REVIEW

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The autoimmune pathogenesis of multiple sclerosis

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One of the major goals of biomedical research is to reveal the pathomechanisms that lead to a disease on a level on which diagnostic criteria and causal therapies can be designed. The understanding and treatment of multiple sclerosis (MS) is still far from this goal, but exciting developments are on the way. MS is thought to be an autoimmune disease that is mediated by brain tissue-reactive lymphocytes, T cells and B cells, but so far these lymphocytes could not be reliably detected. This article highlights recent developments that permit the detection of autoreactive B cells in MS, the implications of this finding for early diagnosis of the disease, monitoring its activity, and eventually for gaining insight into the specific immune pathology that drives MS.

1. Introduction

In 1885 Luis Pasteur engaged in developing a nervous tissue-derived rabies vaccines. The vaccine worked well, but some of the recipients developed neurological complications that clinically resembled multiple sclerosis (MS) (Wu et al. 2011). It was not until 1935 that Rivers and Schwentker (1935) elucidated the mechanisms underlying these complications by showing that injection of proteins isolated from brain tissue suffice to induce a MS-like demyelinating disease in monkeys. These observations prompted the notion of autoimmunity, the hypothesis that MS could be an autoimmune disease in which myelin proteins are attacked by the immune system, and introduced the animal model for MS, experimental allergic encephalomyelitis, EAE. While tremendous progress has been made since, and in particular in the last two decades in elucidating the autoimmune mechanisms involved in the pathogenesis of EAE (Kuerten and Lehmann 2011), still very little is known about the immune pathomechanisms involved in MS. Why?

We still have no disease specific diagnostic markers for MS. The diagnosis is being made based on exclusion of other neurologic diseases following the so-called McDonald criteria (Polman et al. 2011). These criteria are suited to identify advanced stages of MS reasonably well, but they typically fail to detect early MS, or to identify the predisposition to develop the disease. This has been a major setback for scientists who have been attempting to study immune mechanisms involved in the pathogenesis of MS. The autoimmune processes that are thought to mediate the disease have long flared by the time the clinical symptoms manifest themselves. In spite of many attempts in the past, the detection of the autoimmune response itself in MS patients mostly failed, even leading to skepticism in some scientist whether MS is an autoimmune disease at all. Beyond doubt, MS results from an inflammatory destruction of central nervous tissue, and all changes seen in the affected brains are consistent with downstream consequences of an autoimmune attack.

Acquired immunity and autoimmunity are mediated by lymphocytes. Lymphocytes occur as B cells that can produce antibodies, and as T cells that can engage in a direct cell-mediated attack. The structures (mostly proteins) that lymphocytes specifically recognize are called antigens. In the case of MS, the suspected antigens that are targeted by lymphocytes are proteins of the myelin sheath, such as myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and several other myelin proteins. Each lymphocyte, B cell and T cell alike, is specific for one, and only one antigen due to possessing a unique antigen receptor. With about 10^{12} different antigen receptors expressed in a mutually exclusive way on lymphocytes, there are about 10^{12} lymphocytes with different antigen specificity in our body (Fig. 1). The astronomic number of 10^{12} corresponds to the number of stars in the Milky Way, and just like those stars, every lymphocyte has individual properties. Each lymphocyte is therefore unique with regard to its antigen specificity, and each is diluted within a myriad of other lymphocytes that all have different specificities.

When we encounter a new antigen, e.g. get infected by a virus for the first time, in the first two weeks those lymphocytes that are specific for the antigen undergo vigorous cell divisions while inheriting their antigen receptors and hence their antigen specificity to their daughter cells (Fig. 1). In this process of so-called "*clonal expansion*", lymphocytes that have occurred at infinitely low frequencies before the encounter with the antigen can become as frequent as 1 in 100 within all lymphocytes. This is the time point, around ten days after the antigen encounter that is best suited for the detection of antigen-specific lymphocytes. In the subsequent weeks "*clonal contraction*" occurs: few of the daughter cells survive, and the numbers of the antigen-specific lymphocytes keep dropping all until they settle somewhere in the 1 in 100,000 – to 1 in 1,000,0000 frequency range (Fig. 1) reaching the detection limit for present technology. If the antigen persists, like in chronic infections or in autoimmunity, "overwork" and exhaustion makes the numbers of antigen-specific

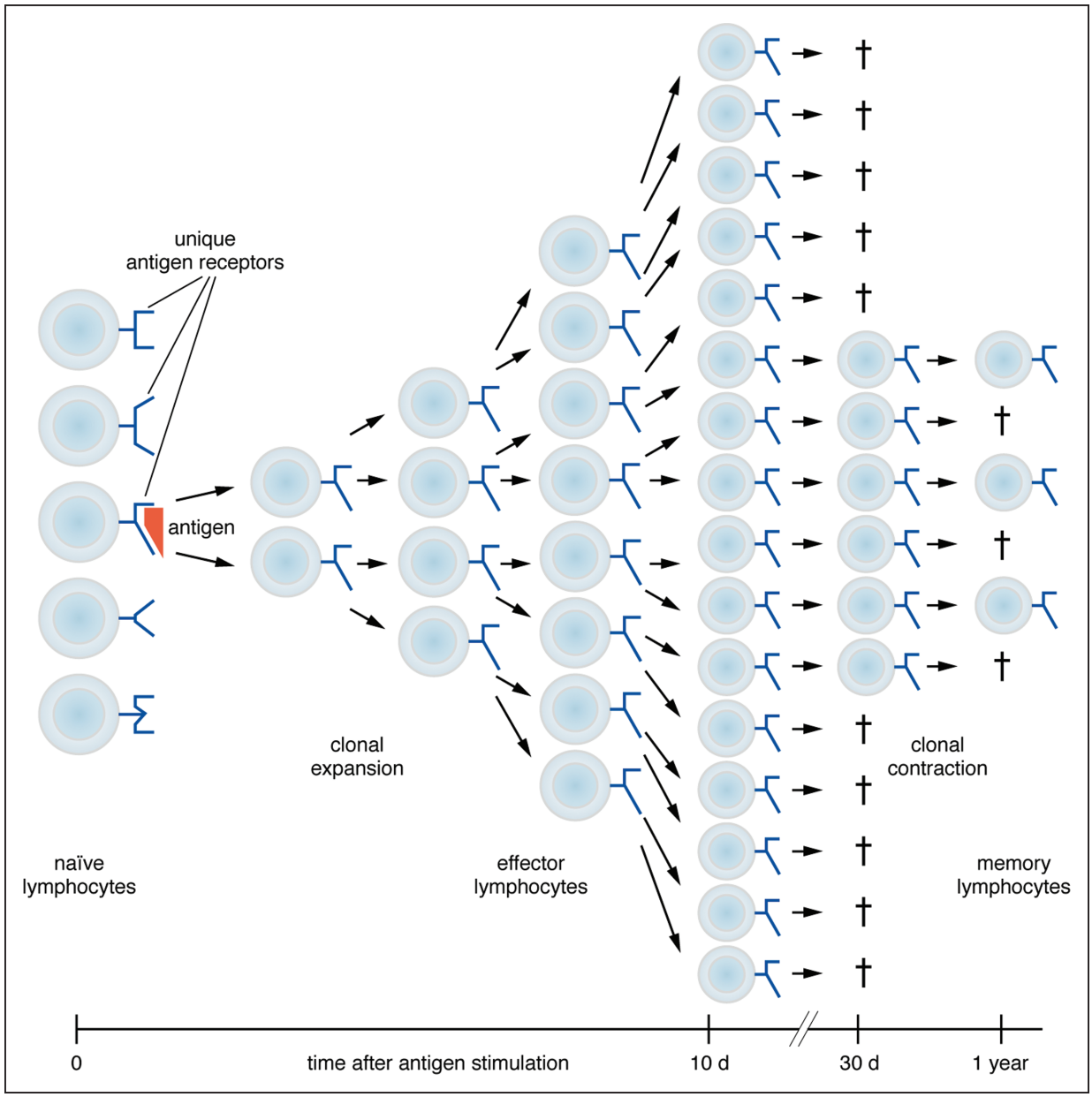

Fig. 1: Basic principle of immune responses. Lymphocytes with unique antigen receptors survey our body. When antigen is encountered under conditions that are stimulatory to naive lymphocytes, those with the matching antigen receptor are triggered to divide. Clonal expansion leads to the generation of effector lymphocytes, peaking around ten days later. Subsequently in a process called "*clonal contraction*" most of the newly generated effector lymphocytes die off, some survive as memory lymphocytes. The frequency of memory lymphocytes with a given specificity is increased relative to that of the naïve cells resulting in a faster secondary clonal expansion upon re-encounter of the antigen. Antigen persistence – like in autoimmunity – however, continues to drive clonal contraction due to the overwork of the autoantigen-specific lymphocytes.

lymphocytes continue to drop to even lower frequencies, below the detection limit of present technology. One can readily detect antigen-specific lymphocytes, for example in acute HIV, hepatitis B and C infection, tuberculosis and borreliosis, but as these infections become chronic the antigen-specific lymphocytes become increasingly difficult to detect, and are eventually undetectable (Kuerten et al. 2008a,b; Anthony et al. 2011; Jin et al. 2013). Also in EAE, the neuroantigen-specific lymphocytes can readily be detected in the first weeks after the injection of the antigen, but within a couple of months the neuroantigen-specific cells become undetectable (Targoni et al. 2001). It is in the well-established nature of the immune system that in chronic immune-mediated conditions it does not leave the hallmark of immune memory behind, that is expanded clonal sizes. Not being able to detect increased frequencies of autoreactive lymphocytes in patients with chronic MS (and in most other suspected autoimmune diseases) therefore does not really argue against an autoimmune etiology, but has made direct studies of the very cells that mediate the disease close to impossible for researchers and has prevented clinicians to use the detection of autoreactivity against the brain as a specific diagnostic marker for MS.

2. iNSBC: a promising new immune diagnostic marker for MS

Most efforts so far have failed to regularly detect neuroantigen-specific autoantibodies in sera of MS patients, or to identify increased frequencies of neuroantigen-reactive T or B cells. However, a breakthrough was reported this year (Kuerten et al. 2014) that not only holds promise for early specific diagnosis of MS, but also provides insight into the immune pathomechanisms operating in MS.

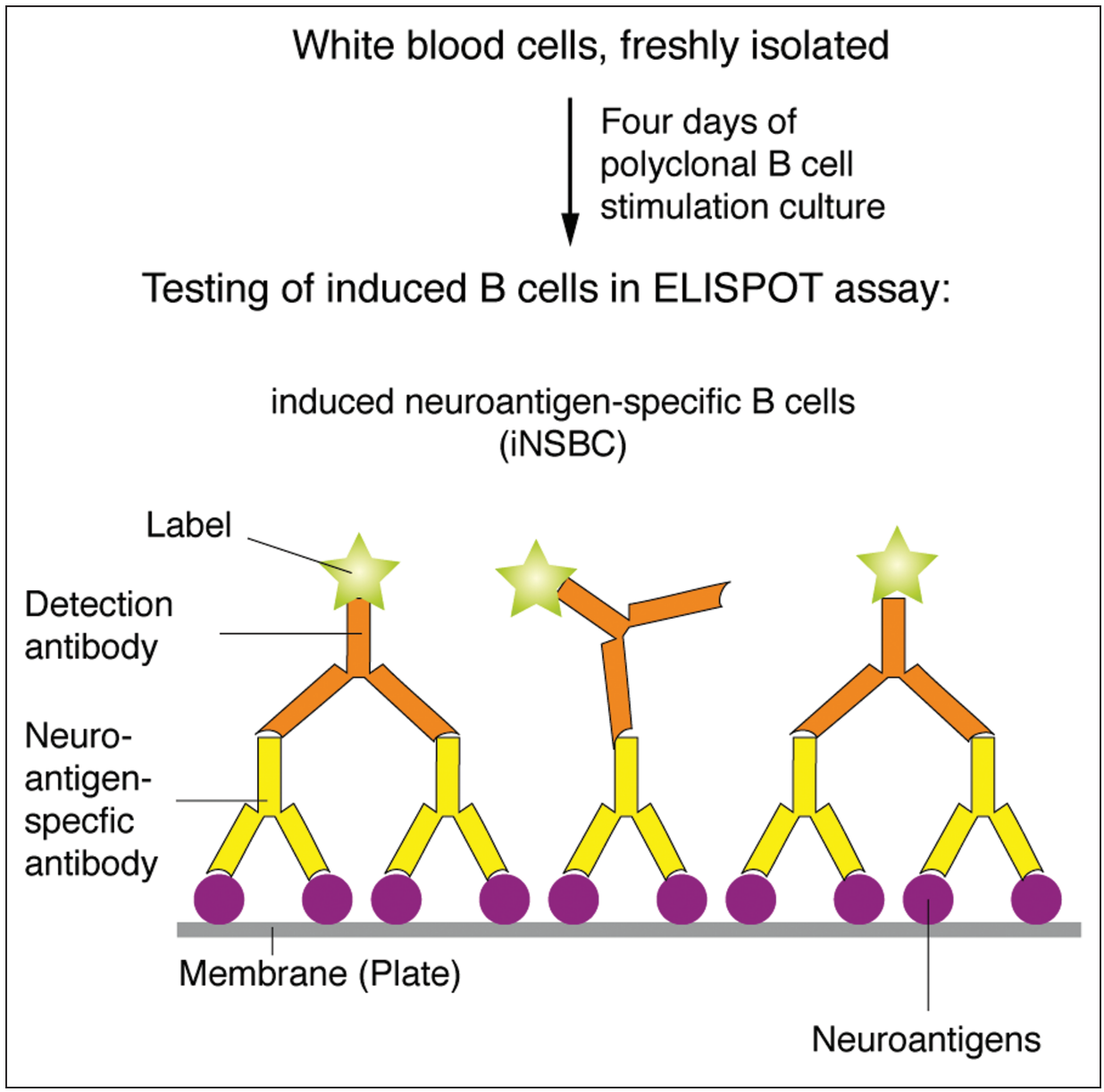

Fig. 2: The test principle for detecting NSBCs. Blood is drawn, and white blood cells are isolated, followed by four days of polyclonal B cell stimulation that induces all resting B cells to secrete antibodies. The induced B cells, including the induced neuro-antigen specific B cells (iNSBC) are plated on a membrane that is coated with neuroantigens. For detecting spontaneous NSBCs (sNSBC), the freshly isolated white blood cells are directly plated without any polyclonal stimulation step. When neuroantigen-specific B cells secrete their antibodies, these antibodies bind around the secreting B cell to the neuroantigens on the membrane. The result is a "spot" of plate-bound human antibody around each NSBC that can be detected by labeled anti-human antibodies. B cells secreting antibodies with different specificities do not bind to the neuroantigen-coated membrane and remain undetected in the test. This ELISPOT test thus provides information on the frequencies of NSBC within all white blood cells of the test subject.

Serum and white blood cells (that include lymphocytes) were isolated from MS patients, healthy controls as well as from patients with other neurological or autoimmune diseases. Reproducing previous findings no definitive signs of reactivity to brain proteins could be detected neither in the serum, nor in B or T cells. This finding dramatically changed after the white blood cells were exposed to four days of polyclonal *in vitro* B cell stimulation, which caused dormant memory B cells to expand clonally moving their frequencies into the measurable range. In addition, these cells became activated to secrete antibodies, which permitted the detection of these otherwise quiescent cells. Subsequently, an ELISPOT (*enzyme-linked immunospot assay*) test was performed that allowed to measure the frequency of induced neuroantigen-specific antibody producing B cells (iNSBC) (Fig. 2). Most of the MS patients (53 of 67 = 79%), but none of the 127 healthy controls or any of the 41 individuals with other neurological or autoimmune diseases displayed increased frequencies of iNSBC. Antibodies that were detected in MS patients were of the IgG immunoglobulin class.

These data indicate that in MS patients (but not in the control subjects) iNSBC have undergone clonal expansion, a process that can exclusively be observed after immune responses. This expansion results in an increased frequency of iNSBC in the MS patients' blood. Also the production of IgG antibodies by the MS patients' iNSBC provides evidence for the fact that these cells have been involved in an immune response *in vivo*, before these cells were isolated from the patients. Naïve B cells (that have not been engaged in immune responses before) always produce IgM antibodies first, and they only switch to IgG production at later time points, which involves fine-tuned interactions with T cells *in vivo*. Therefore, the detection of IgG producing iNSBC in MS patients shows that these B cells have been engaged in an immune response *in vivo* during which they have undergone immunoglobulin class switching. The presence of iNSBC in

a subject holds promise to constitute a marker for predicting who will develop MS. This study included 15 individuals who were diagnosed with a clinically isolated syndrome (CIS), which presents a high risk of developing MS in the absence of a definite diagnosis. Of 15 CIS patients studied, nine (60%) showed increased frequencies of iNSBC. It remains to be elucidated if the occurrence of iNSBC in patients is accompanied with an increased risk of developing definitive MS.
Although the number of MS patients and controls examined in this publication was considerable, it will be important to extend the study to include additional large cohorts of patients and controls. If the data also hold up in those cohorts, the detection of iNSBC will provide the long sought after specific diagnostic test for MS.

3. sNSBC: a promising new marker for activity and progression of MS

There was another exciting finding in the above mentioned report (Kuerten et al. 2014). In some of the MS and CIS patients, but in none of the controls, B cells were detected that produced neuroantigen-specific antibodies at the time point of their isolation from the patients without the need for additional activation of the white blood cells *in vitro*. Spontaneously antibody producing B cells are known to occur in a short window, around ten days after an active immune response has occurred. For example, influenza virus-specific B cells secreting influenza virus-specific antibodies can be detected in freshly isolated blood 8-12 days after an infection or vaccination with the influenza virus, but not at later time points (Saletti et al. 2013). These B cells will produce antibodies only against the antigen that triggered the immune response *in vivo*, in this example against flu virus, but not against other viruses. By inference, detecting B cells that spontaneously produce antibodies against neuroantigens (spontaneous NSBC, sNSBC) suggests that an active immune response has been taking place in the MS and CIS patients at the time point when their blood was tested.
MS has various clinical courses (Lublin et al. 2014). The most common one is a relapsing-remitting form. Clinical exacerbations of the disease are followed by periods of stability ("remission"). Based on extensive animal studies one needs to assume that clinical symptoms are a rather insensitive tool to understand and monitor the underlying immune pathology. Symptoms occur only if the damage is substantial and if it affects those areas of the brain whose dysfunction produces noticeable symptoms, such as paralysis. It would certainly be of clinical benefit to be able to monitor the activity of the very cells that cause the disease. The re-activation of the disease could be observed as soon as the new autoimmune attack builds up and hence before it causes new damage that eventually results in clinical symptoms. Intense immunosuppressive treatments could be administered during such brief time windows of autoimmune flares, without unnecessarily paralyzing the patient's immune system in the long periods of time during which the autoimmune attack is dormant. Because such treatments could be targeted to the relevant brief time frame they could be way more aggressive and thus effective than is conceivable for persistent applications. The detection of sNSBC promises the identification of flares of the autoimmune process in MS.

4. The fine specificity of NSBC: evidence for multiple neuroantigens targeted in MS

The mentioned data has been obtained by testing autoimmune B cell reactivity with a brain protein extract that contained many different neuroantigens. The studies were extended to closer define the autoantigens targeted by the NSBC. Staining of human brain tissue showed that the antibodies secreted by the NSBC specifically bind to the myelin sheath, which matches well with MS as a demyelinating disease. Some of the major myelin antigens were therefore tested, namely MBP, PLP, and MOG. Some of the MS patients' NSBC were specific for MBP, some for PLP, some for MOG, and most recognized yet different neuroantigens. Therefore, the autoimmune attack in MS patients studied so far does not target a single target antigen, but several and to a variable extent.
Defining the target antigen in autoimmune diseases is not only important for diagnostic purposes, but it can also provide leads for specific therapeutic approaches. Based on EAE models it was hoped that a single target antigen or even a short peptide sequence of that autoantigen could be identified in MS. In that case it would be possible to induce immune tolerance to that peptide, altered peptides could be developed that paralyze the peptide-specific T and B cells, the autoantigen-specific T and B cells could be selectively destroyed using therapeutic antibodies or certain molecules involved in antigen presentation could be blocked. All of these are highly attractive therapeutic approaches as they would permit the selective inactivation of the disease-causing autoreactive lymphocytes while leaving the immune system of the MS patients otherwise unaffected. Indeed, in EAE models the efficacy of all of the above approaches has been shown for preventing the development of EAE (but such approaches were largely ineffective in reversing chronic EAE). Since the neuroantigen-specific B cells recognize variable neuroantigens (which also implies that the autoreactive T cells recognize variable antigens, see below), it now seems likely that therapies that target individual myelin antigens, and the autoreactive lymphocytes that recognize such antigens may not hold the promise in MS, that was initially raised by the EAE models. The fact that NSBC target different antigens in MS patients is consistent with an observation that was made already in the 1990's that suggested that autoimmune responses evolve like an avalanche. Even if autoimmunity starts highly targeted, for example being induced through a virus that cross-reacts with autoantigen X through a mechanism called "*determinant/ epitope spreading"*, soon additional autoantigens (U, V, Y and Z) will be drawn into the autoimmune response to a varying degree (Lehmann et al. 1992; Robinson et al. 2003). The diversity of neuroantigen specificity of NSBC observed in MS suggests that also in this disease *determinant/epitope spreading* is a frequently occurring phenomenon. In EAE models spreading occurs in waves, leading to a dynamic lymphocyte repertoire (Lehmann et al. 1992; Robinson et al. 2003). By following NSBC in MS it will be possible for the first time to gain insight into how the autoimmune process operates and what other similarities or differences exist relative to its much studied and well understood model EAE. While this new evidence for the presence of *determinant spreading* reactions in MS might be bad news for antigen-specific therapeutic approaches on the one hand, it will on the other hand offer new treatment opportunities. Each time spreading occurs, the autoimmune response resets itself and a new "avalanche" is started. If one knows when exactly this process happens, it can be stopped before the momentum builds up. sNSBC seem to identify those critical time points of re-flaring immune activity.

5. The assumed pathogenetic role of NSBC in MS

iNSBC promise to provide specific diagnostic markers for MS and sNSBC for activation/reactivation of the disease. But are these autoreactive, myelin-specific B cells participating – or even causing – the pathogenesis of MS or are they just accompa-

nying the disease? For diagnostic purposes the answer does not really matter, but it matters a lot for therapeutic considerations. At this point we can only speculate about the answer, but it is one of those rare questions that can be addressed rather simply experimentally in humans. Rituximab is an antibody-based drug that wipes out most B cells in the human body. It was originally developed to treat B cell-mediated leukemia, but it could be used to treat any condition in which B cells themselves or their products, the antibodies, cause or contribute to the pathogenesis of the disease. What took most immunologists and clinicians by surprise was how little side effects a short-term ablation of B cells had on immune defense. Rituximab-treated individuals, though left largely without B cells, are not much more susceptible to develop infectious diseases than healthy, untreated subjects. Thus, treatment of MS patients with rituximab should be of low risk, and would directly address the question of the pathogenic role of autoreactive B cells and the antibodies they produce in MS.

There is one possible caveat of therapeutic B cell depletion in MS, though. In the EAE model in which autoreactive B cells play a role many B cells reside within the brain. In such animals secondary lymphoid tissues develop in their inflamed brains containing abundant B cells (Kuerten et al. 2012). Such lymphoid structures containing B cells have been also observed in the brains of MS patients (Serafini et al. 2004). Separated by the blood-brain barrier, the brain is normally not penetrated by antibodies present in the serum. The blood-brain barrier occasionally breaks down in MS (which can be visualized by MRI) which would help the penetration of rituximab antibodies, but it remains unclear to what extent such therapeutically administered antibody would have sufficient access to those B cells that reside in the brains of MS patients, particularly in areas of the brain in which the blood barrier is still intact. Clinical trials using rituximab or similar B cell-depleting antibodies will need to take into account these tissue-resident NSBC. The interpretation of the data will also need to take into account that the antibodies that have already been produced by these B cells have a half-life of about 20 days in body fluids that may be prolonged if the antibodies are absorbed by the autoantigens within the tissue.

6. The role of NSBC in EAE

In experimental science the way we ask a question can already influence the result. This has certainly also applied to EAE research over the decades. After Rivers and Schwentker (1935) had made the observation that extracts of brain cause an MS-like disease, the search began to closely define the relevant antigen. In retrospect, that search was biased from the beginning as it favored the identification of water-soluble proteins. MBP has become one of the primary antigens of EAE research. MBP is a major part of the myelin sheath, which consists of lamellas of oligodendrocyte membranes, wound around axons to isolate the axons so they can properly transmit electric signals. MBP is not displayed on the surface of the myelin sheath, but is buried within the lamellae. In this way antibodies have no access to MBP in the undisrupted myelin sheath, and MBP-specific autoantibodies do not cause primary autoimmune destruction. In contrast MBP-specific T cells can cause autoimmune disease, because T cells in general do not bind the antigen itself, but they recognize processed fragments of the antigen that are presented to them on so-called antigen presenting cells (APC). It is well established that MBP-specific T cells, but not antibodies, mediate EAE and from this observation the notion emerged that also MS is primarily T cell-mediated with little contribution of autoantibodies.

This view changed radically when, rather recently, myelin antigens were studied that are present on the surface of the myelin sheath. PLP is also a major constituent of the myelin sheath. It is a transmembrane protein, with two of its seven domains oriented towards the extracellular space and thus being accessible to autoantibodies. Four segments of the protein are highly hydrophobic as required for passing through the cell membrane. Its hydrophobicity has made it very difficult to work with PLP experimentally in EAE models. The situation changed, when a recombinant molecule was created, MP4, in which the transmembrane sequences of PLP were excised, leaving the extracellular (and intracellular) domains of PLP intact (Elliot et al. 1996).

Immunizations with MP4 induce EAE that more closely resembles MS than the MBP-induced EAE model. The MP4-induced disease has a chronic course and demyelination is more profound and lasting (Kuerten et al. 2006). Moreover, autoantibodies in addition to T cells are involved in the pathogenesis of EAE induced by MP4 (Kuerten et al. 2011). Studies of MP4-EAE yielded another surprise: the aforementioned B cell follicles established themselves in the diseased brain and were sites of local antibody production (Kuerten et al. 2012). If these lymphoid structures were the primary site for myelin-specific autoantibody production, this would explain why myelin-specific autoantibodies cannot readily be detected in the serum of MS patients while NSBC can. Myelin-specific autoantibodies produced in the brain would be locally absorbed by the myelin, becoming undetectable in the serum. Memory B cells, in contrast recirculate in the blood, and can be detected with sensitive techniques.

In summary, after decades of focus on autoreactive T cells in MS and its model, EAE, there is a recent shift of interest towards autoantibodies. Such autoantibody producing B cells do not only provide much needed new diagnostic opportunities, but they also permit new ways to gain insights into the contribution of T cells. Autoantigen-specific T cells in large still withdraw themselves from direct observation in MS: they occur at too low frequencies for direct observation. The production of autoantigen X-specific autoantibodies, however, requires the presence of active autoantigen X-specific T cells. Therefore, the newly gained ability to study neuroantigen-specific B cells in MS will provide more information on neuroantigen-specific T cells in MS. As this endeavor has just started, a lot will need to be learned about the pathogenic significance of autoreactive T and B cells in MS.

It is the personal opinion of the authors of this article that T cells are the primary mediators of MS, but antibodies contribute to the pathogenesis (Fig. 3). The histological changes in the MS brain are reminiscent of type IV immune reactions, perhaps better known as "delayed-type hypersensitivity" reactions that are well understood, e.g. in tuberculosis. When antigen-specific T cells of a certain type (T_H17) recognize antigen in a tissue, they start secreting chemokines and cytokines that recruit and activate macrophages to and at that site. Within 48 h a mononuclear infiltrate builds up. Stimulated by the T cell-derived mediators, the macrophages start secreting oxygen radicals, prostaglandins, and other pro-inflammatory molecules. In EAE and MS, oligodendrocytes stop producing myelin, but survive. The resulting demyelination leads to clinical symptoms including paralysis as nerve conductivity is jeopardized, but this stage of demyelination is reversible. The macrophages also become phagocytotically active and attack antibody-coated structures. This is how the autoantibody-coated myelin sheath becomes attacked, and why oligodendrocytes die off, leading to irreparable myelin damage. Axons that are irreversibly stripped of their protective myelin sheath and exposed to pro-inflammatory molecules including oxygen radicals, become

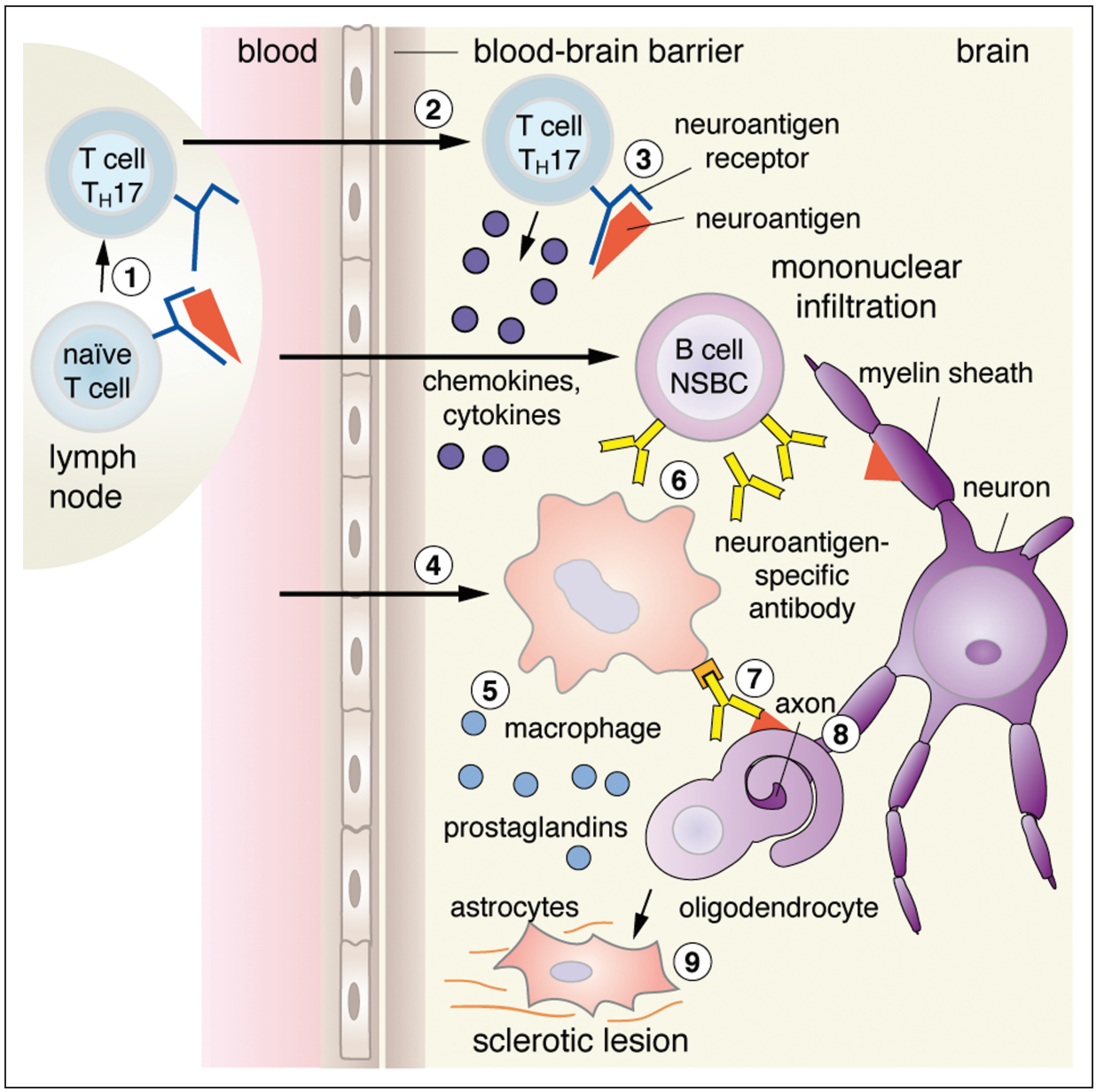

Fig. 3: Critical steps in the pathogenesis of MS. (1) Naïve neuroantigen-specific $CD4^+$ T cells are stimulated by cross-reactive antigen, e.g. a virus, to become a T_H17 $CD4^+$ effector-memory cell that disseminates in the body and also enters the brain (2). This T cell recognizes neuroantigen in the brain (3), which is presented on APC (for simplicity reasons not shown here). This in turn induces the T cell to secrete cytokines and chemokines (4) that attract macrophages, B cells and T cells from the blood. These cells form the initial mononuclear infiltrate. The stimulated macrophages secrete oxygen radicals and prostaglandins (5) causing oligodendroytes to stop producing myelin – temporary demyelination occurs. Neuroantigen-specific antibodies produced by NSBC (6) bind to the myelin sheath marking it for phagocytotic destruction by macrophages (7). Axons get stripped of the myelin sheath (8) causing the degeneration of the corresponding neuron (9). Tissue damage and chronic inflammation stimulate astrocytes to generate local scar formation.

damaged, leading to the death of the neurons. As in tuberculosis and other similar chronic T cell-mediated diseases, including chronic hepatitis, local scar tissue forms if the T cell attack perpetuates. In MS, the results of many type IV inflammatory foci are many scars that gave the disease its name.

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