The common marmoset as an indispensable animal model for immunotherapy development in multiple sclerosis

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Abstract

New drugs often fail in the translation from the rodent experimental autoimmune encephalomyelitis (EAE) model to human multiple sclerosis (MS). Here, we present the marmoset EAE model as an indispensable model for translational research into MS. The genetic heterogeneity of this species and lifelong exposure to chronic latent infections and environmental pathogens create a human-like immune system. Unique to this model is the presence of the pathological hallmark of progressive MS, in particular cortical grey matter lesions. Another great possibility of this model is systemic and longitudinal immune profiling, whereas in humans and mice immune profiling is usually performed in a single compartment, i.e. blood or spleen respectively. Overall, the marmoset model provides unique opportunities for systemic drug-effect profiling.

Introduction

Multiple sclerosis (MS) is a chronic autoimmune neurological disease affecting young adults with an average disease onset in the second or third decade of life. Clinical symptoms involve the motor, sensory, visual, and autonomic systems. MS is pathologically characterized by inflammation, demyelination, and degeneration of the central nervous system (CNS) [1].

For exploratory and applied research into MS the use of an animal model is indispensable. The most commonly used animal model for MS is experimental autoimmune encephalomyelitis (EAE), which has been established in rodents and non-human primates (NHP). EAE is induced in genetically susceptible laboratory animals by active immunization with myelin constituents emulsified in an adjuvant, such as complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) [2-4].

The rodent EAE model has produced an enormous amount of knowledge on the pathogenic process, has led to the development of several immunotherapies and is often used for efficacy tests of potential drugs. However, promising effects of new leads are often not reproduced in MS, contributing to the current high attrition rate due to lack of efficacy in phase II clinical trials [5]. One of the major causes for this translation failure is the immunological distance between humans and young, inbred, specific-pathogen free (SPF)-raised laboratory rodents [6]. Therefore, we propose that the use of EAE models in species that are immunologically and genetically more closely related to humans, such as common marmosets, should be considered more often in preclinical research to examine efficacy of potential drugs. In addition, the marmoset offers a unique ability to investigate the mode of action of drugs in higher species in more detail.

Therapy: From mouse to marmoset to man

The availability of effective drugs for MS has tremendously improved during the last two decades. Some of the current therapies were selected based on efficacy in EAE, others were already being used in other human diseases and translated to MS. For example, glatiramer acetate directly emerged from findings in EAE; it was developed as a mimic of myelin basic protein, but instead suppressed EAE [7]. The observation that blocking of the integrin a4b1 integrin (VLA4) inhibited T-cell trafficking across the blood-brain barrier and thereby prevented the development of EAE in mice [8], led to the development of the anti-VLA4 antibody Natalizumab [9]. An example of an already approved drug that was horizontally translated to MS with unexpected success is rituximab, which had been used since the late nineties as a treatment for B cell lymphoma [10]. However, the list of therapies that cured EAE, but failed in MS, is much longer than the list of successes (reviewed in [11]). For example, an antagonist of tumor necrosis factor (TNF) a, which is a highly effective therapy in rheumatoid arthritis, prevented the development of EAE in SJL/J mice [12], but exacerbated disease in MS patients [13].

Although currently approved drugs are effective in reducing the expanded disability status scale (EDSS) and white matter (WM) pathology in some MS patients, there are still many unanswered questions. Why do some patients respond and others not? Why do some patients develop serious adverse effects as progressive multifocal leukoencephalopathy (PML) or thyroid

disease and others not? What are the long-term effects of current treatments on grey matter pathology? What are the long-term effects of depleting all B cells on the structure and function of the lymphoid organs? These aspects should be investigated in MS models that are sensitive to drugs used in patients. Such models have been established in laboratory primate species: the common marmosets (*Callithrix jacchus)*, rhesus macaque (*Macaca mulatta*) or cynomolgus macaque (*Macaca fascicularis*).

EAE in the marmoset as a preclinical model for MS

The common marmoset is a small-bodied New World monkey with an evolutionary distance from humans estimated at 35 million years. Marmosets are used as a relevant model in several biomedical disciplines, e.g. infectious diseases, neuroscience, and drug development [14]. Advantages and disadvantages of the model are summarized in **Table 1.**

Table 1

Advantages and disadvantages of the marmoset experimental autoimmune encephalomyelitis (EAE) model
Advantages
• Therapeutic experiments require small amounts of test compound because the marmoset is relatively small compared with larger macaques
• The outbred nature of the marmoset reflects human genetic heterogeneity
• Marmosets are born as bone marrow chimeric twins caused by the shared placental bloodstream making fraternal siblings immunologically more similar than siblings from different births. This principle can be used in two-legged therapy trials where one sibling is treated with the experimental compound and the other sibling with placebo
• EAE is induced at adult age when the immune system is fully matured
• Human-specific biological therapeutics often cross-react with the target in marmosets
• The conventional housing implies free exposure to immune-shaping pathogens from the 'milieu exterieur' (environment, gut flora) and the 'milieu interieur' [e.g. latent infection with herpes viruses homologous to cytomegalovirus (CMV) and Epstein–Barr virus (EBV)]
• Systemic and longitudinal immune profiling is possible in the marmoset
• Pathology, including grey matter lesions and iron changes, resembles that of multiple sclerosis (MS)
• The marmoset provides a unique opportunity to study the effect of human skin-derived induced pluripotent stem cells
Disadvantages
• Ethical limitations: when the same information can be obtained in lower species marmosets cannot be used. The number of animals can sometimes be too low to reach statistical significance
• High costs not only of the monkeys themselves but also of the housing and care
• Cross-reactivity of diagnostic reagents, such as FACS antibodies, is limited
• Amount of blood that can be withdrawn is limited, although new techniques enable obtaining relevant data with small blood samples

EAE in the common marmoset can be induced by recombinant human myelin oligodendrocyte glycoprotein (MOG) or a MOG peptide covering residues 34 to 56 (MOG34-56) emulsified in CFA [15]. Refinement of the model led to a clinically and pathologically similar disease when CFA was replaced by IFA, a much milder adjuvant that lacks the mycobacteria [3,4]. The marmoset EAE model has been validated by several immunotherapies or used for reverse translational research (**Box 1**).

Box 1: Forward and reverse translation in marmoset EAE.

- **Anti-IL12p40 mAb:** Blocking of IL-12p40 before immunization with myelin/CFA completely protected against EAE in marmosets. Blocking of IL-12p40 once T2-weighed lesions were present after immunization with rhMOG/CFA delayed the clinical course. Unexpectedly, the same treatment (ustekinumab) lacked clinical efficacy in RRMS [46].
- **Anti-CD20 mAb:** CD20+ B-cell depletion by rituximab, ofatumumab and ocrelizumab reduced the annualized relapse rate, EDSS, and white matter lesions in RRMS [23]. In addition, there are some indications that ocrelizumab can be used for PPMS. Similar results were obtained in the marmoset EAE model: an anti-CD20 mAb, a clonal variant of ofatumumab, prevented the development of clinical symptoms as well as white and grey matter lesions [46].
- **Anti-BLyS/Anti-APRIL mAb:** Atacicept, a fusion protein that blocks the function of BLyS and APRIL, failed in a phase II trial for MS. A reverse translation study was performed in the marmoset in which B cells were depleted by anti-BLyS and anti-APRIL mAbs, but the effect on the clinical scores was minimal. A possible explanation for this effect lies in the depletion of the γ-herpesvirus CalHV3, as described elsewhere in this paper and reviewed in [47].
- **IFNγ**: Administration of interferon (IFN)-γ, the signature and immune-active cytokine of Th1 cells, exacerbated disease activity in RRMS, while treatment with anti-IFNγ antibody had only minor clinical effects. In the MOG34-56/IFA marmoset EAE model, early or late treatment with IFN had no effect on the disease course [48]. Interestingly, Th1-associated humoral and cellular autoimmune parameters were affected, which points to a key role of Th17 cells in this model.

A recently developed EAE model in the marmosets is induced by immunization with a subclinical dose of recombinant rat MOG in IFA followed by stereotactic injection of TNF-α and IFN-γ into the cortex and the corpus callosum at day 70. An advantage of this model compared to models without stereotactic injection is that lesions develop within a predictable time frame (three weeks in 86% of the animals). In addition, confluent lesions develop at the injection sites reminiscent of human MS lesions. A disadvantage is that some marmosets developed small, perivascular foci of demyelination rather than large confluent demyelinating lesions, which makes it difficult to interpret the effect of a new therapy on lesion development [16].

Heterogeneous response to treatment due to outbred nature

Why is it important to test new compounds in higher species than only rodents? Mouse models are 100% inbred, which in essence resembles testing the efficacy of a compound in one MS patient. When the compound does not have an effect in mice, it may still be effective in a subgroup of MS patients, and if a compound cures 100% of the mice from EAE, it may be effective in only a subgroup of MS patients. Evidence for the existence of MS subgroups emerged from the observation that approximately 30% of MS patients did not respond to treatment with IFN-β [17]. It was found that in clinical responders to IFN-β treatment the disease had a Th1-skewed cytokine profile, whereas in non-responders to IFN-β treatment the disease had a Th17 dominated cytokine profile [18]. In contrast to inbred mice, which within one strain all have a similar disease course and response to treatments, outbred marmosets have a heterogeneous disease course and can respond differently to a treatment reflecting their outbred nature. We recently reported that blocking the IL-7 receptor (IL-7R; CD127) was effective only in fast disease progressors, which occurred in three of six marmoset twins; the treated sibling developed clinical symptoms almost 100 days later than the placebo-treated sibling. However, in the three late responder twins, blockade of IL-7R had no effect, suggesting that blocking IL-7 signaling may be a good strategy for a subpopulation of MS patients [19].

A challenge when testing a treatment that lacks a 100% effect is the statistical power analysis and the closely linked ethical constraints. A powered study design for a drug with, for example, a clinical effect in 50% of the animals would lead to group sizes that are ethically unacceptable. When an experiment has been performed in small groups and the clinical outcome is not significantly different, the clinical relevance and biological consequences of a compound should be appreciated as well. It may very well be that a drug has an effect on a subpopulation of MS patients as described above, or that a compound has not significantly changed the clinical outcome, but does induce biological changes, such as a cytokine shift [4]. The ability to assess a magnitude of parameters (i.e. multi-compartment immune profiling, body weight and CNS pathology) and the use of bone marrow chimeric twins provides a controlled system for wide profiling of a drug and thus conclusions should not be based on a single parameter, such as development of clinical symptoms. We propose that scientific journals and pharmaceutical companies should more appreciate such types of studies with small groups of animals, as they can give valuable information about the possible effect in MS patients [20].

Secondary lymphoid organs are required to investigate the mode of action

The mode of action of a compound in MS patients is routinely investigated in blood as lymphoid organs or samples from the target organ are usually not available. However, measurements in blood often inadequately reflect systemic effects on the immune system. A lymphocyte is only transiently present in blood when it circulates from lymph node to lymph node. Lymphocyte generation occurs in primary lymphoid organs (thymus, bone marrow) and the activation, differentiation, and proliferation occurs in secondary lymphoid organs (SLO;

spleen and lymph nodes), while effector functions are exerted within the target organ. SLO are needed to get a complete picture of a treatment effect (e.g. level of B-cell depletion) and the mode of action, but also of the cause of adverse effects.

In marmosets, a sufficient amount of venous blood can be longitudinally collected for analysis of immune parameters during the *in vivo* phase of an EAE experiment; such information can be collected from patients, but not from mouse EAE models. At the end of the experiment, primary and secondary lymphoid organs can be collected for in depth analysis of the activation, differentiation, proliferation and migration of lymphocytes; such information can be collected from mouse EAE models, but not from patients. We have observed in several experiments, that disease parameters measured only in blood insufficiently represent treatment effects and mode of action of a drug. Proliferation and cytokine production of blood mononuclear cells stimulated with myelin antigens used for disease induction are usually low, whereas in spleen and lymph nodes these responses are much higher indicating that the pathogenic cells reside in SLO. In addition, treatment of marmosets with a monoclonal antibody against CD20 led to a 100% reduction of $CD20^+$ B cells in blood, whereas in spleen some $CD20^+$ B cells were still present [21]. In the same studies, we observed that autoreactive T cells were depleted from blood as they were retained in the SLO. Such examples show that results obtained in blood cannot always be extrapolated to SLO.

SLO have been used for in depth investigation of the mode of action of a treatment. B-cell depletion is very effective in reducing clinical symptoms as well as pathology in MS. However, the exact role of B cells is still unknown. The availability of SLO from marmosets treated with an anti-CD20 mAb [21] enabled us to show that B-cell depletion altered the homing of T cells to secondary lymphoid organs and changed the phenotype of T cells within the SLO; these effects were not found in blood [22]. Our current knowledge of the mode of action of B cell-depleting antibodies could never have been gained when only human blood had been analyzed.

In depth knowledge of the working mechanism of already approved broadly immunosuppressive drugs for MS, such as alemtuzumab (lemtrada; Campath-1H), can be useful in the development of more refined treatments with less side effects. Alemtuzumab is a humanized IgG1 mAb against human CD52, which is highly expressed on all T and B cells. In humans, alemtuzumab has received worldwide clinical approval for the treatment of chronic lymphocytic leukemia, and more recently for refractory MS [23]. The profound therapeutic effect of alemtuzumab in RRMS is based on the depletion of all circulating $CD52^+$ T and B cells. However, it has been observed that in a substantial proportion of treated patients (± 30%) rebound autoimmune disease develops, most often of the thyroid gland. This percentage is much higher than the incidence of autoimmune thyroiditis in the human population [23]. The mechanisms underlying this adverse effect are unknown and could very well be investigated in NHP. It has been observed that a few weeks after administration of alemtuzumab to MS patients, repopulation of lymphocytes occurs in the circulation, leading to a rebalance of the immune system. The number of B cells returns to pre-treatment baseline levels three months after infusion of alemtuzumab, whereas the repopulation of T cells in the periphery required 9-12 months. However, the different repopulation kinetics did not correlate with MS disease activity. Some research has been performed in cynomolgus monkeys [24], but a more in-depth analyses of the SLO remains to be performed.

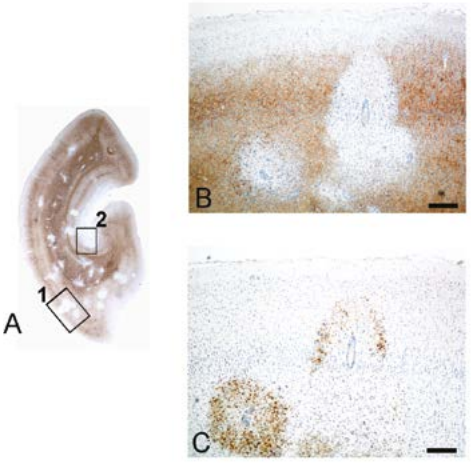

Figure 1. Grey matter lesions in the marmoset EAE model. A shows an overview of a proteolipid protein (PLP) staining of the cortex in which multiple demyelinated areas can be recognized. The rectangle indicated by 1 is shown at higher magnification in **B** (bar = 200µm) and depicts two intracortical lesions. The same intracortical lesions were stained for myeloid-related protein (MRP)14 (**C**). This staining shows an active lesion (left lesion) with many (recently infiltrated) macrophages and an inactive lesion (right lesion) with macrophages only at the rim of the lesion. A subpial lesion is indicated by the rectangle 2 in **A**. Adapted from [36].

Grey matter lesions as a marker for disease progression

The modeling of MS pathology in the marmoset benefits from the similar architecture and anatomy of the marmoset and human CNS. The neuroanatomy and the ratio between WM and GM in the marmoset brain are comparable to humans [25]. Multiple sclerosis was viewed as a mainly white matter (WM) disease for a long time, but since about a decade it has become clear that lesions are also present in the grey matter (GM) and that these play an important role in clinical worsening of patients with progressive MS [26]. In MS, four types of cortical grey matter lesions can be discerned. The most common lesions are subpial (type III), which are extensive and involve several neighboring gyri. Less common lesion types are intracortical and often small and perivascular (type II), large and cortex-spanning (type IV), or leukocortical (type I), which span both the WM and GM [27].

A major advantage of the marmoset EAE model compared to mouse models is the presence of GM lesions that display the characteristic features of MS pathology, including redistribution of iron and mitochondrial degeneration [28]. However, while lesion development in the white matter can be well visualized and characterized on conventional magnetic resonance (MR) images [29-31], this is more problematic for lesions developing in the cortical GM. Three types of GM lesions have been found in marmoset EAE, which are comparable with GM lesions observed in MS [32-35]. Leukocortical lesions accounted for 57% of the total number of cortical lesions found in the marmoset. Intracortical lesions (**Figure 1B-C**) were found in two of the six marmoset brains examined, whereas subpial lesions (**Figure 1A**) accounted for 88% of the total demyelinated cortical area. Activated macrophages and microglia were found in leukocortical and intracortical lesions, but the density was lower than in WM lesions, as has also been described for MS. Subpial lesions expressed only marginal signs of inflammation [32]. Furthermore, the cortical thickness was reduced in marmosets with EAE compared to controls, but no differences were observed

between demyelinated and myelinated areas [34]. Intracortical, but not subpial, lesions displayed Ig leakage and complement deposition [33].

Since GM lesions have a strong impact in disease deterioration, it is expected that therapies targeting mechanisms underlying GM lesion formation have a high impact on MS. The marmoset EAE model can be used to select compounds capable of reducing GM lesion load. Although the role of the immune system in the generation of GM lesions is still unclear, the model predicted that B-cell depletion may prevent the development of these lesions [36]. In line with our prediction, the recently opened trial of the anti-CD20 mAb ocrelizumab showed encouraging clinical benefit in primary progressive MS (presented at the ECTRIMS 2015; http://www.roche.com/investors/updates/inv-update-2015-09-28.htm).

The cause of GM lesions can be investigated in the marmoset EAE model with the final aim to develop a therapy against progressive MS. As not all marmosets develop GM lesions, comparison of animals with and without GM lesions with transcriptomic or genomic techniques can provide valuable information. Unraveling of the underlying mechanism can not only help with the identification of therapy targets, but also provide leads for the development of an animal model for progressive MS in which GM lesion dominate.

The MS-like pathology of the marmoset EAE model is also relevant for studies aiming at repair by, for example, stem cells. We reported previously on the marked clinical effect of human neural stem cells, which were administered via the intravenous route [37]. However, regeneration of damaged CNS tissue was not detectable. A more recently tested approach used oligodendrocyte precursor cells (OPC) that were differentiated from human fibroblast derived-induced pluripotent stem cells (iPS) and injected into the corpus callosum of marmosets with EAE. We observed that the iPS-derived OPC migrated towards the lesions where the contact with demyelinated axons seemed to induce differentiation and production of new myelin [38]. Future studies are warranted to investigate whether repair by iPS-derived OPC can be improved.

A potentially important physiological hallmark of marmosets is the similar metabolism of iron as in humans, which contrasts with rodents [39]. In humans, oligodendrocytes in the aging brain accumulate iron, which is liberated by demyelination of MS lesions [40]. Iron can amplify oxidative stress, which is also seen in MS brains. Similar to MS, iron accumulation and liberation has been observed in the marmoset EAE model [28], but not in rodent EAE [41]. This makes the marmoset a relevant model for the development of therapies that target iron and oxidative stress; which is particularly relevant to progressive forms of disease.

EBV-infected B cells play a crucial role in disease mechanism

Infection with Epstein-Barr virus (EBV), a human herpesvirus, is suggested to increase the risk for MS, albeit with contradictory results (reviewed in [42]). Symptomatic infection with EBV (infectious mononucleosis) later in life increases the risk for MS compared to non-symptomatic infection early in life [43]. All MS patients have serum immunoglobulin (Ig) G antibodies (Ab) against EBV, whereas anti-EBV IgG levels are detected in 80-90% of healthy individuals, and in MS patients the anti-EBV Ab titers are higher than in controls. Anti-EBV IgG levels in cerebrospinal fluid (CSF) did not differ between MS patients and patients with non-MS inflammatory CNS

disease and EBV RNA could not be detected in the CSF of MS patients. It has therefore been suggested that EBV-infected B cells migrate to the target organ where they become a source of autoantibodies and provide co-stimulatory signals to auto-aggressive T cells. Indeed, EBV-positive B cells were found in the meninges of MS brains, but others could not confirm this [42].

To investigate the role of a human pathogen in MS it is essential that the animal in which the disease is modeled is susceptible to infection with that pathogen or closely related counterparts. Non-human primates (NHP) are naturally infected with viruses that are closely related to their human counterpart. Thus, in contrast to SPF-bred rodents, conventionally held colonies of NHP have experienced the immune shaping effect of environmental pathogens throughout their development and may therefore be a good representation of the situation in humans.

Marmosets are naturally infected with CalHV3, a γ-herpesvirus that shares many similarities to human EBV [44]. The CalHV3 load in SLO was profoundly reduced in animals treated with the anti-CD20 mAb compared to non-treated animals, but not when only B-cell growth factors were targeted [45]. Interestingly, this coincided with the clinical result: anti-CD20 mAb therapy reduces the clinical score and pathology of marmosets with EAE, whereas mAb targeting the B-cell growth and differentiation factors BlyS/BAFF or APRIL were only partially effective. This observation suggests that B cells infected with the EBV-related CalHV3 play a crucial role in the disease. This advantage of marmosets can be of use in future therapy development. Especially for therapies developed for selective depletion EBV-infected B cells, the efficacy can very well be analyzed in the marmoset EAE model.

Concluding remarks

Although the availability of effective drugs for MS has improved during the last two decades, a valid preclinical animal model is still needed. The model should be relevant for tests of the efficacy, the mode of action, or the long-term effects. We propose that the EAE model in the common marmoset provides a useful model for preclinical studies because of its genetic heterogeneity, the lifelong exposure to chronic latent infections and new environmental pathogens, the systemic immune profiling, and the presence of pathological hallmarks of progressive MS, grey matter lesions in particular.

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