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Merits and complexities of modeling multiple sclerosis in non-human primates: implications for drug discovery

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ABSTRACT

Introduction: The translation of scientific discoveries made in animal models into effective treatments for patients often fails, indicating that currently used disease models in preclinical research are insufficiently predictive for clinical success. An often-used model in the preclinical research of autoimmune neurological diseases, multiple sclerosis in particular, is experimental autoimmune encephalomyelitis (EAE). Most EAE models are based on genetically susceptible inbred/SPF mouse strains used at adolescent age (10–12 weeks), which lack exposure to genetic and microbial factors which shape the human immune system.

Areas covered: Herein, the authors ask whether an EAE model in adult non-human primates from an outbred conventionally-housed colony could help bridge the translational gap between rodent EAE models and MS patients. Particularly, the authors discuss a novel and translationally relevant EAE model in common marmosets (Callithrix jacchus) that shares remarkable pathological similarity with MS.

Expert opinion: The MS-like pathology in this model is caused by the interaction of effector memory T cells with B cells infected by the γ1-herpesvirus (CalHV3), both present in the pathogen-educated marmoset immune repertoire. The authors postulate that depletion of only the small subset (<0.05%) of CalHV3-infected B cells may be sufficient to limit chronic inflammatory demyelination.

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1. Introduction

Animal models of human disease are essential tools in the preclinical development of new therapeutics and for translational research into pathogenic mechanisms and therapy targets. A well-validated animal model is strategically important for the selection of promising drug candidates from development pipelines and provides important information about potential toxicity, efficacy as well as pharmacokinetic and pharmacodynamic parameters of the drug candidate. The central theme of this discussion is the autoimmune neuroinflammatory disease multiple sclerosis (MS) which, with a prevalence of 1 per 1,000, is the second-most important neurological disease in young adults in Western societies.

The pathological hallmark of MS and the most likely cause of the neurological deficits is the demyelinated lesion. Lesions are usually well-defined abnormalities located in the white and gray matter of the central nervous system (CNS), comprising brain and spinal cord, where the myelin layers around axons are damaged. Inside lesions, a variable degree of inflammation, remyelination, astrogliosis, and degeneration of neuro-/axonal complexes can be observed.

The MS clinical course can be divided into three phases, which can vary in length between patients: (1) In the pre-symptomatic phase, presence of lesions in the CNS white matter can be detected via magnetic resonance imaging (MRI), but evident neurological symptoms are not detected; (2) The relapsing-remitting (RR) phase is characterized by episodes of neurological deficits (relapses), occurring on average once per 2 years, which alternate with recovery (remission); (3) Transition to the progressive phase (=secondary progressive (SP)MS) can occur after 5–20 years and is characterized by the disappearance of relapses and progressive decline of neurological functions without intermittent recovery (Figure 1(a)). Relapse-onset MS affects ≥ 85% of MS patients, of which 60% develop SP disease [1]. In primary progressive (PP) MS, affecting ≥ 15% of the patients, the disease is progressive from the onset without antecedent relapses. Whether SPMS and PPMS are different or identical clinical entities is debated. A noticeable difference between RR and progressive MS exists with respect to the localization and histological features of lesions. In RRMS, lesions are mostly formed around blood vessels in the CNS white matter, while in progressive disease also the gray matter is affected. Moreover, while lesions in the white matter are usually filled with different types of infiltrated immune cells (T and B lymphocytes, macrophages), lesions in the gray matter are usually paucicellular with only a rim of activated microglia.

2. Current MS animal models

A wide range of animal species is used in the preclinical research of MS, including nematode worms, fruit flies, zebrafish, mice, rats, guinea pigs, and nonhuman primates (NHP). A similar wide range of methods is employed for disease
induction, including intracerebral injection of cytotoxic agents (e.g. lysolecithine, ethidium bromide) or neurotropic viruses (e.g. Theiler's murine encephalitis virus, Semliki Forest Virus), feeding the copper chelator cuprizone, gene knock-in/knock-out methods, active immunization with CNS myelin antigens or adoptive transfer of anti-myelin T cells and antibodies. For an in-depth discussion of these models and their relevance for our current understanding of pathogenic mechanisms contributing to the MS pathogenesis, we refer the reader to comprehensive reviews published elsewhere [2–5].

The current discussion will be confined to the experimental autoimmune encephalomyelitis (EAE) model, which is the most widely studied preclinical MS model. EAE has been induced in genetically susceptible strains of inbred/specific pathogen-free (SPF) mice (e.g. C57BL/6, SJL, Biozzi ABH), rats (e.g. Lewis, Dark Agouti), guinea pigs, and NHP (common marmoset, rhesus monkey, cynomolgus monkey). An important motivation for the development of the EAE model has been that clinical and pathological features of the human disease are reproducible in a model animal (face validity). Important for drug development, however, is also that the mechanisms causing the pathology and disease are to a high degree comparable between MS and its animal model (construct validity). The difficulties encountered in the translation of scientific discoveries in the models into an effective treatment for the patient and the fact that current treatments are only effective in RRMS indicates that at the mechanistic level, a wide gap exists between the current rodent EAE models and the patient. In the next paragraphs, we will discuss an EAE model in a small-bodied Neotropical primate, the common marmoset (Callithrix jacchus), that may help to bridge this gap.

**Figure 1. Clinical and pathological aspects of MS.** A. the prevalent outside-in view on the MS course in the majority of patients. Exposure of genetically-prone individuals to an as yet unidentified micro-organism (EBV) activates naïve autoreactive T and B cells present in the immune repertoire. The combined autoimmune attack of T cells and antibodies on myelin sheaths in the CNS white matter (C.2) elicits focal inflammation (orange bar) centered around blood vessels. Oligodendrocytes (ODC) are spared, enabling lesion repair by remyelination. The blue line represents an independent pathological process involving the progressive loss of ODC and neuro-axonal complexes. In presymptomatic MS, lesion activity can be detected via imaging, but as it remains under a (hypothetical) clinical threshold (dotted line), clinical signs are absent. In the RR phase, episodic lesion activity surpasses the clinical threshold and causes relapses. In late stage MS relapses occur less frequently and ultimately disappear. B. primate view on MS. EBV-infected B cells recruit autoaggressive effector memory CTL from the repertoire which attack and kill oligodendrocytes (C.3) eliciting lesions in the white and grey matter (blue field; EAE pathway 2). Myelin antigens (MOG in particular) released from these lesions trigger the activation of pro-inflammatory Th1/17 cells, which induce episodic exacerbation of lesion activity (orange bars; EAE pathway 1) superimposed on the progressive degeneration. C. An oligodendrocyte (ODC) forming multiple axon-enwrapping myelin sheaths (1); a macrophage attacking a myelin sheath opsonized with antibody and complement, but sparing the ODC (2); a CTL attacking an ODC inducing concomitant degeneration of the myelin sheaths.
3. Treatment status

The latest genome-wide association studies reveal that the susceptibility and/or course of MS are influenced by around 200 genes, that the vast majority has a role in the immune system and that there is a considerable genetic overlap between autoimmune diseases [6,7]. It is therefore not surprising that most of the currently developed treatments target the immune system.

The inflammatory activity of immune cells can be suppressed to some extent with immunomodulatory agents, such as the cytokine interferon-β or the synthetic polymer glatiramer acetate [8]. These agents have the advantage of low toxicity, but display moderate therapeutic activity and do not work equally well in all patients [9].

In another therapeutic approach, access of immune cells to the CNS is prohibited. This can be achieved with the orally applicable small molecule fingolimod, which prevents the release of T cells from the lymph nodes, where they are activated, into the circulation [10].

Natalizumab is a blocking monoclonal antibody (mAb) directed against the α4β1 integrin VLA4 that prevents T cell binding to VCAM-1 on blood brain barrier (BBB) endothelial cells for transmigration [11]. In a small group of patients treated with this mAb, fatal progressive multifocal leukoencephalopathy (PML) occurred. PML is a rare condition caused by the reactivation of latent CNS infection with John Cunningham virus, which in the healthy brain is controlled by the immune system [12]. Moreover, after stopping treatment with this mAb severe rebound MS has been observed [13].

In yet another approach, immune cell subsets are depleted from the system. Alemtuzumab is a depleting mAb directed against CD52, a molecule mainly expressed on the surface of T and B lymphocytes and less on innate immune cells such as monocytes/macrophages and natural killer cells [14]. It has become clear that the adverse effects of this treatment are less than expected, albeit not irrelevant. Patients treated with alemtuzumab can experience reactivation of latent cytomegalovirus (CMV) infection [15]. A serious complication that is rather frequently observed after stopping treatment with alemtuzumab administration is antibody-mediated autoimmunity against the thyroid gland [16]. The underlying cause is not exactly understood.

More refined treatments selectively target only certain immune cells. The central pathogenic role of autoreactive CD4+ T helper (Th)-1 or -17 cells in the EAE model has spiked the development of a plethora of treatments aiming at the functional or physical elimination of these T cell subsets. Unexpectedly, depletion of Th cells with an anti-CD4 mAb (cM-T412) or blockade of the cytokines IL-12 and IL23 to prevent differentiation of CD4+ T cells toward a proinflammatory phenotype (Th1/Th17) with an mAb binding the shared p40 subunit of IL-12 and IL-23 (ustekinumab), failed to show convincing efficacy in RRMS patients [17,18]. Moreover, blockade of IL-17 with the mAb secukinumab inhibited inflammation activity in lesions on brain magnetic resonance images, but lacked a marked clinical effect [19].

Many, often sophisticated, procedures have been developed to re-instate the nonresponsiveness of auto-reactive T and B cells against self, such as by the strengthening of regulatory mechanisms. However, despite sometimes promising effects in the EAE model, none of these treatments has reached clinical practice.

The failure of antibodies targeting CD4+ T cells to show a convincing therapeutic effect indicate that the strong clinical effect of alemtuzumab in RRMS cannot be explained by an effect on CD4+ T cells. Hence, the effect might be exerted via CD8+ T cells or B cells. To our knowledge, treatments targeting CD8+ T cells, which per recent insights are more prevalent in MS lesions than the CD4+ subset and are oligoclonally expanded [20,21], have not yet reached the clinic. With respect to B cells, it was found that their depletion with rituximab, a chimeric mAb against the B cell lineage-restricted CD20 marker, exerted a brisk and persistent clinical effect in RRMS [22]. The observation that antibody levels were not affected by the treatment raised questions on the exact role of B cells in the disease, as until then their main contribution was thought to be production of antibodies that mediate demyelination via complement or macrophage-dependent cytotoxicity (Figure 1(c)). In later studies, similar results were obtained with other anti-CD20 mAbs, such as ofatumumab and ocrelizumab [23]. Intriguingly, ocrelizumab is the first therapeutic mAb showing relevant, albeit moderate, clinical effects in PPMS [24].

These obvious successes are contrasted by a long list of treatment candidates where promising effects observed in animal models could not be reproduced in patients; sometimes even detrimental effects were observed [25].

4. Modeling MS in primates

When considering the relevance of laboratory animal models for the preclinical research of MS, an important quote from the statistician George Box should be kept in mind: ‘all models are wrong, but some are useful’ [26]. Indeed, the relevance of an animal model depends entirely on its intended usage. In the vast majority of preclinical MS studies, the mouse EAE model has been used, which is exploited by immunologists also as a generic animal model for studies on autoimmunity and tolerance. It would be inappropriate to downscale the scientific relevance of this model, as it forms the basis of most of our current, albeit still very incomplete, understanding of human autoimmune disease. However, an EAE model in young adolescent (10–12 weeks old) inbred/SPF laboratory mice induced with antigen formulated with a bacterial adjuvant that causes systemic immune activation, has shortcomings that limit its usefulness for drug development. The shortcomings are less apparent in the clinical and pathological similarity of the models with man, than in the pathogenic mechanisms that are thought to lead to disease. Besides species-related differences between the mouse and human immune system [27,28], young adolescent inbred SPF mice lack the effects of aging, genetic variation, and lifelong interaction with endogenous and exogenous micro-organisms on their immune system. Several recent studies document the important influence of these factors on the immunocompetence of the mice [29,30] and their susceptibility to EAE [31].
The EAE model in marmoset monkeys, which first appeared in the literature only 22 years ago [32], provides an exciting new model with high face validity for human MS. In addition, therapies used in the clinic often exert a comparable clinical effect in the model, indicative of a high construct validity.

**Figure 2** shows a representative picture from the archetypical chronic progressive EAE model, which was elicited by the immunization of marmosets with myelin isolated from the brain of an MS patient (obtained via the Netherlands Brain Bank in Amsterdam) formulated with the bacterial adjuvant CFA [33]. Noteworthy is the more widespread inflammatory demyelination in cortical gray matter compared to the white matter. Follow-up studies over the next two decades were dedicated to unraveling of the core pathogenic mechanism and the model validation with clinically relevant immunotherapies. As these explorations have been described in detail in reviews published elsewhere, they will only be briefly summarized here [34–41].

The quantitatively minor CNS myelin constituent myelin oligodendrocyte glycoprotein (MOG) has an immunodominant role in the induction of chronic EAE in marmosets. This was demonstrated in an experiment where bone marrow chimeric marmoset twins were immunized with CNS myelin from a wild type mouse inducing chronic EAE in 5 out of 5, or with CNS myelin from a MOG deficient mouse, inducing acute EAE in 1 out of 5 cases [42]. In a parallel study in Biozzi ABH mice, it was shown that the chronic-relapsing EAE course could be restored by the addition of recombinant MOG extracellular domain (MOGED, residues 1–120) to the MOG-deficient myelin inoculum [43]. We assumed that this finding could be extrapolated to the marmoset and thus tested whether epitopes localized in the MOGED (residues 1–125), which has been expressed as non-glycosylated recombinant protein (rhMOG) in *Escherichia coli* bacteria [44], can elicit immune reactions capable of driving chronic EAE in marmosets.

In marmosets immunized with rhMOG formulated with CFA, we found activation of two pathogenic pathways. One pathway involves the MHC class II/Caja-DRB*W1201 restricted activation of proinflammatory CD4 + T helper cells specific for rhMOG an epitope encompassing residues 24–36. Sensitization against this epitope elicits small perivenular inflammatory lesions. In addition, B cells are involved, which produce antibodies against a conformational epitope at the membrane-distant apical part of homodimeric MOG complexes, inducing demyelination. In an atypical EAE model induced with rhMOG in the mild mineral oil incomplete Freund’s adjuvant (IFA), the synergistic action of cellular and humoral autoimmune factors induced a variety of small and large confluent lesions in the white matter, while demyelination of cortical gray matter was hardly detectable (Dunham et al., in preparation). Of note, this pathogenic mechanism essentially replicates the immunopathogenesis of mouse EAE models.

The second pathogenic mechanism involves the activation of CD3+CD8+CD56+ cytotoxic T cells by CalHV3-infected B cells presenting an epitope encompassing residues 40–48 via MHC class Ib/Caja-E molecules. CalHV3 is a γ1-herpesvirus which just like EBV, its counterpart in humans, exerts a strong influence on the disease, probably via a similar mechanism [40]. In a novel model induced with a peptide encompassing residues 34–56 of rhMOG (MOG34-56) in IFA, these CTL are directly activated and appear to be associated with the demyelination of cortical gray matter via the depletion of oligodendrocytes [41]. This pathway has not been found in any mouse EAE model, but seems particularly relevant for MS [45].

A longitudinal analysis of T2 brain lesion development in the model induced with rhMOG/CFA revealed that long before clinical signs are detectable, dynamic lesion formation disseminated in time and space can be observed [35]. Lesion formation usually starts in the white matter and spreads at a later stage to the cortex. We were unable at that time to visualize with MRI lesions in the subpial region of the brain. However, with PLP staining profound demyelination in different regions of the cortical gray matter can be observed [46].
5. A new concept for therapy development

A widely adhered pathogenic concept for MS, which is for a large part based on the mouse EAE model, implies that immunologically naive autoreactive T and B cells present in the immune repertoire of individuals genetically prone to MS, are activated by exposure to exogenous microorganisms, which express look-alike (mimicry) antigens of self-antigens present in the CNS [47]. Importantly, injection of genetically susceptible mice (e.g. C57BL/6 or Biozzi ABH) with a myelin antigen in the weak adjuvant IFA usually does not elicit autoimmunity but instead enforces immune tolerance against the injected antigen. This is explained by the requirement of danger signals encoded by microorganisms for triggering innate immune mechanisms necessary for the activation of autoimmune reactions by T and B cells [48]. In the EAE model, this danger signal is provided by the mycobacteria emulsified in the adjuvant CFA. This 3-signal dogma of autoreactive T cell activation forms the basis for many new therapies [49,50].

A long list of viruses and bacteria have been proposed as possible trigger of MS, but most evidence points to the common γ1-herpesvirus EBV [51,52]. Although the EBV-MS association is supported by strong evidence, it remains difficult to envisage how the high prevalence of EBV infection in the adolescent human population (>60%) can be reconciled with the much lower incidence of MS (0.1%).

There is a plausible alternative concept, according to which the cause of autoimmunity in MS lies inside the CNS, namely the formation of a primary lesion from which self-antigens are released [37]. Indeed, several studies indicate microglia aggregates a.k.a. microglia nodules in the white matter as the earliest histological manifestation of a lesion [53]. Such microglia nodules are associated with axonal degeneration [54], which is a pathological hallmark of progressive MS. Such observations indicate that the classical ‘outside-in’ concept of MS may need to be reformulated, as the basis of MS may be progressive degenerative pathology and that relapses may be induced by hyper-reaction of the immune system against released self-antigens [37,55]. Conceptually, the immune hyper-reactivity is caused by the interaction of genetic and environmental risk factors, infection in particular [37]. Data obtained in EAE mice, EAE marmosets, and MS patients show that the released self-antigens may be captured by APC in CNS draining cervical and lumbar lymph nodes where immune activation can take place [56,57]. Indeed, surgical removal of these lymph nodes from Biozzi ABH mice impairs the chronic relapsing EAE course induced with myelin/CFA [58].

The essential difference between the two pathogenic concepts is that in the ‘outside-in’ paradigm autoimmunity is triggered by an infection, whereas in the ‘inside-out’ paradigm infection induces a hyper-responsive state of the immune system. This is not a trivial issue, as in the outside-in concept T and B cell activation takes place in peripheral lymphoid organs, which are easily accessed by intravenously-injected therapeutic mAbs. By contrast, in the inside-out concept, T and B cell activation more likely takes place in the lymph nodes that drain fluids from the brain and spinal cord, which are less easily accessed by therapeutic mAbs. This distinction may explain why a mAb against the shared p40 subunit of IL-12 and IL-23 was much more effective when administered around the time of immunization than when treatment was started in late stage disease [59,60].

As will be discussed in following paragraphs, the inside-out concept is strongly supported by data from the marmoset EAE model.

6. A conflict between the R’s in the refined marmoset EAE model

An important aspect in animal model development for preclinical research, nonhuman primates in particular, is compliance with the 3R principles, representing Replacement, Reduction and Refinement [61]. A recent publication provides a comprehensive overview of resources and practical guidelines to implement the 3R principles in animal models of neuroinflammation [62].

The Replacement principle stimulates scientists to use research methods that avoid or replace the use of live animals. Although in vitro models with cells or tissues and in silico models of human pathology are increasingly used in preclinical research, it is generally felt that the interaction of a complex neuro-pathological process in conjunction with other physiological body systems is only displayed in live animals. In that case investigators must choose the lowest animal species from which relevant information can be obtained, such as mice (Mus musculus), zebrafish (Brachydanio rerio), fruit flies (Drosophila melanogaster) or nematode worms (Caenorhabditis elegans). However, although important insights into novel disease mechanisms can be obtained from these models, it is felt that model systems more closely related to the patient are needed for the integration of all information into a coherent pathogenic concept that can be translated into new therapies. These models need to accommodate the emerging modulatory influence of gut microbiota on the disease process [63].

The Reduction principle encourages scientists to use research methods that provide more information from fewer animals. Power calculations are applied to determine the minimal size of experimental and control groups, so that the effect of an experimental variable can be statistically tested. Critical variables in the power calculation are the disease incidence, the anticipated treatment effect and the variation in clinical read-out parameters, such as the time to clinically evident disease or the disease severity. The reduction principle stimulates the usage of inbred/SPF animals where the variable influence of genetic background and environmental factors, which ironically include dominant autoimmune disease risk factors, is precluded. In studies using NHP from outbred and conventionally held colonies, these factors are obviously less well standardized and are an important cause of interindividual variation in disease presentation (see below).

The Refinement principle encourages scientists to use experimental procedures with minimal discomfort to the animals. A major concern with respect to the EAE model is that for reproducible disease induction a strong adjuvant needs to
be used to pepper the immunogenic potency of self-antigens by disrupting the regulatory mechanisms that keep autogressive T and B cells under control [64]. The most frequently used is CFA, which is an emulsion of heat-killed mycobacteria \((M.\text{tuberculosis or butyrium})\) in mineral oil. The mineral oil, which is separately used as IFA, forms a depot for the finely dispersed solution of antigen in aqueous buffer; the mycobacteria provide danger signals needed for ‘awakening’ of the tolerized T and B cells. However, CFA is notorious for its seriously detrimental side effects, in particular the induction of severe ulcerative skin lesions at the injection sites and of a systemic cytokine storm, which are clearly caused by the mycobacteria. Usage of CFA in NHP is therefore discouraged.

Surprising, a highly relevant 4th R, referring to the Relevance of the model for the human disease on which it is projected, is missing. Clearly, the relevance of an animal model for drug development depends on whether the clinical and pathological appearance (face validity) and the pathogenic mechanism (construct validity) of an animal model sufficiently mirror the human disease. The necessity to use CFA for EAE induction in most rodent strains, introduces a mechanistic bias, as immune responses against antigens formulated with CFA are skewed toward a proinflammatory profile dominated by CD4 + T cells [65]. This may not necessarily reflect the immunogenetic predisposition of the individual strain for EAE. The poor translation record of experimental therapies targeting CD4 + T cells from EAE to MS indicates that this cell subset may be less relevant in the human disease than in the animal model [66].

The main aim of our research in the past two decades has been to achieve compliance of the marmoset EAE model with the 3rd R (Refinement) and the here introduced 4th R (Relevance). This was done by stepwise refinement of the EAE model induced with MS myelin in CFA \([37]\). Where possible, the consecutive steps of the refinement process were validated with therapeutic mAbs that worked or did not work in the clinic \([67]\). As discussed above, this strategy led us to the remarkable insight that the complex pathology of RRMS as well as SPMS was replicated in marmosets immunized with the MOG34-56 peptide in IFA. Of note, in this model the only antigenic instruction to the marmoset immune system comes from the MOG34-56 peptide \((\text{GM}E\text{V}G\text{W}Y\text{R}P\text{P}P\text{S}R\text{W}H\text{L}Y\text{R}\text{N}GKD}; \text{with the MOG40-48 epitope of the effector memory (EM)-CTL underlined})\).

As discussed elsewhere, the highly refined MOG34-56/IFA model shares relevant pathological similarity with relapsing-remitting as well as progressive MS \([41]\). So, the compliance with the Relevance criterion worked out well. Moreover, the replacement of CFA, which is notorious for the detrimental side effects, by IFA implied a substantial reduction of discomfort. This means that the model complies also very well with the Refinement criterion. Finally, the possibility to use immunologically comparable twins and the fact that the activation of two dominant EAE pathways is restricted by invariant MHC class II \((\text{Caja-DRB8}^{*}\text{W1201})\) and class I \((\text{Caja-E})\) alleles, implies that meaningful experiments can be set up in a limited number of monkeys. This complies with the Reduction criterion.

In conclusion, this new model implies a considerable improvement at 3 of the 4 R criteria. Nevertheless, unforeseen complications emerged when the model was used in preclinical trials. We will discuss a well-designed and sufficiently powered \((7\text{ twins})\) efficacy study testing a novel mAb against the interleukin-7 receptor \(\alpha\)-chain (CD127) \([68]\). The observation that certain polymorphisms in the gene encoding the interleukin-7 receptor \(\alpha\)-chain (CD127) are associated with enhanced risk to develop MS indicates potential relevance of the mAb as treatment for MS \([69,70]\). Unexpectedly, we observed a dichotomous response of the model to the therapeutic anti-CD127 mAb, as the mAb was effective in 3 twins with short time interval to clinically evident EAE \((<70\text{ days})\) fast responders, while it was ineffective in 3 twins with a late EAE onset \((>105\text{ days};\text{ slow progressors})\). This dichotomous response and the fact that one twin failed to develop clinical EAE killed the statistical evaluation of our well-designed experiment that was powered on a 100% response-to-treatment. In a subsequent study in which the effect of targeted dietary intervention on EAE susceptibility in marmosets was tested, a similar heterogeneous response was detected (Kap et al.; manuscript submitted 2017). At this moment, we have no other explanation for the variation in the response to immunization and in the response to treatment than the genetic diversity of this outbred model. It is important to emphasize here that in the clinical trials of currently registered drugs, such as interferon-\(\beta\) for RRMS, a variable response to treatment had been observed as well \([9]\).

There are at least three ways to deal with the heterogeneity in the response to immunization and in treatment in the outbred marmoset EAE model:

1. To use the much stronger adjuvant CFA instead of IFA. In earlier marmoset EAE studies where CFA was used, a clinical response was always observed in all monkeys \((100\%\text{ incidence})\). However, we find this modification unacceptable as it implies violation of the Refinement criterion.
2. To substantially increase the size of test groups (see Table 1). We also find this unacceptable option as it violates the Reduction criterion.
3. To accept that in certain circumstances statistical evaluation of a study is not possible.

The latter option has been discussed in depth by Bacchetti et al. in several publications \([71-73]\). The authors essentially state that there is a break-even point for group size above
which the projected value of additional study participants (read enlargement of group size) does not outweigh the projected burden of discomfort. The above discussed preclinical test of the anti-CD127 mAb shows that the mAb is clinically effective in 3 out of 3 fast progressor monkeys and ineffective in 3 out of 3 slow progressor monkeys. It is questionable whether a statistically powered study with 40 marmosets per group (see Table 1) would yield a different conclusion. Instead it could be considered to repeat the experiment.

We like to posit here that reviewers of projects or regulatory bodies and editors of scientific journals should do justice to the special nature of NHP models of human disease and free themselves from the traditional sample size dogmas. Heterogeneity in the incidence and course of a disease and the response to treatment is inherent to a model that is exposed to the variable influence of genes and environment. However, this can in part be compensated by using bone marrow chimeric twins, which are immunologically high similar and can therefore be expected to respond more similar to the immunization and an experimental treatment than nonrelated monkeys. Nevertheless, an investment in the clinical relevance and refinement of a NHP disease model, with the aim to enhance the predictive validity for clinical success of a new treatment, is inevitably associated with enhanced sensitivity to variation in genetic and environmental factors.

7. Concluding remarks

EAE in marmoset monkeys is a relevant preclinical model that displays aspects of classical rodent EAE models on the one hand and with MS on the other. It is therefore ideally suited for forward translational research into pathogenic mechanisms and therapy development for the human disease on which it has been projected, i.e. MS. Marmoset EAE has also been a highly useful model for reverse translation analysis of the reasons why certain treatments fail to reproduce promising effects in rodent EAE models, when they were tested in the clinic [67]. However, in the currently used highly refined models a variable influence emerged of genetic and environmental factors on the response to EAE induction and to an experimental treatment.

8. Expert opinion

The current immunomodulatory/immunosuppressive therapies for MS are either safe, but moderately effective (interferon-β or glatiramer acetate) or strongly active, but associated with considerable side effects (fingolimod, alemtuzumab, natalizumab). There clearly is a high unmet need for therapies that have selective activity against pathogenic immune functions, while keeping immune functions needed for protection against infections or tumors intact. We argue that the marmoset EAE model offers a unique system in which such therapies can be developed.

The remarkable pathological similarity between marmoset EAE and MS indicates that disease mechanisms identified in the model are likely relevant for the human disease. The ‘heart’ of the model is formed by the cross talk of CalHIV3-infected B lymphoblastoid cells (BLC) with EM-CTL, which are present in the normal repertoire where they seem to have a role in the control of cytomegalovirus (CMV) latency [39,74]. As shown in the highly refined MOG34-56/IFA marmoset EAE model, the T–B cell interaction elicits complex MS-like pathology in the white as well as the gray matter, which can be abolished by the depletion of B cells with an anti-CD20 mAb [75]. For this reason, we propose that molecules mediating the interaction of BLC with autoaggressive EM-CTL are potential targets of therapy.

We asked which of these two players should preferably be targeted. A score of studies in transplantation shows that systemic T cell depletion in graft recipients may lead to serious clinical problems due to the reactivation of CMV, as seen in immunosuppressed transplant recipients [76] as well as in immunosuppressed NHP used as recipients of allografts [77]. Targeting therapy on the virus-infected B cell seems less perilous [39]. As only 1–50 per 10^6 CD20 + B cells (= <0.005%) carries the virus [78], more than 99.995% of the noninfected B cells in the repertoire is left intact to protect the host against infections and cancer. To gain insight into the pathogenic role of the EBV-infected B cells, we set out a series of experiments in B lymphoblastoid cell (BLC) lines derived from blood mononuclear cells of humans, rhesus monkeys and marmosets by infection with EBV or a suitable EBV-related lymphocryptovirus (LCV).

We assessed the effect of LCV infection on the B cells via comparative RNA sequencing of marmoset B cells infected with human EBV (strain 95–8) and rhesus monkey B cells infected with the related LCV Herpesvirus papio as well as noninfected splenic B cells from both species [79,80]. This analysis revealed multiple alterations in gene expression levels, with a prominent involvement of genes operating in the antigen processing and presentation pathway. In addition, we examined the ex vivo interaction of EBV-infected marmoset B cells with T cells isolated from the lymphoid organs of MS marmosets, which revealed involvement of antigen-dependent and nondependent factors [79]. Among the many dynamic changes, two observations were particularly noteworthy. The production of IL-17A, being the signature cytokine of the EAE model induced with MOG34-56/IFA [81], was the only factor requiring cognate recognition of the immunizing peptide (signal 1). In addition, we observed that incubation with EBV-BLC induced reduced expression of CCR7 only on T cells derived from animals that had developed clinical EAE. Interestingly, an earlier study in marmoset EAE showed that depletion of LCV-BLC with anti-CD20 mAb prohibits the down-regulation of CCR7 on autoreactive T cells, which likely impairs their release from lymphoid organs into the circulation, although this was not formally tested [82].

In summary, these experiments indicate a core pathogenic role of LCV-BLC in the EAE immunopathogenesis as antigen presenting cells (APC). There are some therapeutic options targeting this pathogenic role of LCV-BLC. Physical depletion can be achieved by: (1.) Nonselective depletion of all CD20 + B cells, such as with the anti-CD20 mAb rituximab; (2.) Antiviral drugs, which appear to be moderately effective in MS patients; (3.) Boosting T cell immunity against EBV, which appears to be diminished in MS [83]. Pender et al. published an exciting case study, in which remission of SPMS could be achieved using...
autologous EBV-specific CD8+ cytotoxic T cells [84]. However, such a personalized approach is very labor intensive and may not work equally well in all patients, as is often experienced in dendritic cell vaccination trials for cancer [85].

As an alternative approach, we explored the possibility to intervene in the antigen-presentation process. As a first step toward this ambitious goal, we first wanted to gain more mechanistic insight into the APC function of B cells and examined how the MOG34-56 peptide is handled in noninfected and LCV-infected B cells.

We reported the puzzling observations that in the rhMOG/IFA marmoset EAE model, T and B cell reactivity against all MOG peptides that contain known epitopes could be detected, except against MOG34-56. However, in the MOG34-56/IFA model T and B cell immunity against the same peptide could be detected, indicating that the T and B cells reactive with this peptide are present in the repertoire [81,86]. This indicates that reactivity against the MOG34-56 peptide may be lost during processing of the rhMOG protein, but not during processing of the peptide. Figure 3 summarizes the concept that has been reviewed elsewhere [40].

We analyzed the handling by uninfected and LCV-infected B cells of rhMOG and a truncated version of the MOG34-56 peptide, namely MOG34-51 which lacks the Arg [52] residue. It was observed that rhMOG is degraded in lysates of CD20− and CD20+ spleen cells, while the protein remains largely intact in lysates of LCV-infected primate and human BLC [80]. For the MOG35-51 peptide, citrullination of the Arg [46] residue is essential for the protection of the peptide against degradation by the serine protease cathepsin G via association with autophagosomes, which are induced by the virus [80,87]. This may indicate that the LCV-induced activation of the autophagy pathway in B cells is the Achilles heel of the marmoset EAE model and therefore an attractive target of therapeutic intervention.

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Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.
This hallmark publication shows a dramatic impact of environmental factors on the development of multiple sclerosis. This publication describes the induction of progressive MS through a B cell depletion with rituximab, demonstrating unexpected efficacy of B cell depletion in primary progressive MS.

This publication discusses fundamental immunological differences between mouse and man. It highlights the importance of understanding the role of B cells in multiple sclerosis, with a focus on rituximab as a therapeutic option.

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**This publication shows that autoimmunity the quantitatively minor CNS myelin component MOG is essential for chronic EAE induction.**


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**This review discusses the concept that the cause of MS may not be infection (outside-in), but a degenerative event inside the CNS (inside-out).**


**An authoritative review of the concept that the immune system reacts against antigens recognized in the context of danger, while these are ignored when danger signals are absent.**


**We introduce here the concept that guidelines for the improvement of preclinical animal models can be obtained from investigating the reasons why translation of a scientific concept into an effective therapy failed.**


**This paper critiques the overzealous focus on statistical significance in preclinical studies.**


**In this publication pros and cons on a pathogenic role of cytomegalovirus infection in MS are reviewed.**


**This publication demonstrates that in EBV-infected human individuals only a very small proportion of the B cells contain the virus.**


- This paper presents a case study where secondary progressive MS was effectively suppressed by the elimination of EBV-infected B cells.

