Monitoring anti-B cell immunotherapies in autoimmune diseases: Go with the flow.
A Position Paper of the Italian Society for Clinical Cell Analysis (ISCCA)

Bruno Brando,1 Arianna Gatti,1 Alfredo Maria Lurati,2 Paola M.L. Faggioli2
1Hematology Laboratory and Transfusion Center; 2Rheumatology Unit, Western Milan Area Hospital Consortium, Legnano (MI), Italy

Abstract

During the past decades autoimmune diseases have been usually treated with immunosuppressive drugs mostly active on T-Cell mediated responses. Only in recent years, with our extended knowledge of the pathogenic mechanisms of autoreactive disorders and the tremendous development of new therapeutic monoclonal antibodies, anti-B-Cell therapies have emerged as a new option for treating autoimmune diseases.

The rationale for this changeover from T-Cell to B-Cell targeted therapies resides in the recently accumulated evidence of the role of B-Cells in the pathogenesis of autoimmune diseases and in the generation of tissue damage. Targeting memory and effector B-Cells may then disrupt the production of pathogenic antibodies, counteract the role of B-Cells in sustaining antigen presentation to T-Cells and block the synthesis of B-Cell activation cytokines.

The anti-CD20 monoclonal antibody Rituximab was first introduced more than 20 years ago for the treatment of CD20+ chronic B-lymphoproliferative disorders, and was then successfully experimented in the treatment of an ever-increasing spectrum of autoimmune diseases. Newer anti-CD20 monoclonal antibodies have been introduced more recently, which vary in their biological effects.

The need for laboratory indicators that may help the rational usage and follow-up of anti-CD20 treatments has now emerged, due to the high variability of individual response, to the markedly different outcomes in the various diseases and to the controversial role of pathogenic autoantibodies as indicators of disease activity.

Flow cytometric (FCM) analyses to identify and enumerate the B-cell functional subsets in the peripheral blood have been developed in recent years. They can be used to assess the degree and the persistence of memory B-Cell depletion, the quality and the timing of B-Cell reconstitution, along with the highly sensitive FCM counting technique needed for the detection of extremely low cell levels.

The long-term aim of this innovative approach is to provide clinicians with a tool for a safer and more rational usage of anti-CD20 agents.

Phenotypic and functional B-cell ontogenesis

B-Cells play an important part in immune response, being the precursors of immunoglobulin (Ig)-secreting plasmacells, also acting as antigen-presenting cells to T lymphocytes and as producers of a number of effector and regulatory cytokines.1

B-Cell progenitors originate from bone marrow CD34+ CD10+ CD38++ CD45low precursors and undergo a maturation process that includes the orderly expression of differentiation markers, such as CD19, CD20, CD21, CD22, CD23, CD24, cytoplasmic and surface (s)IgM, slgD, slgG and the gradual disappearance of markers of immaturity (Figure 1). Various terms have been proposed over time to define the earlier B-Cell differentiation steps, sometimes causing confusion in terminology.2 B-Cell precursors in the bone marrow show typical morphological features, with large round nucleus, highly condensed chromatin, absence of nucleoli and scanty cytoplasm. Such cells, collectively called hematogones, are often indistinguishable from neoplastic lymphocytes of B-ALL.2,4 The local maturation process is nurtured by bone marrow stromal cells and by a series of cytokines and growth factors.5,6 Young B-Cells are then released from the bone marrow at a stage defined as ‘Transitional’, which is characterized by the CD45+ CD19+ CD10+ CD24high CD38high slgM+ slgD+ phenotype.6,7 From this stage on slgD, CD20 and CD21 tend to be gradually up-regulated, while B-Cells keep their original naïve antigen-inexperienced status. During this process the B-Cell surface antigen receptor (µ chain) is assembled with other membrane co-receptors to ensure the proper intra-
cellular signaling. The B-Cell antigen receptor is edited to provide the generation of a functional surface immunoglobulin μ chain and to provide the clonal deletion of potentially autoreactive clones, with the development of tolerance induction.9

Once released in the peripheral blood, B-Cells experience a complex recirculation pathway in lymph nodes and secondary lymphoid organs to undergo the somatic hypermutation process and acquire antigen specificity and memory. The acquisition of the surface CD27 marker defines the memory B-Cell phenotypically.10 At this stage two different pathways are possible: the ‘classic’ T-dependent instruction process occurring in germinal centers, giving rise to sIgG switched memory CD27+ B-Cells responsible for the acquired immunity and the ‘T Cell-independent’ differentiation mechanism stimulated by CpG via the Toll-Like Receptor 9, that generates IgM unswitched CD27+ memory B-Cells involved in natural immunity.8,11

After exposure to antigen, memory B-Cells rapidly enter the cell cycle and undergo multiple rounds of division, differentiating into antibody-secreting plasmablasts and plasmacells.8 Plasmablasts mostly of mucosal origin can be found in the circulation in low numbers in normal individuals. They retain for a while some surface Ig chains, while losing most B-Cell differentiation markers except CD19, HLADR and partly acquiring the plasmacell marker CD138.12 Soon plasmablasts find their niche in the bone marrow and in other lymphatic organs where they complete their maturation into fully functional CD38++ CD138++ CD19+ cytoplasmic Ig+++.12

Mature plasmacells can be further divided into short-lived and long-lived elements. Short-lived plasmacells are usually generated in extrafollicular sites, proliferate and can extend their lifespan only under certain favorable conditions. Long-lived plasmacells are non-proliferating post-mitotic elements generated during germinal center reactions, and account for a lifetime production of antibodies independent of further antigenic stimulation.13-16

**Targeting B-cells in the treatment of autoimmune disorders: rationale and consequences**

The concept of T Cell-dependence of both adaptive and autoreactive immune responses has been central to classical immunology for many decades.17 This led to the development of therapeutic strategies to control alloimmune and autoimmune reactions in which T Cells and their soluble products were the major target,17-22 along with the almost ubiquitous corticosteroids.

The aim of T Cell-targeted therapies in autoimmune disorders is the restoration of self-tolerance, which can be sometimes achieved, but only using highly intensive drug protocols.19

Anti-T drug regimens proved overall effective though not entirely satisfactory, due to the high rate of side effects related to over-immunosuppression, opportunistic infections, organ toxicity and cancer.

Although the role of T Cells and their soluble products remains
well established in the pathogenesis of rheumatoid arthritis (RA),23-27 systemic lupus erythematosus (SLE)28,29 and other autoimmune disorders.30-33 the almost incidental evidence that B-Cell depletion can also be effective in such diseases has prompted the re-evaluation of the B-Cell role in the underlying biological mechanisms and as a possible therapeutic target.

Ectopic germinal centers can be demonstrated in the synovium of RA patients, where T-Cell-independent mechanisms of CD27+ memory B-Cell differentiation take place and perpetuate the local autoimmune response.21,25,34,35

Evidence has been accumulated over the last decade concerning the rationale of anti-B-Cell treatments in autoimmune disorders.36 as well as in alloimmune responses in solid-organ transplantation.37

In the majority of autoimmune disorders, autoantibodies against native or modified autologous targets can be usually detectable, and are mostly used as diagnostic indicators at disease onset. Not in all cases, however, do the serologic profiles change with disease activity or under treatment, even in patients with a favorable clinical response to therapy. It is therefore unclear whether such autoantibodies always play a direct pathogenetic role, whether they can be taken as indicators of disease activity or are mere by-products of the ongoing inflammatory processes.13,31,38-46

The rationale for using anti-CD20 monoclonal antibodies (MoAbs) in B-Cell targeted therapeutic protocols mostly resides in the particular kinetics of expression of this marker, which is detectable only in the central period of B-Cell differentiation. Curiously, the ligand of CD20 is still unknown. CD20 is a calcium-dependent stimulatory receptor of B-Cells.38 B lymphocytes can act as antigen-presenting cells to T Cells, express Toll-like receptors for innate pattern recognition and express co-stimulatory and cytokine receptors, which regulate the concerted B-Cell activation during the immune response. As for T cells, not all B-Cells exert effector functions with potential pathogenetic role. Regulatory B-Cells (Bregs) and IL-10 producing subsets with suppressive activity have been also characterized.39,40 Anti-CD20 regimen spare B-Cell precursors, thus ensuring the survival of immature cells that can regenerate new antigen-naïve B-Cells. At the same time mature plasmacells are spared, with the preservation of long-term antibody memory. Moreover, the high level of expression of CD20 (around 150,000 copies per B-Cell)47 and the closeness of its relevant epitopes to the B-Cell surface,48 favor a high concentration of MoAb on target and the development of cytotoxic interactions.

The more pronounced effects on autoimmune serology and the highest clinical efficacy of anti-CD20 treatments seem to be achieved when the reservoir of the pathogenic antibody is within the antigen-primed CD27+ memory B-Cells and the short-lived plasmablasts. Long-lived plasmacells are in fact refractory to immunosuppression and hard to eliminate without undertaking therapy regimens suited for myeloma.13,44,46 The often-disappointing results with anti-B-Cell regimens in SLE seem in turn to indicate that - among other mechanisms - a complete differentiation to autoreactive long-lived CD20-resistant plasmacells takes place in this disease and possibly also in other conditions.49-50 A ‘window of opportunity’ for treatment should be therefore sought in every patient, with the help of innovative cell indicators and biomarkers, to optimize the expected therapeutic effects of anti-CD20 regimen.

Anti-CD20 treatments may also induce changes in the T-Cell compartments, possibly because a few CD20 molecules are expressed by some activated T lymphocytes51,52 and also due to the abrupt reduction of B-Cells in target organs, which can modify the homing patterns of T lymphocytes. An absolute increase of Tregs has been also documented in SLE patients treated with Rituximab.53

### The interaction of anti-CD20 therapeutic antibodies with cell targets

In the late 90s the chimeric anti-CD20 MoAb Rituximab was released as the prototype of anti-B-Cell targeted therapies, and proved to be highly effective in B-Cell non-Hodgkin lymphomas and chronic lymphocytic leukemia.54,55 Rituximab was thereafter experimented in the treatment of some autoimmune diseases, with variable efficacy: it proved efficient in rheumatoid arthritis, ANCA-mediated vasculitis with polyangiitis and pemphigus, whereas it did not show a similar effectiveness in SLE.42

Whatever the clinical application, Anti-CD20+ MoAbs exert their effects with three main immune mechanisms:56 complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC) mediated mostly by NK cells, and antibody-dependent cell phagocytosis (ADCP) mediated by macrophages and granulocytes.

ADCC and ADCP depend on the presence of Fcγ receptors on effector cells to be effective. An allelic form of the CD16, Fcγ Receptor IIIA-V158F has been described in its V/V, V/F and F/F genotype variants. The homozygous V/V form is present in about 10% of the population, and is related to a remarkably stronger ADCC function and better antiviral response, as compared with V/F and F/F genotypes.57-59 The higher CD16 expression on NK Cells in subjects carrying the V/V form can be easily estimated by FCM during the pre-treatment assessments.57 A good representation and function of Fcγ Receptors-positive cell subsets is thus a prerequisite for patients planned to be treated with anti-CD20 MoAbs.56,60

Fcγ receptors are a complex family including at least six members, namely: Fcγ RI (CD64), Fcγ RIIA (CD32A), Fcγ RIIB (CD32B), Fcγ RIIC (CD32C), Fcγ RIIDA (CD16A) and Fcγ RIIBB (CD16B), with different patterns of cell expression and variable affinity for monomeric IgG. It is important to note that the Fcγ RIIB is the only receptor that triggers inhibitory intracellular signals instead of effector mechanisms upon engagement.61

The heterogeneity of Fcγ receptors, which also implies differences in cell-antibody relationship, seems therefore an important factor to explain at least partly the patient-related and disease-related variability when therapeutic anti-CD20 MoAbs are administered.

The different therapeutic anti-CD20 MoAbs have been developed by chemically modifying the Fc portion (glycoengineering) to modulate the binding affinity with Fc receptors, to enhance a specific killing mechanism, to reduce the internalization/modulation of the antigen target and to improve the pharmacodynamics and half-life.56,62,63

The CDC is best elicited by the so-called ‘Type I’ anti-CD20 MoAbs (i.e. Rituximab, Ocrelizumab and Ofatumumab), which readily redistribute in the cell surface lipid rafts upon binding and induce a rapid calcium flux in the cell target.38 ADCC and ADCP are equally exerted both by ‘Type I’ and ‘Type II’ anti-CD20 (i.e. Tositumomab and Obinutuzumab) (Table 1).

Other direct killing mechanisms have been also described more recently, which are specific for Type II MoAbs, that involve homotypic adhesion of the antibody and apoptosis of the target.38,56,62,63 ADCP, phagocytosis and direct apoptotic effect are greatly enhanced in Obinutuzumab.56,64-67
Flow cytometric assays to enumerate B-cell subsets during anti-CD20 therapies

The reference levels of peripheral B-Cell subsets in normal subjects are characterized by a high level of variability, with coefficients of variation approaching or overtaking 100%.\(^6\) This variability makes it difficult to establish clear cut cutoff values especially in pathological conditions and under treatment. A basic prerequisite of immunotherapies with MoAbs is verification of the disappearance of the cell target during treatment, to confirm the correctness of the expected mechanism of action of the drug. Moreover, the actual cell target disappearance should be clearly distinguished from phenomena related to surface antigen modulation/down-regulation, that can mimic the disappearance of the target cell but sometimes may account for escape mechanisms.

The specific case of anti-B-Cell therapies also includes the need for accurate characterization and enumeration of the functional B-Cell subsets affected by the treatment (i.e. naïve, memory, memory-switched cells and plasmablasts) and for monitoring of the timing and quality of cell repopulation after the drug is discontinued.

These challenging requirements can be nowadays accomplished by multicolor FCM (8 to 10 colors) using high-sensitivity techniques developed for rare event analysis, since the relevant B-Cell subsets to be monitored are usually well below 0.1% or <5 cells per microliter of peripheral blood.

Several comprehensive conjugated antibody panels have been developed and validated to characterize and enumerate the functional B-Cell subsets in bone marrow and peripheral blood, using just one multicolor tube for practical usage. The composition of the panels can be adjusted in order to focus on the earliest steps of B-Cell development, when studying bone marrow, or in order to evaluate the later steps of B-Cell differentiation in peripheral blood. Moreover, as discussed, a look at T cell subsets and Fcγ receptor expression by NK cells is also recommended, which can be accomplished using additional dedicated tubes.

The minimum information to be obtained at each clinical control when repeated cycles of anti-CD20 MoAbs have to be administered includes: full blood count with absolute lymphocyte level, percent and absolute total B-Cell level, total memory and naïve B-Cells, IgG-switched memory B-Cells and plasmablasts. B-Cell subsets can be thus reported as individual absolute counts and as percentage of the parent total B-Cell population.

A 9-color panel that can enable the detection of the 11 steps of B-Cell maturation from early precursors to plasmacytomas has been described.\(^7\) It includes CD19 / CD38 / CD24 / CD34 / CD45 / CD10 / slgM / slgD / CD27. This combination is well suited to focus on earlier B-Cell development, but does not include CD20 and slgG, which may be important in anti-CD20 monitoring.

An 8-color panel deriving from research studies that is configured more ‘to the right side’ of B-Cell maturation sequence may include slgD / CD45 / slgM / CD24 / CD38 / CD19 / CD27 / slgG. This approach covers transitional cells, mature, IgM memory and IgG-switched memory B-Cells, plasmablasts and plasmacytomas,\(^7\) with the possible addition of CD20 as the ninth color for sake of completeness.

Another synthetic 8-color panel has been developed by the ISCCA (Italian Society for Clinical Cell Analysis) to assist anti-

Table 1. Anti-CD20 therapeutic monoclonal antibodies that have passed at least a phase-II trial in the treatment of autoimmune diseases.\(^6\)\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\)

<table>
<thead>
<tr>
<th>Molecule (brand names, if available)</th>
<th>Type</th>
<th>Source and Ig class</th>
<th>Mechanisms of action</th>
<th>Clinical Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rituximab (Rituxan, MabThera, Zytux)</td>
<td>I</td>
<td>Chimeric IgG1k</td>
<td>CDC, ADCC, ADCP</td>
<td>RA, SLE, MS, NO, NE</td>
</tr>
<tr>
<td>Ofatumumab (Arzerra)</td>
<td>I</td>
<td>Fully human IgG1k</td>
<td>CDC, ADCC</td>
<td>RA, SLE, MS, LN, PE</td>
</tr>
<tr>
<td>Ocrelizumab (Ocrevus)</td>
<td>I</td>
<td>Humanized IgG1</td>
<td>ADCC</td>
<td>RA, SLE, MS, LN</td>
</tr>
<tr>
<td>Ocaatuzumab</td>
<td>I</td>
<td>Humanized IgG1</td>
<td>ADCC</td>
<td>RA</td>
</tr>
<tr>
<td>Ublituximab</td>
<td>I</td>
<td>Chimeric</td>
<td>ADCC</td>
<td>MS</td>
</tr>
<tr>
<td>Veltuzumab</td>
<td>I</td>
<td>Humanized IgG1</td>
<td>CDC, ADCC</td>
<td>RA, SLE, ITP, PE</td>
</tr>
<tr>
<td>Obinutuzumab (Gazyva)</td>
<td>II</td>
<td>Humanized IgG2k</td>
<td>ADCC, APO</td>
<td>SLE</td>
</tr>
</tbody>
</table>

Major mechanisms of action: CDC, complement-dependent cytotoxicity; ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cell-mediated phagocytosis; APO, apoptosis by direct killing. Clinical applications: RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; MS, multiple sclerosis; SS, systemic sclerosis; NO, neuromyelitis optica; LN, lupus nephritis; NE, autoimmune nephropathies; ITP, immune thrombocytopenia; PE, pemphigus.
CD20 therapies in the clinical practice. It includes the essentials of B-Cell differentiation steps and also an evaluation of T cell subsets: CD20 / CD45 / CD3 / CD38 / slgG / CD19 / CD27 / CD4 (Figure 2). This approach can restrict the analysis to a single tube with a maximum of 8 markers, in order to comply with the limitations in test order and reimbursement policy that are in place in the public health care systems of several countries. It is possible to enrich the clinical information provided by this analysis by also applying the ‘add-on’ mixing of two different indicators in the same fluorescence channel. Taking advantage from the mutually exclusive expression of disparate markers, for instance CD3 and slgM in the FITC channel (Figure 2) and/or CD19 and CD16 in the PE-Cy7 channel, a more extensive phenotyping panel can be obtained without the need to increase the instrument complexity and cost.

Other panel variants or additional tubes can be also devised, to study more specific details of B-Cell development for research purposes or to include the evaluation of Fcγ receptor expression on NK cells, at least before therapy is started.

Whenever the cell surface Ig chains have to be studied, bulk lysis of whole blood, followed by extensive washings with protein-containing buffers is recommended before incubating the cell pellet with conjugated antibodies, also to enrich cell-poor samples and increase the cell collection.

The very low total B-Cell and subset levels typically encountered in patients treated with anti-CD20 MoAbs require the acquisition of at least >1 million total ‘clean’ CD45+ cell events, often more, in order to obtain an adequate representation of the relevant cell populations. This approach is defined as ‘high-resolution FCM’ and ensures an assay sensitivity of <1 cell out of 10,000 or 10⁻⁴.

Figure 2. Example of a baseline analysis of B-Cell subsets using the ISCCA 8-color panel (CD20-V450 / CD45-V500 / CD3-FITC / CD38-PE / slgG-APC-H7 / CD19-PE-Cy7 / CD27-APC / CD4-PerCP) and bulk lysis. Logical gatings are set from left to right in each display row. B-Cell subset levels are defined as percentage of total B-Cells. Upper row: A= Time monitoring of the sample run; B= Doublet exclusion; C= Clean CD45+ cells are included; D= Lymphocytes are included; Middle row: E= CD19+ events are captured; F= CD19+ CD38++ events are defined to be excluded; G= CD19+ CD38++ events are divided into CD27+ Plasmacells and CD27- Early B-Cell pre-curcursors; H= CD19+ CD20+ clean B-Cell events are included; Lower row: I= B-Cells are divided into CD27+ Memory and CD27- Naive cells; J= Within Memory B-Cells slgG+ Memory-switched cells are defined; K= With an anti-slgM-FITC ‘add-on’ and a modified gating Memory B-Cells can be further divided into slgM-Memory and slgG-Memory switched elements; L= An appropriate logical gating also defines CD3+ T Cells and CD4+ and CD8+ subsets.
with an acceptable reproducibility if at least 50 relevant cell subset events are collected.

It is important to stress here the need to ensure both a large total cell collection (typically 1-2 million cells) and the capture of an adequate number of relevant events (>20-30) to allow the minimum detectability level of each studied subset. This prerequisite enables the calculation of reliable percent and absolute cell levels above the lower level of quantitation (LLOQ) or conversely the definition of the operational ‘absence’ of a given population if below the lower level of detectability (LLOD).\(^{77}\)

In any case, the conjugated antibody panel and the high-resolution approach must be used along with a specific, carefully designed gating syntax, aimed at the elimination of the undesired populations and at the characterization and enumeration of the lymphocyte subsets with clinical relevance, as depicted in the example in Figure 2.

It is important to note that during the repopulation phase after anti-CD20 treatments the majority of CD19+ cells detectable in peripheral blood are small plasmablasts/plasmacells and not B-Cells. The application of a well-designed gating syntax enables the accurate discrimination of these two cell subsets.

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**Depletion and repopulation of B-cell subsets under anti-CD20 treatment**

All the anti-CD20 MoAb cause a deep and persistent depletion of peripheral levels of B-Cells, which may last up to 8-10 months after a single therapeutic dose in the majority of cases.

The evaluation of the depth of B-Cell depletion, as well as the timing and quality of repopulation, may vary greatly among diseases and patients, and attempts to correlate such parameters to the individual clinical response have been made, albeit with a certain degree of uncertainty.

At the beginning of the new era of RA treatment with Rituximab the abrupt ‘disappearance’ of peripheral B-Cells represented the expected biological correlation of the correct mechanism of action of the drug, whereas the clinical relapse was invariably preceded by the reappearance of B-Cells.\(^{78}\) However, with the inaccurate and insensitive FCM methods in use at that time, the quantification of the actual degree of B-Cell depletion showed a wide variability.\(^{79}\) Moreover, little attention was initially paid to the quantitative analytical requirements enabling the delicate definition of ‘disappearance’ of a given cell subset. More sensitive, comprehensive and reproduducible methods, validated in chronic lymphoproliferative disorders were then introduced, which shed light on the differential kinetics of repopulation of B-Cell subsets. This evidence formed the basis of a new immune monitoring approach, that linked the pattern of B-Cell changes to the disease course with the aim of predicting the patient’s outcome.\(^{79,80}\)

The multicolor FCM analysis then provided a new type of serology-independent immune monitoring, aiming at the evaluation of the disease course on the basis of B-Cell subpopulation analysis.

Initially a quick B-Cell depletion at 2 weeks from anti-CD20 administration was associated with favorable outcome in RA patients, whereas the persistence of CD19 + CD27 + CD38++ plasmablasts/plasmacells was considered as an indicator of a poorer prognosis.\(^{79}\)

With the ever-increasing use of anti-CD20 treatments in various autoimmune disorders, attention was shifted, rather, to the repopulation of memory B-Cells, which can be considered as the functional reservoir of the effector mechanisms of tissue damage and a sign of impending relapse. The relative and absolute decrease of memory B-Cells, along with an increase of naïve cells, was repeatedly confirmed as a favorable laboratory sign of response in RA,\(^{46,79,81}\) juvenile RA (RA3), SLE,\(^{47,29,43}\) multiple sclerosis,\(^{41,83,84}\) systemic sclerosis,\(^{85,86}\) Sjögren’s syndrome,\(^{12}\) glomerulonephritis\(^{87,89}\) and neuromyelitis optica spectrum disorders\(^{39,50}\) as well as in allogeneic transplantation response.\(^{91,92}\)

The achievement of a deep and durable B-Cell depletion has been then taken as an indicator to define the effectiveness of anti-CD20 therapies. However, in each disease setting quite variable and arbitrary target levels have been established over the years, often ignoring the cytometric requirements for a correct analysis of rare cellular events. For instance, in RA and SLE a target value of <0.01 B-Cells/μL after the first Rituximab dose was established,\(^{46,79,81}\) in membranous glomerulonephritis the ‘disappearance’ of CD27+ plasmablasts was indicated,\(^{89}\) whereas in optic neuromyelitis a level of CD27+ memory B-Cells <0.05% of ‘mononuclear cells’ was recommended.\(^{90,97}\)

It is clear that ‘disappearance’ is a term that mandates the application of stringent technical procedures for the FCM counting, which only seldom occur in the clinical literature, and the heterogeneous ‘mononuclear cells’ are definitely not a suitable cell denominator for a reliable analysis.

The same kinetics of B-Cell depletion and repopulation can take place both in Rituximab-naïve patients and in subjects receiving multiple courses, indicating that in every cycle the B-Cell recovery is recapitulated and that anti-CD20 treatments do not induce permanent changes in the B-Cell repertoire.\(^{81,98}\)

In a seminal paper by Leandro et al., all AR patients showing a memory B-Cell recovery higher than 3/mL experienced a relapse at the time of B-Cell return.\(^{98}\) In a series of RA patients the satisfactory depletion of peripheral B-Cells was set at <5 B-Cells/μL,\(^{1,99}\) a level above which the functional subsetting of B-Cells may become meaningful. It seems however that 5 B-Cells/μL is a cell level already indicating a sustained repopulation, especially if more accurate and stricter FCM counting requirements on whole unseparated blood are applied. Patients with B-Cell depletion periods shorter than expected or with a quicker repopulation phase may be checked for the possible occurrence of anti-rituximab immunization.\(^{79,99,100}\)

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**Timepoints for immune monitoring - The ISCCA proposed scheme**

The long-term aim of this innovative monitoring approach is to provide clinicians with a tool for a more rational usage of anti-CD20 MoAbs, with the hope of increasing the effectiveness and the safety profiles.

Setting the correct timepoints of immune monitoring tests is an important detail in the planning of the follow-up of patients with autoimmune diseases treated with anti-CD20 MoAbs. The wide disease-related and individual variability has however hampered the development of consensus guidelines on that matter. Here is the proposal formulated by the ISCCA working group (www.iscca.eu).

A first baseline test before anti-CD20 administration is recommended to assess the status of the T Cell compartment (which may be altered by previous therapies), the level of expression of Fcy receptors on NK Cells and the starting level of total and memory B-
Cells. All blood immunophenotyping testings should be always performed along with a full blood count to establish absolute cell levels.

After 3 months from the completion of the anti-CD20 administration schedule the expected level of B-Cells must be around zero in the majority of cases. At three months another test is suggested, to detect precautionarily patients with an accelerated MoAb clearance or who repopulate quickly. If total B-Cells are confirmed as virtually absent (i.e. < 0.1/μL with high-resolution FCM), no further B-Cell subsetting is technically possible (or meaningful), and patients can be safely retested after 2 more months.

Conversely, if at three months the total B-Cell count is higher than 0.3-0.5/μL, B-Cell subsetting is possible, and the subsetting data become informative, with focus on the quantification of memory B-Cells. In such instances, if memory B-Cells start to be clearly quantifiable (i.e. more than 30-40% of total B-Cells), another test after one more month may help in better following the type of repopulation that is taking place.

Whenever a clearly sizable total B-Cell population is present (i.e. >1/μL), subsetting must focus on the proportion between naïve and memory B-Cells. In this case a B-Cell memory percentage level >65% of total B-Cells may herald a clinical picture of non-responsiveness or an increased probability of a forthcoming relapse (Figure 3). Such observations derive from preliminary ROC curve data calculated in our institution, that however need further confirmation.

At 8-10 months from the last anti-CD20 MoAb dose the B-Cell repopulation can be considered as virtually complete in the majority of patients. In the long term a state of clinical remission associated with a favorable B-Cell subset pattern (i.e. memory B-Cells < 30-40%) may not require further phenotyping tests in the routine, unless clinical changes take place. Conversely, patients with poor response to therapy and an associated unfavorable B-Cell subset pattern (i.e. memory B-Cells >60-65%) may require the restart of the immune monitoring routine, especially if new cycles of therapy are scheduled or if the administration of different anti-CD20 MoAbs is being considered.

Taking for granted the wide individual variability, these recommendations can be taken as a starting point to disseminate the use of a rational and affordable immune monitoring programme in all the clinical settings, to assist the clinicians in their decision-making process and possibly to optimize the usage of the delicate anti-CD20 MoAbs.

Limitations, open issues and future developments

The complexity of the immune system and of the pathogenesis of autoimmune diseases, along with the redundancy of the regulatory mechanisms, make it difficult to assume that a drug acting on a single molecule may be able to stop permanently a pathological process.28,36,49 In this respect, the overall lower responsiveness to Rituximab in SLE, as compared to RA and other disorders, is a good example of the need to control the disease through a wider immunosuppressive coverage.101,102 Also acting more downstream from CD20 signaling and with a different engagement of Fcγ receptors,28 Other therapeutic approaches such anti-TNFα and Abatacept are also known to reduce memory B-Cells.83,100,104

The B-Cell changes occurring in peripheral blood with anti-CD20 MoAbs may only partly reflect the modifications that take place in the target organs, such as the synovium, kidney or blood vessels, which are also known to occur at a different pace in the various body districts.29

Some controversial clinical results obtained with Rituximab in certain autoimmune disorders105 may be ameliorated with the use of newer anti-CD20 MoAbs,106 such as Obinutuzumab in SLE.65 Ocrelizumab for multiple sclerosis,107 as well as with other antibodies under development.108,109

The knowledge of the Ig heavy and light chains type of each MoAb is of help in the correct interpretation of the serum protein electrophoresis and immunofixation readouts, since MoAb blood levels as low as 0.1-0.2 g/dL may be misinterpreted as serum monoclonal components.110-112

The treatment with Rituximab and other anti-CD20 MoAbs has proved basically safe, also when repeated courses are administered.110-112 Rarely, severe and sometimes fatal Progressive Multifocal Leukoencephalopathy can develop in treated patients54,96,97,106 as a consequence of over-immunosuppression. The risk of developing a severe secondary post-treatment hypogammaglobulinemia has also been described as occurring in a sizable fraction of patients receiving multiple doses of anti-CD20. This condition is in some cases irreversible and can require long-term replacement therapy with intravenous Ig.113-115

Infusion-related reactions have been described for therapeutic MoAbs, and can occur in about 3-4% of patients treated with Rituximab. Reactions are usually mild (fever, chill, urticarial rash) and can be partly attributed to the occurrence of immunization against the injected protein (ADA, Anti-Drug Antibodies) or to other non-specific mechanisms, especially in patients that have received multiple therapy courses.100,110

![Figure 3. Schematic flow chart describing the timepoint for testing B-Cell subsets during anti-CD20 treatments.](Image)
The wide variability of peripheral B-Cell subset levels is at present a major limitation in establishing and validating clear cut cutoff levels of universal usage in the clinical practice. The figures indicated in this review may serve as a preliminary starting point, to be better specified with further studies.

The respective roles of memory slgM+ and memory-switched slgG+ B-Cell levels in the immune monitoring of autoimmune disorders is still to be fully elucidated and warrants additional investigation to evaluate its importance in the clinical practice.

Additional investigation is required to explore in greater detail the B-Cell differentiation steps at which the autoimmune memory is lost, to focus more precisely the involved subset, as promising evidence has been obtained in allogeneic response to solid-organ transplants. 23,24

The analytical approach proposed here should be applied in the various diseases in which anti-CD20 treatments are indicated, to draw a clearer picture of the cellular findings in the different conditions and to establish more robust cell level targets and endpoints. The B-Cell phenotype before the first anti-CD20 cycle may thus be evaluated along with the disease stage and activity index to guide a more rational usage of the drug. Specific investigation should focus on the slgM/slgG switch of memory B-Cells, in order to classify more precisely the cases in which the terminal differentiation of B-Cells has taken place, thus questioning the appropriateness of further anti-CD20 cycles.

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