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Dissociation of the Protection from Experimental Autoimmune Encephalomyelitis and the Allergic Side Reactions in Tolerization with the Neuroantigen MP4

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List of abbreviations

ADEM	acute disseminated encephalomyelitis
ADP	adenosine diphosphate
BSA	bovine serum albumin
CD	cluster of differentiation
CFA	complete Freund's adjuvant
CNS	central nervous system
CPM	counts per minute
drLN	draining lymph node
EAE	experimental allergic/autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
Fig.	figure
GFAP	glial fibrillary acidic protein
GM-CSF	granulocyte monocyte colony stimulating factor
GvHD	graft versus host disease
HEL	hen egg lysozyme
IFA	incomplete Freund's adjuvant
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kD	kilo-Dalton
MAG	myelin associated glycoprotein
MBP	myelin basic protein
MHC	major histocompatibility complex
MOBP	myelin oligodendrocyte basic protein
MOG	myelin oligodendrocyte protein
MP4	MBP-PLP fusion protein
MS	multiple sclerosis
OD	optical density
PAF	platelet-activating factor
PBS	phosphate buffered saline
PBST	PBS containing 0.025% Tween 20

PLP	proteolipid protein
PLPp	peptide 139-151 of proteolipid protein
PTX	pertussis toxin (toxin produced by virulent strains of Bordetella pertussis)
s.c.	subcutaneous
SEM	standard error of the mean
sol	soluble
STAT	signal transducer and activator of transcription
TGF	transforming growth factor
Th cells	T helper cells
Thpp cells	T helper primed precursor cells
TLR	toll-like receptor
TNF	tumor necrosis factor
Tr1 cells	T regulatory 1 cells
vs.	versus

1 Introduction

1.1 Experimental allergic/autoimmune encephalomyelitis (EAE)

1.1.1 History and significance of EAE

Experimental autoimmune or, as it was originally named, allergic encephalomyelitis is an inflammatory, demyelinating disease that causes acute, relapsing-remitting, or chronic-progressive paralysis. It is characterized by perivascular inflammatory lesions in the white matter of the central nervous system (CNS) and has become one of the most important animal models for human inflammatory demyelinating diseases of the CNS and autoimmune diseases in general.

The roots of EAE can be traced back to efforts to develop a vaccine for the rabies virus in the late 19th century: soon after Pasteur had introduced the vaccine consisting of fixed rabies virus grown in rabbit CNS [111], it was noted that several patients receiving it developed paralysis and other neurological dysfunction. The analysis of more than a million cases of patients treated with the vaccine later showed that about one out of two thousand patients were affected by such neurological complications [121]. Histopathological examinations demonstrated perivascular infiltrates of mononuclear cells and focal areas of demyelination within the CNS. This picture is very different from the brain of patients that were infected with and died of rabies [9].

At the beginning of the 20th century, Remlinger hypothesized that the development of this disease was due to brain components in the vaccine, and not to the virus itself [120]. His theory was supported when an acute form of disseminated encephalomyelitis was successfully induced in rabbits by repeated injections of normal brain tissue. Its clinical and histological features were similar to those observed in individuals experiencing postvaccinal paralytic attacks [60,147]. Glanzmann proposed an “allergic” basis for this pathologic reaction [48], hence the disease became known as experimental *allergic* encephalomyelitis.

Some years later, it was shown that EAE could be induced in nonhuman primates by multiple intramuscular injections with homogenates or concentrated alcohol-ether extracts of rabbit brain [124,125]. But only after Freund developed a new technique of adding various adjuvants like paraffin oil and heat-killed tubercle bacilli to the antigens [37,39], it was

possible to induce the disease with a considerably reduced number of injections and a high reproducibility [40,64,71]. In 1949, Olitsky succeeded in eliciting EAE in mice and thus established murine EAE as a model for demyelinating autoimmune diseases [109].

Within a short time period, many investigators began to study EAE, with a major focus on the identification of the specific CNS tissue components which are encephalitogenic (see 1.1.2). Kabat in 1947 suggested that EAE may have an autoimmune etiology [64]. In the 1980s, when more was learned about cellular immunology and the dichotomy of T helper cells type 1 and 2 (Th1/Th2 dichotomy, see 1.2), this hypothesis was further strengthened, and EAE became known as experimental *autoimmune* encephalomyelitis.

Today, EAE is one of the best-studied organ-specific experimental autoimmune diseases, used to gain very general as well as very detailed knowledge about a broad variety of immunologic and inflammatory mechanisms. More specifically, EAE also serves as an animal model for human inflammatory demyelinating diseases of the CNS like acute disseminated encephalomyelitis (ADEM) and multiple sclerosis (MS) [46,144].

This animal model has shown remarkable success, although results of studies in the different EAE models cannot always be transferred directly to human diseases. They rather have to be checked critically with actual findings in patients [80]. Over the years, basic research utilizing the EAE model led to the development of three out of six medications approved for use in MS (glatiramer acetate, mitoxantrone, and natalizumab). Several new approaches tested in clinical trials are based on positive indications in preclinical work relying on the EAE model. And finally, new clues to the pathogenesis of MS as well as new potential surrogate markers for this disease can be concluded from research on EAE [144]. Thus, the significance of this animal model for research on MS as well as autoimmunity in general can hardly be overestimated.

1.1.2 Induction of EAE

Nowadays, EAE can be induced in a wide variety of species, including mice, rats, guinea pigs, rabbits, and non-human primates. In principle, there are two ways to induce the disease: actively, by immunization with CNS antigens in adjuvants, often with additional use of pertussis toxin, or passively, by the transfer of in-vitro cultured, CNS-specific activated T cells, usually type 1 T helper cells (see 1.2.2). Apart from that, spontaneous EAE can occur in unmanipulated animals of certain strains due to their genetic background, especially in animals transgenic for a myelin-specific T cell receptor [78].

As the model used for this project is based upon an immunization protocol, this chapter will be confined to the respective way of disease induction. Some basic information will be given about the variety of antigens that can be used, the adjuvants facilitating the immunization, the necessity of pertussis toxin as a supplement, and, last but not least, EAE susceptible mouse strains. This information is crucial since it provides the frame for the experimental work described herein.

Autoantigens

Over the years, autoantigen preparations used for EAE induction ranged from whole CNS homogenates and myelin preparations over purified proteins to peptides derived thereof. Kabat already presumed that the active encephalitogenic substrate was a myelin component, because white matter was shown to be more encephalitogenic than grey matter, and neonatal tissue, which contains little or no myelin, was inactive [64].

Further studies in the following decades revealed that the most abundant proteins within myelin are myelin basic protein (MBP) and proteolipid protein (PLP). The rest of the protein fraction of myelin consists of more recently discovered molecules. Examples are myelin oligodendrocyte glycoprotein (MOG), which is oligodendrocyte-specific and preferentially incorporated into the outermost surface of the myelin sheath, myelin oligodendrocyte basic protein (MOBP), which is abundantly expressed in CNS myelin, myelin associated glycoprotein (MAG) found in the periaxonal space as an adhesion molecule, glial fibrillary acidic protein (GFAP), and a protein named S100β [46].

MBP is a hydrophilic, highly charged protein and therefore relatively easy to isolate, purify, and study. It is found intracellularly in oligodendroglial cells, constituting about 30% of total myelin protein. Due to differential splicing of seven exons from a single gene, the protein occurs in different molecular forms, although it is highly conserved among most species. MBP has long been shown to be encephalitogenic [29,70]. However, the respective epitopes vary among certain species and even within different strains of a given species [41]. For instance, the amino acid sequence MBP:1-11 is an immunodominant epitope in the B10.PL mouse strain, while MBP:89-100 is immunodominant in SJL mice.

PLP, on the other hand, is a hydrophobic membrane protein which contains both positively and negatively charged regions and which spans the oligodendrocyte membrane four times (see Fig. 1A). It is found in a number of tissues, most abundantly in CNS myelin. While representing approximately 50% of total myelin protein, it was nevertheless un-

known until 1951, when Folch and Lees coined the term to describe a group of substances within myelin that are soluble in chloroform-methanol and insoluble in water or salt solutions [34]. Soon after, first hints on its encephalitogenic characteristics were found [108]. Because of its chemical characteristics, however, methods allowing better purification of the protein were only developed in the early 1980s. Final evidence that PLP itself is encephalitogenic and that the disease induction seen before was not due to trace amounts of MBP in the protein preparation could be supplied exclusively by using synthetic peptides without homology to MBP [152,153]. Since then, numerous additional encephalitogenic epitopes of PLP have been identified in various strains of mice [31,154] and other experimental animals. So far, all of them are located within the more hydrophilic regions of PLP that are proposed to be extramembranous (hydrophilic domains I to III in Fig. 1A and 1B).

Thus, MBP and PLP traditionally are the most important antigens in EAE models. In order to comprise within a single molecule all the epitopes of both proteins which are primarily immunodominant or to which the immune reaction might spread, a recombinant chimeric fusion protein was generated and named MP4 (Fig. 1C). It encompasses the 21.5 kD isoform of MBP (MBP21.5) and a genetically engineered form of PLP (ΔPLP4), the latter encoding the three hydrophilic domains of PLP [30]. Though primarily intended for the induction of tolerance (see 1.2.3), this fusion protein can also be used to induce EAE, as was remarked in a side note for SJL mice [30] and marmoset monkeys [63]. Because of its broad spectrum of possible T cell determinants, this model seems more realistic of human disease than peptide-specific EAE. It was chosen to be used for the experiments of this thesis (see 4.1 for more detailed information on MP4 and the discussion of its usage).

Adjuvants

The use of adjuvants for immunization purposes has a long history. In 1899, Grassberger found that the cellular reaction to mycobacteria was enhanced by the addition of butter and even more by paraffin oil [50]. Their mode of action, however, is still not completely clear, even more than a hundred years later. Freund proposed that the effect of paraffin oil is to bring a relatively large amount of antigen into contact with phagocytic mononuclear cells. Additionally, the creation of numerous foci, remote from the site of injection, namely in the lymph nodes and lungs, might be important as they may act as sources of antigenic stimuli [38]. Another important effect is to locate the antigen in its initial depot, resulting in a slow release with a half-life of about 90 days [55].

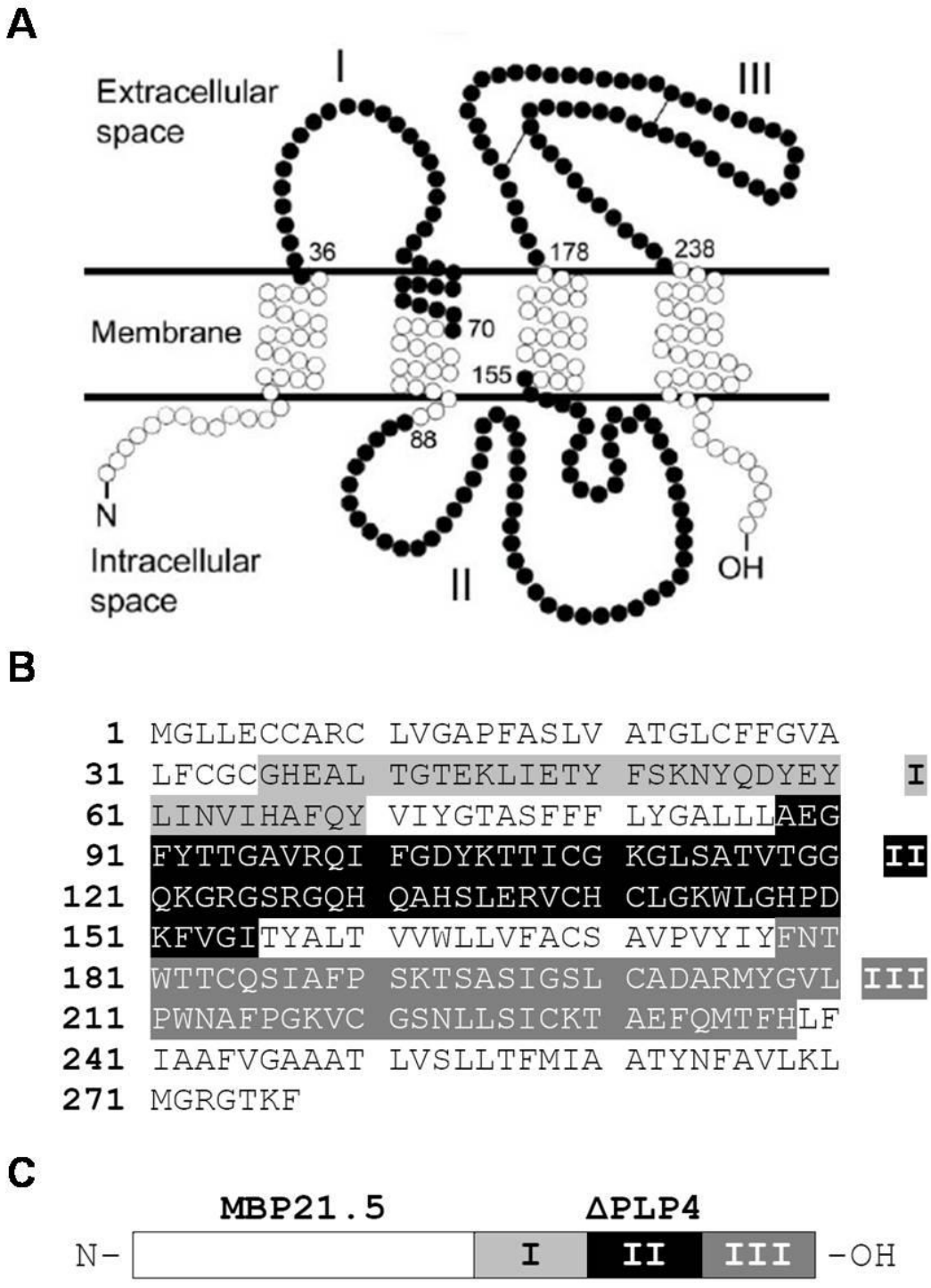

FIGURE 1 Molecular structure of the MBP-PLP fusion protein MP4

(A) Structure of PLP (proteolipid protein). PLP is a transmembrane protein that consists of two extracellular (I and III) and one intracellular (II) hydrophilic domains, and four hydrophobic transmembrane sequences. (B) Amino acid sequence of PLP. The three hydrophilic sequences used for constructing ΔPLP4 are highlighted. (C) Structure of MP4. The three hydrophilic PLP domains have been fused to create ΔPLP4, which has been linked to the 21.5 kD isoform of human MBP (myelin basic protein).

Killed mycobacteria tuberculosis are a very potent adjuvant [88]. Their activity could be due to the recognition of defined microbial components by phylogenetically ancient receptors present on accessory cells. Paraffin oil together with killed mycobacteria tuberculosis is called complete Freund's adjuvant (CFA) [39]. After its discovery, this adjuvant soon became popular for rapid induction of EAE and other experimental autoimmune diseases [64]. Later, when the Th1/Th2 dichotomy (see 1.2) was discovered and studied, it was shown that CFA induces a type 1 response and the respective cytokines, while incomplete Freund's adjuvant (IFA, paraffin oil alone, without killed mycobacteria tuberculosis) induces type 2 cytokines and generally promotes the development of tolerance [164].

Pertussis toxin

Pertussis toxin is the major protein produced by virulent strains of Bordetella pertussis. It belongs to the A-B structure class of bacterial toxins, its B-subunit binding to a receptor on the cell surface, and the enzymatically active A-subunit disrupting intracellular signaling by irreversible adenosine diphosphate (ADP) ribosylation of the G_i-subclass of G proteins [18,161]. It has multifaceted effects that include induction of lymphocytosis, stimulation of insulin secretion, enhancement of vascular permeability, inhibition of lymphocyte recirculation, mitogenic effects on T and B cells, enhancement of IgE production, and sensitization to histamine [42].

Pertussis toxin has long been used in the enhancement of experimental autoimmune diseases elicited by the immunization of animals with the appropriate tissue antigens [82]. Most models actually depend on this supplement. Still, the way in which this effect is caused has not yet been fully understood, and different hypotheses are being discussed.

First, the toxin might disrupt the blood brain barrier, which subsequently facilitates the infiltration of inflammatory cells into the target organ [90,91]. This hypothesis, however, is contradicted by findings that pertussis toxin is most effective when given during the early phase of the development of T cells, and not during effector cell migration to the CNS. Besides, the addition of pertussis toxin cannot be replaced by an aseptic cerebral injury that opens the blood brain barrier directly [57].

Second, pertussis toxin has type 1 adjuvant effects, boosting a pathogenic T cell response [2,141]. Finally, the toxin might be necessary to license the antigen presenting cells for recognition by the effector T cells, meaning that the toxin provides an additional activation of the cells via stimulation of toll-like receptor (TLR) 9 [25,155].

Susceptible mouse strains

Among mice, the strains that have been most commonly employed in studies of EAE are SJL mice that express the MHC alleles H-2^s and B10.PL or PL/J mice expressing H-2^u MHC alleles. On the contrary, most common mouse strains are resistant to MBP-induced disease. This includes C57BL/6 mice (H-2^b), which constitute the most widely used inbred strain and have become very important for mechanistic studies because a wide array of genetic modifications is available on this background [11,43]. However, there is a MOG peptide-based EAE model for that mouse strain [98], and only recently, after the work done for this thesis, a MP4-based model for C57BL/6 mice was introduced [75].

In SJL mice, which were used for this project, PLP is the predominant autoantigen, although T cell responses to MBP are also generated. Male SJL mice are resistant to actively induced EAE, while females readily develop EAE [24]. Susceptible animals commonly exhibit a relapsing-remitting course of the disease. This could be due to the spreading of T cell responses from PLP to MBP epitopes, with each wave of new T cells causing another relapse. The synthetic peptide identical to PLP residues 139-151 was the first shown to be strongly encephalitogenic in SJL mice [152], and this model has been extensively used ever since. For the experiments of this project that require peptide-induced EAE to compare the allergic side effects, this model was chosen as well (see 3.5).

1.1.3 Treatment of EAE

The inhibition and treatment of EAE has long drawn broad attention because of its model character for other autoimmune diseases. During decades of extensive study, various approaches have targeted multiple steps in the pathogenesis of EAE. For instance, it has been tried to diminish the cytokine activity by using antibodies directed against pro-inflammatory cytokines, soluble cytokine receptors, or inhibitors of specific cytokine activity [132,140]. Alternatively, prevention of EAE by blocking antigen presentation has been studied using antibodies to MHC class II molecules [143], antibodies specific for antigen-presenting molecules pulsed with encephalitogenic epitopes [3], or, more recently, altered peptide ligands [16,66,134], the latter also having tolerogenic potential. The T cell function, finally, can be altered very generally by immunosuppressants, by antibodies against CD4 (cluster of differentiation 4) [156], or, more specifically, by antibodies against V genes expressed on encephalitogenic T cells [166].

Among many possible ways of therapy, the induction of specific tolerance to self-antigens has played an outstanding role in the treatment of autoimmune diseases. The presence of self-reactive lymphocytes in the blood of healthy individuals [19] implies that self-antigen-specific regulatory mechanisms are physiological and usually prevent pathological autoimmunity. Therefore, the prime goal of immunotherapy is to elicit a protective form of immunity that is targeted and free of the systemic side effects of conventional immunosuppressive agents. However, there are several mechanisms of antigen-specific tolerance, namely apoptosis, anergy, regulatory T cells and immune deviation, the inter-relation of which has not yet been completely understood (reviewed in [53], see also chapter 4.5).

It has been found that the administration of antigen in the absence of TLR stimulation does not lead to EAE, but rather protects mice from the subsequent induction of the autoimmune disease [61]. Classical protocols for this kind of disease prevention include mucosal administration of antigen via oral [13,56] or naso-respiratory routes [8,99], systemic administration (especially intravenous injection of soluble antigen) [20,100], and injections of the antigen with IFA as an adjuvant [4,119,148].

Besides other potential effects, these treatments all result in a T cell response of type 2/3 cytokine signature and a boosting of the antibody response [23,127]. This effect is called immune deviation, because an originally unpolarized immune response is shifted towards a type 2 response (see 1.2.1).

To better understand this kind of treatment and its potentially dangerous side effects, the next chapter of this introduction will be devoted to the Th1/Th2 dichotomy, putting a special focus on its role in autoimmune diseases and their therapy. Immune deviation as one of the mechanisms involved in the treatment of autoimmune diseases is further discussed in chapter 4.5.

1.2 Th1/Th2 dichotomy

1.2.1 Differentiation of T helper cells

In 1986, it was reported that $CD4^+$ T cell clones could be classified, based upon the cytokines they secrete following activation, into two groups that were named Th1 and Th2 [102]. Th1 cells mainly produce IL-2, IFN-γ, and TNF-β, to some degree TNF-α and GM-CSF, support macrophage activation, trigger delayed-type hypersensitivity, and direct im-

munoglobulin class switching towards IgG2a. Th2 cells, on the contrary, mainly secrete IL-4, IL-5, and IL-6, to some degree IL-10 and IL-13, provide efficient help for B-cell activation, induce eosinophil differentiation and activation, trigger type I hypersensitivity reactions, and direct immunoglobulin class switching towards IgG1 and IgE [103,128]. Both T cell subsets are regulated by different transcription factors, Th1 among others by signal transducer and activator of transcription-4 (STAT-4), Th2 by STAT-6 [149].

Subsequent studies established that there are far more than just those two subsets of $CD4^+$ cells: T cells called Th3 were shown to produce TGF-β and to regulate immunoglobulin class switching towards IgA [87]. They are induced by oral antigen administration and are considered one subset of regulatory T cells, exerting their suppressive activity via TGF-β [159].

Another subset of regulatory $CD4^+$ T cells called T regulatory 1 (Tr1) cells secrete large amounts of IL-10, low amounts of IL-4, and TGF-β in amounts similar to those secreted by Th1 and Th2 cells. These cells were shown to prevent the onset of colitis in an adoptive transfer model [51].

Th0 cells are characterized by production of cytokines of both the Th1 and Th2 type, and are thought to be obligatory precursors of Th1 and Th2 cells [1].

Yang et al. stated that there are already primed ($CD44^{high}$) $CD4^+$ T cells that produce IL-2, but neither IFN-γ nor IL-4, home selectively to lymph nodes, and retain the ability to differentiate into either Th1 or Th2 cells. These cells are called T helper primed precursor (Thpp) cells [162,163].

Quite recently, another distinct subset of Th cells, named Th17 for their production of IL-17, has been suggested to play a major role in the pathogenesis of autoimmune diseases including EAE [79]. The differentiation of naïve precursors towards this phenotype is induced by IL-6 and TGF-β, and further expansion is dependent from IL-23 produced by dendritic cells [12].

Importantly, naïve $CD4^+$ T cells are neither of Th1 nor Th2 type. When they are first stimulated by antigen and antigen presenting cells, they adopt the phenotype of Th0 or Thpp, secreting low amounts of cytokines not specific for either Th1 or Th2. Both subsets of T helper cells originate from these originally uncommitted cells [126,133]. Polarization occurs within the first 48 hours after the stimulation, but only upon restimulation does the cytokine secretion pattern become evident [105].

The differentiation of naïve T cells towards either the Th1 or the Th2 pathway is influenced by many factors [129]: one of the most important of these is the cytokine milieu in which T cell differentiation occurs, itself being strongly influenced by the innate immune system. IL-12 produced by dendritic cells, for instance after interaction of their TLR with mycobacteria (see CFA, 1.1.2), activates STAT-4 and therewith promotes the development of Th1 cells [59], while IL-4 induces the differentiation of naïve T cells towards type 2 T helper cells [81].

Notably, Th1 and Th2 mutually antagonize each other's development by blocking the generation of the other cell type. Thus, IL-12, IL-18, IFN-γ, and IFN-α not only favor the development of Th1 cells, but also inhibit the development of Th2 cells, while IL-4 inhibits the development of Th1 cells and shifts the immune response towards type 2. This leads to the domination of one side or the other, an effect that was named immune deviation, after the term originally coined by Asherson and Stone [6].

While a fraction of Th1 and Th2 cells can still be reverted directly after priming, the cytokine profiles of established Th clones after three or more restimulations are irreversible [104]. This commitment might be of great importance for the development and prevention of autoimmune diseases. The next chapter will roughly outline this connection between T helper cell differentiation and autoimmune diseases.

1.2.2 Th1/Th2 dichotomy in autoimmunity

Direct evidence that EAE is cell-mediated was provided by the demonstration in the rat model that the disease could be adoptively transferred by immune cells, but not by serum [112]. The fact that neonatal thymectomy prevents EAE suggested a critical role for thymus-derived lymphocytes [5]. With the availability of T-cell subset markers, it became clear that the T helper cell population mediates EAE [10,116].

In parallel, early evidence favoring the pathogenicity of Th1 cells came from histopathological studies in the CNS of animals with EAE, showing delayed-type hypersensitivity reactions similar to the ones known to be caused by Th1, but not by Th2 cells [142]. Early evidence for a role of cytokine phenotype in the pathogenesis of autoimmune disease was derived from studies of mercuric chloride-induced lupus erythematodes and chronic graft versus host disease (GvHD), both of which are characterized by elevated levels of IgE [49]. In the following years, many studies showed that Th1 cells play a major role as the effector population in organ-specific autoimmune diseases as well as acute GvHD, while

Th2 cells fail to transfer these diseases. Instead, quite often they even protect from their development [24,36,85,136].

By now, there are extensive reviews concerning the topic of different T helper cell subsets in autoimmunity [22,76,89,96]. In principle, there is consensus among investigators that Th1 cells cause organ-specific autoimmune diseases like EAE, while Th2 cells have a protective effect, a notion called Th1/Th2 paradigm. However, things turned out to be more complicated than expected (see 1.2.4), and more recently discovered T cell subsets are assigned an increasingly important role. Still, the question of whether or not Th2 cells can be employed to prevent or cure Th1-induced diseases has generated a lot of interest.

1.2.3 Immunotherapy by Th2 cells

Immunotherapy of organ-specific autoimmune diseases on the basis of the Th1/Th2 dichotomy has been approached in two different ways: either directly by transfer of Th2 cells, or indirectly by *in vivo* alteration of the Th1/Th2 balance. Many studies in the 1990s showed that transferred Th2 cells are rather ineffective in controlling ongoing Th1 responses, but effectively prevent the development of new responses towards the Th1 pathway. Consistent with this finding, protective effects of Th2 cells have been observed when they were transferred to recipient mice in which EAE was induced with antigen and adjuvant on the same day or up to two weeks later [21,73].

Closer to the methods applied in this project, numerous experiments have been performed with the aim to prevent autoimmune diseases by altering the Th1/Th2 balance in vivo, partly showing impressive protection against disease induction by means of Th1 cells. Approaches taken there include the injection of cytokines, e.g. IL-4 [117,135] or IL-10 [131], or of antibodies directed against cytokines responsible for T cell differentiation [94], the immunization with altered peptide ligands, with soluble antigens, and with antigen in IFA. The latter, on which the experiments of this project are based, have repeatedly been shown to induce type 2 T cell and strong antibody responses and to protect from the development of autoimmune diseases [35,54,84,150,164]. In general, antigenic stimulation of T cells without a costimulatory TLR signal shifts the response towards Th2 [61].

Several studies, however, provide evidence that the Th2 subset can have pathogenic effects in autoimmune diseases under certain circumstances. This result raises strong concerns about the therapeutic value of immune deviation.

1.2.4 Pathogenic type 2 autoimmunity

In contrast to the general notion that type 1 cytokines are involved in disease progression, whereas type 2 cytokines promote disease recovery and protection, evidence is also amounting that challenges the Th1/Th2 paradigm in EAE and other autoimmune diseases. Not only was it shown that Th2 cells might be inefficient suppressors of EAE induced by Th1 cells [69], but they can actually have pathogenic effects. In a marmoset model of EAE, immune deviation therapy induced a lethal demyelinating disorder [45]. Oral administration of autoantigen in mice, applied to shift the immune response towards type 2, induced a cytotoxic T lymphocyte response leading to the onset of autoimmune diabetes [14]. MBP-specific Th2 cells were found to cause EAE in immunodeficient hosts rather than to protect them from the disease [77], and Th2 cells caused an intense and generalized pancreatitis and insulitis [110].

Moreover, various other cells and molecules associated with a type 2 response are apparently involved in the development of autoimmune diseases. Mast cells are essential for early onset and high severity of EAE, suggesting an amplificatory role for these cells in the T cell-mediated immune pathology [15,26,138]; eosinophils were shown to play a critical role in the pathogenesis of EAE [47]. Multiple other elements of allergic immune responses have also been found to be involved in the development of EAE: histamine receptors type 1 and 2 are present on inflammatory cells in brain lesions, and EAE severity is decreased in mice genetically deficient in the receptors FcγRIII and FcεRI [113].

Finally, the induction of type 2 immunity is inherently linked to the appearance of antibodies against the autoantigen administered for accomplishing immune deviation. These antibodies can cause severe anaphylactic reactions in mice as well as in humans and thus provide a challenge for this kind of therapy (see 1.3.3).

Therefore, the application of immune deviation therapy seems to be a double-edged sword. The undeniable protective effects may in certain situations be outweighed by the risks of pathogenic type 2 immunity including anaphylactic reactions. Thus, prevention of allergic side reactions is of considerable importance for the use of immune-modulating therapies.

1.3 Anaphylaxis

1.3.1 History and definition of anaphylaxis

In 1902, Richet and Portier described the experimental induction of hypersensitivity in dogs. In order to confer sting prophylaxis, these animals were immunized with venom from sea anemones. Unexpectedly, they were sensitized to the venom and upon reapplication showed fatal reactions to previously non-lethal doses of the venom. To describe this phenomenon, the authors proposed the term “anaphylaxis”, which was derived from the Greek words “ana” (against) and “phylaxis” (immunity, protection) [122]. In 1913, Richet was awarded the Nobel Prize in Physiology or Medicine for the work on this topic.

Almost a century later, there is still no universally accepted clinical definition for this phenomenon. Some clinicians define it broadly as a syndrome of one or more systemic signs and symptoms without precise specification of these features or their degree [27]. Others have classified anaphylaxis on the basis of its severity [44,123] or demand either dyspnea or hypotension to make the diagnosis [145].

Nevertheless, most clinicians agree that anaphylaxis is an acute life-threatening, allergic reaction in which a physiological process that normally acts in a local and limited manner to protect against infection occurs massively and systematically. According to that opinion, it consists of some or all of the following signs and symptoms: diffuse erythema, pruritus, urticaria and/or angioedema, bronchospasm, laryngeal edema, hyperperistalsis, hypotension, and cardiac arrhythmias. Other signs and symptoms, such as nausea and vomiting, headache, lightheadedness, feeling of impending doom, and unconsciousness, can also occur. The signs and symptoms of anaphylaxis are often produced within 5 to 30 minutes, but sometimes reactions do not develop for several hours [68].

Some authors reserve the term “anaphylaxis” for IgE-dependent events and use the term “anaphylactoid” reactions to describe IgE-independent reactions that are clinically indistinguishable (see 1.3.2). As this possible differentiation is not important for the questions of this project, the term “anaphylaxis” will be used for reactions based on both pathways.

1.3.2 Pathophysiology of anaphylaxis

Anaphylaxis in mice can be induced by two distinct mechanisms. The classical one is also associated with human allergy: antigen cross-linking of antigen-specific IgE bound to mast cell FcεRI stimulates mast cell degranulation, which leads to the rapid release of histamine and serotonin and is followed by the synthesis and secretion of platelet-activating factor (PAF) and leukotrienes [28]. These mediators act on target cells to increase vascular permeability, which causes hemoconcentration and depletes intravascular volume [146]. This pathway is dependent on IL-4, IL-4 receptor, mast cells, FcεRI, and IgE.

A second pathway of murine anaphylaxis has been demonstrated in most studies in which mice, similarly to the respective experiments presented in 3.5, are primed by immunization with an antigen and challenged several weeks later with the same antigen [101,106,146]. This pathway has been shown to be dependent on IgG, macrophages, FcγRIII, and PAF, but independent of IgE, mast cells, FcεRI, and complement.

Responses following both pathways are indistinguishable in their time course, severity, target organ involvement, and challenge dose required. The determinants that influence which of the both mechanisms predominates appear to be the quantity of antigen-specific IgG antibody that is produced and the quantity of antigen used to challenge the immunized mice [146].

1.3.3 Anaphylaxis as risk for immune deviation therapy

As previously mentioned in chapter 1.2.1, B-cell activation, IgG1 and IgE antibody production, and type 1 hypersensitivity reactions are intrinsic parts of type 2 immunity and immune deviation therapy [164]. Such antibodies can cause severe anaphylactic reactions in mice and humans. This risk was outweighing potential benefits in several experimental studies [92,114,115], and has even brought a large clinical trial to a halt [65] (see 4.4).

Even though it has been shown that such anaphylactic reactions are mainly restricted to epitopes that elicit no central tolerance due to lack of expression in the thymus [114], the fear of severe side reactions is a serious setback for current research in this field. More detailed studies of anaphylaxis and its possible dissociation from the protective effect of type 2 immunity seem to be highly important to potentially overcome this problem and to pave the way for immune deviation therapy in the future.

1.4 Aims of this thesis

The major goal of this project was to study, in a murine EAE model, the effects on different parts of the immune system that are produced by a tolerization protocol using multiple antigen injections in IFA. The association between this kind of therapeutic effect and anaphylaxis that several studies had described constituted a major obstacle to the clinical application of this way of treatment in recent years. Therefore, the possibility to dissociate the therapeutic effect of these immunizations from allergic side reactions was to be demonstrated in order to overcome this drawback of immunotherapies.

The EAE model used for that reason was chosen to be as realistic for the situation in clinical studies as possible. Therefore, the first aim was to establish a model for the induction of disease and tolerance using subcutaneous immunizations with the fusion protein MP4. The idea behind the use of this protein is to generate T cell responses to a broad spectrum of MBP and PLP determinants, thus providing a more realistic model of human disease than peptide-specific EAE. The method of subcutaneous immunizations in an adjuvant was chosen because it is also approved for and applied in clinical studies in humans.

The second aim was to understand the effects of repeated versus single immunizations in this model on the response of T and B cells and the protection from subsequent EAE induction. This is of special interest because clinical protocols frequently involve multiple injections of an antigen in order to boost the protective class of T cells. However, it has never been formally established that such repeated injections augment T cell immunity, increase the polarization effect towards type 2 T cells, and thus are of any additional therapeutic benefit.

The third aim was to study some of the factors responsible for the occurrence of anaphylaxis and thus to find a therapeutic regimen that enhances protection by polarizing the immune response towards type 2 T cells without the risk of developing allergic side reactions.

2 Materials and Methods

2.1 Mice, antigens and treatments

2.1.1 Mice

Female SJL (MHC alleles H-2^{s}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained on standard laboratory food and water *ad libitum* in specific pathogen-free animal facilities of Case Western Reserve University (Cleveland, OH). At the time of first injection, all mice were 6–8 weeks old. Paralyzed animals were afforded easier access to food and water. The care of the mice and all treatments were performed under an approved protocol that complied with the institutional guidelines.

2.1.2 Antigens and adjuvants

MP4 (*Apogen*), a fusion protein between the 21.5 kD isoform of myelin basic protein (*MBP21.5*) and a genetically engineered form of proteolipid protein (*ΔPLP4*) (see 1.1.2) was obtained from Alexion Pharmaceuticals Inc. (Cheshire, CT). The PLP peptide 139–151 (amino acids CHCLGKWLGHPDK) was purchased from Princeton BioMolecules Corporation (Langhorne, PA), and hen egg lysozyme (HEL) was obtained from Sigma-Aldrich (St. Louis, MO).

IFA was prepared as a mixture of mannide monooleate (Sigma-Aldrich) and paraffin oil (EMScience, Gibbstown, NJ). CFA was prepared by mixing inactivated Mycobacterium tuberculosis H37RA (Difco Laboratories, Franklin Lakes, NJ) at 5 mg/ml into IFA. Pertussis toxin (PTX) was obtained from List Biological Laboratories Inc. (Campbell, CA).

2.1.3 Immunizations

For immunization purposes, MP4 was used at doses ranging from 37.5 to 300 µg per mouse using CFA or IFA as specified. HEL was administered at a dose of 200 µg per mouse, and the PLP peptide at a dose of 100 µg per mouse. 300 µl of antigen-adjuvant emulsion for the administration of 300 µg of MP4 and 200 µl for all other immunizations were injected per mouse subcutaneously, at two different sites of the flanks.

For EAE induction, 200 ng PTX were injected intraperitoneally in 500 µl of sterile phosphate buffered saline (PBS) directly after the immunization with MP4 in CFA, and then a second time 48 h later.

2.1.4 Assessment of EAE severity

Starting from day 5 after injection of the neuroantigen, the mice were assessed daily for the development of paralytic signs, and the severity of the disease was recorded according to a standard scale: grade 1, floppy tail; grade 2, hind leg weakness; grade 3, full hind leg paralysis; grade 4, quadriplegia; grade 5, death. Mice demonstrating symptoms in between the clear-cut gradations were scored intermediately in increments of 0.5. This score is used as ordinate in Figs. 2 and 7.

2.2 ELISPOT

2.2.1 ELISPOT assay

ImmunoSpot M200 plates (Cellular Technology Limited, Cleveland, OH) were coated overnight at 4°C in a humidifying chamber with the cytokine-specific capture antibodies dissolved in sterile PBS. Antibodies derived from clone R4-6A2 (BD Pharmingen, San Jose, CA) were used at 4 µg/ml for capturing IFN-γ; clone JES6-1A12 (eBioscience, San Diego, CA) was used at 4 µg/ml for IL-2; 11B11 (purified from hybridoma cells in our laboratory) at 8 µg/ml for IL-4; TRFK5 (eBioscience) at 1 µg/ml for IL-5. All of these concentrations had been optimized in preceding titration assays in conjunction with the respective concentrations of detection antibodies. The plates were then blocked for 1 h at room temperature with sterile PBS containing 1% bovine serum albumin (BSA, fraction V, Sigma-Aldrich) and washed three times with sterile PBS.

The mice were sacrificed at specific time points, and the spleen or draining lymph nodes (drLN) were harvested. Single-cell suspensions were prepared, and cells were plated in HL-1 medium (BioWhittaker, Walkersville, MD), supplemented with L-glutamine at 1 mM, without (medium samples) or with antigen (20 µg/ml MP4), in triplicate wells. This antigen concentration had been shown to induce the maximal recall response in preliminary experiments (see 3.1.2). Anti-CD3 antibody (2C11, purified from hybridoma in our

laboratory) at a concentration of 3 µg/ml was used as a positive control. For splenocytes, 10^6 cells were plated per well, whereas for drLN cells, 5×10^5 cells were used per well.

The plates were incubated at 37°C with 7% CO_2 (24 h for IFN-γ and IL-2 assays; 48 h for IL-4 and IL-5 assays). After washing three times with PBS, followed by three times with PBST (PBS containing 0.025% Tween 20, obtained from Fisher Scientific International, Hampton, NH), the detection antibodies were added in PBST for overnight incubation at 4°C in a humidifying chamber. Biotinylated XMG1.2 (BD Pharmingen) was used at 2 µg/ml for detecting IFN-γ, JES6-5H4-biotin (BD Pharmingen) at 2 µg/ml for IL-2, BVD6-24G2-biotin (BD Pharmingen) at 1 µg/ml for IL-4, and TRFK4-biotin (BD Pharmingen) at 2 µg/ml for IL-5. These concentrations for the detection antibodies had also been optimized in titration assays before. The plates were then washed four times in PBST containing 1% BSA.

As a tertiary reagent, streptavidin-alkaline phosphatase conjugate (Dako, Carpinteria, CA) was added at a 1/1000 dilution in PBST containing 1% BSA and incubated for 2 h, followed by three washes with PBST and three washes with PBS. The plates were then developed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphatase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The reaction was stopped by rinsing with tap water the moment when clear spots became macroscopically visible (after 10 to 30 minutes, depending on the cytokine). The plates were air-dried overnight before they were subjected to image analysis.

2.2.2 ELISPOT image analysis

The resulting spots were counted on an ImmunoSpot Series 3B Analyzer (Cellular Technology) specifically designed for morphometric ELISPOT analysis. Digitized images were analyzed for the presence of areas in which color density exceeded the background by an amount set on the basis of the comparison of experimental wells and control wells. After separation of spots that touch each other or partially overlap, additional criteria of spot size and circularity were applied to gate out speckles and artifacts caused by spontaneous substrate precipitation or nonspecific antibody binding. Objects that did not meet these criteria were ignored, and areas that met all criteria were recognized as spots and counted.

All results were normalized to 10^6 cells, and the difference between stimulated and nonstimulated cells was calculated to obtain a number for the antigen-specific spot formation.

2.3 Proliferation assays

For spleen cell proliferation assays, single-cell suspensions were prepared, and 10^6 spleen cells per well were plated in triplicates in flat-bottom, 96-well microtiter plates in serum-free HL-1 medium supplemented with L-glutamine at 1 mM. MP4 was added at a final concentration of 20 µg/ml, which had been established before to induce the maximal recall response (see 3.1.2). During the last 18 h of a 4-day culture at 37°C and 7% CO_2, [^{3}H]thymidine was added (1 µCi/well); incorporation of label was measured by liquid scintillation counting, and the difference in counts per minute (CPM) was calculated with respect to non-antigen-stimulated values.

2.4 ELISA for detection of MP4-specific IgG1 serum antibodies

Mouse serum samples were obtained by storing blood samples for 2 h at 4°C in a non-heparinized Eppendorf tube before centrifuging them for 10 minutes at 10,000 rounds per minute and transferring the supernatant to a new tube.

ELISA plates (Nunc Immunoplate MaxiSorp; Fisher Scientific) were coated overnight at 4°C in a humidifying chamber with 80 µl of MP4 at a concentration of 3 µg/ml in bicarbonate buffer. The plates were washed four times with PBST, blocked for 2 h at room temperature with PBST enriched with 1% BSA, and washed another three times with PBST.

Serial dilutions of mouse serum in PBST containing 1% BSA, starting at 1/200, were added in triplicates to the plate. After overnight incubation at 4°C in a humidifying chamber, the plates were washed three times with PBST.

Plate-bound serum antibodies were detected by alkaline phosphatase-coupled goat anti-mouse IgG1 antibodies (Southern Biotechnology Associates, Birmingham, AL) at 80 µl/well at a dilution of 1:3000. After a 2 h incubation time, the plates were washed three times with PBST and four times with Final wash (distilled water with Tris Base and NaCl at pH 7.5).

100 µl/well of freshly prepared p-Nitrophenylphosphate solution (Research Organics, Cleveland, OH) was used for the development of the colorimetric reaction. The optical density (OD) was read at different time points in an ELISA reader at a wavelength of 405 nm, and values of different plates were adjusted for 1.000 in positive control wells.

2.5 Statistics

Results are presented as mean ± standard error of the mean (SEM). Differences between groups were assessed using the Student's t test or, in case the Normality Test or the Equal Variance Test failed, using the Mann-Whitney U rank sum instead (calculated by Sigma-Stat, version 7.0; SPSS). Statistical significance was set at $p < 0.05$.

3 Results

3.1 EAE induction and antigen recall

3.1.1 EAE induction in the MP4 model

To study the kinetics of EAE development, groups of six female SJL mice each were injected with 300, 150, 75, or 37.5 µg MP4 in CFA, with the addition of PTX according to the protocol (2.1.3), and the clinical EAE score was measured during the days and weeks that followed (Fig. 2).

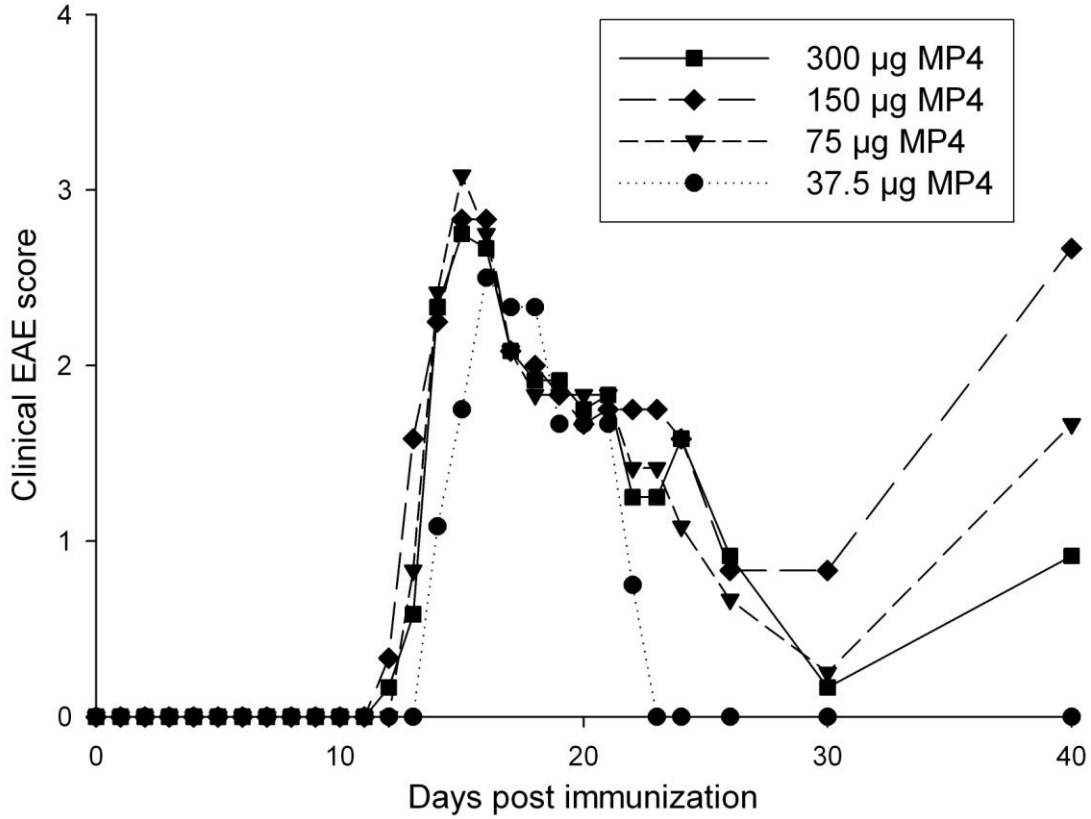

FIGURE 2 EAE induction by immunization with MP4

SJL mice were immunized with different amounts of the MBP-PLP fusion protein MP4 in CFA (complete Freund's adjuvant) and injected with PTX (pertussis toxin) as specified in 2.1.3. During the weeks following immunization, the mice were monitored for clinical signs of EAE (experimental autoimmune encephalomyelitis) and assigned a score according to the standard scale. The mean disease score for each group (n=6) is shown over the course of time. The data shown are from one experiment representative of two performed.

Mice immunized with 300, 150, or 75 µg MP4 all developed EAE with very similar disease characteristics regarding time of onset, severity (mean of individuals' maximal score between 3.0 and 3.2 in all three groups), and time to recover, and mice of all three groups showed a remitting-relapsing disease course. Mice injected with only 37.5 µg MP4, however, developed the disease more slowly, recovered faster, and showed no signs of relapses. Therefore, the dose of 75 µg was elected to be used for induction of disease and tolerance in all subsequent studies.

In contrast to this disease-inducing protocol, SJL mice tolerated an injection of 300 µg (as well as 75 µg) MP4 in IFA without displaying any apparent clinical signs.

3.1.2 MP4 recall titration

To address concentration dependency of cytokine production by T cells, which differs widely from antigen to antigen, two groups of three female SJL mice each were immunized with 50 µg MP4 in CFA or pure PBS in CFA respectively. Spleen cells were collected six weeks after immunization and tested separately for IFN-γ and IL-2 production under stimulation with different concentrations of MP4, ranging from 100 µg/ml in eight steps of 1:2 dilutions down to 0.39 µg/ml, as specified on the abscissa of Fig. 3. The difference between the antigen-specific spot formation (see 2.2.2) in MP4- and mock-immunized mice was calculated (Fig. 3).

The results show that ELISPOT assays with very high or very low recall antigen concentrations are not capable of distinguishing between immunized and unimmunized mice. A high sensitivity and specificity for the detection of cytokine-producing antigen-specific cells, both in the case of IFN-γ and IL-2, was achieved with concentrations of 25 or 12.5 µg/ml MP4. Therefore, 20 µg/ml of MP4 was chosen as the recall concentration for all further ELISPOT assays.

3.2 T cell response to immunization with MP4

3.2.1 Single immunization of MP4 in CFA or IFA

CFA and IFA are common adjuvants widely used to influence the Th1/Th2 balance in immunization protocols (see 1.1.2 and 1.2.3). However, the T cell response profile associated with a subcutaneous injection of MP4 in CFA or IFA has not been established yet.

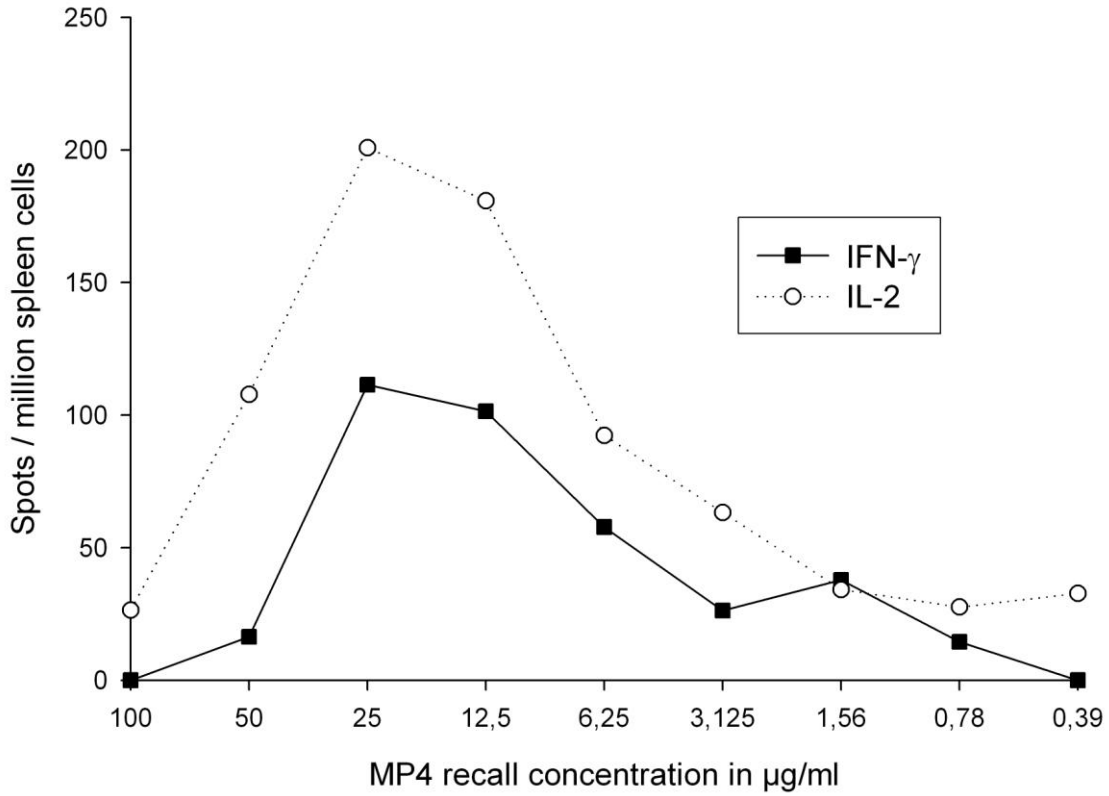

FIGURE 3 MP4 recall titration after immunization with MP4

Two groups of SJL mice were immunized with 50 µg of the MBP-PLP fusion protein MP4 or pure PBS (phosphate buffered saline) in CFA (complete Freund's adjuvant). Spleen cells were tested for IFN-γ (interferon-γ) and IL-2 (interleukin-2) production in ELISPOT assays with different concentrations of recall antigen. Mean numbers are shown for specific cytokine production, calculated as the difference of results in MP4-immunized and PBS-immunized mice. The concentration of MP4 in the recall assay is specified on the abscissa, the number of cytokine-producing cells per million spleen cells on the ordinate.

Two groups of eight female SJL mice each were immunized with 75 µg MP4 in CFA or in IFA, respectively. Draining lymph node cells were collected 9 days after the immunization and tested separately for production of IFN-γ, IL-2, IL-4, and IL-5. The frequency of antigen-specific cytokine-producing cells was calculated (Fig. 4).

The mice injected with MP4 in CFA showed IFN-γ and IL-2, but no IL-4 or IL-5 production, consistent with the induction of a polarized type 1 immunity (see 1.2.1). Draining lymph node cells of mice injected with MP4 in IFA did not generate a clear IFN-γ, IL-2, IL-4, or IL-5 recall response when tested on day 9 after injection (Fig. 4). However,

when the spleen cells of those mice were studied at later time points, a clear IL-2 recall response was detected after 4, 6, and 8 weeks (Fig. 5C), in the absence of significant IL-4, IL-5, or IFN-γ production (Fig.5D, E, and F, respectively).

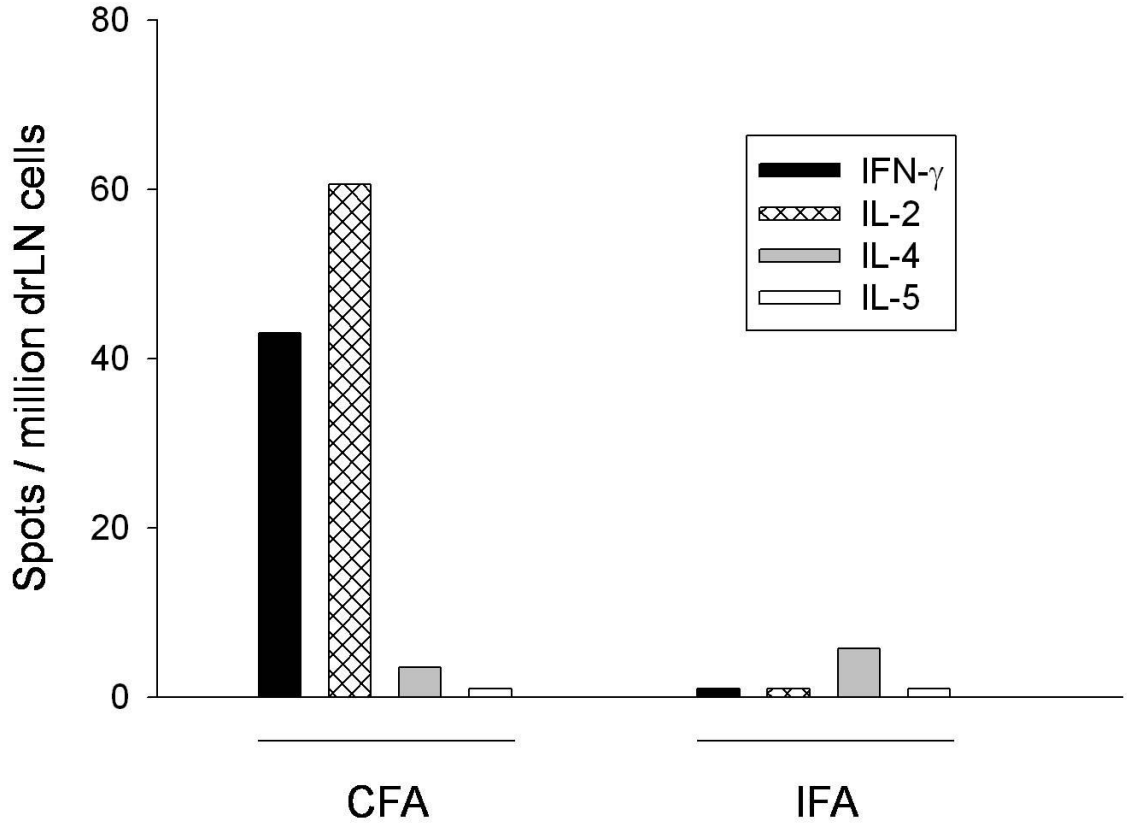

FIGURE 4 Cytokine profile after single immunization with MP4 in CFA or IFA

Two groups of SJL mice were immunized with 75 µg MP4 in CFA (complete Freund's adjuvant) or IFA (incomplete Freund's adjuvant), respectively. On day 9 after injection, draining lymph node (drLN) cells were tested for MP4-induced production of IFN-γ (interferon-γ), IL-2 (interleukin-2), IL-4 (interleukin-4), and IL-5 (interleukin-5) in antigen-specific ELISPOT assays. Cells from eight mice per group were tested individually, each in triplicate wells. Mean values are shown for each group. The number of cytokine-producing cells per million drLN cells is specified on the ordinate. The results shown are from one experiment that is representative of two performed.

Proliferative recall responses were not detected in mice immunized once with MP4 in IFA (groups I-IV in Fig. 5B). This likely results from a lower sensitivity of proliferation assays for the detection of antigen-specific T cells compared to the ELISPOT assays that have a single-cell resolution. When the frequencies of IL-2 producers were higher, e.g. in the group VI that was injected three times, the proliferation assay also picked up a signal (Fig. 5B).

3.2.2 Repeated immunizations with MP4 in IFA

To study the effect of repeated immunizations with MP4 in IFA on cytokine production and immune deviation in detail, groups of fourteen to eighteen female SJL mice were immunized with 75 µg MP4 in IFA up to four times at intervals of two weeks. The mice were tested two weeks after the primary, secondary, tertiary, and quartiary injection, as specified in Fig. 5A (groups I and V-VII). To rule out a pure time effect, another three groups of six mice each were immunized once with 75 µg MP4 in IFA and tested in parallel to the aforementioned groups after four, six, and eight weeks (groups II-IV in Fig. 5A). Finally, to exclude a pure dose effect, one group of mice was immunized with 300 µg MP4 in IFA and tested four weeks later.

At the specified time points, spleen cells were analyzed for the MP4-induced proliferative response by [^{3}H]thymidine incorporation (Fig. 5B) and for MP4-induced cytokine production by IL-2, IL-4, IL-5, and IFN-γ ELISPOT assays (Fig 5, C-F). All mice were tested individually and in triplicates for each assay.

Both after primary and secondary immunization, the proliferative recall response was undetectable, independent from the time point of the testing (Fig. 5B, groups I-V). The tertiary injection induced a strong response (group VI, $p = 0.006$ vs. secondary injection). In mice injected four times, the MP4-induced proliferative response declined again (group VII, decline statistically not significant).

A similar trend was noted for MP4-induced IL-2 production (Fig. 5C). However, this assay proved to be more sensitive, detecting increased frequencies of MP4-specific cells in mice immunized just once (group I, 22/million, 2 weeks after the primary injection). As already stated in section 3.2.1, IL-2 producers were found more frequently at later time points after the single immunization (groups II-IV). The frequencies rose after the secondary injection (group V, 112/million; $p = 0.002$ vs. primary injection) and were further increased after the tertiary injection (group VI, 251/million; $p = 0.002$ vs. secondary injection). Similar to the proliferation assay, a decline of IL-2-producing MP4-specific cells was seen in mice injected four times (group VII, 92/million; $p < 0.001$ vs. tertiary injection).

MP4-specific IL-4-producing cells showed a similar pattern (Fig. 5D). However, their frequencies were considerably lower, 16/million after the primary injection (group I), further decreasing over time (groups II-IV), 15/million for the secondary injection (group V), 48/million for the tertiarily injected mice (group VI), and 32/million for the group that

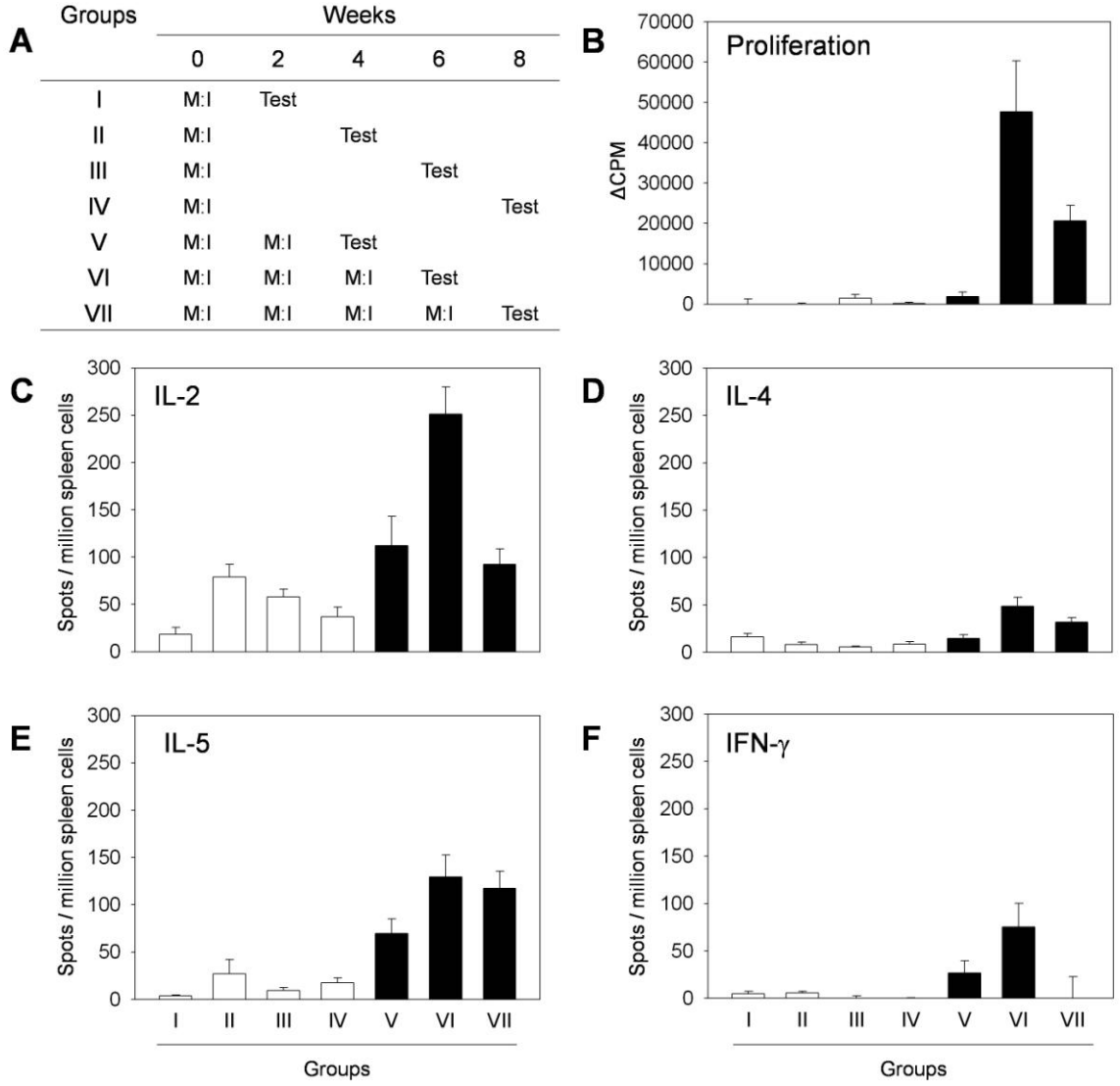

FIGURE 5 Cytokine profile and proliferation after single or repeated immunization with MP4 in IFA

SJL mice were singularly or repeatedly injected subcutaneously with 75 µg MP4 in incomplete Freund's adjuvant (M:I) as specified in A. Groups of mice that had been injected once and were tested at different time points thereafter are represented with open bars, groups that had been injected repeatedly and were tested two weeks after the last injection are represented with filled bars in B-F. At the defined time points (Test), the spleen cells were tested for MP4-induced proliferation by [^{3}H]thymidine incorporation (B) and for cytokine production by ELISPOT assays (C-F). Spleen cells from 6 to 18 mice per group were tested individually (n=6 for groups II-IV, n=14 for groups I, V, and VI, n=18 for group VII), in triplicates each. Mean numbers and the standard errors of mean are shown for each group. The results are pooled from two experiments that have been performed independently.

was injected four times (group VII). Although the overall changes seen were moderate, the difference between the secondary and tertiary injection reached statistical significance ($p = 0.002$).

The IL-5-secreting cells also reproduced this overall pattern (Fig. 5E), their frequencies reaching intermediate numbers between IL-2 and IL-4 producers (the maximal frequency of MP4-induced IL-5-producing cells was 129/million as shown for group VI). The increase from primary to secondary injection was highly significant ($p < 0.001$), the additional increase from secondarily to tertiarily injected mice was still significant ($p = 0.039$). In contrast to the other cytokines, however, the numbers of IL-5 producers did not decrease after the fourth injection (group VII, 117/million).

Low frequency IFN-γ recall responses became detectable in secondarily injected mice (group V in Fig. 4F, 27/million; $p = 0.015$ vs. primary injection); these increased to 75/million after the tertiary injection ($p = 0.041$ vs. secondary injection), and declined to undetectable levels after the fourth injection ($p < 0.001$ vs. tertiary injection).

For all measurements, the application of the 300 µg dose in a single immunization yielded the same results as a single immunization with 75 µg MP4, therefore this group was excluded from figure 5 for reasons of better clearness.

Overall, the frequency measurements of cytokine-producing MP4-specific T cells after immunizations with antigen in IFA showed that a type 0/2 response was induced whose magnitude peaked after three immunizations; after the fourth injection, the T cell response became entirely type 2 polarized with a prevalence of IL-5 over IL-4 producers. This development could neither be attributed to the time course after the injection nor to the increased antigen dose, but was due to the repeated injections (see 4.3 for further discussion).

3.3 B cell response to immunization with MP4

To study the effect of multiple reimmunizations with antigen in IFA on the B cell response and antibody production, groups of eight female SJL mice each were immunized with 75 µg MP4 in IFA up to four times at intervals of two weeks. The mice were tested two weeks after the primary, secondary, tertiary, and quartiary injection, as specified in Fig. 5A for groups I, V, VI, and VII, respectively. The mice were bled, serum was obtained, and the MP4-specific IgG1 antibody titers were measured by ELISA (see 2.4). The results of this experiment are presented in Fig. 6.

No antigen-specific IgG1 antibodies could be detected in mice immunized just once, undistinguishable from unimmunized control mice. However, as expected, the serum levels clearly increased with each booster injection, up to very high titers after the fourth immunization.

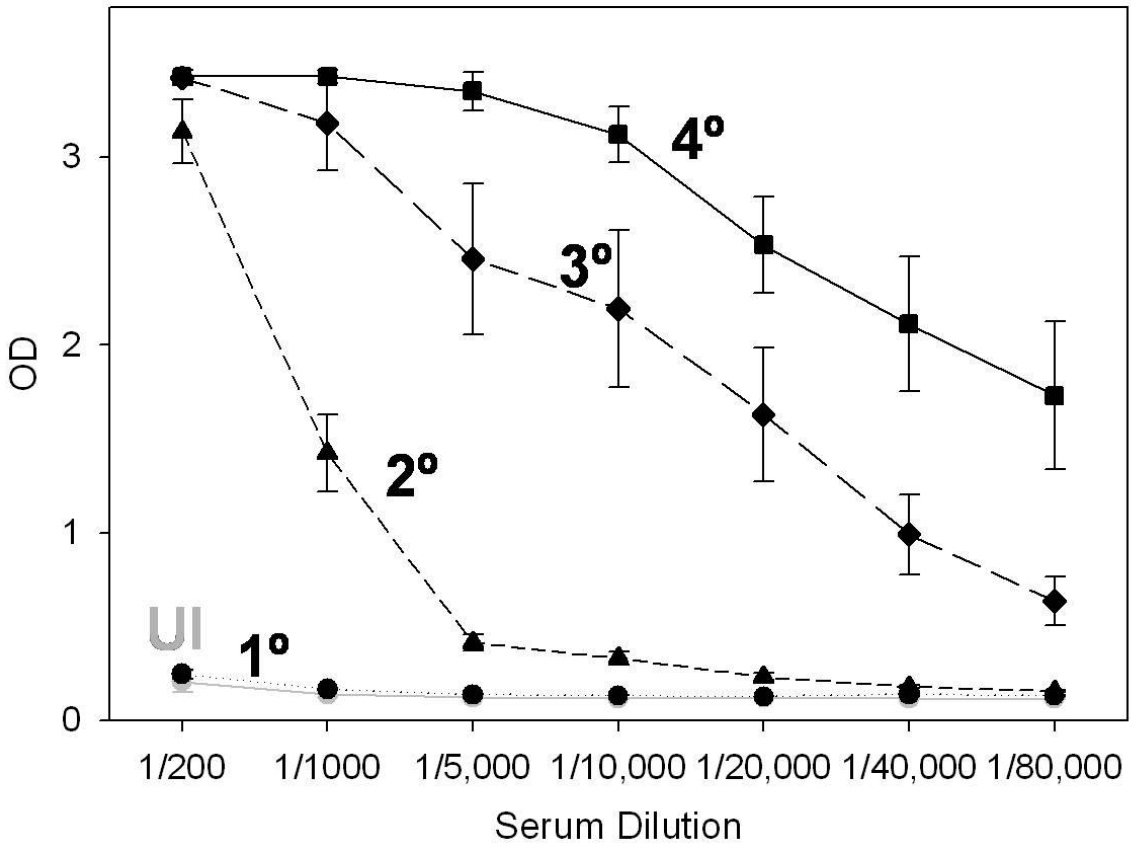

FIGURE 6 IgG1 antibody production after single or repeated immunization with MP4 in IFA

SJL mice were repeatedly injected subcutaneously with 75 µg MP4 in IFA (incomplete Freund's adjuvant), as specified in Fig. 5A for primary (1°), secondary (2°), tertiary (3°), and quaternary (4°) injections (groups I, V, VI, and VII, respectively). Two weeks after the last injection, the mice were bled, serum was obtained, and the MP4-specific IgG1 antibody titers were measured by ELISA. Sera of eight mice per group were tested in serial dilutions individually in triplicates; the mean and SEM (standard error of the mean) of the OD (optical density) for each group is shown for each dilution, compared with sera of two unimmunized control mice (UI). The results shown are representative of two individual experiments per group.

3.4 EAE protection by tolerization with MP4

Type 2 T cells are generally known to confer protection from organ-specific autoimmune diseases (see 1.2.3). To study whether or not repeatedly injected mice are better protected from MP4-induced EAE than mice receiving just a single injection of MP4 in IFA, groups of ten female SJL mice each were injected with 75 µg MP4 in IFA one to four times at intervals of two weeks, corresponding to groups I and V-VII in Fig. 5A. Another experimental group (six mice) received a single injection of the fourfold dose, meaning 300 µg of MP4 in IFA. Control mice (also ten per group) were injected once or four times with 200 µg HEL in IFA, in parallel to groups I and VII. Two weeks after the last injection of the four times pretreated groups, all mice received the disease-inducing injection of MP4 in CFA with the additional PTX challenge (according to 2.1.3), and the clinical EAE score was measured during the days and weeks that followed. The results of this experiment are shown in Fig. 7.

The mice preinjected four times with HEL in IFA developed severe EAE. The disease course for mice injected once with HEL in IFA was very similar. Single or double injections of 75 µg of MP4 in IFA or the single inoculation of 300 µg MP4 in IFA led to comparable results: these pretreatments did not have a detectable effect on the time point and severity of disease onset. However, all of these treatments ameliorated the subsequent disease to a similar extent.

In contrast, the mice preinjected four times with MP4 in IFA showed a marked reduction of disease severity at onset (maximum score 0.8 vs. 3.0), and by day 22, 8 of the 10 mice had complete recovered, the ninth having a score of 1, and the tenth a score of only 0.5. The disease level of the group that received three injections of MP4 in IFA resulted in intermediate scores between the twice and four times injected groups. The enhanced protection seen in mice preinjected four times vs. just once was reproduced in an independent experiment using 10 mice per group.

Statistical analysis of mice in both experiments showed highly significant differences between once and four times injected mice on day 14 ($p = 0.001$), and significant differences on day 21 ($p = 0.048$). These results provide evidence that repeated injections not only boost type 2 immunity, but also increase the therapeutic efficacy of immune deviation by treatment with MP4 in IFA.

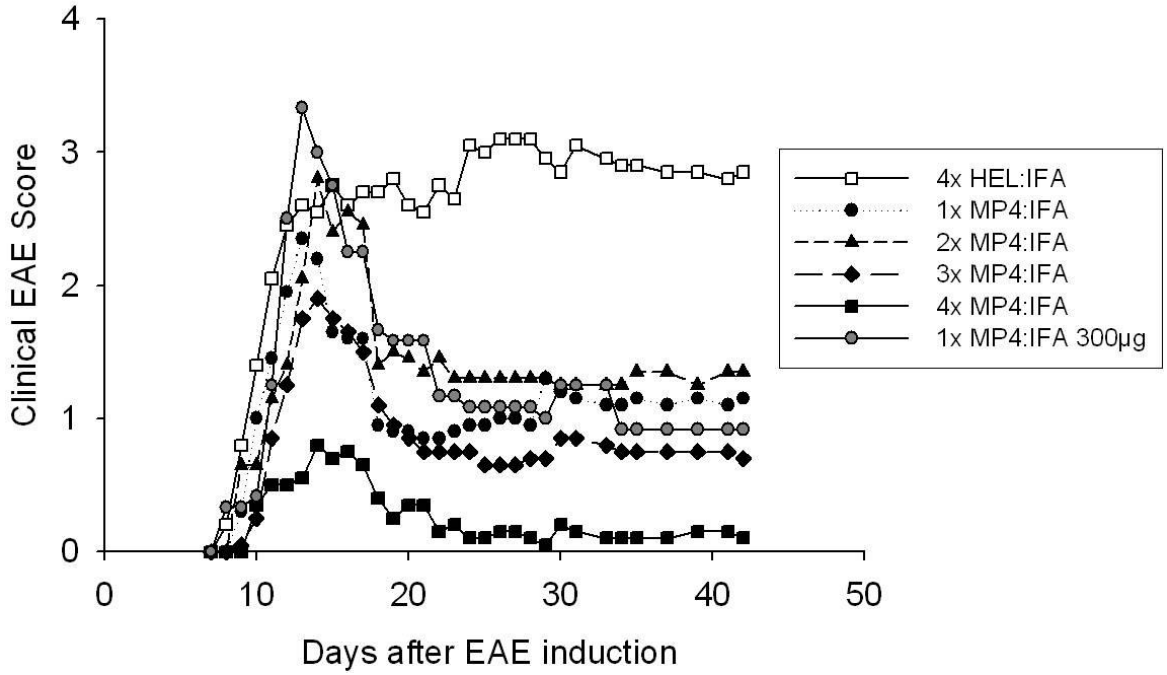

FIGURE 7 Protection from EAE induction after single or repeated immunization with MP4 or HEL in IFA

SJL mice were repeatedly injected subcutaneously with 75 µg MP4 in IFA, as specified in Fig. 5A for groups I, V, VI, and VII, with a single dose of 300 µg MP4 in IFA, or with four doses of 200 µg HEL (hen egg lysozyme) in IFA. Two weeks after the last injection of the four times pretreated groups, all mice received the disease-inducing injection of 75 µg MP4 in CFA with the additional PTX (pertussis toxin) challenge, and the clinical EAE score was measured during the days and weeks that followed, according to the standard scale defined in 2.1.4. The mean disease score for each group (n=6 for the 300 µg MP4 group, n=10 for all other groups) is shown over the course of time. The data shown are from one experiment representative of two performed.

3.5 Anaphylactic side reactions of tolerization with MP4

For the experiments outlined above (3.2.2, 3.3, and 3.4), 38 mice were injected four times with MP4 in IFA, an additional 24 mice received three injections of MP4 in IFA, and another 24 mice received two of these injections. Although those mice developed high titers of anti-MP4 antibodies of the IgG1 class (see 3.3 and Fig. 6) and these antibodies excel in mediating anaphylactic reactions (see 1.3.2), none of the mice showed even mild signs of immediate hypersensitivity that were looked for (piloerection, prostration, erythema, dyspnea, shallow breathing). Presumably, the retention and slow release of the antigen from the adjuvant helps to prevent allergic reactions in this regimen.

Because allergic side reactions have been repeatedly reported during immune deviation therapies (see 1.3.3), this last set of experiments was designed to systematically establish the conditions that favor their occurrence (Table 1).

TABLE 1 Anaphylactic side reactions after antigen challenge with MP4 protein or PLP peptide 139-151 in IFA or solubly in PBS

SJL mice were injected subcutaneously (s.c.) with 75 µg MP4 or 100 µg PLP (proteolipid protein) peptide 139-151 (PLPp) in IFA, one to three times in 2-week intervals, as specified. Four weeks after the last injection, the mice were challenged with the respective antigens in IFA or solubly in PBS (sol), as specified. The development of anaphylactic reactions was closely monitored during the first 90 minutes. The number of mice developing lethal and nonlethal reactions is shown under "Total"; mice developing a lethal anaphylactic shock are specified separately.

		Anaphylactic side reactions	
Pretreatment	Challenge	Total	Lethal
MP4:IFA s.c.			
1x	MP4:IFA s.c.	0/34	0/34
2x	MP4:IFA s.c.	0/34	0/34
3x	MP4:IFA s.c.	0/48	0/48
1x	MP4 sol s.c.	0/10	0/10
2x	MP4 sol s.c.	0/10	0/10
3x	MP4 sol s.c.	0/10	0/10
PLPp:IFA s.c.			
1x	PLPp:IFA s.c.	0/10	0/10
2x	PLPp:IFA s.c.	0/10	0/10
3x	PLPp:IFA s.c.	0/10	0/10
1x	PLPp sol s.c.	10/10	5/10

Type 2 immunity was induced by injecting MP4 in IFA up to three times, and such mice were challenged with either MP4 in IFA or with soluble MP4 subcutaneously. The number of injections thus added up to 4, as for the disease test (see 3.4). No allergic reactions were observed in any of the mice, neither during the first 90 minutes after injection, nor when rechecked after 3 and after 24 hours. Under these conditions, MP4 is first drained through the lymphatics before it can reach the blood circulation, and due to its high molecular weight, it may be trapped in the lymphoid tissues.

Because allergic side reactions were observed after injection of soluble low molecular weight peptides, we repeated these experiments with PLP peptide 139–151 (see 1.1.2). SJL mice were sensitized by a single injection of this peptide in IFA, followed by a subcutaneous challenge with 100 µg of this peptide in PBS. All mice (10 of 10) developed symptoms of immediate hypersensitivity, and 5 of the 10 mice died of anaphylactic shock within 62 minutes. In contrast, up to four injections of 100 µg of the peptide in IFA injected every second week were tolerated without any detectable allergic side reactions. The retention of the peptide in the adjuvant seemed to limit its diffusion into the blood circulation, avoiding systemic antigen concentrations high enough to cause generalized mast cell degranulation and the development of an anaphylactic shock.

4 Discussion

4.1 MP4 as an autoantigen for induction of disease and tolerance

During decades of research with EAE, several proteins and protein components have been found to be encephalitogenic, and various EAE models have been established. It was found that encephalitogenic determinants vary strongly from species to species and even between strains of the same species. Within mice, for instance, MBP and MBP peptides can easily induce EAE in H-2^u strains (B10.PL and PL/J), while most common mouse strains are resistant to MBP-induced disease. SJL mice proved to be susceptible to PLP-induced EAE; because of the highly hydrophobic character of this protein, however, this model was restricted to peptides, especially peptide 139-151. MOG peptide 35-55 and PLP peptide 178-191 can induce disease in C57BL/6 mice.

Each combination of a neuroantigen with the respective susceptible mouse strain results in a characteristic form of EAE. These show fundamental differences in disease characteristics between each other. For example, the MBP-induced disease in B10.PL and PL/J mice is monophasic: the mice completely recover after a single episode of short acute paralysis and become resistant to reinduction of EAE [158]. EAE induced in SJL mice by immunization with PLP peptide 139-151 shows a relapsing-remitting course [58], while the disease elicited in C57BL/6 mice by injection of MOG peptide 35-55 is chronic-progressive [32]. Besides, the EAE models differ from each other by involving diverse regions of the CNS and by showing unique histopathologies [74,75]. None of these disease forms ideally models human demyelinating disease, but joined together, they provide a more accurate picture [137]. Therefore, new EAE models adding an additional aspect are still being searched for.

As described in chapter 1.1.2 and shown in Fig. 1, MP4 encompasses the 21.5 kD isoform of MBP and the three hydrophilic domains of PLP. Thus, T cell responses to various determinants of both proteins can be generated without being confined to specific peptides as in the classical PLP models, providing a more accurate model for human disease. This is especially useful since it has been realized that autoimmune T cell repertoires undergo determinant spreading, rendering models with highly restricted T cell responses unrealistic [62,83,97,165].

Though demonstrated to be encephalitogenic in SJL mice and marmoset monkeys, MP4 has never been formally established as a model antigen for EAE induction. Elliott et al. used 300 µg/mouse MP4 in 300 µl of CFA emulsion, complemented with two injections of 300 ng pertussis toxin each at day 0 and 2, for disease induction in SJL mice in a single experiment [30]. They found a disease course quite similar to the one shown here in Fig. 2 for the doses of 75, 150, and 300 µg of MP4. However, they did not show that such high amounts of antigen were essential to induce the disease. Jordan et al. used MP4 at an unspecified dose with CFA to induce very mild disease in two marmoset monkeys [63]. Aside from that, no experiments inducing active EAE with the help of MP4 immunizations were described in the literature yet.

The experiments presented here established MP4 as an antigen for both disease and tolerance induction in SJL mice. After this work was published, it has been shown that C57BL/6 mice are also susceptible to EAE induction with this fusion protein, providing yet another animal model with different disease characteristics [75] and differing histopathology [74].

4.2 Effects of multiple subcutaneous injections with MP4 in IFA

Although highly effective in mouse models, intraperitoneal antigen injections with IFA are not a conceivable treatment for humans. Subcutaneous injections with mineral oil (or with related lipids), however, are approved for clinical studies. Unlike the immune-modulating effects of intraperitoneal IFA injections that have been closely studied in mice [35,54,95,164], the effects of subcutaneous IFA injections have not been well characterized so far.

Moreover, the exact consequences of repeating these injections have never been established. This is rather surprising, as multiple immunizations are regularly applied in large studies with the intent to enhance type 2 deviation and protection from EAE and other autoimmune diseases [65,92,115]. Seemingly, it has just been assumed that they boost the T cell response and therewith the protective effect, similar to the B cell response and antibody production, where this has long been known. Therefore, an important part of this project was to analyze the effects of single and repeated subcutaneous MP4 injections on the T cell and B cell response. Their consequences on EAE protection will be discussed in the following chapter.

A single subcutaneous injection of MP4 in IFA induced IL-2-producing T cells occurring at a relatively low frequency (<100 antigen-specific cells in 1 million spleen cells; Fig. 5C). Most of these IL-2-producing T cells did not produce IL-4 or IFN-γ; these T cells therefore qualify as uncommitted Thpp cells (see 1.2.1). IL-5 producers, which are prevalent after a single intraperitoneal injection of antigen in IFA [54,164] and which also become prominent after repeated subcutaneous injections (Fig. 5E), were present in low but clearly detectable frequencies, at 27 per million spleen cells in primarily injected mice.

The response induced by a subcutaneous injection had delayed kinetics compared to an intraperitoneal immunization: while the latter triggers peak numbers of antigen-specific memory cells in the spleen by day 10 [164], it took 4 weeks for the response to reach peak levels after the subcutaneous immunization (Fig. 5C and E). The kinetics of the injection in IFA was also delayed compared to a single subcutaneous injection of MP4 in CFA where the magnitude of IL-2 producing cells was in a similar range on day 9 already (Fig. 4).

Unlike primary intraperitoneal injections with IFA that trigger high titers of IgG1 and IgE (but no IgG2a) antibodies [164], the primary subcutaneous injections of MP4 did not induce detectable IgG1 levels (Fig. 6), at least not during the first two weeks after the immunization. Repeated subcutaneous MP4 injections with IFA, however, resulted in increased titers of specific IgG1 antibodies, as expected.

Interestingly, the frequency of the antigen-specific T cells was also elevated after multiple reimmunizations. This outcome has not yet been documented and might seem surprising because the half-life of an antigen in IFA is 90 days, and thus, the reinjection of antigen every 14 days does not add to the continuity of its presence. Although repeated injections increase the net amount of antigen deposited, the booster effect cannot be explained by the antigen dose alone, as a single subcutaneous injection of 300 µg of MP4 in IFA was shown to induce frequencies of MP4-specific T cells similar to the single injection of 75 µg of MP4 in IFA (see 3.2.2). Rather than mere antigen presence or the increased antigen dose, it can be assumed that the booster effect of repeated injections results from the induction of new waves of dendritic cells migrating from the new sites of antigen deposition [33,118].

Secondary and tertiary injections increased the frequencies of IL-2 and to a lesser extent of IL-4, IL-5, and IFN-γ-producing cells, that is to say, the T cell response maintained an unpolarized cytokine expression profile, despite being boosted. By the fourth injection, however, an almost complete type 2 polarization was accomplished: the numbers of un-

committed, IL-2-producing Thpp cells decreased, and IFN-γ-producing T cells were no longer detectable, whereas IL-5 producers prevailed. The IL-5 producers outnumbered the IL-4 producers, consistent with the fact that these cytokines are frequently not coexpressed by T cells [17,67]. This dissociation of Th2-specific cytokines is not surprising because IL-4 and IL-5 expression by T cells underlies independent instructed differentiation and different gene regulation pathways [67,107].

Taken together, it has been shown in the MP4 model that subcutaneous injections in IFA have a similar effect compared to intraperitoneal immunizations, but with a delayed kinetics. Secondly, it has been established that their repetition not only boosts the B cell response, but also the T cell response, with a polarization effect from uncommitted Thpp cells towards a pure Th2 response with prevalence of IL-5 producing cells, especially after the fourth immunization.

4.3 Immune deviation as a protective mechanism in the MP4 model

Repeated subcutaneous injections of MP4 in IFA induced and boosted a type 2-polarized MP4-specific immune response. Despite the high numbers of autoreactive type 2 T cells present and the high titers of autoantibodies induced, the mice repeatedly injected with MP4 in IFA did not develop any signs of neurological or other disease. This result is not self-evident, because MP4 contains extracellular domains of PLP that these antibodies can access, resulting in massive deposits on the surface of Schwann cells [54]. But obviously, that did not cause any detectable immune pathology.

On the contrary, such mice were protected from MP4-induced EAE (Fig. 7). Importantly, mice injected repeatedly with MP4 in IFA showed a more profound protection. Once again, the number of injections rather than the antigen dose alone defined the extent of the protection. Mice that were injected once with 300 or 75 µg of MP4 were similarly protected, whereas the injection of 300 µg of MP4 in four doses of 75 µg each resulted in a much more distinct effect on the disease severity at onset and the time to recover from the disease.

Studying the impact of repeated antigen injections in IFA on the antigen-specific T and B cell response as well as on disease protection provides insight into the protective mechanism. These data show that for the first three injections, boosting of type 2 immunity and of EAE protection went in parallel. After the fourth injection, when the protective ef-

fect was the most pronounced, a marked decrease of IL-2 producers was observed, whereas the number of type 2 T cells was largely unaffected. These IL-2 producers are thought to be memory cells that are yet uncommitted to type 1/type 2 differentiation [162,163]. It appears that these uncommitted cells are increasingly converted into committed type 2 cells by the repeated injections. Because type 2 cells are not capable of autocrine proliferation, the proliferative recall response (Fig. 5B) and the overall clonal sizes of the antigen-specific T cells seem to decline with the subsequent injection of antigen in IFA. By the fourth treatment with MP4 in IFA, the antigen-specific T cell pool was highly type 2 polarized, with IFN-γ producers no longer detectable. Therefore, we can conclude that the extent of EAE protection paralleled the extent of type 2 polarization. That is to say, immune deviation was induced and not clonal anergy or deletion.

The exact mechanism by which immune deviation leads to protection remains unknown. Several possibilities can be envisioned: first, the conversion of naïve T cells and of Thpp cells (that are uncommitted with respect to type 1/2 differentiation) into committed type 2 cells exhausts the pool of precursor cells from which type 1 effector cells can be generated. Second, because type 2 differentiation is under positive cytokine feedback regulation, preexisting autoantigen-specific type 2 T cells will cause a type 2 bias in subsequent T cell responses; this means that they will cause type 2 determinant spreading [151]. Third and last, cytokines secreted by such type 2 cells can create a microenvironment in the target organ that is suppressive to proinflammatory type 1 cells. IL-10 and TGF-β are among the prime cytokine candidates mediating such an effect.

It is unclear whether regulatory T cells producing such cytokines coexpress IL-4 or IL-5, meaning that they are the same cells that we have measured, or whether they are independent lineages engaged in parallel. Single-cell resolution measurements of MP4-specific IL-10 and TGF-β-producing T cells were not conducted because both cytokines are not readily amenable to ELISPOT T cell analysis. The former are masked by activation of cells of the innate immune system [52], whereas the latter are obscured by the present inability to distinguish between the active and passive form of the molecule [7].

In general, there are other methods to measure these cytokines. However, the frequencies of the cytokine-producing antigen-specific cells in most measurements was <100/million (<0.01%). This is below the detection limit of intracytoplasmic cytokine staining by flow cytometry, and hence the detection of such rare cells was dependent on the high sensitivity of ELISPOT measurements.

In conclusion, the repetition of MP4 injections in IFA clearly increased the protection from subsequent EAE induction with the same antigen. The dynamics of this increase paralleled the boost of the T cell response and the polarization from Thpp cells to Th2 cells described in the previous chapter. Among the different mechanisms of tolerance induction, immune deviation played a more pronounced role than clonal anergy or deletion. The emergence of regulatory T cells producing IL-10 or TGF-β could not be analyzed in this setting; therefore, their importance for the protective effect cannot be excluded.

As repeated injections with an antigen not only influence the T cell response, but also the B cell response and their antibody production, the application of this method is always associated with the risk of allergic side reactions. If immune deviation therapy is to play a role in clinical settings, such dangerous or even life-threatening side effects need to be clearly known and evitable. The ultimate goal of this project therefore was to look closely at the conditions under which anaphylaxis occurs and to figure out if there is a way of dissociating the intended protective effect from the appearance of such reactions.

4.4 Dissociation of protective effect and allergic side reactions

It is known that repeated injections with MP4 are critical in order to augment their therapeutic effect. But they are also linked to the boosting of cellular and humoral type 2 immunity, as it has been demonstrated for IgG1 antibodies. This entails the risk of allergic side reactions, particularly of hypersensitivity of the immediate type. Thus, anaphylactic reactions were seen in mice and humans after immune therapy with different peptides [65,92,114,115].

These reactions are mediated by IgE and IgG1 antibodies that cause systemic mast cell degranulation either by the binding of antigen to the sensitized mast cells, or indirectly, by activation of systemic complement, whereby activated complement components (C3a, C4a, C5a) are responsible for the mast cell degranulation. In both cases, for systemic anaphylaxis to occur, the antigen that elicits the reaction needs to disseminate in the body via the blood stream; failure to do so results in mast cell degranulation that is only localized.

When MP4 or PLP peptide 139–151 was injected repeatedly with IFA, not even mild anaphylactic reactions did occur (Table I). This result suggests that systemic antigen concentrations did not reach sufficient levels to elicit generalized mast cell degranulation, probably due to the retention and gradual release of the antigen from the IFA depot. This

antigen retention in the adjuvant seems to favor immunogenicity, thereby enhancing the therapeutic effect, while avoiding the life-threatening allergic side reactions.

In contrast, when soluble PLP peptide 139–151 was injected in mice that had been sensitized before with only one injection of this peptide in IFA, half of the mice died of anaphylaxis, and the other half displayed severe symptoms of immediate hypersensitivity (Table I). Interestingly, the subcutaneous injection of soluble MP4 in mice preinjected even four times with MP4 in IFA did not result in anaphylaxis despite the high titers of MP4-specific IgG1 antibodies present in their blood (Fig. 6).

The most prominent difference between these two antigens is that the PLP peptide measures only 13 amino acids, while MP4 is almost 400 amino acids long and comprises a multitude of B cell determinants. Consequently, when MP4 is injected in a sensitized host, the immune complexes that are generated are of considerably bigger size than the PLP peptide complexes. The larger the immune complexes are, however, the more efficiently the reticuloendothelial system clears them. Therefore, it is conceivable that after a subcutaneous injection, MP4 is filtered from the lymph as it passes the draining lymph nodes and is prevented from entering into the blood stream.

These two mechanisms of antigen retention in the adjuvant and filtering of large immune complexes might be the reason why life-threatening anaphylactic reactions did only occur in a very specific experimental setting, namely after the injection of a small peptide in a soluble form. On the contrary, in the model used in this project to confer EAE protection, both mechanisms may have protected the mice from this kind of adverse reaction.

Therefore, protocols that comprise multiple reimmunizations with an antigen seem to be free of the risk of anaphylactic reactions, even though antibody production is boosted, as long as large proteins are preferred to small peptides and immunizations in a mineral oil to injections of the antigen in a soluble form.

Consistent with this notion, all four studies cited before that showed the association of immune deviation therapy and severe hypersensitivity reactions applied repeated injections with short peptides in a soluble form to boost the immune response. Kappos et al. described the suspension of a clinical trial in relapsing-remitting MS because of hypersensitivity reactions in some patients after repeated immunizations with an altered peptide ligand in a physiologic buffer [65]. Pedotti et al. reported frequent anaphylactic reactions in mice after multiple reinjections of short peptides derived from proteolipid protein, myelin oligodendrocyte protein [114], or glutamic acid decarboxylase [115], each dissolved in

saline. Likewise, Liu et al. observed fatal anaphylaxis in mice after injecting them repeatedly with a small peptide of insulin B chain in saline [92]. If the results discussed here for the immunizations with the PLP peptide could be generalized, maybe these hypersensitivity reactions would not have taken place in models boosting the immune response by means of injections with the antigen in a mineral oil.

4.5 Immune deviation – an ideal therapy for autoimmune diseases?

Among all the approaches to treat EAE and other organ-specific autoimmune diseases (see 1.1.3), one of the most promising is to induce specific tolerance to self-antigens, thus avoiding a generalized immune compromised state by therapies that do not distinguish between events mediating the autoimmune disease and those required for host defense. Treatments either with the autoantigens themselves or with modified T cell ligands are prime candidates for accomplishing this goal.

There are several mechanisms possibly underlying antigen-specific tolerance [reviewed in 53]. Clonal deletion, meaning antigen-induced T cell apoptosis, is often seen after treatment with high-dose soluble protein or peptide given via intraperitoneal, subcutaneous, intravenous, or mucosal routes. The state of clonal anergy, a T cell hyporesponsiveness, is associated with the downregulation of surface T cell receptors and can be induced by altered peptide ligands or by high-dose mucosal or systemic antigen, depending on other determinants. T regulatory cells secreting anti-inflammatory cytokines and possibly competing with effector T cells for antigen presentation develop after low-dose mucosal antigen donation or as an outcome of clonal T cell deletion or anergy. Immune deviation, finally, is a shift from a pathogenic T cell response to a less pathogenic or even protective T cell response, usually from Th1 to Th2 or Th3 immunity.

It is an ongoing discussion which, if any, of these mechanisms plays the most important role in protection from autoimmune model diseases [53,76,130,139,157]. Some studies doubt the relevance of immune deviation for disease suppression, demonstrating paradoxical effects of Th1 cytokines on autoimmune pathogenesis [72,93,160]. Others claim that autoreactive cells are relatively resistant to immune deviation at later stages during their development, or even demonstrate Th2-mediated autoimmunity.

Immune deviation as the underlying mechanism for the treatment of organ-specific autoimmunity, however, has some advantages, compared to highly selective clonal dele-

tion or anergy. In addition to the functional depletion of the effector cells, the “deviated” T cells frequently secrete cytokines in the target organ that inhibit Th1-like effector T cells of other antigen specificities in an organ-specific manner. Thus, the need to know if the antigen used to induce tolerance is the major or primary pathogenic autoantigen is obviated. This strategy is also promising in cases where the autoimmune response has undergone determinant spreading, now targeting a wide array of second wave autoantigens [83,86]. Both features could be extremely useful in a clinical setting, where the exact autoantigen causing a disease is often unknown and determinant spreading seems to play an important role.

Therefore, immune deviation is still a very attractive approach to the treatment of autoimmune diseases, especially if its application can be dissociated from potentially dangerous side effects. Its ideal therapeutic application would probably be the prophylactic vaccination of susceptible individuals, as the protective effect is the most pronounced with a very early onset of therapy.

Similar to the discussion about the mechanism of disease protection, there is some disagreement about the best way to elicit this effect, and whether these methods can be transferred to clinical therapy in human disease.

As outlined before, protocols for disease prevention include mucosal administration of autoantigen via oral or naso-respiratory routes and systemic administration of antigen in a soluble form, either intravenously, intraperitoneally, or subcutaneously. Besides, injections of autoantigens in IFA, typically intraperitoneally in neonatal mice, have been a classical means of preventing autoimmune disease. Intraperitoneal injections with IFA at adult age have also been shown to be protective, both operating by the engagement of type 2 immunity [35,54,150].

Still, the repeated administration of antigen in a soluble form entails the risk for potentially dangerous allergic reactions, and intraperitoneal antigen injections with IFA cannot be used for treatment of humans. These experiments helped to fill the gap of knowledge about multiple subcutaneous injections in IFA and proved that immune deviation and protection from autoimmune disease can very well be achieved by this method, without ruling out that other mechanisms also play a significant role for the therapeutic effect.

4.6 Conclusion

In summary, it has been shown here that subcutaneous immunizations of SJL mice with the neuroantigen MP4 in IFA resulted in a T cell response that was boosted and polarized towards type 2 immunity by repeating the injections. The extent of the protection from the subsequent induction of EAE paralleled the T cell boost and its polarization. As a treatment-associated effect on humoral immunity, the repeated injections also induced high titers of antibodies, namely the IgG1 subtype. Still, anaphylactic reactions did not occur when the antigen was a large protein or when it was retained in an adjuvant. Thus, the therapeutic benefit of inducing a type 2 T cell response could be dissociated from the antibody-mediated complications of the treatment.

These findings demonstrated in a murine model of EAE may have important implications for immune therapy in humans. Although immune therapy in mice is well established and the first clinical trials were promising in respect to the possible therapeutic effect, antibody-mediated complications have virtually stopped any further progress. The demonstration of a possibility to dissociate the therapeutic benefit from the dangerous side effects in a model that reflects human autoimmune diseases in terms of antigen determinants and methods of treatment might revive efforts to use autoantigens or altered peptide ligands for the therapy of human autoimmune disease.

Subsequent experiments should investigate the contribution of other T cell subsets to the protective effect seen here after multiple reimmunizations with MP4 in IFA. For instance, details about the frequency of antigen-specific IL-10 or TGF-β producing cells could throw some light upon the role that regulatory T cells like Tr1 and Th3 play. Besides, the protocol should be tested in other models of autoimmune disease, with different antigens as well as in different animal species. The most intriguing question, however, is if clinical trials of immune deviation therapy in the future will show a therapeutic effect without the risk of allergic side reactions when using a protocol similar to the one presented in this thesis for the treatment of human autoimmune diseases.

5 Summary in German

Die experimentelle autoimmune Enzephalomyelitis ist eine entzündliche demyelinisierende Erkrankung des zentralen Nervensystems, die in zahlreichen Spezies durch Immunisierungen mit verschiedenen Neuroantigenen induziert werden kann. Sie stellt ein wichtiges Tiermodell für Autoimmunitätserkrankungen dar. An ihr wurde untersucht, wie durch Injektionen des Antigens in speziellen Trägerstoffen Toleranz diesem gegenüber und damit ein Schutz vor der Erkrankung erzeugt werden kann. In entsprechenden tierexperimentellen und klinischen Studien kam es jedoch gehäuft zu allergischen Reaktionen bis hin zum anaphylaktischen Schock. Deshalb wurde diese Therapieform generell in Zweifel gezogen.

Die Analyse der bisherigen Therapieansätze zeigt aber, dass alle Studien eine identische Methode einsetzen: Durch wiederholte Injektion eines kurzen Peptids in löslicher Form wird der therapeutische Effekt erzielt, aber auch eine Hypersensitivitätsreaktion bewirkt. Möglicherweise beruht die beobachtete Assoziation also auf dem speziellen Immunisierungsschema. Zudem ist noch weitgehend unbekannt, welche Effekte solche mehrmaligen Immunisierungen auf die einzelnen Komponenten des Immunsystems haben.

Die vorliegende Arbeit versucht zum einen, diese Wissenslücke zu schließen, zum anderen, eine Möglichkeit zu präsentieren, wie die genannte Assoziation von gewünschtem therapeutischen Effekt und lebensbedrohlichen allergischen Reaktionen durchbrochen werden kann. Es wird gezeigt, dass die wiederholte Immunisierung mit einem großen antigenen Protein in einem reinen Mineralöl als Adjuvans diese Bedingungen erfüllen kann.

Das verwendete Mausmodell beruht auf subkutanen Immunisierungen mit dem Protein MP4. Dieses ging aus einer Fusion von Myelin-basischem Protein und Proteolipid-Protein hervor und beinhaltet daher antigene Determinanten dieser beiden häufigsten Proteine in der Myelinschicht zentraler Axone. Subkutane Injektionen wurden statt intraperitonealen verwendet, weil nur jene auch für den klinischen Versuch zugelassen sind.

Der Effekt mehrerer solcher Immunisierungen auf die Antigen-spezifische Antwort von T-Zellen wurde hier erstmals detailliert untersucht. Die dazu verwendeten Methoden umfassten Proliferationsexperimente mit radioaktiv markiertem Thymidin und ELISPOT-Untersuchungen für einige ausgewählte Zytokine (IL-2, IL-4, IL-5, IFN-γ), die charakteristisch sind für Subtypen einer T-Zell-Antwort.

Es konnte gezeigt werden, dass eine subkutane Injektion von MP4 in einem Mineralöl als Adjuvans T-Zellen induziert, die IL-2 und teilweise auch IL-5 produzieren. Wieder-

holte Immunisierungen erhöhten die Zahl Antigen-spezifischer T-Zellen. Während die zweite und dritte Immunisierung die Produktion aller Zytokine gleichermaßen verstärkte, führte die vierte Immunisierung zu einem relativen Überwiegen der IL-5-produzierenden T-Zellen. Dies wird als Polarisierung in Richtung einer Typ-2-Antwort bezeichnet.

Es wurde untersucht, wie sich diese Vorimmunisierungen auf den Schutz der Mäuse vor einer anschließenden Induktion der Enzephalomyelitis auswirken. Eine subkutane Injektion von MP4 in dem Mineralöl führte zu einer Reduktion der klinischen Symptome der Erkrankung im Vergleich zu Tieren der Placebo-behandelten Kontrollgruppe. Eine viermalige Vorimmunisierung verstärkte diesen Effekt erheblich.

Mit Hilfe eines ELISA wurde die Auswirkung mehrmaliger Immunisierungen von MP4 in dem Mineralöl auf die Produktion von Antikörpern der Klasse IgG1 untersucht, welche für die Hypersensitivitätsreaktionen verantwortlich sind. Die wiederholte Verabreichung von Antigen führte zu einer deutlichen Steigerung der Konzentration dieser Antikörper im Serum. Dennoch wurde keinerlei allergische Reaktion beobachtet.

Zu anaphylaktischen Reaktionen kam es lediglich bei wiederholter Applikation eines Peptids in löslicher Form. Erfolgten die Injektionen hingegen in dem Mineralöl als Adjuvans, so blieben unerwünschte Reaktionen aus. Die Verwendung des Proteins MP4 erwies sich sogar bei Applikation in löslicher Form als ungefährlich. Diese Ergebnisse können so interpretiert werden, dass das langsame Entweichen des Antigens aus dem Adjuvans beziehungsweise die erhöhte Filterwirkung bei Immunkomplexen mit großen Proteinen verhindern, dass das Antigen systemisch hohe Konzentrationen erreicht. Trotz des Vorhandenseins von Antikörpern werden anaphylaktischer Reaktionen damit vermieden.

Zusammenfassend zeigte es sich, dass die wiederholte Immunisierung von Mäusen mit dem Fusionsprotein MP4 in einem Mineralöl als Adjuvans zu einer Polarisierung der spezifischen T-Zell-Antwort führt und einen erhöhten Schutz der Tiere vor der Induktion der Enzephalomyelitis bewirkt, und dass dieser therapeutische Nutzen von den möglichen Komplikationen der Therapie dissoziiert werden kann.

Die Ergebnisse der Arbeit könnten für die Durchführung zukünftiger tierexperimenteller und klinischer Studien bedeutsam sein, bei denen Immunisierungen mit einem Autoantigen vor dem Auftreten einer Autoimmunerkrankung schützen oder die Ausprägung der Symptome reduzieren sollen. Das verbesserte Verständnis der immunologischen Effekte solcher Immunisierungen und die Möglichkeit, therapeutische Effekte von unerwünschten Reaktionen zu trennen, könnten entsprechende Bemühungen wieder intensivieren.

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8 Curriculum vitae

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EDUCATION

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WORK EXPERIENCE

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06 to 08/2006	Clinic of internal medicine, University of Freiburg Subinternship. Endocrinology and Infectious Diseases
05 to 06/2006	Memorial Sloan-Kettering Cancer Center, New York City Elective. “Clinical Problems in the Treatment of Solid Tumors” Subinternship. “Clinical Medicine and Oncology“
11/2002 to 03/2004	Institute for Molecular Medicine and Cell Research, Univ. Freiburg Scientific assistant. Undergraduate biochemistry course

10/1999 to 03/2004	Clinic for tumor biology, Freiburg Scientific assistant. Database analysis and computer programming.
03/2004	Clinic of urology, University of Freiburg "Famulatur". Assistance in outpatient clinic
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04/2003	Clinic of cardiology, "Barmherzige Schwestern" hospital, Vienna "Famulatur". Assistance on ward and ICU

RESEARCH EXPERIENCE

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10/2001 to 03/2004	Laboratory of Christof v. Kalle, Institute for Molecular Medicine and Cell Research, University of Freiburg MD student. Independent study. Lentiviral gene transfer into hematopoietic stem cells after mobilization into peripheral blood.

PUBLICATIONS

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