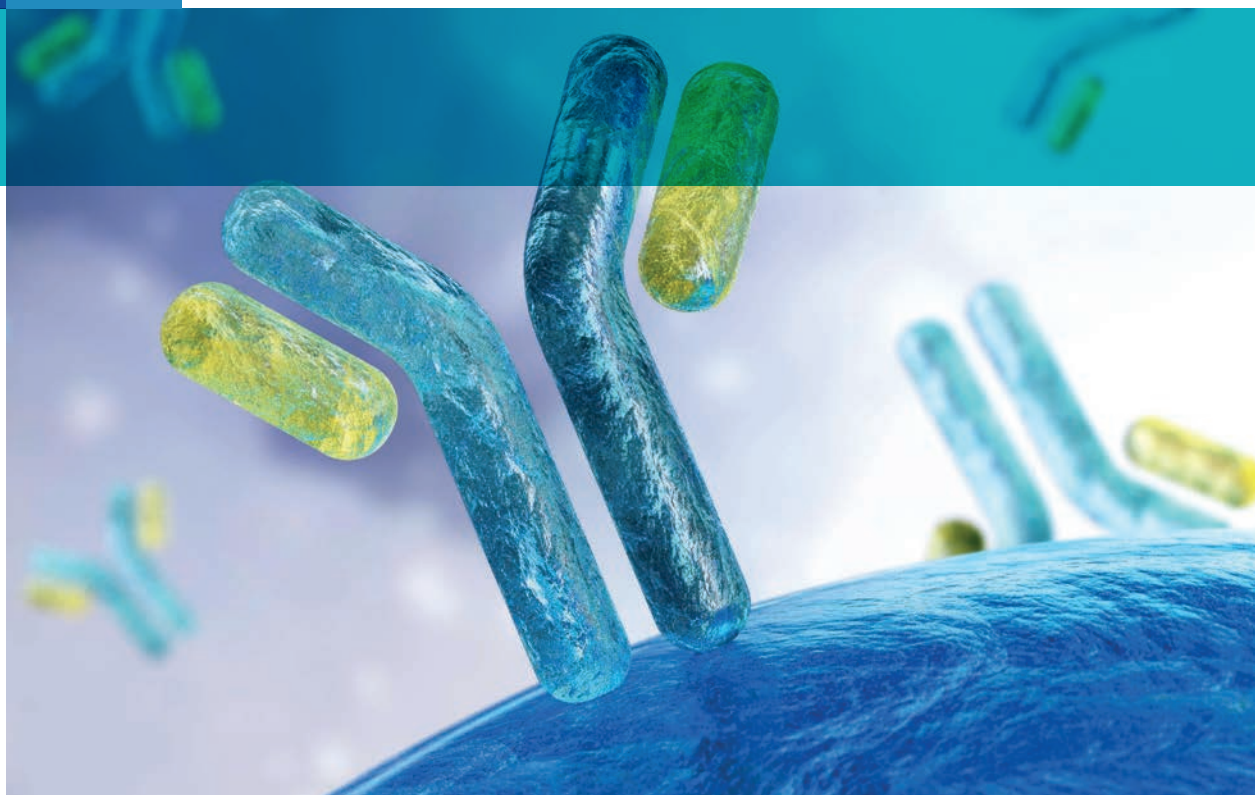

APPLICATION OF IMMUNOLOGICAL MONITORING

Prospects for development

Con il patrocinio di

FADOI Società Scientifica di Medicina Interna

SCCA Italian Society for Cytometric Cell Analysis

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Prospects for development

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Foreword

This document is dedicated to the specialists who treat autoimmune diseases in the *rheumatology, neurology, haematology* and *nephrology* sectors and its purpose is to define the setting for use of immunological therapies and their monitoring in the aforesaid areas, as a professional development tool supported by the most recent international literature.
The basic characteristic of the use of biological medicinal products is that they involve the patient's immune system in response to the disease, consequently making the follow-up of treated patients an aspect of paramount importance.
In this setting, by presenting vast potential for use in the diagnosis and follow-up of the diseases in question, the practice of **immunophenotyping by flow cytometry** is able to provide valuable information that result in useful practical guidance on its use in good clinical practice.
We therefore believe that these topics, which are less well known than immunotherapy in the oncology sector, may be of interest and use to clinicians who wish to improve their knowledge on the developments regarding immunophenotyping methods.
The contents are organised as follows:

- **First part** dedicated to the immunotherapy setting in a general sense, starting with the oncology sector and progressing on to matters regarding autoimmune disease in the rheumatology, neurology, haematology and nephrology sectors.
- **Second part** focussing on the basic principles of immunomonitoring with a description of the rationale underlying the administration of immunomodulating therapies, and subsequently defining a model for immunophenotyping common to all the conditions addressed, with a description of flow cytometry as the laboratory technique of election. This section also provides monitoring schedules to be used to study the immune B- and T- cell populations considered.
- **Third part** dealing with autoimmune diseases in the rheumatology, neurology, haematology and nephrology sectors, providing, for each one, guidance on the role of immunomonitoring, with practical hints for implementation.
- **Fourth part** with conclusions and key points.

1. Immunotherapy: a new era

1.1 Background on immunotherapy

BACKGROUND BOX The Immune System

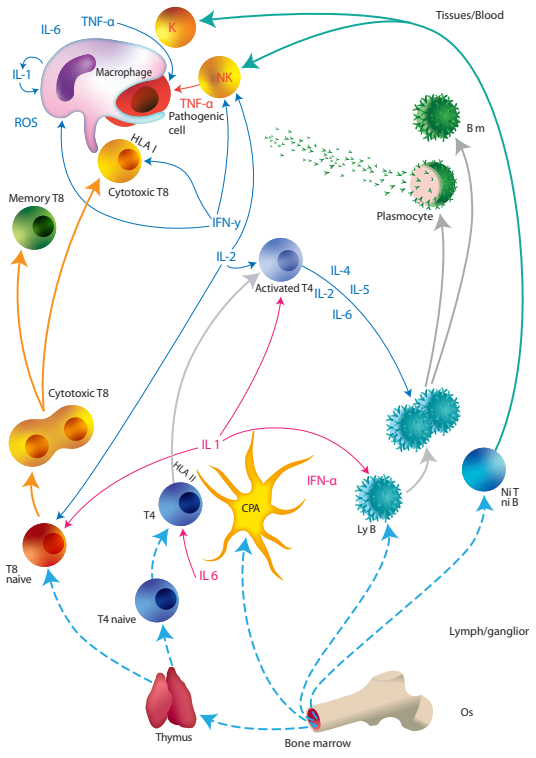
The immune system is a very complex, dynamic, interconnected and non-compartmental organ that is always active and continuously searching for a balance between the various circuits in an alternating succession of activation/inhibition. The failure of this balance leads to immune dysregulation, which can result in various pathological states. Recent research has established that the immune system plays a complex role in order to fine-tune the many interactions that help combat many pathological events, from inflammatory phenomena to autoimmunity, tumours to infectious diseases.

Overview of the Immune System

Inflammatory site
Place of engagement:
- Macrophages (phagocytosis)
- CTC (cytotoxicity)
- Cytokines (TNFα)
- NK (ADCC)
- Plasmocytes (antibodies)

Secondary lymphoid organs
Places of:
- Antigen presentation between APCs and CD4+ and CD8+ T cells
- Clonal expansion

Primary lymphoid organs
Places of:
- Immunocompetent cell production



Source: Institut Français de Micro-immunothérapie

Immunotherapy is a revolutionary treatment approach that is mainly and was first applied in the oncology field and includes a number of different strategies that share the ability to activate the patient's immune system and direct it against tumour cells.[1] Hundreds of clinical studies have confirmed the safety and efficacy of immunotherapies, as well as treatment combinations including both immunotherapy and conventional cancer treatments.

Four therapeutic approaches based on immune system modulation (active immunotherapy) are currently approved for clinical use.

The first is based on therapies with **monoclonal antibodies** (**mAb**), and subsequent evolutions (antibodies combined with chemotherapy or radiotherapy), which represent one of the first forms of immunotherapy strategy for which an effective clinical benefit was demonstrated for adult and paediatric patients. These molecules act by recognising the target antigen identified on the tumour cell (**Figures 1 and 2**).

The second approach is based on **bi-specific antibodies** or BiTE (*bispecific T-cell engagers*), such as *blinatumomab* (anti-CD3/anti-CD19 antibody), which have dual specificity in that they bind simultaneously with 2 antigens, one present on the immune system effector cell, the other on the pathological target cell, to create a kind of immunological synapsis able to cause the destruction of the tumour cell[1] (**Figure 2**).

The third regards *immune checkpoint inhibitors*, i.e. monoclonal antibodies able to reactivate the immune system against the tumours. Checkpoints are molecules located on the surface of the T lymphocytes and on that of the pathological target cells. The binding between checkpoint inhibitors and their ligands inhibits immune response.[1,2] The basic concept is to develop agents able to inhibit the checkpoints – the most commonly used is an anti-PD1 (*programmed cell death 1*) antibody – in order to allow the T lymphocytes to annihilate the tumour cells. This discovery earned James P. Allison and Tasuku Honjo the 2018 Nobel Prize for Medicine.

The fourth approach is based on engineered cells known as chimeric antigen receptor T (**CAR-T**) cells.[1]

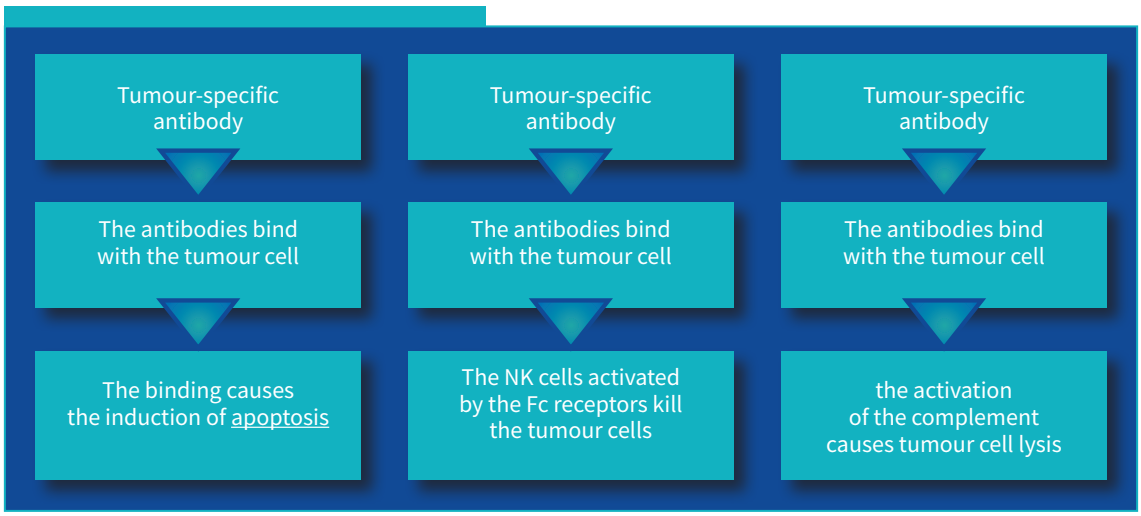


Figure 1. Certain mechanisms by means of which the monoclonal antibodies used in oncology act. Adapted from Foltz et al., Circulation 2013;127(22):2222-30.

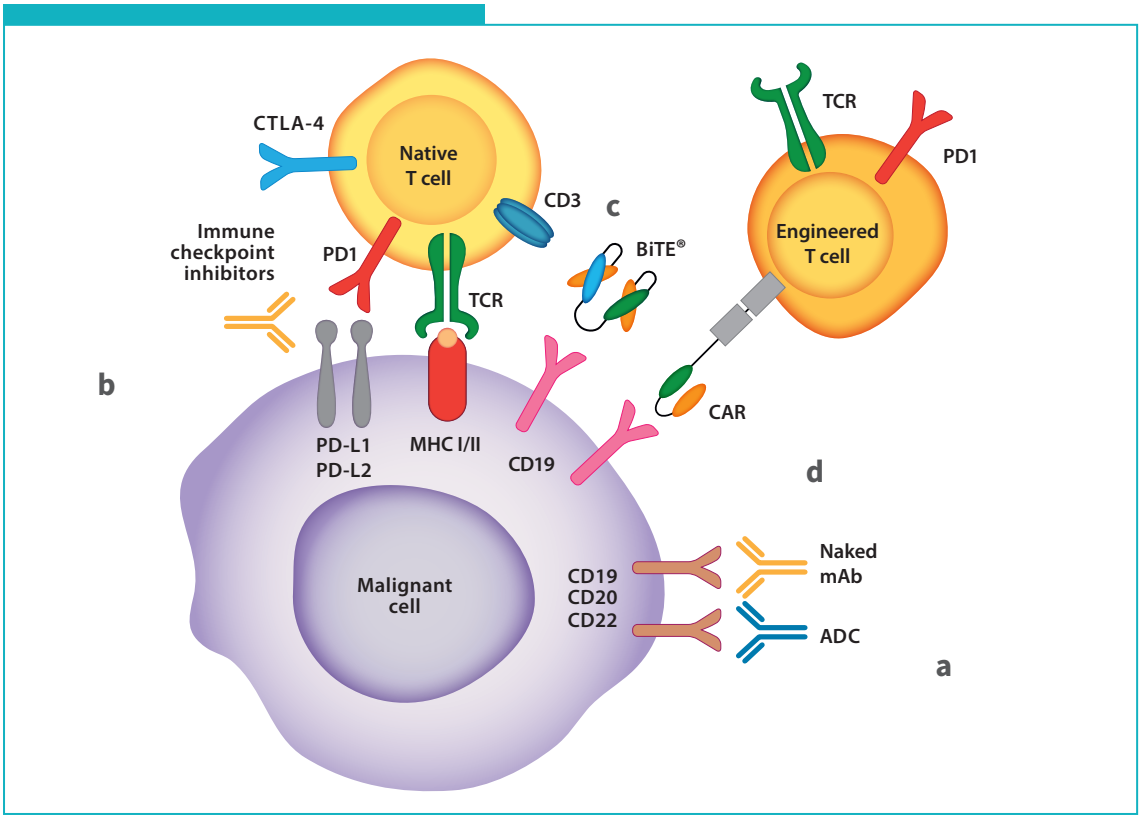


Figure 2. Main mechanisms of action of immunotherapy. a. Monoclonal antibody conjugated to toxins or radionuclides; b. monoclonal antibody with immune checkpoint inhibitor activity that blocks the effects of PD-1 and CTLA-4 activation; c. BiTE bi-specific antibody that acts as a "bridge" between the tumour cell to be destroyed and the T cells that must destroy it through a double bond with the two antigens; d. CAR-expressing engineered T cells able to recognise the surface proteins of tumour cells.
ADC, antibody-drug conjugates; BiTE, bi-specific T-cell engager; CAR, chimeric antigen receptor; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; mAb, monoclonal antibody; MHC, major histocompatibility complex; PD-1, programmed death protein 1; PD-L1, programmed dealth ligand 1; TAA, tumour-associated antigen. TCR, T-cell antigen receptor. Adapted from Batlevi et al., Nat Rev Clin Oncol 2016;13(1):25-40.

1.2 Focus on CAR-T cells, the most revolutionary of immunotherapy approaches

CAR-T cells are therapies based on T lymphocytes expressing a chimeric receptor for the target antigen and they represent a new form of personalised immunotherapy for cancer that acts directly on the patient's immune system to make it able to recognise and destroy the tumour cells. CAR-T cell therapies use specific immune cells (T lymphocytes) that are extracted from the patient's blood, genetically modified and cultivated in a laboratory ("engineered") before being re-infused into the patient, in order to potentiate his immune system's response against the disease. After the infusion, the patient is hospitalised for a few days and constantly monitored for any adverse reactions to the treatment. (**Figure 3**)[3].

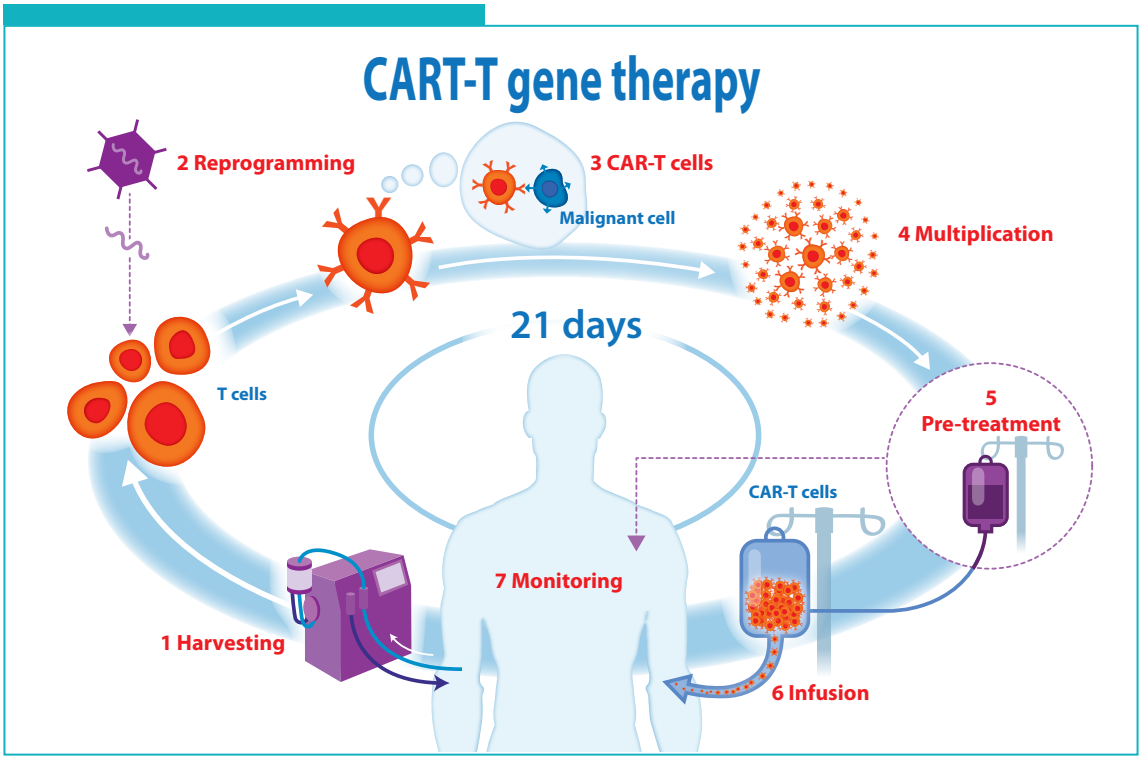


Figure 3. Immunotherapy with CAR-T cells. Lymphocytes do not recognise tumour cells because they are devoid of specific receptors for their antigens. Lymphocytes are therefore harvested from the patient (or a donor) and engineered to express the right receptors. The lymphocytes are re-infused into the patient and attack the tumour cells. Adapted from https://www.aifa.gov.it/-/aifa-approva-la-rimborsabilita-della-prima-terapia-car-t

1.3 Monoclonal antibodies (mAb) as a therapeutic strategy and subsequent evolutions of their use

One of the first mAb to be introduced and to have revolutionised clinical practice, more than twenty years ago now, was **rituximab**, a monoclonal antibody directed against surface protein CD20 that was approved by the FDS in 1997 for the treatment of lymphatic system tumours such as leukaemias and B-cell lymphomas and, more recently, certain autoimmune diseases. CD20 is a protein that is normally expressed on the surface of B cells. The agents binding with the target cells eliminate the latter, acting in a very similar way to what happens when the body's antibodies interact with bacteria or viruses. On account of these characteristics, agents such as rituximab were invented and found their main domain of application in the treatment of cancers involving mature B cells, primarily non-Hodgkin's lymphomas caused by the uncontrolled proliferation of a pathological B cell clones.

1.4 Expansion of the use of anti-B-cell therapies to autoimmune diseases

Given the mechanism of action of the medicinal products described above, in recent years, a number of studies have been initiated in the immunology field to evaluate their use also in a series of diseases associated with the presence of **non-neoplastic clone B cells**, able to produce antibodies directed against antigens of the same organism (autoantigens), the phenomenon underlying the presentation of a number of medical conditions known collectively as autoimmune diseases. The results of these studies were so encouraging that the use of anti-CD20 agents expanded its approved therapeutic indications and those currently being trialled, to a number of autoimmune conditions.[4]

2. Immunomonitoring

The **term immunomonitoring** is used to refer to a set of tests aimed at:

- identifying prognostic immunological biomarkers, with a predictive value, that reflect the evolution of the condition and the response to treatment;
- finding new potential treatment targets;
- conducting a dynamic analysis of the immune system before, during and after a treatment;
- optimising the frequency of administration and dose of the medicinal product;
- evaluating immunotherapeutic vaccines.

In recent decades, autoimmune diseases have been treated with immunosuppressive drugs that act primarily on T-cell-mediated response.[4] It was not until recent years, with the improvement in knowledge of the pathogenetic mechanisms of autoimmune disorders and the extensive development of new therapeutic monoclonal antibodies, **that therapies targeting the B cells emerged as a new option for the treatment of these diseases.** Following the change in therapeutic targets, it has become increasingly necessary to **identify laboratory** markers to enable a rational use and follow-up of the new biological treatments, given the vast variability in individual response, the very different results obtained in the different diseases and the controversial role of pathogenic autoantibodies as markers of disease activity.
Recently, immunological phenotyping techniques based on **flow cytometry** have been developed, in response to the need to identify and quantify the functional subgroups of B cells in peripheral blood, an area in which they are able to provide considerable support. These tests can be used to establish the grade and persistence of B-cell depletion, and the quality of healthy B-cell reconstitution also in terms of timing, together with the highly sensitive quantitative parameters relating to the absolute count of the individual subpopulations that flow cytometry can produce. These values can provide very important cut-off information for subsequent treatment decision-making.[4]

2.1 Basic principles of immunological monitoring

We have already seen that anti-CD20 mAb are commonly used in the treatment of many autoimmune diseases. The aim of therapies with mAb is to limit the damage in the target tissue by eliminating the memory B cells responsible for more rapid immune response

in the case of renewed exposure to the same antigen and that support the production of pathogenic autoantibodies, thereby sparing the reserve of the precursor population, naïve B cells and plasma cells.

The immunosuppressive action is not limited to the reduction in the production of pathogenic autoantibodies, but also has effects on various stimulatory and regulatory functions performed by the B cells on the T cells and on the cells expressing the antigen, with a final somewhat complex outcome of which certain aspects are yet to be clarified (**Figure 4).**

The rationale for the use of anti-CD20 therapies in the treatment of autoimmune diseases, their characteristics and the need to develop a specific immunomonitoring schedule were recently reviewed. The maximum clinical efficacy of anti-CD20 treatments in autoimmune disorders is obtained when the reserve of the pathogenic antibody is limited to the memory CD27+ B cells and the plasmablasts, immature plasma cells.

After the deep B-cell depletion phase induced by the anti-CD20 mAb, memory B cell and plasmablast repopulation was initially considered as being associated with an imminent relapse or treatment resistance. Besides, lasting memory B-cell depletion and repopulation in the peripheral blood and secondary lymphoid organs with naïve B lymphocytes were interpreted as **signs of clinical response** in a large number of autoimmune diseases, such as rheumatoid arthritis, lupus erythematosus, multiple sclerosis, neuromyelitis optica, glomerulonephritis and others, and they represent a desirable event for the full restoration of the patient's immunocompetence with elements at least initially devoid of harmful memory towards autoantigens.

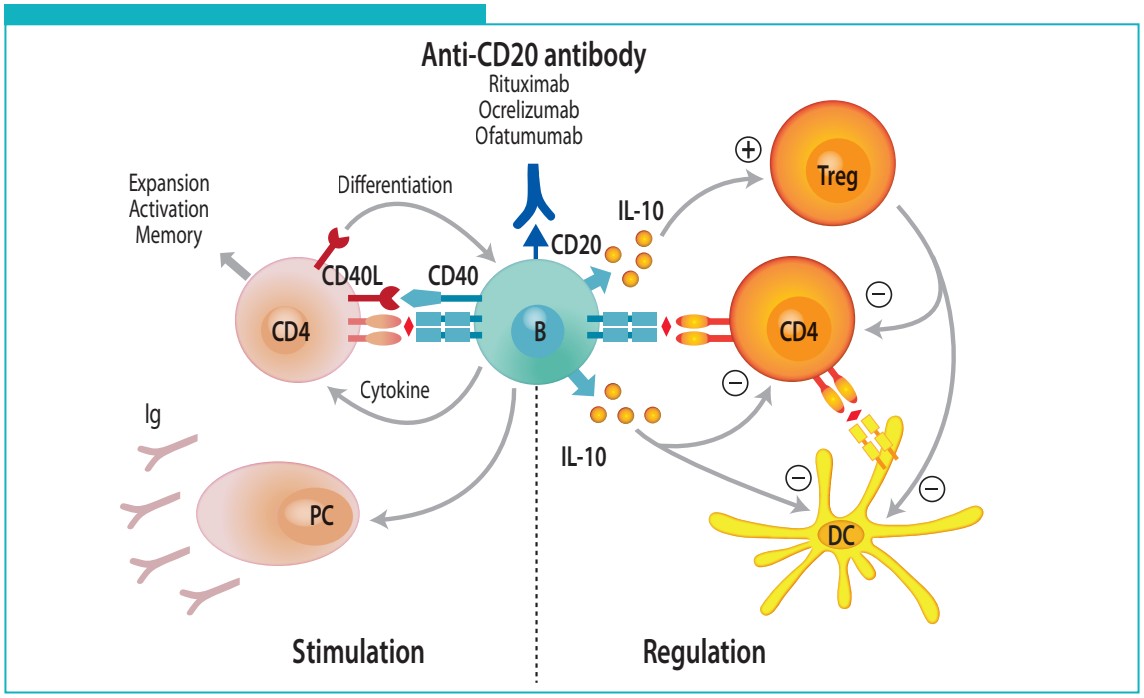


Figure 4.The depletion of the B cells by anti-CD20 monoclonal antibodies, such as rituximab, not only causes the suppression of immunoglobulin (Ig) production, but also a reduction in certain actions performed by the B cells on the T cells and on dendritic cells, with opposing mechanisms. Adapted from Gallo, AboutOpen 2015;1(1):10-7.

In addition, the great phenotypical and functional complexity of B cells has not always been dealt with in a thorough manner by means of the monitoring strategies proposed to date in the literature, in which certain important information is most likely lacking. The traditional identification of memory B cells by CD19+ CD27+ coexpression alone can be considerably improved by more thorough typing, by adding the study of surface immunoglobulin (sIg) expression, which further differentiates memory B cells into sIgM+ of sIgG+ subsets, with different functions and probably a different pathogenic role in autoimmune diseases.
Differing degrees of susceptibility to anti-CD20 agents have been observed in each autoimmune disease, due to the disease-specific inflammatory or immunological mechanisms. It is also known that patients with the same disease can show a vast individual variability regarding the duration of the B-depletion phase and the need for repeated cycles of therapy. The different characteristics of the disease, previous treatments and the patient's immune status have a huge impact on the patient's capacity to respond to a treatment.

Understanding how different treatments affect the response of the immune system and against the disease is the basis for the optimisation and personalisation of therapy regimens.

In the absence of reliable biomarkers, physicians usually use the patient's clinical response as a guide in treatment protocols. However, the great variability that characterises autoimmune diseases can be kept under better control by a standardised immunomonitoring system, designed to provide information on the mechanism of action of anti-CD20 mAbs. The use of mAbs as a therapeutic strategy provides a unique opportunity to verify the correctness of the mechanism of action and to monitor individual biological response. The techniques for immunological monitoring, in particular immunophenotyping by flow cytometry, have vast potential for use for diagnosis and monitoring in a number of clinical sectors, including that of autoimmune diseases in the rheumatology, haematology, neurology and nephrology fields. This approach can be of fundamental importance in the management of the vast patient- and disease-related variability, in support of the clinical criterion of the simple conventional assessment of response. Unfortunately, at the current time, there is very limited evidence of reliable biomarkers to support the follow-up of these diseases that tend to relapse frequently.
The first attempts at immunomonitoring were impacted by the poor sensitivity of the flow cytometry protocols used in the past and a certain confusion regarding the markers and the targets. The Official Gazette recmmendations[5] for neuromyelitis optica, for example, indicate the therapeutic target as being a lowering of circulating memory B cells to under 0.1% and 0.05% of total mononuclear cells, which can present methodological problems at laboratory level. In addition, the great phenotypical and functional complexity of B cells has not always been dealt with in a thorough manner by the monitoring strategies proposed to date in the literature, which probably overlooked certain important information regarding the maturation and functional status of the memory B cells.

2.2 Immunomonitoring protocol

The availability of a standardised immunomonitoring protocol for detecting deviations from expected response and the repopulation of favourable or highly pathogenic cell groups can help clinicians prevent both insufficient and exaggerated immunosuppression. It seems reasonable to consider use of this approach as a useful aid for defining the most appropriate type of therapy for supplementing the limited and subjective information associated with clinical response. An example of a protocol for high-definition monitoring of the B cell subsets in the peripheral blood of patients treated with anti-CD20 monoclonal antibodies, that identifies the naïve B cells associated with the efficacy of response and the memory B cells associated with resistance, is provided in **Figure 5**.

The Italian Society for Clinical Cell Analysis (ISCCA) recently developed a standardised high-resolution flow cytometry protocol with an 8-colour, 10-marker immunophenotyping panel, to be applied in the follow-up of patients with autoimmune diseases treated with anti-CD20 antibodies, in order to identify and accurately quantify the major subsets of functionally-relevant B lymphocytes. This immunological protocol was developed to provide laboratory analysis support for the clinical decision-making process in the treatment of autoimmune diseases treated with anti-CD20 mAbs (**Figure 6**).[4]

Flow cytometry represents the technology of election for evaluating and monitoring many diseases, proving to be a highly effective tool thanks to its ability to quantify and simultaneously analyse dozens of biological and functional parameters in each individual cell. The new knowledge on the biological behaviour of inflammatory and tumour cells obtained in the next few years could have a very significant impact on the current criteria for

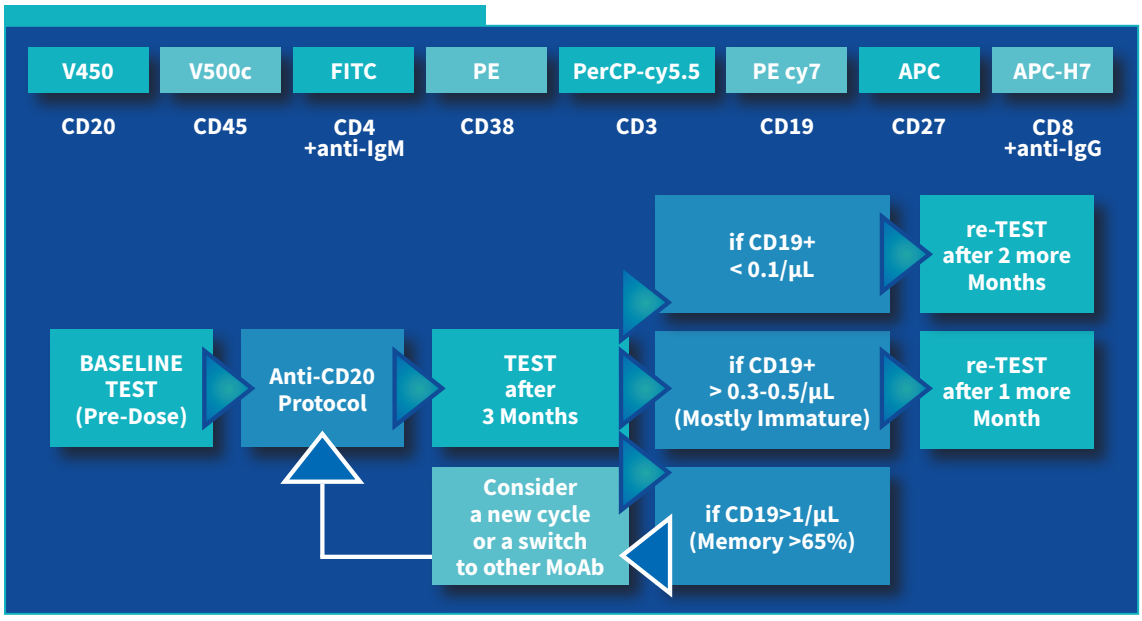


Figure 5. ISCCA protocol for high-resolution monitoring of the B-cell subsets in the peripheral blood of patients with autoimmune diseases treated with anti-CD20 monoclonal antibodies.

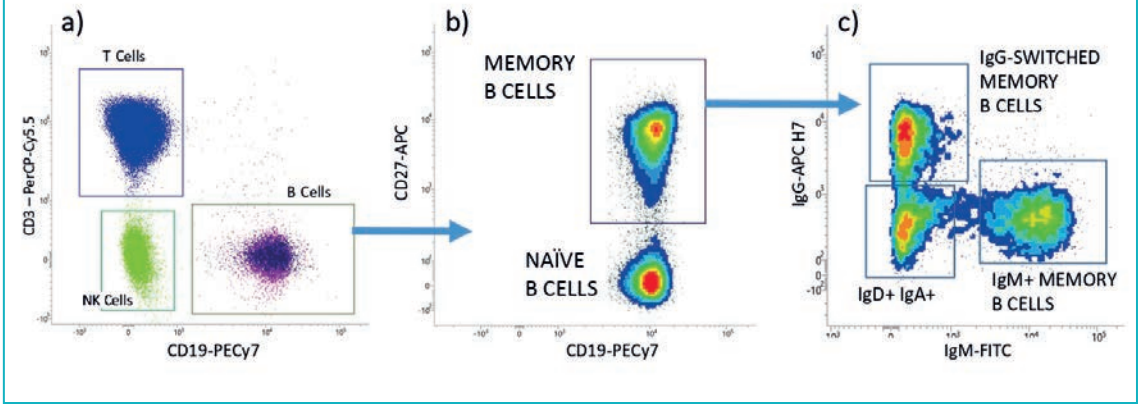


Figure 6. Example of baseline B-cell subpopulation analysis using the 8-colour, 10-marker IS-CCA immunophenotyping panel. Adapted from Brando et al., Beyond Rheumatol 2019;1:26.

risk stratification and consequent definition of therapy. A path that certainly must not be neglected. These procedures will hopefully be used in future prospective studies in order to confirm the validity of this analytical approach.[4]

2.3 Specific fields of application

Immunomonitoring provides support to the clinician's evaluation to corroborate clinical response. It can provide important information regarding the response of patients treated with biologicals, the possible development of phenomena of resistance to the current therapy, the development of toxicity phenomena for their containment, the re-assessment of the therapeutic strategy in order to consider a possible switch to other treatment options and to monitor the onset of any excessive immunosuppression phenomena. There are however, a number of open questions, for example for how long patients should be monitored and at what frequency.

Rheumatology

- Immunomonitoring is a supportive method in the management of the better-known diseases, such as rheumatoid arthritis and systemic lupus erythematosus (SLE). At the current time, it is less used for rarer diseases, such as Takayasu arteritis.
- In patients with rheumatoid arthritis, a decisive role is played by clinical monitoring and the monitoring of serum IgG, whose low values, in turn an expression of B-cell depletion, correlate with a greater risk of infection.
- In patients with severe or treatment-refractive SLE, treated with rituximab, immunomonitoring of the different clinical forms could help us understand the individual variability that is still observed despite the good and lasting response seen in many of these patients.

- The immunomonitoring of lupus patients receiving treatment with rituximab is also necessary in view of the infection risks that can be attributed to the period of maximum drug-induced B-cell depletion, the related hypogammaglobulinaemia, and the frequent co-administration of cortisones and/or cytotoxic drugs.
- In patients with idiopathic vasculitis of the central nervous system that is resistant to treatment with rituximab, a large-scale immunomonitoring study could provide the key for the interpretation of this clinical resistance.
- In patients with idiopathic inflammatory myopathy, a partially heterogeneous group of vasculites, adequate immunomonitoring, together with a thorough knowledge of the pathogenetic mechanisms, could make it possible to tailor therapeutic choices to the individual patient or specific clinical and immunological subsets.

Nephrology

- Can the nephrotic condition cause an excessive urinary loss of the monoclonal antibody administered, thereby reducing its clinical efficacy?
- Immunological monitoring can help clarify the variability of patient response to a biological treatment. For example, there are patients who do not respond to treatment with rituximab.
- At the current time, immunomonitoring represents a “refined” tool that supports clinicians in their review of the treatment strategy: when it is appropriate to continue a given treatment, with what frequency and at what doses, and when, on the other hand, it is necessary to suspend it or adjust the treatment protocol.

Neurology

- In the case of neuromyelitis optica treated with rituximab, immunological monitoring can reveal an increase in memory B cells (CD19, CD27), which is associated with a greater risk of disease relapse.
- In this case, it is appropriate to perform monitoring every two months (intervals defined in accordance with the guidelines set forth in Official Gazette Resolution no. 330/2018).
- The management of multiple sclerosis requires biomarkers able to guide clinicians in the management of therapy, especially in order to promptly change the treatment when patient response is inadequate.
- For myasthenia gravis, on the other hand, there are no specific guidelines for the most appropriate timing of immunological follow-up.

Haematology

- For autoimmune diseases in the haematology domain, rituximab (like other mAbs) is used in the treatment of a number of conditions. Once again in this setting, being able to adequately monitor immunological markers over time is essential for verifying whether and how the patient responds to therapy and therefore in order to intervene swiftly in the redefinition of the treatment strategy.

- Important results have been obtained in the treatment of thrombocytopenic thrombotic purpura and Coombs-positive haemolytic anaemias supported by “warm” IgG autoantibodies.
- In autoimmune idiopathic thrombocytopenic purpura, treatment with rituximab can be used successfully alongside thrombopoietin agonists in refractory cases.
- Positive evidence regarding the use of rituximab has been obtained also in the rarer but severe conditions of acquired haemophilia and catastrophic anti-phospholipid syndrome.
- Immunomonitoring can also be important in cancer patients receiving CAR-T cell therapy in order to assess the memory effect and therefore the persistence of the CAR-T cells, to study their function, understand immune system reaction, and monitor the state of T-cell “exhaustion” and activation.

3. Autoimmune diseases

3.1 Background

Autoimmunity occurs when the mechanisms underlying autotolerance are altered or eluded.[6] The failure to maintain immunological tolerance is caused by the activation of both the T and B cells, which produce **chronic inflammatory reactions in the target tissues**. Autoimmune disease is a condition in which immune tolerance towards one or more self-antigens is lost. The loss of tolerance results in the formation of autoantibodies and/or autoreactive T cells that cause tissue damage and disease.

Every year, more than 4 thousand per 100 thousand people are affected by autoimmune diseases in Europe; these patients - almost 5% of the population in Western countries - experience phases of exacerbation and remission, are placed under constant medical observation and require drug and dose adjustments in line with the evolution of the disease. More than **80 immune diseases** are described in literature, from Crohn's disease to rheumatoid arthritis, multiple sclerosis and neuromyelitis optica.[7]

For many years, the only remedies for autoimmune patients were cortisones and anti-inflammatory drugs; however, over the past two decades, there has been a gradual increase in the number of "immunospecific" drugs, the latest frontier in immunomodulatory agents, which have an impact on the activity of the immune system by blocking certain critical points of immune response. Monoclonal antibodies have changed the approach to autoimmune diseases and patients' quality of life. Although the immunopathogenesis of systemic autoimmune diseases is yet to be completely clarified, the effector mechanisms involved in organ-specific autoimmunity have been primarily associated with the activity of CD4+ helper T cells (Th) and CD8+ cytotoxic T cells (Tc).[6]

3.2 Immunotherapy in autoimmune disease

In autoimmune diseases, the aim is to promote immune suppression and stem inflammation.[8] One very recent study, published by Chen et al. in *Nature Biomedical Engineering* in 2019,[9] demonstrated that, in mice, a single chain of the anti-PD-1 antibody, conjugated to a bacterial toxin, is able to selectively kill autoreactive T cells, in other words, it is able to induce diabetes or autoimmune encephalitis. The authors conclude that the targeted lysis of the autoreactive T cells that express PD-1 could be efficacious in the treatment of a vast range of autoimmune diseases.

CAR-T cell therapies can also act on autoantigens to suppress autoimmunity.[8] Back in 2016,

Ellebrecht et al. in a study published in *Science*,[10] devised an artificial CAR-like receptor potentially able to induce the patients' T cells to attack only the B cells that produced harmful anti-Dsg3 antibodies in the cutaneous autoimmune disease *pemphigus vulgaris*. The results of the study show that CAR cells can be developed to recognise and selectively eliminate autoreactive B cells, providing an efficacious and universal strategy for the specific targeting of autoreactive B cells in antibody-mediated autoimmune diseases.

One study published by Kansal et al. in 2019,[11] based on a trial in mice that yielded promising results, confirmed that CAR cell therapy could also be efficacious for autoimmune diseases.

4. Focus on autoimmune diseases in haematology, nephrology, neurology and rheumatology

This chapter will provide a short description of the main autoimmune diseases and of the immunotherapy strategies adopted.

4.1 Use of monoclonal antibodies in haematology

Autoimmune haemolytic anaemias (AIHA)

Warm autoimmune haemolytic anaemia (wAIHA) is characterised by the destruction of red blood cells (haemolysis) that are attacked by warm autoantibodies, belonging to the IgG class, active at an optimum temperature of 37°C (temperature range 25-37°C) and directed against the antigens (proteins) expressed on their surface. The annual incidence of wAIHA is 1 to 3 cases per 100,000 people.[12,13] In randomised, controlled trials, glucocorticoid plus rituximab therapy was seen to be superior to glucocorticoid monotherapy, which is the first-line treatment.[14,15] In addition, recent UK guidelines recommend rituximab as **second-line therapy** over splenectomy, indicate that it is efficacious in approximately 80% of cases and specify that rituximab can be repeated various times while maintaining the results obtained.[16]

Rituximab has also shown efficacy in **cold autoimmune haemolytic anaemia** (cold AIHA, cAIHA), which is characterised by the presence of antibodies that are referred to as cold because they are active at 4-20°C and in **cold agglutinin disease** (CAD), with efficacy rates of between 45% and 55%.[12,17] CAD, a rare, chronic and severe blood disease in which the complement system attacks healthy red blood cells by mistake, has been treated successfully with bortezomib[18] and eculizumab[19,20] in non-responders to rituximab.

Autoimmune idiopathic thrombocytopenic purpura

Autoimmune idiopathic thrombocytopenic purpura is an autoimmune disease characterised by an acute deficiency of platelets (thrombocytopenia) in the absence of other associated disorders. In patients who do not respond to or relapse after treatment with glucocorticoids, rituximab has shown efficacy in 60% of cases, with complete response in 40% of cases.[21,22]

Thrombocytopenic thrombotic purpura (Moskowitz syndrome)

In thrombocytopenic thrombotic purpura, an aggressive multisystemic thrombotic microangiopathy, rituximab acts as a suppressant of anti-ADAMTS13 antibody production by B-cell

depletion, and it is indicated in patients who are refractory to or relapse after treatment with glucocorticoids, with good response in 60% of cases at 6 months and of 30% at two years; the treatment may be repeated.[23] Rituximab has also been seen to be highly efficacious in the **prophylaxis of acute relapse.**[24]

Rituximab has also proven efficacious as first-line treatment for **acquired haemophilia in adults** in single-centre studies or case reports[25,26] and in **catastrophic antiphospholipid syndrome** (CAPS), a rare systemic autoimmune disease associated with the formation of blood clots, in which "catastrophic" refers to the high mortality (50%) of patients with CAPS. In CAPS, recent case reports or expert opinions, supported by the most recent EULAR guidelines (2019), suggest **eculizumab**, a monoclonal antibody that inhibits the activation of the terminal part of the complement, as a new treatment.[27,28]

4.2 Use of monoclonal antibodies in nephrology

In nephrology, a number of monoclonal antibodies directed against membrane proteins expressed on B cells, against B-cell-stimulating cytokines or against complement components are currently being studied.[29,30]

Membranous nephropathy

Membranous nephropathy (MN) is a chronic glomerular disease characterised by structural alterations of the glomerular capillary walls, with the deposition of immunocomplexes on the epithelial face of the glomerular basement membrane, that causes proteinuria and can progress to chronic kidney disease. It is one of the main causes of nephrotic syndrome in adults.[31] When the aetiology is unknown, it is referred to as **idiopathic**. Approximately one quarter of cases of MN can have secondary causes: systemic lupus erythematosus, viral infection (for example, hepatitis B) or tumour.

MN is a chronic disease whose evolution involves spontaneous remissions (in approximately 30% of patients with milder forms, usually within the first 2 years of diagnosis) and frequent relapses, through to chronic kidney failure.[32] A systematic review of the literature from 1980 to 2010 showed that it has a global incidence of 1.2 cases per 100,000 people per year, with a high prevalence amongst Caucasian males over 40 years.[33] The disease's exact pathogenetic mechanisms were defined in the past 10 years, thereby paving the way for new treatment scenarios.

Rituximab has proven efficacious as a novel treatment in idiopathic and secondary MN and a number of international RCTs (randomised controlled trials) (GEMRITUX) and observational studies have shown its efficacy and safety on the depletion of the PLA_2R antibodies that cause the disease.[31,32,34,35]

Fervenza et al.[36] published in the New England Journal of Medicine the results of the MENTOR RCT conducted in the USA, confirming that therapy with rituximab is superior to that with cyclosporine in the treatment of idiopathic MN, with complete or partial remission of proteinuria after 12 months and is superior in maintaining it up to 24 months. Other trials report new directions in research on specific (belimumab)[37] and non-specific inhibition mechanisms (ACTH), versus cyclophosphamide and other calcineurin inhibitors. More occasional reports consider new potential treatment options, such as ofatumumab[37], bortezomib[38] and eculizumab[37].

Other glomerulopathies

HCV-associated membranoproliferative glomerulonephritis is characterised by changes in the glomerular basement membrane associated with immunocomplex deposits on the endothelial face and mesangial cell proliferation. It is associated with acute cryoglobulinaemic syndrome in HCV-positive patients and presents with haematuria and nephrotic proteinuria. Its course can lead to a progressive deterioration in renal function.[39-41]

Steroid-dependent or steroid-resistant nephrotic syndrome secondary to minimal change glomerulopathy or focal segmental glomerulosclerosis is another kidney disease in which rituximab has been used, as in the forms of vasculitis associated with the presence of ANCA, both P-ANCA and C-ANCA. Studies have shown that rituximab is as efficacious as cyclophosphamide in inducing acute phase remission and is even more so in the treatment of refractory and/or relapsing forms of this disease.[42-57]

4.3 Use of monoclonal antibodies in neurology

In the past 20 years, the use of mAbs in neurology has revolutionised the treatment of a number of different neuroimmunological diseases. Although **multiple sclerosis** was the trail-blazer, with the introduction of natalizumab, a humanised mAb directed against alpha-4-integrin VLA4, and subsequently immunodeleption mAbs such as alemtuzumab, rituximab and ocrelizumab, more recently, the use of mAb was extended to many other immunomediated diseases of the central and peripheral nervous systems. More specifically, the use of anti-B mAb (rituximab) has consolidated its position in the treatment of neuromyelitis optica, in diseases associated with anti-MOG antibodies (which were only nosographically classified recently), autoimmune and paraneoplastic encephalitis, myasthenia gravis and inflammatory polyneuropathies.[58,59]

Multiple sclerosis

Multiple sclerosis (SM) is a progressive, degenerative and chronic autoimmune disease affecting the central nervous sytem.[60] MS is the major cause of non-traumatic disability in young adults: in Italy, the ASM estimates that over 122,000 people are affected, with a diffusion that is twice as high amongst women as amongst men; it is estimated that there are over 3400 new cases every year, with an estimated incidence of between 5.5 and 6 per 100,000 people (12 per 100,000 in Sardinia).[60]

Over 80% of patients have a relapsing-remitting clinical phenotype, which is followed after 10-years of disease by a secondary progressive form, characterised by an accumulation of neurological disability in the absence of obvious inflammatory activity. Between 10 and 15% of patients, on the other hand, present a variant with a primary progressive evolution from the outset, which remained a therapeutic orphan for many decades.[61]

Following a first historical phase during the 1990s characterised by treatments with biologicals, such as interferon, the approval, in 2003, of the use of natalizumab revolutionised the treatment of this condition.[62] Natalizumab blocks the interaction between VLA4 and ICAM1 thus inhibiting lymphocyte transmigration across the blood-brain barrier, which very significantly reduces the disease's inflammatory activity, the rate of clinical relapses and the progression of disability.[62]

In recent years, the introduction of alemtuzumab, a humanised, long-acting anti-CD52 mAb,

has demonstrated that immunodepletion followed by immunoreconstitution can represent a treatment strategy especially for those forms of disease that are highly refractory to conventional therapies or as an induction strategy at onset.[62] However, this therapeutic strategy is burdened by a high risk of side effects, including induction of secondary autoimmune diseases (thyroiditis, autoimmune thrombocytopenic purpura, nephropathy) most likely associated with the different kinetics of B- and T-cell immunoreconstitution.[62] The recent finding that B cells play a non-marginal role in the immunopathogenesis of MS has led to extensive off-label use of rituximab.[58]

Ocrelizumab, a new humanised anti-CD20 antibody, was recently approved for both the relapsing-remitting forms and the primary progressive forms of MS and other mAbs directed against the B-cells (ofatumumab) and agents that interfere with B-cell regulatory and activation mechanisms, such as ibrutinib (an inhibitor of Bruton tyrosine kinase, BTK), are now being studied.[58,62,63]

In a number of different clinical trials ocrelizumab has shown excellent efficacy in reducing the relapse rate, but also, for the first time, the progression of disability in the primary progressive forms, becoming the first medicinal product to be officially approved for the treatment of this disease phenotype.[62-64]

Neuromyelitis optica and MOG antibody-associated diseases

Neuromyelitis optica spectrum disorders (NMOSD), previously known as Devic's disease or neuromyelitis optica, are inflammatory diseases of the central nervous system characterised by severe immune-mediated demyelination and axonal damage that predominantly affects the optic nerves and bone marrow.[65,66] The pathogenesis is secondary to the development of antibodies directed against the aquaporine-4 (AQP-4) water channel protein that plays a fundamental role in the regulation of the concentration of intra- and extracellular water in the central nervous system.[65,66] These antibodies cause astrocyte damage in the brain and spinal cord by complement activation and involvement of the adaptive and innate immune system, in which interleukin-6 (IL6) would appear to play a fundamental role.[65] The discovery of specific antibodies (anti-AQP4) led, in 2015, to a review of the diagnostic criteria, with the inclusion in addition to the classic longitudinally extensive settings of optic neuritis and myelitis, of new characteristic clinical and radiological states such as area postrema syndrome, diencephalic syndrome and brainstem syndrome.[66]

Even more recently, a number of clinical phenotypes meeting the definitions of the latest diagnostic criteria, but in the absence of anti-AQP4 antibodies, were correlated with the presence of antibodies directed against myelin oligodendrocyte glycoprotein (MOG).[67] The subsequent acknowledgement of other clinical syndromes associated with the presence of these antibodies and the characterisation of primary demyelinising damage allowed the definition of a new nosological group known as MOG-antibody diseases (MOGAD).

Both the disorders described here are usually treated in the first line with rituximab and in particular with a redosing approach secondary to circulating B-cell monitoring: CD27 monitoring in particular would appear to bring advantages in terms of clinical response over that of CD19, to the extent that a recent AIFA resolution suggests using this parameter as a marker of clinical response. Treatment with rituximab also correlates with a decrease in the titre of anti AQP4 and anti-MOG antibodies.[65,67]

The literature provides extensive evidence of a close relationship between increases in circulating B cells, increases in antibody titre and the risk of relapse. If therapeutic response is

not adequate, use of tocilizumab, the monoclonal antibody directed against the IL-6 receptor, represents the rescue therapy strategy.[68]
Moreover, very recently, three new monoclonal antibodies have shown efficacy in the control of NMOSD with or without anti-AQP4 antibodies, in different clinical trials, as both monotherapy and as an add-on. These agents are a new monoclonal anti-B-cell antibody directed against the CD19 antigen (inebilizumab), satralizumab an antibody directed against the IL-6 receptor, a bioengineering product, and eculizumab, an antibody directed against complement factor 5.[69] The imminent placement on the market of these drugs will therefore inevitably generate a need for specific immunomonitoring kits for verifying both therapeutic response and the potential adverse events of the various medicinal products.

4.4 Use of monoclonal antibodies in rheumatology

Biologicals have profoundly changed the life of many patients with rheumatic diseases. These drugs entered the Italian rheumatology scenario more than 20 years ago and after an initial trial and "compassionate use" phase, many molecules are now a consolidated part of the rheumatologist's weaponry, with increasingly vast indications. They were introduced in 1998 for the treatment of rheumatoid arthritis, and led to a complete overhaul of the available therapeutic scenario, bringing excellent outcomes in terms of quality of life, disability, morbidity and mortality, thanks also to a gradual expansion of the indications of new and old molecules. And research is extremely active in this field, with the introduction of new biologicals.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease that affects the joints, it has an erosive, deforming and sometimes ankylosing nature and a chronic, progressive course characterised by the presence, in the vast majority of cases, of IgG anti-immunoglobulin auto-antibodies (rheumatoid factors).[70] The prevalence of the disease in the worldwide population is between 0.3% and 1.5%. It has a uniform distribution around the world and it does not appear to be impacted by meteorological, geographic or socioeconomic factors. In Italy, it is estimated to affect approximately 0.5% of the general population (0.6% of women and 0.25% of men); and therefore approximately 170,000 women and 60,000 men suffer from RA, with a total prevalence of 230,000 adult individuals. Although the prevalence of RA is far lower than that of other conditions, such as osteoarthritis, the frequent severity of the clinical situation and its high invalidating potential make it an illness with a considerable socioeconomic impact in terms of costs, disability and loss of productivity.
Patients with RA are initially treated with one or more background agents (disease-modifying anti-rheumatic drugs, DMARDs), such as methotrexate, sulfasalazine, leflunomide and hydroxychloroquine. Biologicals with different therapeutic targets have been available to patients with RA for some time.[71]
RA is characterised by a synovial inflammation that results in osteoarticular destruction. In RA, autoreactive B cells, together with the T cells, form the basis of the aetiology and maintenance of the autoimmune inflammation of the disease.[72-74] Copious clinical evidence shows an interruption of processes governed by the B lymphocytes in the pathogenesis of RA and other autoimmune disorders, which provides the rationale for B cell depletion therapy in RA.[72,74]
Rituximab is classically indicated in the treatment of the refractory forms of RA and, albeit at reduced doses, also in maintenance of clinical control at a low risk of infection. As a matter of

fact, in many clinical studies rituximab has been seen to be efficacious in reducing the signs and symptoms of RA together with the inhibition of progression of the joint damage, with a significant effect on the depletion of the peripheral B cells and, to a lesser extent, in tissues, in adult patients who have shown inadequate response or intolerance to other DMARDs, comprising one or more inhibitors of tumour necrosis factor (TNF).[71,74]
The efficacy of response and its long-term duration are related to the entity of B-cell depletion, which is more commonly observed in subjects who are positive for anti-citrullinated protein antibodies and rheumatoid factors, in nonsmokers and in subjects receiving combined treatment with other immunosuppressive drugs.
Administration of rituximab causes a rapid and almost complete depletion of positive B cells in the peripheral blood and only a partial exhaustion in the synovial tissue and bone marrow.[71]
Treatment with rituximab induces a clinical improvement in most patients with positive autoantibodies for RA; however, a high) percentage of patients (>50%) relapse or do not respond to rituximab. The reason for resistance may also be due to the fact that approximately 50% of patients have decreased levels (when not completely absent) of B cell infiltration into the synovial tissue.
A recent study evaluated treatment with **tocilizumab** vs rituximab. Tocilizumab was found to be more efficacious than rituximab in achieving significant decreases in disease activity in patients with RA classified according to the presence of low levels of B cells in the synovial tissue, who do not respond to DMARDs and TNF inhibitors. These results suggest the path for more appropriate **therapy personalisation** in the setting of this disease.[75]

Systemic lupus erythematosus

SLE is a chronic inflammatory rheumatic disease with an autoimmune pathogenesis and it represents the most significant of all connective tissue diseases, due to both its potential extension to many organs and systems and the severity of the lesions. In Italy, SLE affects approximately 240,000 patients, with a F:M ratio of 9:1. The most common age of onset is between 25 and 40 years. Its multifactorial aetiology includes genetic, endocrine and, environmental factors (infectious, physical, chemical, pharmacological). The recent updating of the EULAR guidelines for the management of SLE (2019)[76] redefined the aims of the disease's treatment:

1. remission or decreased disease activity (2b/B);
2. prevention of relapse in all organs (2b/B);
3. use of maintenance therapy with the lowest possible dose of glucocorticoids (GC).

Randomised controlled trials on SLE are limited and the treatment usually includes GC and hydroxychloroquine for mild to moderate disease and immunosuppressants for the more severe forms. Rituximab, available since 2004 and used off-label, is indicated by the EULAR guidelines in refractory cases or those who are intolerant to or have contraindications for standard immunosuppressive drugs. Early treatment of SLE patients with rituximab has been seen to be safe and efficacious and allows a reduction in the use of steroids.[77]
Aguiar et al.[78] demonstrated, in a retrospective analysis of a cohort of patients in London treated with rituximab between 2000 and 2013, that treatment with rituximab is able to guarantee in the long term (>14 years of follow-up) an efficacious reduction in disease activity and in the incidence of adverse events in patients with severe SLE or who are refractory

to treatment. The results showed at least 25% increases in complement protein C3 levels in 36.5% of participants in the study after the first cycle of treatment with rituximab, whereas an at least 50% reduction in dsDNA levels was observed in 38.2% of patients. CD19+ cell depletion was achieved in 94% of participants in the study. The authors of the study conclude that "rituximab represents the most commonly used biological characterised for off-label use in the treatment of refractory SLE".[78]

Belimumab, a completely humanised monoclonal antibody directed against BLyS, a cytokine that is particularly increased in patients with active-phase lupus with the function of increasing the differentiation, proliferation and maturation of B cells, has been available in Italy since 2013. In SLE, as in other autoimmune diseases, high BLyS values can favour the production of autoantibodies that attack and destroy bodily tissues. Administration of belimumab therefore inhibits the mechanism that maintains and increases damage to the organs and systems of patients with SLE with high-grade disease activity.[76]

Recently, in a pilot study presented in Madrid during the EULAR 2019 Congress, it was demonstrated that the adoption of the combination regimen including rituximab and belimumab appears to be very efficacious in severe SLE that does not respond to the current treatment.[79]

Sjögren's syndrome

It is a chronic inflammatory disease, with an autoimmune pathogenesis, that potentially involves various organs and systems, but can particularly affect the exocrine glands (salivary and tear glands, pancreas, bile ducts and renal tubules). Like other systemic autoimmune diseases, the aetiology is multifactorial and the prevalence appears to be no lower than that of RA. The disease is associated with a high risk of lymphoma, whose standardised incidence rate ranges between 7.1 and 15.57%. In addition to systemic signs and symptoms (asthenia, fever, joint pain) and the signs of organ involvement, in the advanced stages of the disease it is possible to detect the signs and symptoms of functional insufficiency of certain exocrine glands (xerostomia and xerophthalmia, parotid gland tumour).

A great many studies seem to confirm the possible "inducer" role of cytomegalovirus and EPV, both of which find their natural location in the salivary glands. Pathogenesis would appear to be associated with an autoimmune mechanism that is de-latentised by a viral infection, directed towards the exocrine glands, with a molecular mimicry and/or polyclonal non-specific activation type. Hypergammaglobulinemia, rheumatoid factor and potentially also cryoglobulin positivity, autoantibody positivity and the high incidence of B-cell lymphoma are clear evidence of the key role played by the B cells in the pathogenesis of Sjögren's syndrome. Despite being accompanied by T cells, B-cell infiltration increases with the severity of the inflammation and therefore also organ damage.

B-cell hyperactivity is the key for treatment with rituximab, which has shown beneficial effects on gland morphology, on dry eye and mouth, on asthenia and other extra-glandular symptoms, although at the moment there is no unanimous consensus in the scientific community regarding this type of treatment.[80] Certain recent studies, although not controlled and with limited cohorts, have recorded an efficacious response to treatment with rituximab, especially in patients with more considerable B-cell infiltrates and germ centres at onset, particularly when characterised by CD20+.[81]

Various DMARDs (hydroxychloroquine, mycophenolate mofetil, methotrexate, cyclophosphamide) have been used to treat Sjögren's syndrome with varying and in any case non-significant results in the various studies.

Idiopathic inflammatory myopathies

Idiopathic inflammatory myopathies are a heterogeneous, acquired, systemic group of connective tissue diseases, including polymyositis, dermatomyositis, juvenile dermatomyositis and cancer- or other connective tissue disease-associated myositis, as well as inclusion body myositis.[82]

Limited studies with rituximab have been conducted in dermatomyositis and polymyositis, chronic autoimmune diseases characterised by muscle asthenia and, also, by pulmonary, joint, skin and gastrointestinal involvement in some patients. Patients with myopathy refractory to previous treatments appear to respond efficaciously and persistently to rituximab, although the results involve differing population percentages in the various studies (in part due to the absence of homogenisation criteria for the populations studied). Clinical responsiveness would appear to be more limited in the case of skin involvement, young subjects and in dysphagia. However, although the results for rituximab were efficacious, it is still not clear when it should be used and with what treatment regimen.[82,83]

Other rheumatological diseases

Rituximab has also been evaluated in a number of rheumatological diseases.

Systemic scleroderma[84] is a connective tissue disorder characterised by micro- and macroangiopathy of varying degrees in the same patient, associated with the histopathological hallmark of the disease, namely fibrosis of the parenchymal interstitia with typical but not exclusive clinical expression in the skin and lungs. There is a limited cutaneous form and a diffuse form, the latter being more frequently associated with pulmonary fibrosis, anti-Scl70 autoantibody positivity and a worse prognosis, whereas the limited cutaneous form is more frequently associated with pulmonary arterial hypertension and frequent anti-centromere antibody specificity. Raynaud's phenomenon in the hand and feet acro-sites characterises its onset. Acro-site digital ulcers, on the other hand, are more or less delayed expressions of a condition that was not adequately treated from the outset. Aetiopathogenesis is complex and not completely clear. The role of the B cells would nevertheless appear to be central to the extravascular pathogenetic mechanisms, which guide the clinical status towards fibrosis. For this reason, rituximab is used in patients who are non-responders to conventional therapies, especially in the case of interstitial lung disease and the diffuse cutaneous form,[85,86] although the improvement in respiratory parameters was not confirmed in other studies.[87]

Takayasu arteritis[88] is a vasculitis of the large arteries involving the thoracic aorta and the abdominal aorta and its major branches, as well as the pulmonary arteries. It typically occurs in females, with the highest incidence between 15 and 40 years of age, although earlier and later ages of onset cannot be excluded. It is most common amongst Asians. The annual incidence in the USA is 2.5 cases per million inhabitants. The aetiology is unknown, whereas the pathogenesis is at least partly cell-mediated. The efficacy of response to rituximab in some cases refractory to conventional therapies nevertheless highlights the possible pathogenic role of the B cells. In recent years, anecdotic data in literature have reported cases of patients who respond to rituximab after being resistant to traditional immuno-

suppressive drugs (T-cell co-stimulation modulators, TNF-alpha inhibitors, IL-6 inhibitors).
Kawasaki disease,[89] a rare, usually self-limiting febrile inflammatory disease, pertaining to the systemic vasculitis family, affects the paediatric population from the first months of life to 8 years. Slightly more common amongst males, it is characterised by an acute onset with fever, laterocervical lymphadenomegaly, oropharyngeal and conjunctival swelling, and erythema of the palms of the hands and the soles of the feet that tends towards desquamation of the fingertips. Cardiac, and especially coronary, involvement is possible (15-30% of cases) with the development of aneurysms or thrombi. Therapy with acetylsalicylic acid, especially when associated with high-dose IGVENA is also efficacious for reducing coronary complications. However, a significant percentage of patients do not respond to IGVENA. Given the importance of this vasculitis at autoantibody level, and the B-cell activity in the acute stages of the illness,[90] the use of rituximab would appear to be a valid alternative to traditional therapy.[89]
Behçet's disease[91] is a rare (1:500,000) chronic inflammatory disease with an autoimmune pathogenesis characterised by an alternation between small vessel inflammatory and vasculitic mechanisms. More common in the Mediterranean basin than in Asia, Behcet's disease is the most common cause of acquired blindness in Japan, where it is not rare (1:1000). Various organs and systems can be affected. The risks for life are usually associated with central nervous system, intestinal and arterial involvement. Common signs are painful oral and genital sores, involvement of the anterior and/or posterior segments of the eye, skin signs and symptoms (furuncles, erythema nodosum, pathergy reaction) and joint symptoms (joint pain/arthritis). B51 positivity is common. There are a number of treatment options, including both conventional DMARDs and synthetic biologicals. In the forms of vasculitis involving the posterior segment of the eye that are refractory to traditional therapies, there is evidence, albeit anecdotic and with non-significant numbers, of an efficacious and rapid response to rituximab, which supports a B-cell pathogenesis.[92-94]
Idiopathic CNS vasculitis is often refractory to various cytotoxic agents. By means of B-cell depletion, rituximab has proved to be efficacious in inducing clinical remission and reducing clinical exacerbations, as well as reducing the doses of cortisones. Despite these results that make rituximab the treatment of choice for both the induction of response and for exacerbation phase management, the evidence of phenomena of clinical resistance to treatment with rituximab even in the case of B-cell depletion cannot be explained. Few cases have been studied in literature.
ANCA-associated vasculites (granulomatosis with polyangiitis – Wegener's disease[95] – and microscopic polyangiitis) with B-cell pathogenesis are diseases characterised by the presence of circulating ANCA and leukocyte vascular infiltrates, as well as fibrinoid necrosis; they are burdened by a high mortality rate at 1-2 years and frequent clinical exacerbations. In this clinical setting, rituximab, also in combination with low-dose steroids, now represents one of the strategies of election, as its efficacy is similar to that of cyclophosphamide in the induction of remission in the acute phases, and superior to this latter cytotoxic agent in the control of frequent clinical exacerbations.

Conclusions

Despite having considered the advantages offered by immunological monitoring, it is important to stress that at the current time, the focus is limited to the scope of anti-CD20 monoclonal antibodies. The considerable potential of this method, which has thrown new light on the management of challenging diseases, can be further studied and extended, but we also need to identify other markers and other types of cell to analyse. One of the challenges in this domain is the development of new monitoring schedules to be tailored to suit each biological used, along the path forged by the now consolidated experience of the use of rituximab. This calls for the need to investigate each biological model underlying the various autoimmune diseases and the new treatment approaches with the increasing sophisticated technologies now at our disposal (receptor occupancy analysis, genetic signatures for autoimmune diseases, identification of new types of cell of pathological significance, such as circulating dendritic and mesenchymal cells bearing signs associated with tissue damage).

KEY POINTS

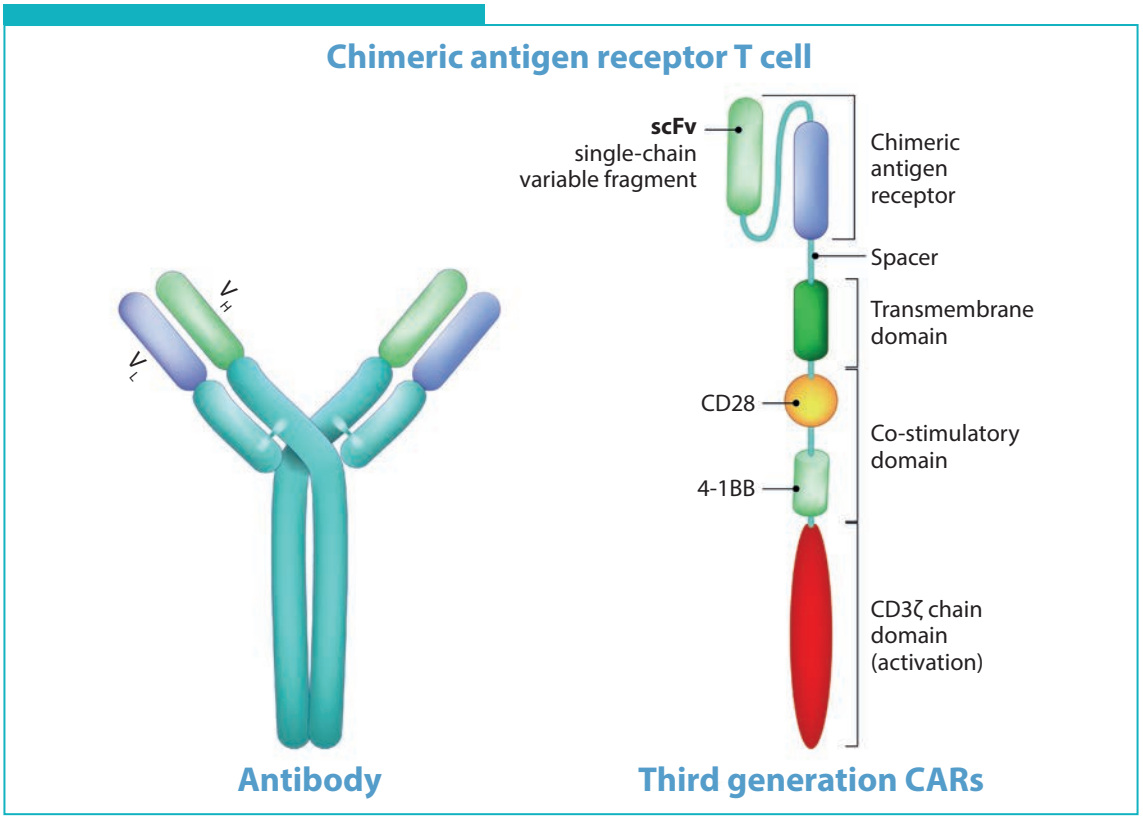
- ***Immunomonitoring of immunosuppressive treatments with rituximab and other biologicals in autoimmune diseases brings the added value of objective measurements to the mere observational clinical criterion of the status of disease remission.***
- ***Immunological monitoring makes it possible to promptly identify subjects who are refractory to specific therapies or who for various biological reasons present phenomena of resistance to the treatment.***
- ***Appropriate immunological monitoring could make it possible to tailor treatment choices to the individual patient or to specific clinical and immunological subsets.***
- ***Immunological monitoring keeps under control the global effect that the treatments have on immune response. Indeed, it is important not to forget the risks associated with excessive immunosuppression, which in some patients can generate a state of secondary immunodeficiency that can be severe.***

Appendix - Immunotherapy with CAR-T cells

CAR-T cells, whose development commenced in the late 1980s, are engineered cytotoxic T cells with a chimeric antigen receptor, composed of a single chain of the variable fragments of VL and VH immunoglobulins (able to bind to the antigen) fused with the transmembrane and intracellular domains of the TCR (able to transmit the CAR-T activation and amplification signal).

Structure and function of CAR-T cells

In unmodified T cells, antigen recognition and the formation of immunological synapses require the presence of various molecules and ligands in addition to the TCR, and are influenced by a multitude of molecules with an inhibitory or stimulatory action. In CAR-T cells, the chimeric receptor recognises the specific antigen and sends a signal to the modified T cell, which reacts as if it had recognised its target. During the development of the various generations of CAR cells, costimulatory signals (CD28 or 4-1BB) fused to the CD3ζ (third-generation CAR have two costimulatory domains) have been added to the chimeric structure.
One important aspect in the construction of an efficacious CAR cell is the choice of the ideal antigen, i.e. the best target. Identifying the right target is not always easy, considering that the cell simultaneously possesses its own endogenous TCR and there can be problems of competition between it and the CAR. CAR-T cells persist indefinitely in the patient's immunological repertoire, making it possible to provide the latter with a memory cell that continues to develop, thereby also providing protection for the future.



After infusion, the CAR-T cells act by migrating from the blood stream to the tumour sites, where they identify and kill neoplastic cells, which release specific antigens, thus activating the immune system and the non-CAR T cells. The major toxicities are associated precisely with the massive tumour lysis (neurotoxicity, cytokine release syndrome, or CRS).
Clinical trials based on the use of CAR-T continue to increase worldwide (approximately 450 trials at January 2020), with China leading the field in terms of the number of active trials (followed by North America).1 It is important to point out that Italy has played a fundamental role in the development of CAR-T cell therapy.
On the subject of our country, we cannot overlook the work done by the group led by Prof. Andrea Biondi, Professor of Clinical Paediatrics at Università degli Studi Milano-Bicocca and Director of the Paediatric Clinic of S. Gerardo di Monza, which, back in 2016, treated the first Italian child with acute lymphoblastic leukaemia (LLA), the most common paediatric tumour in children, adolescents and young adults up to 25 years of age, affecting approximately 450 individuals each year. Together with the Haematology Group of ASST Papa Giovanni XXIII di Bergamo, directed by Prof. Rambaldi, several years ago now, Prof. Biondi started using non-genetically-modified T cells in phase 1 and 2 studies (lymphocytes known as cytokine induced killers - CIK, because they are activated in vitro using certain cytokines). In addition, the Monza centre was the only site in Italy to take part in the pivotal study for tisagenlecleucel. At the current time, having received authorisation from the AIFA, the “S. Verri” Cell and Gene Therapy Laboratory of Ospedale S Gerardo di Monza, together with the Haematology Group of ASST Papa Giovanni XXIII di Bergamo, is conducting a phase 1 clinical study with an anti-CD19 CAR produced in their academic Laboratory.2-5

Anti-CD19 CAR-T cells

The most extensively used CAR-T cells to date are anti-CD19 (therefore not a specific tumour antigen, but one that is characteristic of B cells as a whole). In the treatment of acute lymphoblastic leukaemia (ALL) in children and young adults and in non-Hodgkin’s lymphoma, treatment with anti-CD19 CAR-T cells has yielded very positive results, with high complete remission rates in patients with multiple previous lines of therapy. Nevertheless, it is necessary to bear in mind the limitations to their use, first and foremost the difficulty of the entire procedure. Indeed, in one study on 107 patients with ALL, just 92 were enrolled and just 75 actually received the infusion, with only 48 patients continuing to the final follow-up visit.6 Another problem is the variability of the efficiency of the transfection required for chimeric receptor insertion, which can be very low in some cases. To date, the indications for treatment with CAR-T cells is limited to INN-tisagenlecleucel (ALL and diffuse large B-cell lymphoma) and Gilead’s INN-axicabtagene ciloleucel (mediastinal and diffuse large B-cell lymphoma). Both products are derived from the same anti-CD19 mAb.

Monitoring of the infused CAR-T cells

As the infused CAR-T cells become memory cells and persist in the body, it would be very interesting to be able to use immunological phenotyping after infusion, in order to analyse how these cells proliferate, differentiate and persist in the patient. This type of monitoring could also make it possible to differentiate the different phenotypes and to control the toxicity phenomena.7 At the current time, however, this would appear to be far from simple, as immedi-

ately after infusion the CAR-T levels decrease and they remain as memory cells at infinitesimal levels. The monitoring studies conducted are based on PCR, and state that the levels of transduced cells increase again if the disease reappears; however this kinetic is yet to be demonstrated. It is very interesting that, in the Italian experience conducted with engineered cells in which the CAR cells were based on a transposon, and therefore not on retroviral vectors, it was possible to monitor the genetically-engineered cells for long periods of time with standard flow cytometry, using the same CD19 molecule as a marker.5

The limitations of CAR-T cells

The limits of treatment with CAR-T cells are associated predominantly with production difficulties and the toxicity phenomena they generate. The production of autologous CAR-T cells is a complex and costly process, which reduces the number of patients who actually receive the treatment. One possible solution could be off-the-shelf CAR-T cells, produced using umbilical cord CIK cells, which could be transduced with the vector containing the CAR and do not cause GvHD toxicity when infused by haploidentical transplantation. The production of a biobank of umbilical cord blood CIK cells, to be infused with a 3/6 matching criterion, is feasible assuming the in vitro expansion of a limited number of banked cord blood units, such as to cover cord compatibility (evaluated as 3/6 low-resolution loci) of approximately 70% of patients, in order to have disposal of ready-to-use CAR cells. One alternative strategy could be endogenous TCR deletion; however, this procedure is very difficult.
The most dramatic adverse events associated with CAR-T cell treatment are related to massive tumour lysis: CRS, neurological damage and macrophage activation syndrome (MAS). CRS is now treated with tocilizumab, an IL-6 receptor antibody, although other cytokines are involved in the process.
Lastly, a further limitation is the loss or modulation of the target antigen when the disease reappears. It is a relatively frequent event, favoured by the fact that CD19 can present surface expression variants that can cause the loss of susceptibility to the CD-19 antibody. For this reason, clinical trials using multi-antigen CAR-T cells (CD19 and CD22 or CD19 and CD20) are currently being conducted.

Transposon-based CAR-T cells

Transposons can be used as an alternative to the viral vectors (retrovirus or lentivirus) usually used for the production of CAR-T cells. In this case, the CAR cell is inserted inside a plasmid and introduced into the T cell together with another plasmid containing transposase: the chimeric receptor is therefore inserted at a random point of the T cell genome.
A phase 1/2a trial has been designed involving the treatment of patients with ALL who relapsed after allogeneic transplantation with donor CIK cells, in which the anti-CD19 CAR cell was inserted by nonviral transfection. The chimeric receptor used includes in addition to the variable regions of the Igs, also CH2 and CH3, making it possible to monitor the CIK CAR cells by flow cytometer. The protocol has been approved and complete remissions and reduced toxicity (possibly due to the type of CIK cell) have been observed in the first treated patients.5 Possible scope for immunophenotyping in this domain is associated with the typing of the CIK CAR cells for evaluating the product and in vivo monitoring, which are, however, difficult at the current time.

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