time chosen for the control synovial biopsies. On the basis of previous results, the authors suggested that depletion of synovial B cells was not sufficient in and of itself for inducing clinical response. Other B-cell-related processes (such as B-cell Ag presentation) and non–B-cell-dependent mechanisms (cytokine production) may also have been contributing factors (Kavanaugh et al., 2008).

RTX is increasingly used in patients with systemic autoimmune diseases, mainly SLE, systemic vasculitis, and primary SS (Dörner et al., 2009a). However, it is also used in other systemic autoimmune diseases, including antiphospholipid syndrome, mixed connective tissue disease, thrombotic thrombocytopenic purpura, Behçet disease, SSc, and inflammatory myopathies (Ramos-Casals et al., 2008, 2010).

b. Systemic lupus erythematosus. The off-label use of RTX in patients with SLE was first reported in 2002 (Perrotta et al., 2002), and it has since increased progressively. Although the pathogenesis of SLE is not yet fully understood, a growing body of experimental evidence indicates that B lymphocytes play a central role (Chan et al., 1999; Looney et al., 2004; Driver et al., 2008). Moreover, the efficacy of B-cell depletion using anti-CD20 monoclonal antibodies in murine models of SLE has been demonstrated (Ahuja et al., 2007; Bekar et al., 2010). In human studies, RTX substantially lowers $CD20^+$ B-cell levels in peripheral blood within days to weeks (an effect sustained for up to 6 months), reduces anti-dsDNA and anti-nucleosome antibodies (Cambridge et al., 2008), and reverses B-cell homeostasis abnormalities (Anolik et al., 2004).

Substantial clinical experience in the off-label use of RTX in patients with severe, refractory SLE has accumulated during the last decade; nearly 200 cases have been included in open-label studies and small case series being reported through 2008 (Ramos-Casals et al., 2009b). In addition, recent studies have described the use of RTX in large series of patients. Lu et al. (2009) reported the largest series of patients with SLE treated with RTX from a single center; of 50 patients with refractory SLE, only 11% did not respond [no change in British Isles Lupus Assessment Group (BILAG) A/B score after treatment]. The BILAG index is a computerized index for measuring clinical disease activity in SLE that was developed according to the principle of the physician's "intention to treat." The index allocates

separate alphabetic scores to each of eight organ-based systems.

Another retrospective study reported the results of RTX treatment in 107 patients with SLE (Ramos-Casals et al., 2010), 77% of whom achieved a complete or partial response. Although RTX should not be used as first-line treatment in SLE or in patients with a predominantly mild form of the disease, the results of its off-label use in patients with severe, refractory SLE seems to be sufficiently positive to warrant its use in this subgroup of patients.

The results observed in uncontrolled studies of patients with refractory SLE are in clear contrast to the poor results of two recently completed RCTs. The EXPLORER trial was a 52-week phase II/III RCT designed to evaluate the efficacy and safety profile of RTX treatment in patients with active SLE while excluding those with lupus nephritis (Merrill et al., 2010). A total of 257 patients from approximately 55 sites in the United States and Canada were randomized to RTX ($n = 169$) or PBO ($n = 88$). Baseline immunosuppressive agents were continued and patients received a 10-week course of high-dose corticosteroids (0.5–1.0 mg/kg) with a subsequent progressive reduction in the dose until the end of the trial. RTX 1000 mg or PBO was administered on days 1 and 15 of month 0 and again at month 6. The two arms of the trial showed a significant reduction in clinical activity compared with baseline, although the differences in the primary and secondary endpoints were not statistically significant; however, a subanalysis did reveal some benefits of RTX in African Americans and Hispanics compared with other subjects. The use of RTX was associated with a significant reduction in anti-dsDNA titers and a significant increase in C3 and C4 levels. With respect to adverse events, neutropenia (3.6 versus 0%) and herpesvirus infection (15 versus 8%) were more frequent in the RTX arm. The second RCT was the *LUNAR* trial, a phase III trial that evaluated the efficacy and safety of RTX treatment in patients with proliferative lupus nephritis. RTX or PBO was added to standard-of-care therapy (mycophenolate plus high-dose corticosteroids). One hundred forty-four patients were not only randomized (72 to RTX and 72 to PBO) but were also stratified by ethnicity (African Americans versus others). The preliminary results presented at the 2009 ACR meeting (Furie et al., 2009) showed that the trial did not achieve its primary or secondary endpoints.

Before concluding that RTX is not a good therapy for SLE, careful evaluation of the designs underlying the *EXPLORER* and *LUNAR* trials is required, including considerations of disease severity, concomitant therapies, ethnic factors, and primary endpoint definitions (Ramos-Casals et al., 2009a). The patients included in the two recently completed RCTs of RTX in SLE seem to be completely different from the individual patients who had been receiving off-label RTX since 2002. Thought should be given to the design of future trials in SLE; compared with RA (the paradigm for the licensed use of biological therapies), SLE is more clinically heterogeneous and infrequent and occurs in younger populations.

c. Sjögren's syndrome. The emergence of biological therapies has increased the array of treatments available to clinicians when faced with the most severe situations in primary SS, even despite the lack of specific formal licensing (Tobón et al., 2010). At present, B-cell targeted therapies seem to be the most promising agents, especially RTX, which has been used in more than 100 reported cases. As suggested by some recent preliminary studies (Jousse-Joulin et al., 2007; Lavie et al., 2007; Pijpe et al., 2009) it seems plausible that this type of agent may play a role in modifying the etiopathogenesis of primary SS, a disease characterized by B-cell hyperactivity.

Several uncontrolled studies have reported successful off-label use of RTX in small series of patients (<20) with primary SS. The first open-label study was reported in 2005 and included 15 patients (including 7 patients with B-cell lymphoma) who received four weekly infusions of 375 mg/m^2 of RTX (Pijpe et al., 2005), with a significant improvement in subjective symptoms and increased salivary gland function in patients with residual glandular function. Devauchelle-Pensec et al. (2007) studied 15 patients with primary SS who received two weekly doses of RTX (375 mg/m^2). After 12 weeks, depletion of peripheral B cells was complete in all patients save one, without significant changes in the levels of natural killer, T helper, and cytotoxic T cells. In a retrospective study of 16 patients with primary SS and systemic features, treatment with RTX was associated with decreased serum levels of RF, γ-globulins, and β2-microglobulin (Seror et al., 2007), with B-cell depletion showing an inverse relationship with serum BLyS levels. Three patients achieved sustained B-cell depletion lasting 9 to 18 months. More recently, a multicenter Spanish register (BIOGEAS) reported 15 patients with primary SS treated with RTX (Ramos-Casals et al., 2010). Ten (67%) achieved a complete response, three (20%) a partial response, and 2 (13%) were classified as nonresponders [one patient had glomerulonephritis, another central nervous system (CNS) involvement].

Two recent RCTs involving 47 patients have found significant improvements compared with PBO in sicca features, salivary flow, ocular tests, fatigue, and some quality-of-life scores (Dass et al., 2008; Meijer et al., 2010). The first RCT included 17 patients with primary SS with severe fatigue score on fatigue visual analog scale (VAS) >50 who were randomized to receive either two infusions of 1 g of RTX or PBO. Both arms received 100 mg of methylprednisolone on day 1, 60 mg of prednisone on days 2 to 7, and 30 mg on days 8 to 14. There was significant improvement from baseline in fatigue VAS in the RTX group ($p < 0.001$), although a trend

toward significant improvement was also observed in the PBO group ($p = 0.147$). However, SF-36 social functioning scores were significantly higher in the RTX group at 6 months ($p = 0.01$). The SF-36 is used as a variable in the quality-adjusted life year calculation to determine the cost-effectiveness of a health treatment. It is not clear, however, whether the differences can be exclusively attributed to RTX or if they stem from the synergistic effect of RTX and the high doses of corticosteroids prescribed. The second RCT (Meijer et al., 2010) included 30 patients with active primary SS and stimulated whole saliva secretion ≥0.15 ml/min who were treated with either RTX (1000 mg) or PBO infusions at days 1 and 15. In the RTX group, a significant improvement was found for stimulated whole saliva secretion and for various laboratory parameters (RF), subjective parameters (multidimensional fatigue inventory scores and VAS scores for sicca complaints), and extraglandular manifestations compared with PBO. Moreover, RTX treatment significantly improved stimulated whole saliva secretion, RF, unstimulated and stimulated whole saliva, lissamine green test, VAS scores, and questionnaires of quality of life and fatigue compared with baseline values.

Similar to the studies on immunosuppressive agents in SS, those involving RTX have centered on evaluating its effects on sicca syndrome. However, RTX has shown promising improvements in extraglandular features in a recent small RCT (Meijer et al., 2010). In addition, 15 (83%) of 18 patients with extraglandular involvement included in three separate uncontrolled studies responded to RTX (Pijpe et al., 2005; Devauchelle-Pensec et al., 2007; Seror et al., 2007). The amount and the quality of the reported evidence on the off-label use of RTX in SS is clearly higher than that for the standard options (glucocorticoids and immunosuppressive agents). However, while we await the results of larger trials, RTX should only be considered as a rescue therapy in patients who exhibit involvements refractory to standard treatment (Ramos-Casals and Brito-Zeron, 2007).

d. Systemic sclerosis. SSc is an autoimmune disease characterized by excessive extracellular matrix deposition in the skin and other visceral organs. The molecular basis for SSc is unknown; however, a number of studies have focused on the pathogenic mechanisms of immune activation and tissue fibrosis in SSc.

The presence of specific circulating autoantibodies (including anti-topoisomerase I antibody, anti-centromere antibody, and anti-RNA polymerase antibody) is a common identifying feature and usually precedes disease onset (Hasegawa, 2010). B cells from patients with SSc overexpress CD19 and are chronically activated, compared with B cells from healthy subjects and disease control patients (Sato et al., 2004).

Analysis of gene expression in SSc skin lesions through DNA microarrays has revealed the up-regulation of genes related to B cells (Whitfield et al., 2003). B-cell infiltration is found in the lesional skin of patients with SSc and in the lungs of persons with SSc-associated interstitial lung disease. (Whitfield et al., 2003; Lafyatis et al., 2007).

Three open-label clinical studies are currently evaluating the clinical efficacy of RTX in SSc (Lafyatis et al., 2009; Smith et al., 2010; Daoussis et al., 2010). Daoussis et al. (2010) performed an open-label, randomized, controlled, 1-year pilot study with 14 patients suffering SSc (Daoussis et al., 2010). Eight patients were randomized to receive two cycles of RTX at baseline and at 24 weeks [four weekly RTX infusions (375 mg/m^2)] in addition to standard treatment, whereas 6 other patients (control group) received standard treatment alone. After 1 year, there was a significant improvement in the pulmonary function test, including forced vital capacity and diffusing capacity of carbon monoxide in the RTX group compared with baseline, whereas no change was noted in the control group. The improvement of lung function tests in the patients treated with RTX was already evident at the 24-week evaluation.

At the same time, significant skin improvement was recorded in the RTX-treated group compared with baseline, as assessed by the modified Rodnan skin score (mRSS) and by the reduction of collagen deposition in the papillary dermis at 24 weeks. [The mRSS uses clinical palpation to estimate the skin thickness. The skin thickening is assessed by palpation of the skin in 17 areas of the body (fingers, hands, forearms, arms, feet, legs and thighs, face, chest, and abdomen) using a 0-to-3 scale.] In contrast, no significant changes in skin scores or skin biopsies were noted in the control group.

Smith et al. (2010) evaluated the clinical efficacy of RTX therapy in eight patients with diffuse SSc who received a single course of RTX (1000 mg). After 24 weeks, skin thickening as assessed by mRSS, collagen scores, and myofibroblast positivity on skin biopsies improved significantly compared with baseline values. B cells were evident in the skin of four patients and were eliminated after treatment. Parameters of internal organ involvement and function remained stable throughout the study. RTX was well tolerated, and no unexpected adverse events were observed.

Lanzavecchia and Sallusto (2009) conducted an open-label study of RTX treatment in patients with diffuse SSc. Fifteen patients were enrolled; all received 2 doses of RTX (1000 mg) intravenously, administered 2 weeks apart. The mRSS did not change significantly between baseline and 6 months. Nevertheless, histological analysis of skin biopsies revealed a significant reduction in the myofibroblast score and in the elimination of skin-infiltrating B cells after treatment. Pulmonary function tests remained stable at 24 weeks compared with baseline. None of the patients showed evidence of new or progressive major organ involvement. No serious adverse events considered to be associated with RTX

were noted. The potential efficacy of RTX in SSc-associated pulmonary arterial hypertension has not yet been evaluated.

e. Vasculitis.

i. Anti-neutrophil cytoplasmic antibody-associated vasculitis. As mentioned above, evidence derived from both in vitro studies and recent animal models points to a pathogenic role of ANCAs in AAV. Multiple cell types are present in WG granulomas, including $PR3^+$ cell clusters (neutrophils and monocytes), which are surrounded by Ag-presenting cells and abundant Th1-type $CD4^+CD28^-$ effector memory T cells. Maturing B and plasma cells are also present, suggesting the neoformation of lymphoid-like tissue (Lamprecht et al., 2007). Chronic T-cell activation might promote neogenesis of lymphoid-like tissues in which the affinity maturation of autoreactive B cells and plasma cells may take place (the latter secreting PR3-ANCA, which, on reaching the bloodstream, causes vasculitic lesions) (Voswinkel et al., 2005).

Voswinkel et al. (2006) found B-lymphocyte-rich follicle-like aggregates within the granulomatous lesions of endonasal specimens from patients with WG in the vicinity of $PR3^+$ cells and plasma cells. Analysis of the immunoglobulin gene repertoire revealed the presence of autoreactive B cells with a potential affinity for PR3. Chronic secretion of TNFα and interferon-γ in the granuloma might provide a favorable proinflammatory environment for the neogenesis of GC-like structures, in which T and B cells would cooperate to break down tolerance and maintain antibody production against PR3.

The rationale for RTX use in AAV rests on the assumption that elimination of the immediate precursors of CD20 plasma cells could interfere with their replacement, leading to transient pathogenic antibody removal and healing of vasculitis. This presupposes that ANCAs are produced by short- and not by long-lived plasma cells (Sneller, 2005). In addition, RTX might block B-cell-dependent autoimmune arms unrelated to autoantibody production such as cytokine secretion, Ag presentation, and interactions with T cells and other Ag-presenting cells, thus hypothetically producing widespread immunologic effects (Lamprecht et al., 2007). In patients with AAV, the number of activated circulating B lymphocytes correlates with disease activity and with the extent of involvement (Popa et al., 1999).

It seems that granulomatous features (typically found in WG) are resistant to RTX, which confirms the current belief that B cells and ANCAs play negligible roles in initiating or sustaining granuloma-dependent damage (Sneller, 2005). However, RTX seems to effectively heal vasculitis, apparently by preventing the generation of ANCAs, although some patients exhibit B-cell depletion, albeit without ANCA clearance.

Likewise, RTX failed to reduce anti-dsDNA and antiphospholipid antibodies in patients with SLE. This challenges the theory of ANCA production by short-lived plasma cells. The fact that half the cases experienced such a failure suggests that a significant proportion of ANCAs may be produced by autoreactive long-lived plasma cells; in fact, the ANCA isotype (IgG) and the presence of somatic mutations point to long-lived plasma cells as the source of ANCAs (Sneller, 2005). Another plausible explanation is that the beneficial effect of RTX depends more upon the inhibition of B-cell functions rather than upon antibody production (e.g., the B-cell influence on T-cell functions).

The best evidence for the use of RTX in patients with ANCA-associated vasculitis comes from retrospective case series and small prospective uncontrolled studies, mainly involving patients with refractory or frequently relapsing disease. The initial 6-month results of the multicenter, randomized, PBO-controlled RAVE trial of the effectiveness of RTX compared with cyclophosphamide in inducing disease remission in patients with severe WG or microscopic polyangiitis positive for PR3-ANCA or MPO-ANCA have been reported (Keogh et al., 2005; Stone et al., 2010). Ninety-nine patients received intravenous RTX (375 mg/m^2 of body surface, once weekly for 4 weeks) plus daily PBO-cyclophosphamide, whereas 98 control subjects received PBO-RTX infusions plus daily cyclophosphamide (2 mg/kg body weight, adjusted for renal insufficiency). Control subjects achieving remission between 3 and 6 months were switched from cyclophosphamide to azathioprine (2 mg/kg per day). Patients receiving RTX who achieved remission during the same period were switched from PBO-cyclophosphamide to PBO-azathioprine. The glucocorticoid regimen was 1 to 3 pulses of methylprednisolone (1000 mg each), followed by prednisone (1 mg/kg/day) in both groups, with tapering so that all patients achieving remission without disease flares by 5 months had discontinued glucocorticoids. The primary endpoint was a Birmingham Vasculitis Assessment Score for Wegener's Granulomatosis (BVAS/WG) of 0 and successful prednisone tapering at 6 months, and was achieved by 64% of patients receiving RTX and 53% of control subjects, meeting the criterion for noninferiority ($P < 0.001$). [The BVAS/WG score includes both general symptoms (arthralgia, arthritis, and fever) and involvement of eight major organ systems and ranges from 0 (complete remission) to a maximum of 68.] RTX was more efficacious in inducing remission of relapsing disease; 67% of patients receiving RTX and 42% of control subjects reached the primary endpoint ($P = 0.01$). RTX was as effective as cyclophosphamide in treating patients with major renal disease or alveolar hemorrhage. No significant differences in disease flare rates, total adverse events, serious adverse events, non–disease-related adverse events, or patients with ≥ 1 non–disease-related adverse event were found. Thirty-three percent of control subjects had ≥ 1 of the predefined selected adverse events compared with 22% of patients receiving RTX ($P = 0.01$), princi-

pally as a result of more episodes of severe leukopenia in control subjects.

Concurrently, the open-label, two-group, parallel-design RITUXVAS trial involving patients with newly diagnosed ANCA-associated vasculitis and renal involvement found that RTX was not superior to standard intravenous cyclophosphamide in inducing disease remission (Jones et al., 2010). Patients were randomly assigned to standard glucocorticoids plus either RTX (375 mg/m^2 of body-surface area per week for 4 weeks) with two intravenous cyclophosphamide pulses (33 patients) or intravenous cyclophosphamide for 3 to 6 months followed by azathioprine (11 control subjects). Sustained remission at 12 months and severe adverse events were the primary end points. Sustained remission was achieved by 76% of RTX patients and 82% of control subjects ($P = 0.68$). Six RTX patients (18%) and 2 control subjects (18%) died ($P = 1.00$). There were no significant differences in severe adverse events.

ii. Mixed cryoglobulinemia. Mixed cryoglobulinemia (MC) is a systemic disease resulting from small vessel immune-complex-mediated vasculitis. Discovery that the disease has a viral origin [hepatitis C virus (HCV)] led to widespread hope among researchers (Bichard et al., 1994). Indeed, the years since this finding have been characterized by intensive efforts to achieve viral eradication and disease remission. It is worth noting that the use of immunosuppressives has been discouraged for many years because they could favor viral replication. Because lymphoproliferation is one feature of this disease, investigators have focused on the potential benefit of newly targeted therapies specifically directed against B lymphocytes.

The objective of B-cell depletion in MC is to reduce IgM RF synthesis and to halt the proliferation of B-cell clones that sustain the disease (Racanelli et al., 2001; Landau et al., 2007). To date, seven off-label clinical trials involving 74 patients have been carried out (Zaja et al., 2002a,b, 2003; Sansonno et al., 2003; Visentini et al., 2007; Roccatello et al., 2008). In a recent systematic review of 57 patients with MC treated with RTX up to 2007, clinical response was found in 80 to 93% of cases and relapse in 39% of responders (Cacoub et al., 2008). Responders exhibited improvements in skin lesions, neuropathy, arthralgia, and renal function; reductions in cryocrit and RF levels; and normalization of serum C4 levels (Sansonno et al., 2003; Zaja et al., 2003; Roccatello et al., 2008; Saadoun et al., 2008). However, the hepatitis C viral load increased in responders without significantly changing in nonresponders (Sansonno et al., 2003; Roccatello et al., 2008).

Based on the observed increase in viral load during RTX treatment in patients with HCV-related MC, two protocols have been proposed. The first combines RTX with antiviral therapy (pegylated interferon and ribavirin) (Saadoun et al., 2008). Of 16 patients with HCV-related MC treated under this protocol, 10 (63%) had a complete clinical and virological response. The second protocol involves administering a lower dose of RTX (250 mg/m^2 on days 1 and 8) (Visentini et al., 2007). In a study of six patients with HCV-related MC, this schedule led to a complete clinical and laboratory response in four of the five eligible patients.

A prospective study (Petrarca et al., 2010) showed the effectiveness and safety of prescribing RTX for MC syndrome with advanced liver disease. In this trial, 19 HCV-positive patients with MC and advanced liver disease who had been excluded from antiviral therapy were treated with RTX and observed for 6 months. A consistent improvement in MC syndrome was evident at the end of treatment and at the end of follow-up. Once CD20$^+$ B cells were depleted, an improvement in cirrhosis syndrome was also observed, despite the possibility of transient increases in viremia titers.

f. Inflammatory myopathies. Various small open-label studies have used RTX to treat severe, refractory inflammatory myopathies. Chung et al. (2007) described partial remission of muscular involvement in three of eight (37%) patients, with no significant differences in cutaneous involvement or subjective assessments, whereas Levine (2005) reported a clear improvement in six patients (although four had a muscular relapse) with a high rate of adverse events (40%). Sem et al. (2009) reported the use of RTX in 11 patients with antisynthetase syndrome presenting severe, progressive interstitial lung disease, most of whom were refractory to immunosuppressive agents. RTX stabilized and/or improved pulmonary involvement in 7 of 11 (64%) patients 6 months after treatment, although one patient developed a fatal infection (*Pneumocystis jiroveci* infection) 3 months after the last infusion with RTX. Two separate groups reported the successful use of RTX in seven patients with refractory inflammatory myopathies (Noss et al., 2006; Mok et al., 2007). Sultan et al. (2008) studied the use of RTX in eight patients, of whom only two anti-Jo1 antibody-positive patients experienced a successful clinical response. However, histological reevaluation showed that three patients had, in fact, other types of muscular diseases (inclusion body myositis, muscular dystrophy, and nodular sclerosing lymphoma). This underlines the importance of obtaining a definitive diagnosis of inflammatory myopathy before initiating off-label therapies.

The largest series of patients thus far has been that reported by the BIOGEAS Multicenter Study Group (Ramos-Casals et al., 2010), which included 20 patients with inflammatory myopathies (11 with dermatomyositis, 4 with polymyositis, and 5 with antisynthetase syndrome), of whom 11 (55%) achieved a complete response, 6 (30%) achieved a partial response, and 3 (15%) were classified as nonresponders. The therapeutic response was excellent for muscular (94%), cutaneous (80%), and pulmonary involvement (75%). After a mean follow-up of

19 months, 47% of the responders relapsed and one patient died.

2. Other Anti-CD20 Therapies. Two humanized anti-CD20 monoclonal antibodies that target CD20 in RA are in phases II and III of clinical development: ocrelizumab (Genentech, South San Francisco, CA) and ofatumumab (Genmab, Copenhagen, Denmark).

a. Rheumatoid arthritis. In vitro, ocrelizumab demonstrated enhanced antibody-dependent, cell-mediated cytotoxicity and reduced complement-dependent cytotoxicity compared with RTX.

In the phase I/II dose-ranging *ACTION* trial (Genovese et al., 2008), which included 237 patients with active RA, ocrelizumab plus MTX was compared with PBO plus MTX. The ocrelizumab group was divided into five different dose subgroups (10, 50, 200, 500, and 1000 mg). Patients had longstanding and active RA (mean DAS-28 ranging from 6.6 to 7.1), and approximately half had previously taken an anti-TNFα inhibitor.

A higher proportion of patients in all of the ocrelizumab groups achieved an ACR20, ACR50, or ACR70 response at week 24 compared with patients in the PBO group. The best response rates in these categories were observed in the groups receiving 200 and 1000 mg. Safety was evaluated over 72 weeks after the infusions on days 1 and 15. Serious adverse effects were observed in both groups (17.9 versus 14.6%). Ocrelizumab had low immunogenicity.

The *CINEMA trial* (clinicaltrials.gov identifier NCT00808210) is an ongoing phase II study designed to evaluate ocrelizumab plus MTX compared with infliximab plus MTX in patients with active RA who have responded inadequately to anti-TNFα therapy (etanercept or adalimumab).

Ofatumumab binds to a more proximal portion of CD20, closer to the B-cell membrane. In addition, Ofatumumab has a slower rate of dissociation from CD20 than RTX, which results in greater complement-dependent cytotoxicity and lysis of RTX refractory B-cell lines.

Ofatumumab is undergoing clinical trials to assess dosing, efficacy, and safety when used in patients with RA. There are two ongoing phase III trials evaluating the efficacy of ofatumumab in RA in patients who have had an inadequate response to MTX (clinicaltrials.gov Identifier NCT00611455), as well as in patients with an inadequate response to anti-TNFα therapy (clinicaltrials.gov Identifier NCT00603525).

The small modular immunopharmaceutical drugs are highly specific, single-chain polypeptides designed with optimized target-binding and effector functions to overcome the limitations of therapeutic anti-CD20 antibodies. Small modular immunopharmaceutical SBI-087 (Fleischmann et al., 2010) is a humanized version of Trubion's TRU-015 (Stromatt et al., 2009), a single-chain construct containing a single-chain Fv specific for CD20 that is linked to the human IgG1 hinge, CH2, and CH3 domains but is devoid of the CH1 and CL domains. SBI-087 is in phase I studies for RA and SLE and TRU-015 is in phase II studies for RA (Levesque, 2009).

b. Systemic lupus erythematosus. The BELONG trial is a 2-year phase III RCT designed to evaluate the efficacy and safety of ocrelizumab (humanized anti-CD20 monoclonal antibody) in patients with lupus nephritis. The design used was similar to that of the LUNAR trial, ocrelizumab being added to the standard-of-care therapy plus immunosuppressive drugs (cyclophosphamide administered after the maintenance regimen or mycophenolate) and a course of high-dose corticosteroids. In March 2010, Roche and Biogen Idec announced their decision to suspend the ongoing trials of ocrelizumab in patients with RA and SLE (Roche Press Release, 2010). Their decision follows the recommendation of the independent Ocrelizumab RA and Lupus Data and Safety Monitoring Board based on their assessment of four trials involving RA (*SCRIPT*, *FEATURE*, *FILM,* and *STAGE*) and two trials targeting SLE (*BELONG* and *BEGIN*). The Monitoring Board review detected an infection-related safety issue that included serious and opportunistic infections, several of which were fatal in some of the 2400 patients included in these trials in more than 30 countries. Thus far, no details about the rates, types, and geographical distribution of infections have been released.

c. Anti-neutrophil cytoplasmic antibody-associated vasculitis. With regard to other B-cell directed therapies, no trials have been reported thus far in patients with AAV. It is noteworthy that ocrelizumab and ofatumumab (both humanized anti-CD20 antibodies) could provide more effective and long-lasting gains in B-cell depletion without the development of neutralizing anti-chimeric antibodies, as was reported with RTX (Golbin and Specks, 2007; Walsh and Jayne, 2007).

3. Anti-CD19-Directed Therapies. CD19 is a B-cell-specific membrane protein that is broadly expressed during B-cell development, from the pro-B cell to the early plasma cell stage. Although CD19 and CD20 mAb share common effector mechanisms, therapies targeting CD19 might offer several unique advantages for the treatment of RA compared with currently available CD20-directed immunotherapies (Tedder, 2009).

At least two unconjugated CD19 mAbs are being developed for human therapy, including the humanized mouse HB12 anti-hCD19 mAb. In addition, an Fc-engineered, affinity-matured humanized anti-hCD19 mAb (Xencor, Monrovia CA), which boasts increased FcγR binding affinity, has been shown to improve antibody-dependent cellular cytotoxicity.

MDX-1342 (Medarex, Princeton, NJ) is a fully humanized antibody that not only binds selectively to CD19 expressed on B cells (without targeting stem cells or fully differentiated plasma cells, which lack CD19 expression), but that also induces the depletion and elimination of CD19-positive B cells. Preliminary data available from an ongoing phase I study of MDX-1342 in

subjects with active RA (despite treatment with MTX) has demonstrated potent B-cell depletion effects with a single-dose (10 or 30 mg) administration of MDX-1342 (Tedder, 2009).

4. Anti-CD22 Therapy (epratuzumab). Epratuzumab is a monoclonal antibody against CD22 that inhibits B-cell proliferation, reducing their numbers by approximately 35 to 44%. CD22 is a member of the Ig superfamily and binds to sialic acid-bearing molecules on other hematopoietic and nonhematopoietic cells (Engel et al., 1995a).

a. Systemic lupus erythematosus. In an open-label trial, 14 patients with SLE were treated with a dosage of 360 mg/m^2 every 2 weeks (Dörner et al., 2006) with no significant adverse events and with improvements in BILAG scores. A subsequent study of peripheral B cells in 12 of the 14 patients showed that epratuzumab reduced levels of CD27$^-$ B cells (mainly naive and transitional B cells), without having any effect on CD27$^+$ memory cells (Jacobi et al., 2008). In an August 27, 2009, press release, UCB and Immunomedics announced positive results in a phase IIB RCT, in which a difference of 25% in the clinical response between epratuzumab and PBO was found at week 12 using a novel endpoint, which was a composite of improvement in the BILAG score and no worsening in the SLEDAI score or the Physicians Global Assessment score (UCB Press Release, 2009). The trial enrolled 227 patients, 70% with "severely active disease." However, more detailed information is still required to fully evaluate the positive results obtained in this trial.

b. Sjögren's syndrome. In 2006, a prospective open-label study carried out in 16 patients treated with four infusions of epratuzumab 360 mg/m^2 once every 2 weeks found a significant improvement in fatigue and sicca symptoms based on a composite response score (Steinfeld et al., 2006), which included the Schirmer-I test, unstimulated whole salivary flow, fatigue, ESR, and Ig G. B-cell levels were reduced by a mean of 54 and 39% at weeks 6 and 18, respectively, although T-cell levels and Igs remained relatively unchanged. Fifty-three percent of patients achieved a clinical response (>20% improvement) at week 6, and 67% responded at week 32, with significant improvements being observed in fatigue and patient/physician global assessments. However, 10 patients reported adverse events, including four classified as severe events. Since 2006, no other studies have tested epratuzumab in patients with primary SS.

B. Blocking B-Cell Activation and Survival

1. Belimumab. BLyS is a factor used to determine the survival of B cells. This is particularly the case for murine B cells—BLyS has been found to have very little effect on the survival of developing human B cells; this is also consistent with the relatively modest effects of anti-BLyS Ab therapies in humoral autoimmune conditions.

Elevated BlyS levels, either locally or in serum, have been observed in patients with RA and SLE. Belimumab is an anti-BLyS monoclonal antibody (LymphoStat-B) that binds to soluble BLyS (Levine et al., 2000) and inhibits its binding to TACI, BCMA, and BR3. The specificity and affinity of belimumab for BLyS suggests that it may reduce B-cell survival resulting from an excess of BLyS (Cancro et al., 2009).

a. Rheumatoid arthritis. In a randomized, double-blind, multicenter, and PBO-controlled phase II clinical trial, a total of 283 patients with RA (85% with positive RF) with active moderate-to-severe disease activity were studied (McKay et al., 2005). Belimumab at doses of 1 (n = 72), 4 (n = 71), or 10 mg/kg i.v. (n = 71), or PBO (n = 69) was administered on days 0, 14, and 28, and then every 4 weeks through week 24, in addition to standard-of-care therapy (concurrent DMARD). The ACR 20 response at week 24 was 29% in all belimumab-treated groups and 35% in the 1 mg/kg group, which were modest but significantly higher gains than that (16%) observed in the PBO group. In addition, trends suggesting some drug benefit were observed in the 4 mg/kg group, as well as in the 10 mg/kg group, although no significant dose-response relationship was noted. The ACR 50 responses at week 24 were 9 to 14% in the belimumab-treated groups, which were not significantly higher than that recorded in the PBO group (4%).

In the phase II clinical trial of RA, there were no significant differences in the incidences of any adverse events, any related adverse events, or any severe and serious adverse events between the belimumab and PBO groups. The most frequent adverse events observed were arthralgia, upper respiratory tract infections, urinary tract infections, diarrhea, joint swelling, headache, fatigue, peripheral edema, pain in extremities, cough, pruritus, and sinusitis (Ding and Jones, 2006).

b. Systemic lupus erythematosus. Elevated serum levels of soluble BLyS have been observed in patients with SLE (Cancro et al., 2009), suggesting that this molecule may play a key role in the etiopathogenesis of the disease.

The safety and efficacy of belimumab in SLE has been evaluated in several RCTs. In a dose-ranging phase I RCT (Furie et al., 2008) that included 70 patients with mild-to-moderate SLE, treatment with one or two doses of belimumab led to significant reductions in the median percentage of CD20$^+$ B cells. A recent dose-ranging phase II trial (Wallace et al., 2009) evaluated three doses of belimumab (1, 4, or 10 mg/kg) in 449 patients with SLE administered at 0, 2, and 4 weeks and then every 4 weeks for 52 weeks. At study entry, patients were required to be on stable background therapy, including immunosuppressive agents, and 30% had negative immunological markers (ANA and/or anti-dsDNA). There were no significant differences between belimumab and PBO in either of the primary endpoints at any dose used (Wallace et al., 2009). The data from this trial were subsequently used to develop a novel SLE responder index (SRI). An SRI response was defined as

>4-point reduction in the Systemic Lupus Erythematosus Disease Activity Index (SELENA)-SLEDAI score with no new BILAG A or no more than one new BILAG B score and no deterioration from baseline in the physician's global assessment (Furie et al., 2009).

The initial results of two phase III RCTs using belimumab in patients with SLE have been reported (BLISS-52 and BLISS-76). The design of the BLISS-52 trial was standard-of-care therapy plus belimumab (1 or 10 mg/kg) or standard-of-care plus PBO on days 0, 14, and 28, and then every 28 days for 52 weeks. A total of 865 patients with moderate to severe SLE (mean SELENA-SLEDAI of nine) were included. Preliminary results were presented as a late-breaking abstract at the 2009 ACR National Meeting (Navarra, 2009). An SRI response at 52 weeks was achieved by 46% of PBO-treated patients compared with 51% of those receiving belimumab 1 mg/kg and 58% of those treated with belimumab 10 mg/kg ($p = 0.013$ and 0.0006, respectively). A second phase III trial (BLISS-76) randomized 819 active patients with SLE mainly recruited in North America and Europe to PBO or belimumab (1 or 10 mg/kg). The design of BLISS-76 is identical to that of BLISS-52 with the primary endpoint at week 52, although patients and investigators remained blinded for an additional 24 weeks. On November 2, 2009, preliminary results were made public in a press release from Human Genome Sciences (Press Release, 2009). The SRI response at 52 weeks showed a significant effect of belimumab for the high-dose group and a trend toward significance in the low-dose group, although the secondary outcomes were not uniformly positive as in BLISS-52, and there were no statistical differences in the steroid reductions achieved across the groups.

Belimumab may be the first new drug approved for SLE in over 40 years. However, some doubts persist regarding its true effectiveness. The difference in the SRI response between the high-dose arm and the PBO arm at 52 weeks was 14% in BLISS-52 and 9.4% in BLISS-76. These differences are relatively modest and will certainly be weighed against the cost once the price of this medication has been established. In addition, the global response rates in the LUNAR trial (57 versus 46%) closely parallel those of the BLISS-52 (58 versus 44%) and BLISS-76 (43 versus 34%) trials. At present, some questions remain unanswered, including which lupus manifestations might be most responsive to this therapy and at which point during the course of SLE should belimumab be initiated (Dall'Era and Wofsy, 2010).

c. Sjögren's syndrome. Several studies have described higher BAFF expression in SS. Groom et al. (2002) found elevated levels of circulating BAFF, as well as a dramatic up-regulation of BAFF expression in salivary glands, suggesting that the altered differentiation and tolerance of B cells was induced by an excess of BAFF. Szodoray et al., (2003) found reduced levels of apoptosis among BAFF-expressing cells, possibly leading to prolonged BAFF expression in these cells, which had maintained positive signals for the infiltrating B cells to proliferate and mature. Two separate groups have revealed the capacity of epithelial cells to express and secrete BAFF after interferon stimulation (Gottenberg et al., 2006; Ittah et al., 2006). These experimental studies suggest that BAFF plays a key role in the development of autoimmune/lymphoproliferative processes in primary SS. Mariette et al. (2003) clinically demonstrated a correlation of BAFF levels with circulating autoantibody levels (IgG, RF, anti-Ro, and anti-La) in patients with SS. Pers et al. (2005) have recently found increased BAFF serum levels in 43 patients with SLE, 58 patients with primary SS, and 28 patients with RA, compared with 68 control subjects. High levels of BAFF were associated with the presence of autoantibodies (anti-dsDNA antibodies in SLE, anti-SSA antibodies in SS, and RF in RA), suggesting that high levels of BAFF may be directly related to the B-cell hyperactivity/proliferation usually observed in patients with systemic autoimmune diseases. Recent studies have focused on analyzing the influence of BAFF expression in response to treatment with RTX (Lavie et al., 2008; Quartuccio et al., 2008). BAFF-blocking agents may prove to be an effective therapy for primary SS, and a phase II trial is currently under way (SS-BEL-01-1.0, clinicaltrials.gov).

2. Atacicept. Atacicept is a fully humanized, recombinant receptor–Ig fusion protein (TACI-Ig or atacicept). Atacicept binds to the B-cell growth factors BLyS and APRIL. Atacicept inhibits B-cell maturation, differentiation, and survival, as well as immunoglobulin production, by depriving B cells of needed growth and development signals (Dörner et al., 2009c).

a. Rheumatoid arthritis. In a phase Ib clinical trial of 73 patients with RA, atacicept was found to be well tolerated. Adverse events were reported by 32 patients (44%); only three events were considered to be severe. There was no significant difference in the frequency of infection-related events between patients who received atacicept and those who received PBO or between treatment groups. No infection-related events were considered serious or severe (Tak et al., 2008).

Given the positive results in these early studies, several phase II clinical trials are currently ongoing and include: 1) dose-finding study (clinicaltrials.gov identifier NCT00430495), 2) combination study with RTX (clinicaltrials.gov identifier NCT00664521), and 3) study in anti-TNFα-naive patients with moderate-to-severe RA and an inadequate response to MTX (clinicaltrials.gov identifier NCT00595413).

With both of these compounds (belimumab and atacicept), there was a reduction in B-cell numbers, a reduction in RF, and reductions in total immunoglobulin levels, efficacy in these studies was not as robust as that seen with compounds targeting CD20.

b. Systemic lupus erythematosus. Atacicept has been evaluated in several phase I studies in patients with mild-to-moderate SLE (Pena-Rossi et al., 2009; Dall'Era and Wofsy, 2010; Nestorov et al., 2010). The results have shown that atacicept reduced Ig levels, as well as the number of mature and total B cells, with no significant changes in the number of T cells and monocytes. A recent phase II trial of atacicept in lupus nephritis in combination with mycophenolate mofetil was suspended because of a high rate of severe infections, although a phase II/III trial of atacicept for nonrenal lupus with less immunosuppressive concomitant therapy remains ongoing (Looney, 2010). Because of its profound effects on B cells, atacicept may have potential treatment implications for autoimmune diseases, although its concomitant use with other biological agents or with immunosuppressive drugs must be rigorously investigated.

c. Vasculitis. BAFF is found at increased levels in patients with active vasculitis (Krumbholz et al., 2005). It is therefore thought that anti-BAFF antibodies (belimumab) could subtly modulate T- and B-cell interactions in AAV without incurring B-cell depletions. BAFF levels become elevated with RTX therapy, suggesting that the addition of belimumab may enhance B-cell suppression (Golbin and Specks, 2007).

V. Safety of B-Cell-Depletion Agents

Information regarding the safety of B-cell therapies (especially RTX) in rheumatic diseases comes mainly from trials performed in patients with RA. The safety of RTX in the treatment of RA has been reported in RCTs after 6 to 12 months of follow-up, and in open-label extension studies over multiple courses of RTX (Edwards et al., 2004; Cohen et al., 2006; Emery et al., 2006; Keystone et al., 2007). A pooled analysis of safety in RA was recently published (van Vollenhoven et al., 2010). This study included 2578 patients with RA who received at least one course of RTX, thus providing 5013.5 patient-years of observation during these clinical trials. A total of 2244 patients had ≥1 year of follow-up, 851 patients had ≥2 years, 720 patients had ≥3 years, 317 patients had ≥4years, and 97 patients had ≥5 years.

All studies of patients with RA have indicated that circulating B cells are undetectable after a brief dosing regimen of RTX. B-cell numbers in the blood remain low for approximately 6 to 12 months (Roll et al., 2006). Stem cells in the bone marrow are thought to be spared, although depletion of pre-B cells has been noted in a few patients (Leandro et al., 2007). In general, the capacity to regenerate naive B cells, however, seems to remain unaffected.

A. Infusion Reactions

Mild or moderate adverse effects are common in patients treated with RTX and are particularly likely during the first infusion, occurring in up to 30 to 45% of patients (Edwards et al., 2004). Most RTX infusions are accompanied by a mild sensation of tightening in the throat area. Based on two phase II studies (Edwards et al., 2004; Cohen et al., 2006) and one phase III trial (Cohen et al., 2006), the most common infusion-related side effects reported by patients receiving RTX were headache, hypertension, nausea, pruritus, urticaria, and flushing, all of those occurring within the first 24 h.

RTX infusion reactions are thought to largely stem from the degree of B-cell lysis and the amount of cell content release, rather than as a direct reaction to the agent itself. The frequency and intensity of the acute infusion reactions can be reduced by approximately one third if intravenous corticosteroids are given as premedication (Fleischmann, 2009).

From a pooled analysis of 2578 patients treated with RTX, a total of 123 patients (5%) who received RTX withdrew because of adverse events (AE), including 57 (2%) serious AE. The most common AEs leading to withdrawal, excluding arthritis-related events, were infusion-related reactions (IRR) (29 events), malignancies (19 events), infections (15 events), and cardiac disorders (6 events).

The overall rate of AE was 359.6 events per 100 patient-years (95% CI, 354.4, 364.9). Overall AE rates were highest for course 1, declining for course 2, and then remaining stable during the later courses. The most commonly reported AEs were IRR, particularly with course 1 (25% of patients during the first infusion). IRRs were experienced by 915 patients (36%), the incidence being highest during the first infusion of course 1 (25%). The incidence of IRRs in all subsequent courses then fell.

The proportion of patients with IRRs who required slowing, stopping, or interruption of the infusion was highest during course 1 (9%), subsequently declining to 5, 3, 2, and 0% for courses 2 to 5, respectively (van Vollenhoven et al., 2010).

B. Immunogenicity

The prevalence of human anti-chimeric antibodies (HACA) to RTX in RA and patients with SLE treated with this agent proved higher than that previously noted in patients treated for lymphoma. The clinical significance of HACA development in patients with RA remains unclear.

In the above-mentioned pooled analysis (van Vollenhoven et al., 2010), 11% (273 of 2578) of patients had an HACA-positive titer on at least one visit. The proportion of patients with overall IRRs upon retreatment (course 2) was similar between those who were previously HACA-positive (15%; 24 of 157) and those who were previously HACA-negative (17%; 286 of 1733).

C. Infections

The risk of serious infections does not seem to be significantly higher in adult patients with RA treated

with RTX compared with those treated with MTX. The longer-term effects of B-cell depletion and reduction in serum IgG and IgM levels vis-à-vis the risks of infection are uncertain at this stage. A recent *meta*-analysis suggests that treatment of patients with RA with RTX is not associated with an increased incidence of serious infections (Salliot et al., 2009). Serious infections were reported to occur most often during the first 3 months after the infusion, declining thereafter irrespective of treatment course. The most common infections reported were bacterial infections, including nasopharyngitis, pneumonia, urinary tract infections, and cellulitis.

In the three largest controlled clinical trials using RA (Edwards et al., 2004; Cohen et al., 2006; Emery et al., 2006), infections were observed in 123 (16.5%) of the 745 RTX-treated patients and in 74 (18.6%) of the 398 who received PBO, which did not represent a significant difference. In a pooled analysis of safety (van Vollenhoven et al., 2010), a total of 1663 (65%) of 2578 patients experienced infections with a rate of 97.7 per 100 patient-years (95% CI, 95.0, 100.5). During the 6-month PBO-controlled periods, rates of overall infections were similar between the PBO and RTX groups (39 and 40%, respectively).

There were no cases of tuberculosis, disseminated fungal infections, or other serious opportunistic infections during the analysis period made by van Vollenhoven and colleagues. The risk of viral infection during RTX treatment remains unknown, although some case reports have been published, including hepatitis B virus (Dervite et al., 2001), cytomegalovirus (Suzan et al., 2001), varicella-zoster (Bermúdez et al., 2000), and parvovirus B19 (Sharma et al., 2000).

Progressive multifocal leukoencephalopathy (PML) is a severe demyelinating disease of the CNS that is caused by reactivation of the polyomavirus JC (JCV). Asymptomatic primary infections with JCV occur in childhood and antibodies can be found in most adults (Weber et al., 1997). In most individuals, JCV remains latent in the kidneys and lymphoid organs; however, in the context of profound cellular immunosuppression, JCV can reactivate, spread to the brain, and induce a lytic infection of oligodendrocytes, which are the CNS myelin-producing cells.

PML was initially described in patients with lymphoproliferative and myeloproliferative diseases such as chronic lymphocytic leukemia, chronic myeloid leukemia, and Hodgkin's lymphoma. Subsequently, PML was reported in patients with solid organ malignancies, granulomatous and inflammatory diseases, solid organ transplant recipients, and human immunodeficiency virus-infected patients. Rare cases of PML have been observed in patients with CD8-positive T-cell leukopenia with multiple sclerosis and/or Crohn's disease who were treated with the biological therapy natalizumab.

In the context of rheumatic diseases, 38 of the 50 patients with PML reported in the literature until April 2009 suffered from SLE, whereas 18 had other rheumatic diseases. Most patients received immunosuppressive treatment and RTX was used in only two cases (two patients with SLE) (Molloy and Calabrese, 2009).

Molloy and Calabrese (2009), drawing from the Nationwide Inpatient Sample database, examined the different cases of PML reported in rheumatic diseases. Overall, 92 patients were analyzed: 43 had SLE, 25 had RA, and 23 had other connective tissue diseases (including SSc, dermatomyositis, polymyositis, and SS).

A clear association between PML and RTX cannot be established at present. PML had been reported in patients suffering from SLE without previous exposure to RTX therapy. Furthermore, some patients have been described as having a history of moderate-low doses of immunosuppression, suggesting that SLE itself may be a predisposing factor for the development of PML.

VI. Future Directions and Conclusions

B-cell-directed therapies have become the focus of intensive research during recent years. The effectiveness of RTX in patients with RA, which has already been demonstrated in several large clinical trials, has led to an exploration of its efficacy in those autoimmune diseases in which B cells are thought to play a pathogenetic role. For instance, a recently concluded double-blind RCT has shown the effectiveness of this agent in inducing remission in patients with AAV. The results of many noncontrolled clinical trials and case studies suggest that rituximab is effective in SS, SLE, MC, and inflammatory myopathies. Although evidence from large controlled clinical trials is still needed, the superiority of RTX over other B-cell-directed therapies for the treatment of systemic autoimmune disorders will probably decrease as other B-cell-directed therapies have successful trials.

The anti-CD20 B-cell depletion observed in patients with RA, as well as in persons afflicted with other diseases, has increasingly pointed to the possibilities of reactive memory, which seems to be an extraordinary target, particularly for those who are refractory to anti-TNFα therapy. Because the effects on protective immunoglobulin levels are less significant than those on some other autoreactive autoantibodies, it may be time to reconsider former theories and assumptions about "autoantibody-driven autoimmunity."

Further dissection of the function of B-cell subsets, including Breg cells, will likely allow for the development of more efficient and safer B-cell-directed therapies, beyond drastic depletion, for the treatment of rheumatic autoimmune diseases.

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Wrote or contributed to the writing of the manuscript: Engel, Gómez-Puerta, Ramos-Casals, Lozano, and Bosch.

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