Translational multiple sclerosis research in primates
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Chapter 4

General Discussion
Graphical abstract of this thesis

Figure 1. Graphical abstract of this thesis. The development of clinically evident EAE involves both pathogenic mechanisms in the periphery and inside CNS. In the peripheral compartment, auto-reactive T cells are activated with injected antigens presented by APCs in lymphoid organs, such as the lymphocryptovirus infected B cell. During this process, T cells receive 3 signals: I. antigen presentation, II. co-stimulation and III. signaling from cytokines (i.e. IL-7) that direct the fate of the T cell response. In marmoset EAE, the activated auto-reactive T cells cross the BBB and enter the CNS. In this model, injury to the WM and GM of the CNS is variable, and involves activation of resident myeloid cells (microglia) and infiltrated myeloid cells (macrophages), which exert cytopathic effects via the production of reactive oxygen species (ROS) and cytokines. Oxidative injury caused by ROS contributes to axonal degeneration, and has deleterious consequences for neuronal mitochondria.
Preface

MS is a chronic demyelinating disorder of the CNS that is debilitating for patients and represents a major health concern for western societies. Key elements driving neurodegeneration and CNS injury include inflammatory demyelination, microglia activation, oxidative stress and neuronal mitochondrial defects. The animal model for MS, i.e. EAE, has contributed to the understanding of autoimmune mechanisms operating in the disease and the development of therapeutics. While rodent EAE is an appropriate model to answer mechanistic research questions in some situations, the predictive value of rodent models in relation to therapeutic intervention for disease seems to be limited. Primate models offer a unique opportunity to translate principles discovered in lower species (rodents) to a situation that with respect to pathological presentation more closely resembles the human disease. The marmoset EAE model, in particular, replicates many key features of MS and is therefore an attractive model for translational research into pathogenic mechanisms for the human disease.

Leading up to the start of this thesis, several observations and controversies shaped the research direction taken. These include:

- The emergence of a possible role of the IL-7/IL-7R pathway in MS, and a controversy as to whether IL-7 contributes to the development of Th1 or Th17 responses.
- A lack of clarity on if or how EBV could contribute to the development of autoimmunity and notably MS.
- A new understanding of the importance of cortical pathology in MS.
- The recognition that the oxidative stress pathway is only partly reflected in rodent EAE, yet is a key component of MS.
- Growing evidence that mitochondrial defects play a key role in MS disease progression.

Therefore, the aims of this thesis were i) to utilize the marmoset EAE model for gaining a better understanding of pathogenic mechanisms involved in MS and ii) to characterize NHP models to determine how closely they replicate key pathological features of MS. Understanding how similar, or dissimilar, a model is with respect to key features of MS is paramount in the model selection process for basic (i.e. exploratory research) and applied (i.e. therapeutic testing) research.

While a tremendous amount of work had been done over the last 20 years on refining the marmoset EAE model with respect to the adjuvant and encephalitic antigens, refinement of the marmoset EAE model had largely taken place prior to the emergence of the importance of GM pathology. At the start of the current project (this thesis), a comparison between immunization methods with regard to the ability to elicit WM and GM demyelination had yet to occur. Furthermore, key pathological features such as iron accumulation in the NHP brain, or the ability of the NHP EAE models to elicit the oxidative stress pathway and mitochondrial alterations had also not yet been investigated. The key findings of this thesis are summarized in Box 1. These findings, and their contribution to the field, will be discussed in the subsequent sections of this chapter.
Box 1. Key findings of this thesis.

- Blockade of CD127 delays EAE in fast progressor marmosets, but exerts no detectable effect in slow progressor cases.
- The communication process between the LCV-infected B cell and T cell is aberrant compared to non-infected B cells; key markers of activation, Th17 responses and homing are altered.
- Iron accumulates in myelin and oligodendrocytes in the marmoset and rhesus monkey brain.
- Both marmoset and rhesus EAE exhibit MS-like oxidative stress and injury in the brain.
- The extent of mitochondrial injuries observed in MS is incompletely replicated in EAE.
- The extent of GM injury in marmoset EAE is highly variable, yet increased in monkeys immunized with antigen-CFA formulation.

Figure 2. Research strategy. The non-human primate is used for both exploratory and applied research, which are connected in an iterative process. Scientific concepts developed in the exploratory arm are validated in the applied arm with therapies that work or do not work in the clinic. For therapies that failed to reproduce promising effects obtained in the animal model (forward translation), the reason of failure is examined by retesting the treatment in the relevant animal model (reverse translation). This iterative approach led to the discovery that lymphocryptovirus-infected B cells have a central pathogenic role in disease progression. Understanding how well a model replicates key features of human disease is fundamental in determining the translatability of the data derived from this exploratory and applied research.
Peripheral immunopathogenic mechanisms

Therapeutically targeting the IL-7/IL-7R pathway

The IL-7/IL-7R pathway has been the focus of research attention in MS for several reasons. Polymorphisms of the IL7Rα gene are among the more prominent non-HLA- genetic factors to confer risk for MS development. T cells derived from MS patients exhibit enhanced IL-7R expression and IL-7 level is significantly elevated in the CSF of patients. Constitutive secretion of IL-7 by EBV-infected B lymphoblastoid cell lines has also been demonstrated. Therefore, in chapter 2.1 we assessed the role of the IL-7/IL-7R pathway in the most refined version of the marmoset EAE model, namely elicited with MOG34-56/IFA.

One surprising finding in chapter 2.1 was the relatively limited impact of IL-7R (CD127) blockade on T-cell subset distribution and responses. Treatment therapeutically or prophylactically with anti-IL-7Rα mAb has been shown to mitigate EAE severity in a mouse model induced with MOG/CFA by depleting both naïve and effector memory CD4 and CD8 T-cell subsets and altering Th1 responses; something not observed in our study. Despite reports on a (in vitro) role of IL-7 in expansion of MBP-specific T cells in MS patients, in vivo treatment with the anti-IL-7Rα mAb had no detectable effect on ex-vivo MOG34-56-stimulated marmoset T cells.

At the start of the thesis research there was considerable controversy regarding the contribution of IL-7 to the development of Th1 or Th17 responses. An early report, which is now retracted, demonstrated a role of IL-7 in the survival and expansion of mouse Th17 subset. Others failed to replicate these findings, yet demonstrated a role of IL-7 in the development of pathogenic Th1 cells as both murine and human naïve T cells differentiated into Th1 cells when stimulated with IL-7. Blockade of IL-7R in murine EAE also mitigated disease severity at the expense of Th1 responses. In our study, IL-7R blockade had negligible impact on either IFNγ or IL-17A production, regardless of EAE progression, further suggesting that IL-7 has minimal impact on the development and expansion of Th1 or Th17 responses.

We observed that therapeutic intervention in the IL-7/IL-7R pathway with an anti-CD127 antibody was only successful in a subgroup of monkeys whose placebo-treated twin sibling developed EAE in a rapid manner; a phenomenon associated with alterations in the CD20+CD40+B-cell subpopulation. Although these results did not replicate the homogenous response rates observed in mouse EAE, our data would predict heterogeneous responses for the human population. One final caveat to this data is that IL-7 promotes cytotoxicity of the CD8+ T cell in the MS patient, something not seen in rodent models or healthy cohorts; hence the MS T cell may be unique with respect to the IL-7/IL-7R signaling pathway. Thus, although the CD8 cytotoxic T cell appears to play a role in the marmoset EAE model, one might expect responses in the MS population to be higher than we observed.

EBV in autoimmunity

Delineating a mechanistic role for EBV infection with the development of autoimmunity and MS has been difficult. Early reports identifying EBV in the brain of MS patients were met with great enthusiasm, yet could not be replicated by other groups and remain controversial. Despite this, strong correlation between infectious mononucleosis (IM) and MS, and viral-specific Ab titers with relapses suggest an association of EBV with disease development. Hence, a role for
EBV in MS warrants further investigation. A focus of this thesis was to utilize the marmoset EAE model to explore this possible association mechanistically. Prior to the start of the research for this thesis it was demonstrated that the marmoset EBV equivalent, CalHV3, played a role in EAE progression via altering the secondary lymphoid organ milieu. In chapter 2.2, we therefore examined how EBV infection influences communication between B cells and T cells from MOG34-56/IFA-immunized marmosets. These results demonstrated that infection with EBV alters key receptors and molecules of the B cell that are involved in antigen presentation and co-stimulation of the T cell. We demonstrated that the communication process is aberrant between the LCV-infected B cell and T cell compared to non-infected B cells; phenotypically altering expression of critical T-cell markers related to homing, Th17 responses, development and function.

The absence of EBV+ B-cells in target-specific tissue, albeit still controversial, added to the temporal association of EBV with disease activity would suggest that the EBV-disease association either results from dysregulated immune responses to EBV related to disease activity (i.e. IL-10 secretion) or that infection plays a role in disease progression via immunomodulatory effects in the periphery or directly in the CNS in the earliest phase of disease. Recently, Pender and colleagues demonstrated that in MS, CD8+ T cells are defective in their ability to control EBV infection, which they attributed to an exhaustion of the EBV-specific CD8+ T cell repertoire. An intriguing single case study showed that revitalization of the repertoire via vaccination against EBV induced disease remission in a secondary progressive MS patient. Although a direct role in the CNS in the earliest phase of the disease cannot be entirely excluded, EBV has never been assessed in the CIS brain. The data presented in chapter 2.2 indicate that key pathways involved in autoimmunity are dysregulated in the cross talk-between LCV-infected B cells and T cells. Concomitantly to our data, colleagues from our research group demonstrated the ability of the LCV-infected B cell in cross-presenting auto-antigen that would be degraded in non-infected B cells. As demonstrated in the marmoset EAE model, B cell depletion altered the milieu within the secondary lymphoid tissues; T cells in B cell depleted SLO maintained high CCR7 expression and were retained in the lymph nodes. We could show that co-culture with B-LCL altered expression of the homing marker CCR7 in T cells from monkeys that developed clinically evident EAE. As will be discussed in the open question section, it remains unanswered if EBV is a causative or mere correlative factor for MS. The results in this thesis, along with evidence from others, would indicate that if EBV is a causative factor of MS, a possible role in disease progression might be linked to irregular functions of infected B cells as an APC.
Into the CNS

The oxidative injury pathway

From a pathogenic point of view, both MS and EAE disease activity can be viewed as compartmentalized in peripheral and target tissue compartments. For EAE pathogenesis, autoreactive T cells are induced by peripheral immunization with myelin antigen resulting in tissue injury of the CNS. While no unique antigen has been identified in MS, there is an apparent nexus between the immune system and damage that occurs in the CNS. The fundamental basis that EAE models are judged by is their ability to replicate key pathological features observed in the MS brain. It is increasingly clear that a model that reflects key aspects of the oxidative injury pathway will be vital for development of therapeutics. However, this feature is only partially reflected in current rodent EAE models.

In chapters 3.1 and 3.3 we assessed aspects of the oxidative tissue injury pathway in primate EAE models, with a focus on marmoset EAE, as this model most closely reproduces MS pathology. We demonstrated that iron accumulation in myelin and oligodendrocytes in the NAWM and NAGM occurs in both the rhesus monkey and marmoset brain at the earliest ages of monkeys used for EAE experiments, i.e. 4 and 1.5 years respectively. This represents a clear divergence from rodent species used for EAE modeling (rats, mice) and may explain the presence of oxidative stress and injury to the CNS observed in the primate models.

The MS brain is characterized by the presence of respiratory deficient cells that have lost functionality of complex IV (COX-1) due to deletions of mtDNA and/or point base mutations leading to loss of individual subunit proteins. Furthermore, mitochondrial axonal content is increased in the active lesion and differs based upon lesion, myelination and phosphorylation status. The inability of the marmoset model to induce deletions of mitochondrial DNA or respiratory deficiency, yet induce loss of individual subunits in the mitochondrial respiratory chain, indicates that severe mitochondrial injury may require a degree of chronicity that is not likely to be feasible in animal models. The level of oxidative stress exceeds endogenous anti-oxidant capabilities in the MS patient. Testing therapeutics directed at this pathway in models where oxidative stress does not exceed endogenous anti-oxidant activity likely has minimal predictive value for clinical efficacy, underscoring the value of NHP models in this area of research.

Cortical pathology

The importance of cortical pathology in the MS brain with respect to disease progression and clinical symptoms is becoming increasingly clear. This critical feature of disease pathology is frequently absent in mouse EAE models, yet distinctly present in strains of Lewis rats and in marmoset EAE models. In chapter 3.2 we performed a comparative analysis of the ability to elicit GM pathology with the different EAE induction methods in marmosets. We demonstrated that previous estimates of cortical pathology development were much lower than actually observed numbers. This is likely due to reliance on MRI diagnosis. We observed that the amount of demyelination varied between immunization protocols. Although the use of IFA in EAE induction is more animal welfare friendly, it clearly elicited less cortical demyelination than was observed in EAE models based on CFA. Interestingly, all marmoset EAE models exhibited the...
same predilection sites with respect to development of subpial demyelination. Like MS, low flow areas such as deep sulci were frequently affected \(^{22,50,51}\). Furthermore, in marmosets immunized with MOG34-56 emulsified in either CFA or IFA, antibodies directed against conformational MOG were not observed, unlike in rhMOG-immunized animals, confirming previous results \(^{30}\). Whereas histologically we could observe signs of IgG and complement deposition in the early active lesions in the WM or in leukocortical lesions, identification of early active cortical lesions by MOG staining is challenging and clear uptake of IgG and C9neo by macrophages in WM and leukocortical lesions was not easily discernable. It is unclear if this staining pattern represents pathogenic antibodies binding other myelin antigens or if this is a non-specific pattern as described by Prineas and colleagues \(^{52}\). Future studies are warranted to eliminate the possibility of a pathogenic antibody directed against myelin constituents other than MOG.

**Outstanding questions**

**Is EBV a causative or correlative factor of MS?**

One of the most challenging questions that remains to be answered is if EBV is a causative factor for MS, and thus actively contributing to disease progression, or if the correlation is a non-relevant bystander effect (guilt-by-association) \(^{18}\). Proposed roles of EBV in disease progression, including those in this thesis, remain largely speculative and are based on *ex vivo* and *in vitro* cell cultures.

A causative role of EBV for disease initiation or progression would considerably change therapeutic strategies in early stages of disease as immune modulation could be directed against virus-infected cells \(^{53}\). Therapies that compromise the immune system, and have negative side effects, would not be necessary; patients that develop IM could be prophylactically treated, thereby avoiding the development of MS. Yet, significant challenges exist. Once latency is established, anti-viral therapies are ineffective in EBV-related lymphoproliferative disorders and therefore might also not be successful in MS \(^{54}\). Depletion of CD20+ B cells would eliminate the

**Figure 3. Mitochondrial alterations in marmoset EAE.** The axonal mitochondrial (mt) content was assessed in relation to the phosphorylation status of neurofilament, as a marker of inflammation-related pathology \(^{48}\). A-B shows staining of normally phosphorylated (blue; SMI31+) or EAE-related non-phosphorylated (blue; SMI32+) neurofilament with cytochrome C oxidase subunit 1 (red; COX-1) and porin, a voltage dependent anion channel (VDAC) expressed in the outer membrane of all mitochondria (green). Shown in C-D is that while the number of mitochondria did not differ between SMI31+ and SMI32+ axons (injured), the percentage of axonal area occupied by mitochondria was significantly increased in SMI32+ axons. To determine the activity of mitochondria (E-G), we examined the dynamics between COX-1, a mtDNA encoded member of the COX catalytic core, and porin. In relation to SMI31+ axons, SMI32+ axons exhibited significantly lower levels of inactive (= COX-1 negative) mitochondria (absolute deficiency; E), and significantly higher levels of active (= COX-1 positive) mitochondria (F). The percentage of mitochondria deficient axons or mitochondria lacking COX-1 immunoreactivity, was significantly lower in SMI32+ axons in comparison to SMI31+ axons (G). Using COX histochemistry combined with fluorescent labeling, we observed a partial loss of COX activity in astrocytes and neurons (H-M), but not in macrophages (data not shown). Cells that had partial activity loss were observed in EAE lesions, absent in healthy controls, and frequently exhibited a loss of individual complex IV subunits COX-1 (L-M). Non-lesion associated loss of COX-1 was observed in purkinje cells (data not shown).
reservoir for EBV, yet a specific role for EBV and MS cannot be conferred from this treatment as large populations of B cells and minor populations of T cells are eliminated. Furthermore, mAb may have difficulties passing the BBB depending on the integrity of the barrier; thus to entirely eliminate the EBV+ B cell pool the therapeutic must be delivered intrathecally. Recently a case report was published showing that adoptive immunotherapy whereby autologous EBV-specific CD8+ T cells were expanded in vitro and transferred back to a SPMS patient led to the induction of remission. Although the patient benefited from the transfer, a larger sample size is needed to be able to draw information as to how successful this therapy will be. The role of iron in EAE and MS: amplification or remyelination? Iron is an essential element required for normal brain health as a contributing co-factor for mitochondrial respiratory complexes, oligodendrocyte precursor cell proliferation and the (re)myelination process. On the other hand, unbound iron can contribute to oxidative tissue injury by the generation of hydroxyl radicals from hydrogen peroxide via the Haber-Weiss reaction. Iron metabolism is dysregulated in the MS brain and the destruction of myelin and oligodendrocytes is associated with altered expression of iron metabolic markers and perilesional accumulation of iron in myelin, oligodendrocytes and microglia. Recently, it has been demonstrated that lesions forming iron rims are more likely to expand than those not forming iron rims. This finding, however, is correlative and since iron rim lesions were larger at the start of the observation period one cannot deduce if iron itself is attributed to the expansion of the lesion. In rodent EAE, iron deficiency attenuated symptoms of disease. These effects though were associated with immune suppression, and importantly iron overloading did not exacerbate EAE. Unfortunately, clinical trials of iron chelation have been limited in size and number, predominantly based on PPMS or SPMS patients and yielded mixed results. As shown in Chapter 3.1 and 3.3 NHP EAE models offer attractive systems for preclinical testing of iron chelation therapies. Whereas the NHP brain exhibits profound iron accumulation, this is limited in the rodent brain at ages used for experimental modeling. The marmoset model is particularly well suited for this research as twin animals can be used for studies and unlike humans, these monkeys are fed standardized diets; the possible effect of dietary iron intake and variation in basal brain deposition levels of humans could confound studies using too few patients. Is inhibiting oxidative stress a feasible strategy to alter disease activity? Enhancement of anti-oxidant activity, and cessation of oxidative stress mechanisms has been proposed as an attractive therapeutic strategy to delay the conversion from RRMS to SPMS. Recent clinical success of Nrf2 stimulation with dimethyl-fumerate (DMF) indicates that targeting the oxidative stress pathway may yield a favorable outcome. The clinical effect of DMF cannot be entirely attributed to stimulation of the Nrf2 pathway, as innate and adaptive immune response modulation by DMF has been observed independent of Nrf2 stimulation. In MOG-induced rodent EAE models, targeting the NADPH complex function has a significant impact on disease and thus there is clear evidence to support such a strategy. Alleviating oxidative stress and injury could reduce or delay the development of defective mitochondria, spare axons from insult and degeneration, and prevent energy diversion from the remyelination process. Importantly, these strategies are not without risk. For instance, therapeutically targeting NOX2
would likely target other tissues as well and may increase the patient risk to infection. Hence, the ideal effective therapy would be CNS specific. As reduction of stress can also have deleterious consequences for mitochondria and as ROS also play a key role in angiogenesis, oxygen sensing, cell proliferation and signaling, it is vital that this underlying mechanism is well understood; potential therapeutics need therefore to be tested in models that reflect human-like levels of oxidative stress. As presented in chapter 3.1 and chapter 3.3, the NHP EAE models are attractive models for testing therapeutics and underlying mechanisms involving oxidative stress.

Can marmoset EAE be modified to induce 100% incidence of GM pathology?

One of the prominent features of marmoset EAE is the ability of monkeys to elicit GM pathology. As presented in chapter 3.2 the incidence of GM pathology varies per immunization protocol. In our own unpublished data, we have noticed that some therapeutic interventions, regardless of outcome of treatment, result in alterations of GM lesions. For instance, monkeys treated with an antibody against the B cell growth and differentiation factor “a proliferation-inducing ligand” (APRIL) developed GM lesions at a 100% incidence rate, yet the treatment had little effect on disease outcome (own unpublished observations). This suggests that the EAE induction method can be altered in a way that would favor the development of cortical pathology.

Cortical demyelination in the Lewis rat can only be observed in the LEW.1AR1 (RT1$^{r2}$) and LEW.1W(RT1$^{u}$) strains that contain RT1$^{B^D}$ MHC-II and RT1$^{A^u}$ MHC-I alleles. In these strains, extensive cortical demyelination develops and does so in a reproducible fashion. The MHC allelic variation has yet to be assessed for the marmoset in relation to development of cortical pathology; however it should be noted that marmoset MHC equivalent (Caja) alleles are fairly conserved. As oxidative stress in the marmoset model was not confined to the immunization method but rather dependent upon demyelinating activity, effort should be placed in refining the model to develop higher incidences of cortical pathology.

Future perspectives

Arguably, few phrases better embodies the state of MS research activities than that attributed to French Neo-Platonist philosopher Bernard of Chartes: “Standing on the shoulder of giants”. Through success and failures, knowledge of pathogenic mechanisms has been greatly expanded and now therapeutic regimens can modify disease course. Yet still, the cause of MS is unknown and current therapeutics only variably modify disease; development of effective therapeutics for progressive forms of disease is critical and refinement of treatment regimens for RRMS is greatly needed.

Despite this pressing need for therapeutic refinement and development, optimism is warranted. Efforts to develop tissue banks across Europe and the US has granted researchers access to tissues that would have previously been difficult to obtain. Technological advances such as single cell technologies, multiplexing and high throughput technologies allow screening not previously possible. Animal models will undoubtedly play a role in research efforts. As shown in this thesis, the NHP models replicate many key features of disease and will be valuable models in these efforts.
References


