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Myelin-specific T cells also recognize neuronal autoantigen in a transgenic mouse model of multiple sclerosis

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We describe here the paradoxical development of spontaneous experimental autoimmune encephalomyelitis (EAE) in transgenic mice expressing a myelin oligodendrocyte glycoprotein (MOG)-specific T cell antigen receptor (TCR) in the absence of MOG. We report that in *Mog*-deficient mice (*Mog*$^{-/-}$), the autoimmune response by transgenic T cells is redirected to a neuronal cytoskeletal self antigen, neurofilament-M (NF-M). Although components of radically different protein classes, the cross-reacting major histocompatibility complex I-A^b–restricted epitope sequences of MOG$_{35–55}$ and NF-M$_{18–30}$ share essential TCR contact positions. This pattern of cross-reaction is not specific to the transgenic TCR but is also commonly seen in MOG$_{35–55}$–I-A^b–reactive T cells. We propose that in the C57BL/6 mouse, MOG and NF-M response components add up to overcome the general resistance of this strain to experimental induction of autoimmunity. Similar cumulative responses against more than one autoantigen may have a role in spontaneously developing human autoimmune diseases.

Organ-specific autoimmune disease is a key group of inflammatory disorders that includes rheumatoid arthritis, type 1 diabetes mellitus, thyroiditis and multiple sclerosis. The prevailing thinking is that the pathogenic changes are typically initiated and driven by T cells, which express receptors for autoantigens restricted to, or enriched within, the particular target tissues.

Unfortunately, it has been impossible so far to identify, with certainty, which autoantigens are the targets in individual humans. One reason for this limitation is the complexity of the human autoimmune response. Indeed, there is evidence that in one person more than one self antigen may be the target of the autoimmune attack and that, in addition, the profile of target autoantigens may fluctuate over time[1]. Furthermore, the peripheral immune repertoire of healthy humans contains a large number of T cells specific for many, if not all, autoantigens potentially related to autoimmune diseases[2]. There is no practical assay to distinguish T cells with high pathogenic potential from nonpathogenic counterparts and, moreover, to identify in humans the T cells participating in the pathogenesis from those that are uninvolved.

We report here a new mechanism of autoimmunity, 'cumulative autoimmunity', that may provide a solution to this dilemma. Cumulative autoimmunity designates an autoimmune response that targets more than one particular cognate autoantigenic target at the same time, and the accumulation of these responses results in a tissue attack of enhanced vigor.

We observed a cumulative autoimmune response in transgenic mice with a TCR selected for reactivity to MOG peptide$_{35–55}$, who develop spontaneous EAE in the presence of MOG and, unexpectedly, also in its absence. We found that in *Mog*-deficient mice, the transgenic T cells recognize a peptide fragment of the medium-sized neurofilament NF-M.

RESULTS

Spontaneous EAE in the absence of MOG

In an experiment designed to detail the role of the autoantigen in spontaneous autoimmunity, we bred transgenes encoding the MOG-specific TCR 2D2 (ref. 3) and immunoglobulin heavy chain specific for MOG (IgH^{MOG}) (ref. 4) either separately or together into *Mog*$^{-/-}$ mice[5] (**Fig. 1**). To our surprise, spontaneous EAE developed in *Mog*-deficient 2D2 transgenic mice (2D2 × *Mog*$^{-/-}$) with incidence and kinetics indistinguishable from those of wild-type (WT) counterparts. Between 15% and 20% of 2D2 × *Mog*$^{-/-}$ mice developed spontaneous EAE (**Fig. 1a** and **Supplementary Table 1** online). *Mog*$^{-/-}$ mice and the *Mog*-deficient IgH^{MOG} mice (IgH^{MOG} × *Mog*$^{-/-}$), whose B cells, but not T cells, are specific for MOG, remained healthy (**Fig. 1a**

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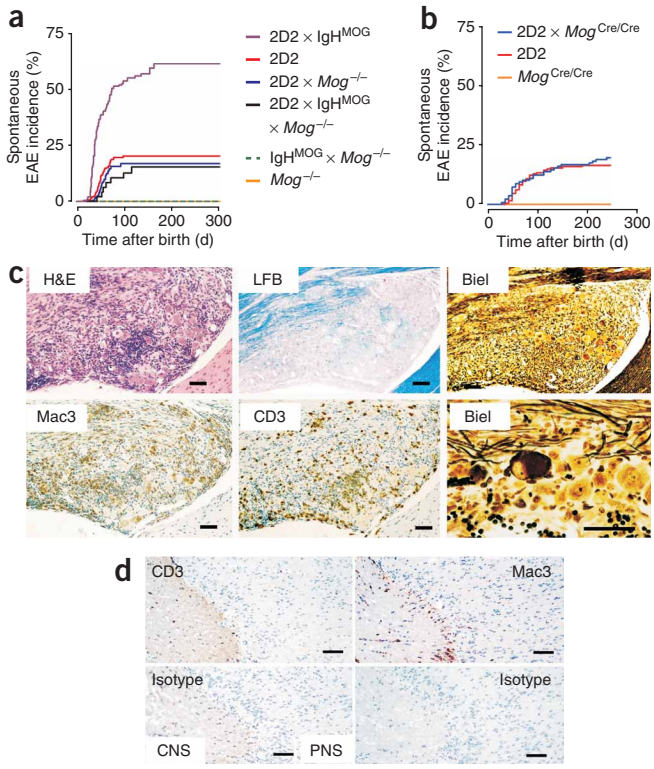


Figure 1 Paradoxical development of spontaneous EAE in MOG-specific 2D2 TCR–transgenic mice in two different *Mog*-deficient strains. (**a**) Spontaneous incidence of EAE-like disease observed in transgenic mice carrying MOG-specific TCR (2D2), B cell receptor (IgH^{MOG}) or both, on *Mog*-sufficient and *Mog*-deficient C57BL/6 backgrounds. Shown is the survival curve analysis of the mice that were observed for a minimum of 7 weeks after birth. 2D2, $n = 440$; 2D2 × $Mog^{-/-}$, $n = 218$; 2D2 × IgH^{MOG}, $n = 258$; 2D2 × IgH^{MOG} × $Mog^{-/-}$, $n = 48$; IgH^{MOG} × $Mog^{-/-}$, $n = 63$; $Mog^{-/-}$, $n = 279$. (**b**) Incidence of spontaneous EAE in MOG-specific 2D2–transgenic mice on a *Mog*-deficient background (different than in **a**). 2D2, $n = 199$; 2D2 × $Mog^{Cre/Cre}$, $n = 140$; $Mog^{Cre/Cre}$, $n = 23$. (**c**) Nervous system pathology of 2D2-transgenic mice with spontaneous EAE. Infiltration, demyelination and axonal damage in trigeminal ganglia were revealed by H&E, luxol fast blue (LFB) and Bielschowsky silver impregnation (Biel), respectively. The infiltrates are composed of macrophages (Mac3) as well as $CD3^+$ T cells. Scale bars, 100 μm. (**d**) Nervous system pathology of C57BL/6 mice immunized with $MOG_{35–55}$. The acute EAE lesions of CNS and PNS parts of the trigeminal nerve and ganglion were visualized by staining for macrophages (Mac3) and $CD3^+$ T cells. The bottom images show the respective isotype control antibody staining. Scale bars, 100 μm.

and **Supplementary Table 1**). Fifty percent of double-transgenic 2D2 × IgH^{MOG} mice (also known as OSE/Devic mice[6]) spontaneously develop opticospinal myelitis[3,6], but, in a limited cohort of MOG-deficient 2D2 × IgH^{MOG} mice, fewer than 15% of the mice developed spontaneous EAE, a proportion similar to the one seen with *Mog*-deficient 2D2 mice but substantially lower than that in 2D2 × IgH^{MOG} *Mog*-sufficient counterparts (**Fig. 1a** and **Supplementary Table 1**).

Clinically, spontaneous EAE was indistinguishable between *Mog*-sufficient and *Mog*-deficient transgenic mice. In all groups, disease started between 7 and 10 weeks of age, with classical paralytic EAE signs and, in a minority of cases, with a spastic component (**Supplementary Table 1** and **Supplementary Movies 1–6** online).

The lesions in *Mog*-sufficient and *Mog*-deficient groups were indistinguishable (data not shown), and they were restricted to the optic nerve and spinal cord[6,7]. In addition, we have now observed inflammatory infiltrates in the trigeminal ganglia, spinal ganglia, spinal roots and peripheral nerves, despite the absence of MOG within these tissues (**Fig. 1c** and **Supplementary Table 2** online). However, in mice immunized with $MOG_{35–55}$, the acute EAE lesions were present only in the central nervous system (CNS) and not in the peripheral nervous system (PNS; **Fig. 1d**).

2D2-transgenic T cells recognize a non-MOG CNS autoantigen

EAE in the $Mog^{-/-}$ mice might be explained by the incomplete deletion of *Mog*. The *Mog* knockout strain initially used in this study was created by the insertion of a cassette containing the LacZ and neomycin resistance genes behind the *Mog* promoter, leaving the MOG coding sequence intact[5], which could leave some aberrant MOG expression in 2D2 × $Mog^{-/-}$ mice. However, in line with the original description of the $Mog^{-/-}$ mice[5], western blot analyses with monoclonal as well as polyclonal antibodies did not detect any residual MOG protein expression in $Mog^{-/-}$ mice (**Supplementary Fig. 1** online). To further exclude faulty MOG expression, we bred 2D2 TCR–transgenic mice with another line of *Mog*-knockout mice in which the 5′ end of MOG exon 2 encoding the immunodominant $MOG_{35–55}$ epitope was deleted and replaced by the *Cre* recombinase gene ($Mog^{Cre/Cre}$)[8] (**Supplementary Fig. 2** online). 2D2-transgenic mice crossed onto the $Mog^{Cre/Cre}$ background also developed spontaneous EAE at the same rate as 2D2-transgenic mice (**Fig. 1b**).

Spontaneous EAE in 2D2 × $Mog^{-/-}$ mice could also have been caused by T cells recruited from the endogenous repertoire or by T cells with dual TCR expression—expressing both 2D2 and endogenous receptor chains. Indeed, whereas in 2D2-transgenic mice most $CD4^+$ T cells use the transgenic TCR, there is also a considerable population with endogenous receptors (data not shown). In the absence of MOG, alternative TCRs might be stimulated to mount an attack against an alternative CNS target autoantigen. However, FACS analysis of 2D2 and 2D2 × $Mog^{-/-}$ thymus and spleen did not reveal any considerable differences in cell number or in activation markers such as CD25, CD44 and CD62 ligand (**Supplementary Fig. 3** online) or MOG-specific forkhead box P3–positive T regulatory cells (**Supplementary Fig. 4** online). In addition, the CNS infiltrates were predominantly composed of transgenic T cells with only a minor population of endogenous T cells (**Supplementary Fig. 5** online). Furthermore, we noted spontaneous EAE in two out of six triple-transgenic *Rag2*-deficient 2D2 × $Mog^{-/-}$ mice (2D2 × $Mog^{-/-}$ × $Rag2^{-/-}$), whose T cells express exclusively the transgenic TCR, indicating that transgenic T cells, not endogenous T cells, are the principal agents in the observed EAE.

Finally, the transgenic TCR might recognize an endogenous cross-reactive epitope. We tested some of the known encephalitogenic proteins and peptides such as myelin basic protein (MBP), S-100 calcium-binding protein, beta chain (S100β) and proteolipid protein (PLP) amino acids 139–151, but none of them activated the 2D2-expressing T cells (**Fig. 2a**). Then we compared crude myelin preparations from $Mog^{+/+}$ and $Mog^{-/-}$ CNS isolated by classical protocols[9,10]. Both preparations activated MOG-specific 2D2-expressing cells to a comparable degree when presented by syngeneic bone marrow–derived dendritic cells (BMDCs) (**Fig. 2a**) but did not stimulate ovalbumin-specific OT-II TCR transgenic T cells, which we used as negative controls. Furthermore, myelin-induced proliferation of 2D2-transgenic T cells was blocked by antibodies to CD4 and major histocompatibility complex (MHC) class II but not by antibody to CD1d or control rat antibodies (**Fig. 2b**).

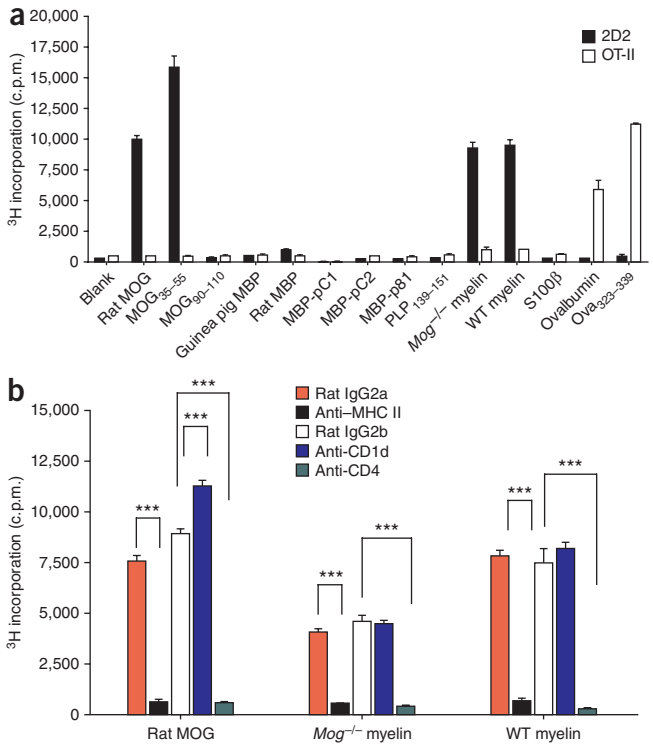


Figure 2 MOG-specific T cells respond to myelin from *Mog*$^{-/-}$ mice. (**a**) Proliferation, as measured by ^{3}H-thymidine incorporation assay, of spleen cells from 2D2-transgenic or control OT-II mice (2×10^5 cells per well) together with BMDCs (5×10^4 cells per well) cultured with the indicated proteins, peptides (20 μg ml^{-1}) or myelin preparations (1 μl per well) from *Mog*$^{-/-}$ and WT mice. pC1, rat $MBP_{68–84}$; pC2, guinea pig $MBP_{45–67}$; p81, guinea pig $MBP_{69–83}$. (**b**) Proliferation, as measured by ^{3}H-thymidine incorporation assay, of 2D2-transgenic spleen cells together with BMDCs incubated with rat MOG (20 μg ml^{-1}) or myelin suspension (1 μl per well) preparations from *Mog*$^{-/-}$ and WT mice. The antibodies to MHC II (Anti-MHC II), CD1d (Anti-CD1d) or CD4 (Anti-CD4) or control rat IgG2a and rat IgG2b antibodies were added at 10 μg ml^{-1}. Proliferation of T cells from **a** and **b** was measured by labeling with ^{3}H-thymidine during the last 16 h of a 72-h assay. Shown is the mean ± s.e.m. of triplicate measurements. Statistical significance was analyzed by analysis of variance. ***$P < 0.001$.

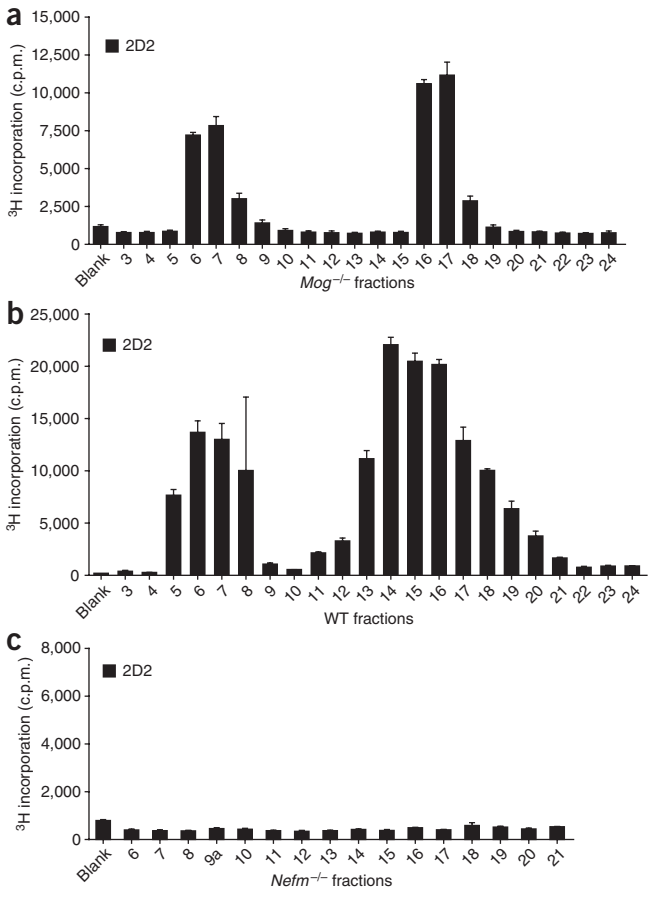
NF-M peptide cross-reacts with MOG-specific T cells

We systematically fractionated CNS tissue from *Mog*$^{-/-}$ mice (**Fig. 3**). We employed a purification regime composed of homogenization, lipid extraction and purification of the urea-dissolved proteins by ion-exchange and gel chromatography (**Supplementary Fig. 6** online). Among the chromatography fractions presented to 2D2-expressing T cells by syngeneic antigen-presenting cells (BMDCs), we eluted several antigenic fractions from the anion-exchange column (**Fig. 3a**). Fractionation of *Mog*-sufficient CNS tissue led to a similar profile (**Fig. 3b**). Parallel fractionation of extracts from WT and *Mog*$^{-/-}$ CNS with the cation exchange column yielded similar results (**Supplementary Fig. 7a,b** online).

Gel filtration chromatography of both pooled positive fractions (fractions 16 and 17) from the anion exchange column resulted in one fraction (fraction 9) that was recognized by 2D2-expressing T cells (**Fig. 4a**). SDS-PAGE analysis revealed two prominent bands with molecular masses of approximately 68 kDa and 150 kDa (**Fig. 4b**). We excised both bands from the gel, in-gel digested them with trypsin and subjected them to matrix-assisted laser desorption-ionization–time-of-flight mass spectrometry. We identified the 68-kDa protein as the light chain of mouse neurofilament (NF-L) and the 150-kDa protein as NF-M.

Figure 3 Fractionation of CNS proteins from *Mog*$^{-/-}$, WT and *Nefm*$^{-/-}$ mice. (**a**–**c**) Proliferation, as measured by ^{3}H-thymidine incorporation assay, of 2D2 spleen cells in response to anion exchange column fractions of CNS tissue extracts from *Mog*$^{-/-}$ (**a**), WT C57BL/6 (**b**) and *Nefm*$^{-/-}$ (**c**) mice. ^{3}H-thymidine incorporation was measured during the last 16 h of the 72-h assay. Shown is the mean ± s.e.m. of triplicate measurements. Shown is a representative of two individual protein purifications (with different pools of *Mog*$^{-/-}$ mice, WT mice or *Nefm*$^{-/-}$ mice) and stimulation experiments. The narrow elution profile of the *Mog*$^{-/-}$ CNS extract is due to the use of Mono Q column material instead of Source Q material, which was used for WT and *Nefm*$^{-/-}$ experiments.

We tested the exclusive cross-reactivity of the NF-M with MOG-specific 2D2-transgenic T cells by an analysis of CNS proteins from NF-M–deficient (*Nefm*$^{-/-}$) mice. None of the ion-exchange chromatography fractions from *Nefm*$^{-/-}$ mice activated 2D2-transgenic T cells (**Fig. 3c** and **Supplementary Fig. 7c**). In addition, we also found that the crude myelin preparations from both *Mog*$^{-/-}$ and WT mice that contained abundant amounts of NF-M (**Supplementary Fig. 8a** online), and not those from the *Mog*$^{-/-}$ × *Nefm*$^{-/-}$ mice, activated 2D2-transgenic T cells (**Fig. 2a** and **Supplementary Fig. 9** online). Full-length MOG, which is highly hydrophobic owing to its three membrane-spanning helices, was found mainly in the urea-insoluble fraction (data not shown). We detected only trace amounts of MOG in the urea-soluble extract but not in any of the 2D2-transgenic T cell–activating fractions from *Mog*-sufficient mice (**Supplementary**



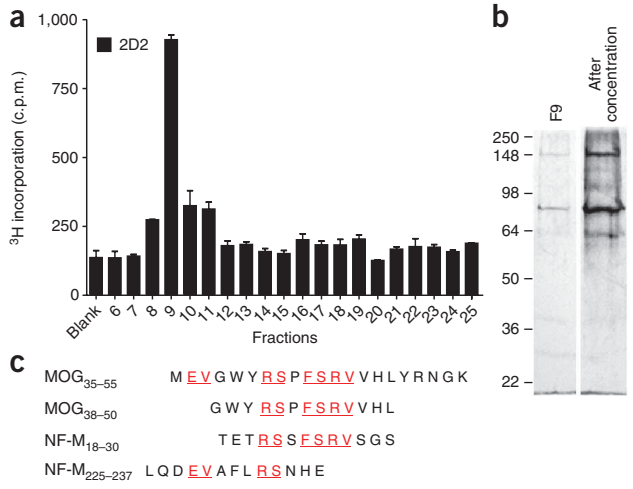


Figure 4 Identification of a protein that cross-reacts with MOG-specific 2D2–transgenic T cells. (**a**) Proliferation, as measured by ^{3}H-thymidine incorporation assay, of 2D2 spleen cells in response to the T cell–activating fractions (fractions 16 and 17 from **Fig. 3a**) from *Mog*$^{-/-}$ mice. The fractions were pooled and further separated by gel filtration chromatography. Proliferation was measured during the last 16 h of the 72-h assay. Shown is the mean ± s.e.m. of triplicate measurements. (**b**) Silver staining of a 2D2 T cell-activating fraction from *Mog*$^{-/-}$ mice. T cell-activating fraction (fraction 9) from several similar gel filtrations were pooled and concentrated by re-chromatography with a Mono Q column. These fractions were resolved on a 10% Tris-glycine gel and stained by silver staining. F9, gel filtration fraction before concentration. (**c**) Amino acid alignment of the immunodominant epitopes of MOG and NF-M. MOG$_{35–55}$ and the minimal epitope MOG$_{38–50}$ were aligned with NF-M. Shown in red and underlined is the amino acid identity of MOG and NF-M peptides.

Fig. 8b). Hence, MOG is lost quantitatively during lipid extraction and urea solubilization, and these data exclude the presence of additional cross-reactive antigens, at least in the urea-soluble fraction we examined.

An *in silico* search identified a seven–amino acid peptide of NF-M nearly identical to the core region of the antigenic peptide MOG$_{38–50}$ (**Fig. 4c**), which spans from Tyr40 to Val47 and contains the amino acids Arg41, Phe44, Arg46 and Val47, which are known to be the crucial contact amino acids for the 2D2 TCR and other MOG-specific T cell lines[11,12]. These amino acids are completely preserved at identical positions in NF-M$_{18–30}$; that is, positions Arg21, Phe24, Arg26 and Val27. Another candidate peptide, NF-M$_{225–237}$, also showed some homology to MOG but lacked the essential residues of the core region (**Fig. 4c**).

2D2 T cells responded vigorously to the synthetic peptide NF-M$_{18–30}$ but not to NF-M$_{225–237}$ (**Fig. 5a**). In addition, NF-M$_{18–30}$ induced strong proliferation of 2D2 × *Rag2*$^{-/-}$ transgenic T cells, indicating that the cross-reactivity is intrinsic to the transgenic 2D2 TCR (**Fig. 5b**). This cross-reactivity is not limited to the synthetic MOG and NF-M peptides. 2D2 T cells also recognized the naturally processed recombinant MOG and NF-M proteins produced in *E. coli* (**Fig. 5a,b**). Notably, NF-M and MOG peptides mixed in a 1:1 ratio induced proliferation and cytokine secretion of 2D2-expressing T cells isolated either from the spleen or from the CNS of mice with spontaneous EAE without any signs of tolerance or anergy (**Fig. 5a** and **Supplementary Fig. 10** online).

2D2-transgenic T cell responses to MOG and NF-M peptides were similar but not identical. In dose-dependent proliferation tests, NF-M$_{18–30}$ peptide was superior to MOG$_{35–55}$ and MOG$_{38–50}$ peptides in inducing proliferation (**Fig. 5a,b**). We confirmed this finding in cytokine assays, in which NF-M stimulated larger interferon-γ (IFN-γ), interleukin-17 (IL-17), IL-2 and IL-10 releases than did MOG (**Fig. 5c**).

To clarify whether the cross-reactivity of 2D2-expressing T cells with NF-M reflects a 'private' clonotypic response (a response by a single T-cell clone) or represents a more general cross-reactivity between MOG$_{35–55}$- and NF-M–specific T cells, we isolated fresh MOG$_{35–55}$-specific T cells from C57BL/6 mice, expanded them and tested them for reactivity with NF-M. Indeed, a polyclonal MOG$_{35–55}$-specific

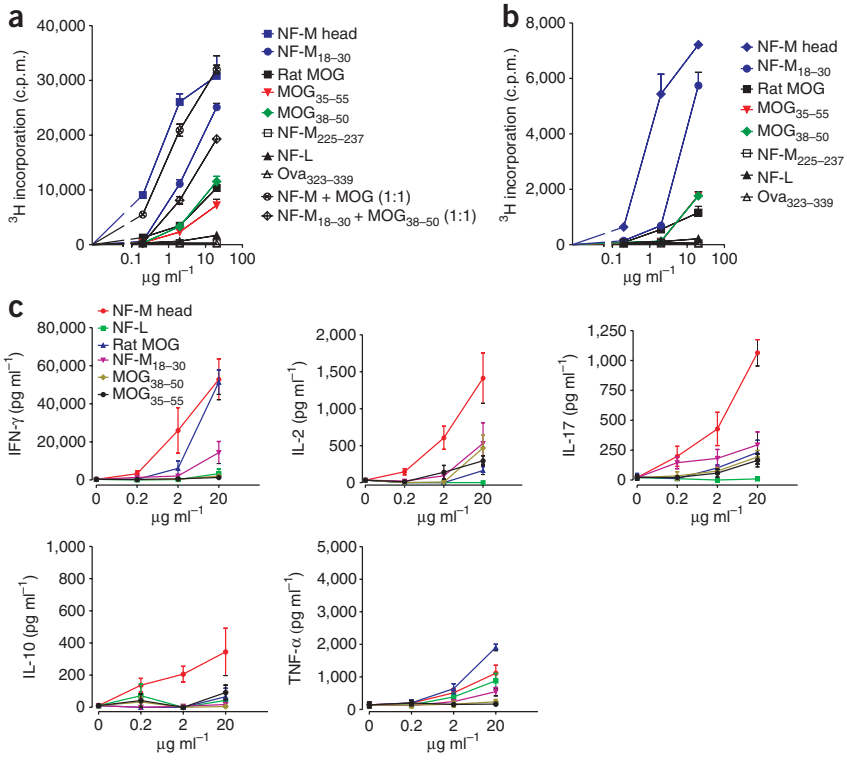


Figure 5 NF-M reacts specifically with 2D2-transgenic T cells. (**a**) Proliferation, as measured by ^{3}H-thymidine incorporation assay, of 2D2 splenocytes cultured with increasing concentrations of the indicated proteins, peptides or mixtures. Shown is a representative of more than three individual experiments consisting of more than six mice. (**b**) Proliferation, as measured by ^{3}H-thymidine incorporation assay, of splenocytes from 2D2 × *Rag2*$^{-/-}$ mice cultured with the indicated proteins and peptides. Means ± s.e.m. of triplicate measurements are shown. (**c**) Quantification of cytokines released by MOG-specific 2D2-transgenic T cells in response to cross-reactive NF-M peptide and protein. 2D2 splenocytes were cultured for 3 d with the indicated peptides and proteins in a dose-dependent fashion. The concentrations of the cytokines secreted by the T cells were measured in the supernatants in a sandwich ELISA composed of specific antibody pairs. The data were combined from three independent experiments. Each data point represents two to seven mice per group. Means ± s.e.m. are shown.

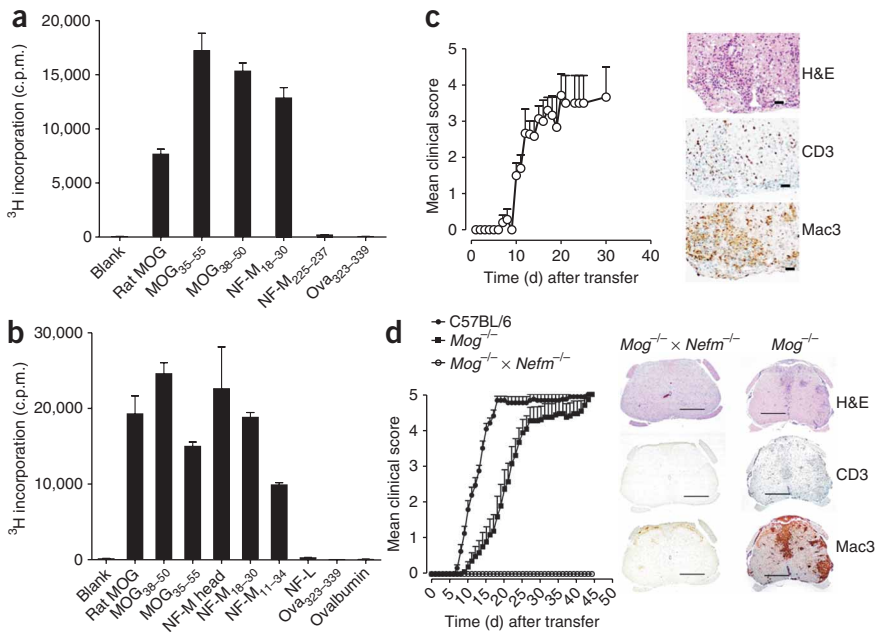


Figure 6 *In vitro* and *in vivo* cross-reactivity between NF-M– and MOG-specific T cells. (**a**,**b**) Proliferation, as measured by ^{3}H-thymidine incorporation assay, of MOG- and NF-M–specific T cell lines. A $MOG_{35–55}$-specific T cell line (**a**) and NF-M–specific T cell line (**b**) generated from WT mice primed with its respective antigen were tested with the indicated protein and peptides (20 μg ml^{-1}). The proliferation was measured during last 16 h of the 72-h assay. Shown is the mean ± s.e.m. of triplicate measurements. (**c**) Clinical course of EAE induced by the NF-M–specific T-cell line. Naive WT C57BL/6 mice were lightly irradiated (400 rad) and injected intravenously with 12×10^6 ($n = 3$ mice) or 6×10^6 ($n = 7$ mice) activated T cells specific for $NF\text{-}M_{15–35}$ peptide. The T-cell line was derived from $NF\text{-}M_{15–35}$–immunized WT C57BL/6 mice. Shown is the mean clinical score of mice from three experiments. Histopathology analysis of spinal cords (right) shows typical EAE pathology, with inflammatory infiltrates comprised of polymorphonuclear cells, T cells and macrophages or activated microglia. Scale bars, 100 μm. (**d**) EAE induced by 2D2 T cells in WT C57BL/6 and *Mog*$^{-/-}$ but not in *Mog*$^{-/-}$ × *Nefm*$^{-/-}$ double-knockout mice. We transferred 15×10^6 2D2 *Rag2*$^{-/-}$ T helper type 1 cells into lightly irradiated (300 rad) syngenic WT ($n = 15$), *Mog*$^{-/-}$ ($n = 15$) and *Mog*$^{-/-}$ × *Nefm*$^{-/-}$ double-knockout ($n = 9$) C57BL/6 mice. Left, EAE clinical score of mice from three (WT, *Mog*$^{-/-}$) or two (*Mog*$^{-/-}$ × *Nefm*$^{-/-}$) independent experiments. EAE frequency is significantly lower in double-knockout recipients as compared to either of the two other groups (X^2; $P < 1 \times 10^{-6}$). Kinetics of EAE differs significantly (Log-rank test; $P < 10^{-4}$) between WT and *Mog*$^{-/-}$ mice. Data represent mean ± s.e.m. Right, representative images comprising inflammatory infiltrates in the spinal cord of *Mog*$^{-/-}$ and *Mog*$^{-/-}$ × *Nefm*$^{-/-}$ mice revealed by H&E, CD3 and Mac3 staining. Scale bars, 1 mm.

T cell line, which used V_α and V_β regions distinct from the 2D2 TCR (**Supplementary Fig. 11a** online), readily responded to $NF\text{-}M_{18–30}$ but not to $NF\text{-}M_{225–237}$ or ovalbumin (Ova) amino acids 323–339 (**Fig. 6a**). Conversely, T cell lines from NF-M–immunized mice, also with V_α and V_β gene segments different from those in the 2D2 TCR (**Supplementary Fig. 11b**), cross-reacted with MOG protein and peptides (**Fig. 6b**).

In vivo recognition of NF-M by MOG-specific T cells

To determine whether 2D2-transgenic T cells find their alternative target (that is, NF-M) *in vivo* during EAE, we transferred MOG peptide–activated, 2D2-transgenic CD4$^+$ T cells to *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$ × *Mog*$^{-/-}$ mice. Whereas both recipient groups developed EAE, in *Mog*-deficient *Rag2*$^{-/-}$ mice the disease was delayed (**Supplementary Fig. 12** online).

We next examined whether NF-M–activated 2D2-T cells can also induce EAE. NF-M–activated 2D2-expressing T cells triggered EAE in WT hosts, which by incidence and kinetics was comparable to EAE caused by MOG-activated 2D2-expressing T cells (**Supplementary Fig. 13a,b** online). To assess the impact of soluble MOG peptide tolerization, we transferred *in vitro* MOG peptide–activated 2D2-transgenic T cells into irradiated WT or *Mog*$^{-/-}$ mice and injected $Ova_{323–339}$ or $MOG_{35–55}$ peptides intravenously. We observed that treatment with MOG peptide delayed the onset of EAE in WT C57BL/6 recipients and, more notably, also in *Mog*$^{-/-}$ mice (**Supplementary Fig. 13a**). Similarly, MOG peptide tolerization lowered the encephalitogenic activity of 2D2-expressing T cells activated *in vitro* with NF-M peptide (**Supplementary Fig. 13b**).

Furthermore, we tested the encephalitogenic potential of 'genuine' NF-M–specific T cell lines, that is, CD4$^+$ T cells isolated from NF-M–primed C57BL/6 mice and propagated by serial NF-M–specific activation. T cells from an NF-M–specific line transferred to C57BL/6 mice induced severe EAE (**Fig. 6c**). The pathology in these mice was closely similar to that seen after transfer of 2D2-expressing cells in MOG-deficient mice. Lesions were most pronounced in the spinal cord and featured inflammation by T cells and macrophages (**Fig. 6c**), confluent demyelination and severe axonal loss (data not shown). We transferred NF-M peptide–activated transgenic T cells derived from 2D2 × *Rag2*$^{-/-}$ mice into both *Mog*$^{-/-}$ and WT mice. These monoclonal 2D2 × *Rag2*$^{-/-}$ T cells induced EAE in *Mog*$^{-/-}$ mice, albeit with a delayed onset as compared to WT recipients, confirming the autonomous capability of 2D2-expressing T cells to recognize the alternative target, NF-M (**Fig. 6d**).

The lesions developing in *Mog*$^{-/-}$ recipient mice injected with 2D2 × *Rag2*$^{-/-}$ T cells were severe, with large infiltrates in the spinal cord and cerebellum (**Fig. 6d**) and, remarkably, also in the trigeminal ganglia and in peripheral nerves (data now shown). These findings are reminiscent of the pathology seen in the spontaneous EAE of 2D2 × *Mog*$^{-/-}$ mice.

To confirm that the 2D2-transgenic T cell–mediated lesions in the *Mog*$^{-/-}$ recipient mice are due to *in vivo* recognition of NF-M, we transferred activated 2D2 × *Rag2*$^{-/-}$ T cells into *Mog*$^{-/-}$ × *Nefm*$^{-/-}$ double-knockout mice. None of the double-knockout mice showed any clinical signs of EAE, and there were no lesions in the CNS (**Fig. 6d**).

DISCUSSION

C57BL/6 mice are resistant to the induction of most T cell–mediated organ-specific autoimmune diseases. In these mice, immunization with classical autoantigens elicits vigorous T cell responses but commonly fails to produce a clinical autoimmune disease. In contrast, immunization with $MOG_{35–55}$ peptide reliably induces clinical EAE, thus overcoming resistance of C57BL/6 mice[13]. Our observations may provide an unexpected clue explaining the unusual autoimmune potential of $MOG_{35–55}$. We show that CD4$^+$ T cells selected from C57BL/6 mice for reactivity to $MOG_{35–55}$ also respond to an epitope

of the medium-sized neurofilament, NF-M. We propose that the combined response to the two target structures may overcome the innate resistance of C57BL/6 mice to autoimmune diseases.

It has previously been reported that the 2D2-transgenic C57BL/6 mice that harbor large populations of MOG-specific $CD4^+$ T cells tend to spontaneously develop optic neuritis and EAE[3]. This trend is markedly increased in the presence of MOG-specific transgenic B lymphocytes[6,7]. Paradoxically, however, as reported here, 2D2 mice develop spontaneous EAE also in the absence of MOG, the primary encephalitogenic target.

This was discovered in mice with disrupted exon 1 of the *Mog* gene[5], and it was confirmed in another cohort of *Mog*-deficient 2D2-transgenic mice, whose *Mog* gene was deleted by an independent knock-in strategy[8], excluding residual, atypical MOG material in these knockout animals as a possible encephalitogenic target.

We fractionated CNS tissue from *Mog*-knockout mice and found material that was presented to and recognized by 2D2-transgenic T cells. We identified the autoantigenic component, by classical biochemical methods and subsequent mass spectrometry, as NF-M. The salient target epitope of NF-M was finally determined by an *in silico* search for sequences related to the $MOG_{35–55}$ motif recognized by the 2D2 clone[11]. Given the marked degeneracy of peptide recognition by T cells[14], cross-reaction of NF-M by 2D2 at the peptide level may not be very unexpected. However, less trivially, we confirmed NF-M as the stimulatory autoantigen at the protein level using both CNS white matter protein extracts and recombinant proteins.

Neurofilaments, including NF-M, are produced by neurons and also by some glial cells[15]. They were characterized recently as autoantigens in actively induced EAE and as possible targets in multiple sclerosis, too. Immunization of Biozzi ABH (antibody high, AB/H, ABH) mice with the light form of neurofilament, NF-L, causes EAE in a moderate proportion of treated animals[16]. Also, autoantibodies to NF-M have been detected in the cerebrospinal fluid of some individuals with multiple sclerosis[17].

The $CD4^+$ T cell repertoire of 2D2-transgenic mice is dominated by the transgenic, MOG-reactive TCR but is by no means monoclonal. The NF-M–specific response could have been effected either by T cells from the residual endogenous repertoire or by T cells that escaped allelic exclusion and use endogenous TCR chains along with the transgenic one. We ruled out both possibilities, as 2D2-expressing T cells from *Rag2*-knockout mice showed a similar heteroclitic (stronger) cross-recognition of NF-M *in vitro* and *in vivo*, indistinguishable from their *Rag2*-sufficient counterparts. Furthermore, we observed spontaneous EAE in 2D2 × $Rag2^{-/-}$ × $Mog^{-/-}$ mice, suggesting the autonomous role of transgenic T cells in the cross-recognition.

EAE was readily mediated by T cell lines selected for reactivity to either MOG (2D2) or NF-M and by 2D2-expressing T cells activated by NF-M. In contrast, we were unable to induce disease by immunization with NF-M using protocols that allow active disease induction by $MOG_{35–55}$ (data not shown). This discrepancy between active and passive EAE induction is, however, not exceptional. It has been previously described for other models, including EAE induced in Lewis rats by glial fibrillary acidic protein[18] and S100-β[19] and MBP-induced EAE in BALB/c mice[20].

Autoimmune cross-reactivity between MOG and NF-M has been discovered and analyzed in one clonal model, 2D2-transgenic T cells, but has been confirmed in other I-Ab–restricted MOG- and NF-M–specific $CD4^+$ T cells. MOG- or NF-M–primed polyclonal T cell populations isolated from WT C57BL/6 mice show extensive cross-reactivity between NF-M and MOG proteins and their salient epitopes, respectively. Of note, these populations rarely use $V_{\alpha 3.2}$ and $V_{\beta 11}$, the variable chains used by the 2D2 clone, indicating that MOG and NF-M cross-reactivity is not limited to the 2D2 TCR.

Our *in vitro* results formally establish the cross-reactivity of 2D2 and other $MOG_{35–55}$ peptide–specific T cells with NF-M, but do these T cells respond to NF-M *in vivo*, and might there be additional cross-recognized autoantigens? *In vivo* NF-M–specific responses are suggested by several lines of evidence. We found lesions in trigeminal and spinal ganglia, tissues which even in WT mice are devoid of MOG autoantigen but contain NF-M. Furthermore, 2D2 × $Rag2^{-/-}$ T cells transferred into $Mog^{-/-}$ × $Nefm^{-/-}$ double-knockout recipients failed to develop EAE. This latter observation, together with the loss of autoantigenic potential of myelin from double-knockout white matter, also rules out unknown autoantigens acting in addition to MOG and NF-M.

How would cross-reactive T cells respond to the simultaneous presence of both MOG and NF-M? Would the response components add up or would there be tolerization? Several observations suggest that $MOG_{35–55}$-specific T cells respond both to the MOG epitopes as well as to the cross-reactive NF-M epitopes at the same time. *In vitro*, T cells isolated from CNS and spleen respond to both antigens in an additive fashion. *In vivo*, transfer of activated 2D2-expressing T cells caused substantially earlier appearance of EAE in *Mog*-sufficient mice compared to $Mog^{-/-}$ mice. Also, the clinical picture of *Mog*-sufficient and *Mog*-deficient 2D2-transgenic mice is very similar. However, a selective anti-MOG response component seems to prevail in transgenic mice with transgenic TCRs plus B cell receptors. In the presence of MOG, in T-B double-transgenic mice the incidence of spontaneous EAE rises to rates of 50% and more. In the absence of MOG, these double-transgenic mice develop EAE at proportions similar to their single-TCR transgenic counterparts. The MOG-dependent elevation of spontaneous EAE frequency, noted in double-transgenic 2D2 × IgH^{MOG} mice[6], is not seen in the absence of MOG.

To our knowledge, this is the first description of immunological self-mimicry, that is, the response of one T cell population to two independent target autoantigens in the same tissue, MOG and NF-M. Is such a response an exception to the norm or is it common? As mentioned, the MOG and NF-M response does not seem to be unique to the MOG-reactive 2D2 clone studied here but is also noted in polyclonal MOG- and NF-M–reactive T cell populations from C57BL/6 mice. Furthermore, another case of self mimicry, though between structures from different tissues was reported by another group, who described in the Dark Agouti (DA) rat cross-reaction between MOG-specific T cells and an epitope of the milk protein butyrophilin[21].

The dual response of T cells against two target autoantigens expressed within the same target tissue could have major implications for organ-specific autoimmune disease. It could have an additive role in determinant spreading (development of an immune response distinct from the initial disease-causing epitope) in the course of an autoimmune response. Beyond this, we propose that in C57BL/6 mice autoimmune response components directed against MOG and NF-M may accumulate to overcome the general resistance of these mice to induction of EAE. T cells with similar cumulative double self-reactivity could act as dominant pathogens in human multiple sclerosis, and genetic factors favoring bireactive T cells would enhance susceptibility to the disease. This study should provide a way to identify such T cells in humans and appreciate their role in the pathogenesis of multiple sclerosis.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

G.K. performed most of the experiments. G.K. and H.W. designed the study and wrote the manuscript with input from co-authors. A.S., L.T.M. and R.S.L. contributed EAE and T cell data. K.D. supervised protein purification and mass spectrometry and performed *in silico* searches. R.M. performed mass spectrometry. H.S.D. assisted in EAE experiments. A.B.-N. performed T cell line transfer EAE experiments. H.L. performed and interpreted histology. F.C.K. designed experiments and performed protein purification.

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ONLINE METHODS

Transgenic mice. We bred MOG-specific TCR transgenic mice (2D2)[3] and B cell knock-in IgH^{MOG} (also known as Th)[4] on a C57BL/6 background into $Mog^{-/-}$ mice[5] to obtain 2D2 × $Mog^{-/-}$ and IgH^{MOG} × $Mog^{-/-}$ and 2D2 × IgH^{MOG} × $Mog^{-/-}$ mice. We obtained WT C57BL/6 mice from the animal facility of the Max Planck Institute of Biochemistry. We bred $Rag2^{-/-}$ mice and OT-II mice (Jackson Laboratories) in the conventional animal facilities along with other transgenic mice. We bred a second $Mog^{-/-}$ strain ($Mog^{Cre/Cre}$) harboring the insertion of the gene encoding Cre recombinase in the first exon of *Mog*[8] with the 2D2-transgenic mice in the specific pathogen–free animal facility of Institut Fédératif de Recherche (IFR30). We also maintained $Nefm^{-/-}$ mice[22] in the specific pathogen–free animal facility of IFR30.

We routinely monitored a cohort of transgenic mice of the above genotypes at least one or two times a week for clinical EAE signs. The EAE disease scores were according to the classic scale[6]. To determine other neurological abnormalities, we lifted the mice by their tail and allowed them to grab the grid of a cage by their front limbs (see **Supplementary Movies 1–6**). We noted the clasping and hyperextension of hind limbs within 10–30 s of holding them by the tail.

All animal procedures were in accordance with guidelines of the Committee on Animals of the Max Planck Institute of Neurobiology or the Midi-Pyrénées Ethic Committee on Animal Experimentation and with the license of the Regierung von Oberbayern (or from the French Ministry of Agriculture).

Peptides and proteins. Mouse $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK), mouse $MOG_{38–50}$ (GWYRSPFSRVVHL), mouse NF-$M_{18–30}$ (TETRSSFSRVSGS), mouse $PLP_{139–151}$ (HSLGKWLGHPDKF), $Ova_{323–339}$ (ISQAVHAAHAEINEAGR), mouse $MOG_{90–110}$ (SDEGGYTCFFRDHSYQEEAA), rat MBP pC1 ($MBP_{68–84}$) (HYGSLPQKSPRSQDENPV), guinea pig MBP pC2 ($MBP_{45–67}$) (GSDRAAP KRGSGKDSHHAARTT) or guinea pig MBP p81 ($MBP_{69–83}$) (YGSLPQKSQR SQDEN) were synthesized either by BioTrend or by the core facility of Max Planck Institute of Biochemistry. We obtained mouse NF-$M_{225–237}$ (LQDEVA FLRSNHE) from Metabion. We purified the peptides by HPLC to >95% purity and analyzed them by mass spectrometry.

We purified recombinant soluble rat MOG protein ($MOG_{1–125}$)[23], mouse full-length NF-L and mouse head domain fragment of NF-M (NF-M 'head'; NF-$M_{1–102}$) (**Supplementary Methods** online) from bacterial inclusion bodies. We purchased S100β and ovalbumin from Sigma. We purified guinea pig MBP and rat MBP using standard protocols.

Histology. We perfused mice with 4% paraformaldehyde in PBS and stored them in the same fixative for 24 h. We stained adjacent serial sections of CNS and PNS with H&E, luxol fast blue or Bielschowsky silver impregnation. We also stained some sections with CD3-specific (Serotec) and Mac3-specific (BD Biosciences) antibodies. We stained adjacent sections with the respective isotype controls.

Adoptive transfer EAE. For 2D2 × $Rag2^{-/-}$ T cell transfer, we purified $CD4^+$ T cells from 2D2 × $Rag2^{-/-}$ mice and stimulated them *in vitro* with 20 μg ml^{-1} of NF-$M_{15–35}$ peptide in the presence of 20 ng ml^{-1} IL-12 and 1 ng ml^{-1} IL-2 (both from R&D Systems) and irradiated syngeneic splenocytes. On day 6, we re-stimulated viable cells with splenocytes and 20 μg ml^{-1} of NF-$M_{15–35}$ in the presence of 20 μg ml^{-1} IL-12 and 1 μg ml^{-1} IL-2. On day 9, we injected Ficoll-purified T helper type 1 cells into lightly irradiated (300 rad) syngeneic recipients.

Myelin purification. We purified crude myelin from $Mog^{-/-}$, WT C57BL/6 or $Mog^{-/-}$ × $Nefm^{-/-}$ CNS tissues according to previously published protocols[9,10]. Briefly, we pooled brain and spinal cord and homogenized it in 0.32 M sucrose in 10 mM Tris HCl, pH 7.4. Then we centrifuged at 15,000*g* and washed the pellet twice with 0.32 M sucrose solution. Finally, we suspended the pellet in 0.32 M sucrose and overlaid on to 0.85 M sucrose and centrifuged at 26,000*g*. We collected the myelin at the interface, washed it twice and suspended it in 1 ml of sterile PBS.

Proliferation assay. For the analysis of fractions from biochemical separations, we mixed spleen cells from 2D2 or OT-II mice (2×10^5 cells) with LPS-activated BMDCs from WT C57BL/6 mice (5×10^4 cells) together with 1 in 50-diluted fractions.

Unless otherwise mentioned, in all other T cell proliferation experiments, we cultured 2×10^5 spleen cells with 20 μg ml^{-1} peptides and proteins. We performed all proliferation assays in triplicate. We measured the T cell proliferation by the incorporation of ^3H-labeled thymidine during the last 6 h of a 48-h culture or the last 16 h of a 72-h culture.

Enzyme-linked immunosorbent assay. We assayed cell culture supernatants with antibody pairs or kits for IFN-γ, IL-2 (both from BD Biosciences), TNF-α (Peprotech), IL-10 (R&D Systems) or IL-17 (eBioscience) according to the manufacturer's instructions.

T cell lines. We established antigen-specific T cell lines from C57BL/6 mice immunized with $MOG_{35–55}$, NF-$M_{15–35}$ or NF-M 'head' in complete Freund's adjuvant supplemented with 5 mg ml^{-1} *Mycobacterium tuberculosis* (strain H37Ra) using established protocols. We collected spleen and draining lymph nodes 10–12 d after immunization and stimulated them with respective antigen at 20 μg ml^{-1}. We supplemented T cell cultures with recombinant mouse IL-2 (Peprotech) and supernatant from concanavalin A–stimulated mouse spleen cells on days 0, 3 and 5. We purified live T cells by Nycoprep gradient (Progen Biotechnik) and repeated stimulation every 7–10 d.

Statistical analyses. We analyzed spontaneous EAE incidence by Kaplan-Meier survival curve analysis, and we analyzed adoptive transfer EAE data and proliferation assays by analysis of variance. We used GraphPad Prism for all statistical analyses. We considered *P* values less than 0.05 to be significant.

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