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THE JOURNAL OF IMMUNOLOGY

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J Immunol 2010; 184:2839-2846; Prepublished online 17 February 2010;
doi: 10.4049/jimmunol.0903639
http://www.jimmunol.org/content/184/6/2839

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Contribution of Myelin Autoantigen Citrullination to T Cell Autoaggression in the Central Nervous System

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Breakdown in immunological self tolerance, leading to autoimmune diseases such as multiple sclerosis, might arise from immune recognition of self proteins that have undergone heightened posttranslational modification under pathophysiological conditions. A posttranslational modification of particular interest is the deimination of Arg to citrulline, catalyzed by peptidylarginyl deiminase (PAD) enzymes. As a CD4$^+$ T cell-driven model of multiple sclerosis, we used experimental autoimmune encephalomyelitis (EAE) induced with the immunodominant 35–55 peptide of myelin oligodendrocyte glycoprotein (pMOG) in C57BL/6 mice to test whether citrullination of a T cell epitope can contribute to disease etiopathology. Immunization with an altered peptide ligand (APL) of pMOG with an Arg→citrulline conversion at a TCR contact (residue 41) led to the activation of two populations of APL-responsive T cells that either did, or did not cross-react with the native pMOG peptide. This APL could induce EAE. However, this reflected the activation of T cells that cross-reacted with the native pMOG epitope, because prior tolerization of these T cells using pMOG prevented APL-induced EAE. Using a passive transfer model, we found that T cells that responded specifically to the citrullinated form of pMOG were neither necessary, nor sufficient to initiate the EAE lesion. Nevertheless, these cells could provoke exacerbation of pathology if transferred into mice with ongoing EAE. The PAD2 and PAD4 enzymes were markedly upregulated in the inflamed CNS. Therefore, once inflammation is established, citrullination of target autoantigens can allow an expanded repertoire of T cells to contribute to CNS pathology. ***The Journal of Immunology*****, 2010, 184: 2839–2846.**

Although the mammalian genome encodes only 20 amino acids, analyses of mature proteins have identified >100 diverse amino acids (1). These arise from post-translational modifications (PTM) that can occur spontaneously (2), or as a result of enzymatic activity. The de novo recognition of self-Ags that have been modified as a result of pathophysiological disturbance (3, 4) is an appealing basis for breakdown in immunologic self tolerance; namely, the immune system would target the modified self-Ag as if it were pathogen derived. The literature provides various examples of Ab- and/or T cell-reactivity against antigenic epitopes that contain PTM such as glycosylation, deamidation, and citrullination (5–8). Citrullination, the deimination of Arg to citrulline (Cit) (9), has received particular interest as an autoantigen modification. This reaction, by which the imino group of the protein arginyl residues is removed giving rise to Cit, is catalyzed by a family of calcium-binding enzymes termed peptidylarginine deiminases (PADs; 3.5.3.15). A set of paralogous genes encode five PAD isoforms in mammals, *Pad1–4* and *-6*, which have a tissue-specific expression as well as substrate specificity. Expression of PAD1, PAD3 and PAD6 appears tightly restricted (10), whereas PAD4 is expressed in monocytes and granulocytes and PAD2 can be found in the CNS (11, 12). The association between citrullination and rheumatoid arthritis (RA) appears well established. Both PAD2 and PAD4 are elevated in inflamed joints (13), autoantibodies are found that specifically recognize citrullinated self-proteins (14), and a citrullinated form of type II collagen (CII) displayed enhanced arthritogenicity in rats (15). Citrullinated self-peptides have been shown to display enhanced binding to RA-associated HLA-DR4 molecules (16) and DR4 transgenic mice can develop arthritis after immunization with citrullinated, but not unmodified, fibrinogen (17).

Interest in citrullination in multiple sclerosis (MS) first developed from studies showing increased citrullination of myelin basic protein (MBP) in patients, and particularly in the hyperacute Marburg's syndrome (18, 19). MS patients have been reported to show enhanced T cell reactivity to citrullinated MBP leading to the suggestion that this may contribute to disease induction or perpetuation (20). This has prompted some studies on the contribution of citrullination to pathogenesis of the primary preclinical model for MS, experimental autoimmune encephalomyelitis (EAE). Citrullinated MBP is encephalitogenic in both rats and mice (21, 22). Within the CNS, PAD2 has been found in oligodendrocytes, microglia, and astrocytes (12, 23, 24) providing the opportunity for modification of myelin autoantigens. Immunohistochemical analyses revealed that, although citrullinated proteins were not readily detectable in the healthy CNS, these were clearly evident in mice with EAE (25, 26). SJL/J mice with chronic EAE induced by immunization with a proteolipid protein peptide mounted autoantibody responses against citrullinated MBP (27). However, although autoantibodies may contribute to pathology, EAE is primarily driven by the actions of proinflammatory CD4$^+$ T cells reactive against myelin. Thus far, no analysis of the contribution of T cells recognizing citrullinated

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Received for publication November 12, 2009. Accepted for publication January 11, 2010.

This work was supported by grants from the Medical Research Council (U.K.). A.C.-V. was supported by a long-term fellowship from the Federation of European Biochemical Societies.

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Abbreviations used in this paper: APL, altered peptide ligand; Cit, citrulline; EAE, experimental autoimmune encephalomyelitis; Iso, cells stained with the relevant isotype control Ab; MBP, myelin basic protein; Med, cells cultured in medium alone prior to staining; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PAD, peptidyl arginyl deiminase; pMOG, 35–55 peptide of MOG; PTM, posttranslational modification; RA, rheumatoid arthritis; Treg, regulatory T cell; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0903639

myelin Ags has been performed in EAE. In this study, we describe the contribution of T cells recognizing citrullinated forms of the immunodominant 35–55 peptide of myelin oligodendrocyte glycoprotein (hereafter referred to as pMOG).

Materials and Methods

Mice, Ags, and immunizations

C57BL/6 (CD45.1$^+$ or CD45.2$^+$) mice were bred under specific pathogen-free conditions at the University of Edinburgh. The 6–8-wk-old sex-matched mice were used for all experiments. pMOG (MEVGWYRSPFSRVVHLYRNGK) and the pMOG(41Cit), pMOG(46Cit), and pMOG(41,46Cit) APL were obtained from Peptide 2.0 (Chantilly, VA). Mice were immunized with 100 μg peptide emulsified in CFA (Sigma-Aldrich, Poole, U.K.). A total of 100 μl emulsion was injected s.c., 50 μl into each hind leg. Primed lymphoid populations were derived either from spleens or from draining inguinal and para-aortic lymph nodes at the indicated time. Active and passive EAE were induced using previously described protocols (28, 29) Clinical signs of EAE were assessed daily with the following scoring system: 0, no signs; 1, flaccid tail; 2, impaired righting reflex and/or gait; 3, partial hind limb paralysis; 4, total hind limb paralysis; 5, hind limb paralysis with partial front limb paralysis; and 6, moribund or dead. Tolerance was induced by injection of 200 μg peptide in 0.2 ml PBS i.v. at the indicated time before immunization. All experiments were approved by the University of Edinburgh ethical review panel and were conducted under United Kingdom legislation.

Spleen recall responses

Spleen cell suspensions were cultured in 96-well flat-bottom microtiter plates (Becton Dickinson, Oxford, U.K.) at 8×10^5 splenocytes/well using X-vivo 15 serum-free medium (BioWhittaker, Maidenhead, U.K.) supplemented with 2 mM L-glutamine and 5×10^{-5} M 2-ME (all from Invitrogen, Paisley, U.K.). Cultures were stimulated with a dose range of peptides for 48 h prior to addition of [^{3}H]dThd (0.5 μCi/well) (Amersham, Amersham, U.K.). After an additional 18 h, dThd incorporation was measured using a liquid scintillation β-counter (LKB Wallac, Turku, Finland). Results are expressed as mean cpm of triplicate cultures. Supernatants from similar 72 h cultures were tested for IFN-γ and IL-17 production by ELISA.

PAD2 and PAD4 mRNA expression

Total RNA from brain and spinal cord or lymph node and spleen was extracted using RNeasy Lipid Tissue Mini Kit or RNeasy Mini Kit (Qiagen, Hilden, Germany), respectively. Single-strand cDNA was then synthesized from 1 μg RNA according to the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen). Real-time quantitative PCR was performed by the Chromo4 real-time system (MJ Research, Waltham, MA). Reactions were performed in a 50 μl volume containing 10 μl RT product as template DNA (100 ng), 2× Platinum SYBR Green quantitative PCR SuperMix-UDG (Invitrogen), and 200 nM for each primer set. A UDG incubation step before PCR cycling was carried out to destroy any contaminating dU-containing product from previous reaction. UDG was then inactivated by the high temperatures during the first PCR step (95°C for 2 min.). After that, a denaturation (95°C for 15 s), annealing (60°C for 30 s) and extension (72°C for 15 s) steps were repeated 30 times with fluorescent data acquisition after each extension step. Finally, a melting curve program from 60°C to 95°C, followed by a cooling step at 20°C were performed. Primers (5′ to 3′): *Pad2* (241-bp product): 5′ TAC AGC AAG CAA GAC CTC CA 3′ (exon 6) and 5′ CCA CGA AGA ACA GCA ACT CC 3′ (exon 7); *Pad4* (217 bp product): 5′ GCT GCC TGT GGT CTT TGA CT 3′ (exon 9) and 5′ GTA ACC GCT ATT CCC GAT GA 3′ (exon 11). All reactions were performed in triplicate. The *hprt* gene was used as the control for the calculation of ΔCt. Analysis of relative gene expression was determined by evaluating the expression of $2^{-\Delta\Delta CT}$.

T cell hybridomas

Draining lymph node populations were stimulated in vitro with 10 μM peptide for 3 d and blasts were then fused with the TCR-deficient BW5147 thymoma as described previously (30). All hybrids were cloned and recloned by limiting dilution. IL-2 production in response to 24-h culture with peptide presented by irradiated (30 Gy) syngeneic splenocytes was determined by ELISA.

CNS-infiltrating mononuclear cells

Mice were perfused with cold PBS, brain and spinal cords were removed, digested, and mononuclear cells retrieved from a 30%:70% discontinuous percoll gradient as described previously (28).

Flow cytometry for surface phenotype and intracellular cytokine staining

Cells were stained for FACS using the following Abs and reagents: anti–CD4-AlexaFluor 700/allophycocyanin/PE/PerCP (BD Pharmingen, San Diego, CA), anti–CD45.1-biotin, anti–IFN-γ–FITC, anti–IL-17 PE, rat IgG1 FITC, rat IgG1 PE, and streptavidin-PerCP (eBioscience, San Diego, CA). TCRVβ usage by hybridomas was assessed using a panel of anti-Vβ Abs (BD Pharmingen). FACS data were collected on a LSR II flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (TreeStar, Ashland, OR). For intracellular cytokine staining, cells were cultured at 1×10^7 cells/ml in the presence or absence of 5 μM peptide. After overnight culture, 1 μg/ml brefeldin A (eBioscience) was added for the last 4 h of culture. Cells were permeabilized, stained, and fixed as previously described (29).

Results

The core epitope of pMOG has the potential to be citrullinated at two TCR contact residues

A series of studies, including our own, have characterized T cell recognition of pMOG in C57BL/6 mice (30, 31). The core epitope lies within residues 40–48 (31) and a combination of MHC-binding studies and analyses of T cell responses to altered peptide ligands (APLs) have defined MHC- and TCR-contact residues (32, 33). As shown in Fig. 1*A*, and prompting this study, both residues 41Arg and 46Arg are available for TCR recognition, providing the opportunity for PAD-mediated deimination to Cit at either or each of these residues, thereby modifying T cell recognition of MOG. We therefore synthesized three APLs based on the pMOG sequence to provide pMOG(41Cit), pMOG(46Cit), and pMOG (41,46Cit) (Fig. 1*B*) for analysis of their biological function.

PAD2 and PAD4 are upregulated in the inflamed CNS

If citrullination of native MOG was to play any role in the pathogenesis of EAE, we would expect to find expression of the PAD2 and/or PAD4 enzymes within the CNS, and perhaps particularly within the inflamed CNS. Quantitative RT-PCR revealed that expression of PAD2 was elevated in mice with EAE, compared with healthy control mice, specifically within the CNS and not in the spleen (Fig. 2*A–C*). In contrast, PAD4 was upregulated in mice with EAE in all organs tested, and in the CNS this appeared most marked in mice with higher clinical scores (Fig. 2*D–F*). We

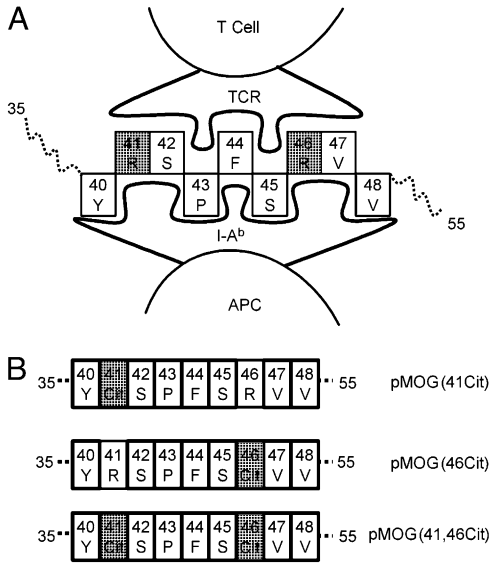


FIGURE 1. Citrullination of pMOG TCR contact residues. *A*, Interaction of pMOG with I-A^b and TCR, based on previous studies (30–33). *B*, WT pMOG peptide and APLs used in this study.

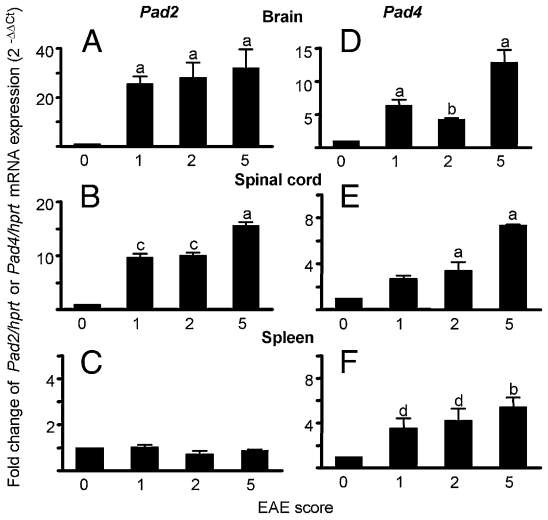


FIGURE 2. *Pad2* and *Pad4* are upregulated in the inflamed CNS. Quantitative RT-PCR for *Pad2* and *Pad4* mRNA expression was performed on samples taken on day 12 after EAE induction with pMOG, and on those taken from healthy control mice (0). mRNA levels were normalized against those for the *hprt* housekeeping gene. Data are from triplicate experiments performed. Statistics (Student *t* test): a, $p < 0.001$; b, $p < 0.005$; c, $p < 0.01$; and d, $p < 0.05$ compared with the control (0) group.

therefore concluded that there was the opportunity for MOG to be citrullinated within the CNS during the inflammatory process that drives EAE. PAD2 may be most important within the CNS, because mice specifically lacking this isoform have been previously shown to have greatly reduced expression of citrullinated proteins in the CNS (34).

Citrullinated APLs are immunogenic

Our next question was whether the mice could mount a T cell response against the citrullinated APLs. Analysis of splenocyte responses from mice immunized against wild-type (WT) pMOG revealed that both the pMOG(41Cit) and the pMOG(46Cit) APLs were functional as weak agonists, with the pMOG(41Cit) APLs being the strongest of these (Fig. 3*A–C*). In contrast, the pMOG (41,46Cit) APL behaved essentially as a null peptide, failing to trigger any clear recall response from pMOG-primed cells. All three APLs were effective immunogens. However, only immunization with either the pMOG(41Cit), or the pMOG(46Cit) APL [and not the pMOG(41,46Cit) APL] could prime an immune population capable of responding to the WT pMOG peptide (Fig. 3*D–L*). Consistent with its higher antigenicity on cells primed against pMOG, the pMOG(41Cit) APL provided a population with stronger responsiveness for pMOG than did the pMOG (46Cit) APL. Notably, pMOG-driven production of effector cytokines (IFNγ and IL-17) was more evident in pMOG(41Cit)-primed populations (Fig. 3*E–F*). The conclusion from these polyclonal analyses was that immunization either with the WT pMOG peptide, or with the pMOG(41Cit) APL would effectively prime a population that contained T cells with some degree of cross-reactivity against the other peptide. This conclusion was supported by a clonal analysis of $CD4^+$ T cell hybridomas (using IL-2 production as a measure of TCR stimulation of the hybridomas). This showed that ~10% of hybridomas that were raised against pMOG would respond to pMOG(41Cit), and vice versa (Fig. 4). A previous study of TCR usage by pMOG-reactive T cells has shown that around 50% express TCRVβ8.1/2, with the "public" TCR rearrangement using Vβ8.2-Jβ2.1 (35). Serological analysis of our two panels of T cell hybridomas revealed that 42% of those primed with pMOG used Vβ8 (11/26), whereas only 18% of pMOG(41Cit)-primed hybridomas (4/22) used Vβ8 (Fig. 4*B*). Therefore, after immunization with pMOG(41Cit), there was a dominant population of T cells that responded only to pMOG (41Cit), but there was also a small fraction that could cross-react against native pMOG.

Immunization with pMOG(41Cit) can initiate EAE, but this requires cross-reactive T cell recognition of noncitrullinated pMOG

Our next step was to test whether immunization with either of the APLs could induce EAE. Totally consistent with the data described previously, it was only the pMOG(41Cit) APL that could reproducibly provoke an encephalitogenic response, giving an equivalent incidence to that seen with the WT pMOG peptide, although the severity of the disease attained was significantly lower (Fig. 5*A*). Analysis of recall responses from the CNS revealed effector responses (IFN-γ staining) against both pMOG(41Cit) and pMOG in those mice immunized either with pMOG, or with pMOG (41Cit) (Fig. 5*B*). Interestingly, in pMOG-immunized mice, the response to pMOG(41Cit) appeared to be concentrated in the inflamed CNS and could not be detected in the spleen (Fig. 5*B*), suggesting that PAD-mediated citrullination of MOG specifically within the CNS was providing a target for these T cells. The frequencies of IL-17^+ cells were always lower, but followed the same patterns of responsiveness as found with IFN-γ (data not shown).

From the previously described data, we could not tell whether those T cells that could recognize native pMOG were required for the initiation of the EAE lesion, or whether those cells that specifically recognized only the citrullinated form of MOG could do this. We tested this by tolerizing mice either against WT pMOG or against pMOG(41Cit) by i.v. injection of peptide in saline, prior to immunization with pMOG(41Cit). This tolerance protocol is demonstrated to lead to deletion of peptide-reactive T cells (36) and prevented the development of EAE in each of the tolerized groups (Fig. 6*A*). This indicated that the response to the WT peptide was required to initiate EAE; namely, the group tolerized against pMOG could only mount a (noncross-reactive) response to pMOG (41Cit) after immunization with the APLs but did not develop disease. Those mice tolerized against pMOG(41Cit) also did not develop disease, indicating the need for activation by the APL to provoke the cross-reactive response against pMOG. In the reverse experiment, mice pretolerized against pMOG(41Cit) were fully susceptible to EAE induced with pMOG (Fig. 6*B*), underlining that a response to citrullinated MOG in vivo was not required for EAE induction. Furthermore, passive transfer experiments revealed that disease could only be transferred using lymph node populations from mice immunized with pMOG. This required their reactivation in vitro with pMOG. If the same lymph node populations were triggered in vitro with pMOG(41Cit), they were unable to transfer disease (Fig. 6*C*, 6*D*).

T cells recognizing citrullinated pMOG can contribute to ongoing disease

Our conclusion from the EAE experiments described previously was that specific recognition of pMOG(41Cit) was neither required nor sufficient to initiate the EAE lesion. This would be consistent with our finding that both PAD2 and PAD4 were markedly upregulated within the CNS of mice with ongoing EAE (Fig. 2); namely, mice in which the CNS is not already inflamed would have little or no citrullinated MOG available to act as a substrate for those T cells that respond only to pMOG(41Cit). However, we speculated that this upregulation of PAD2 and PAD4, associated with CNS inflammation, might provide sufficient citrullinated

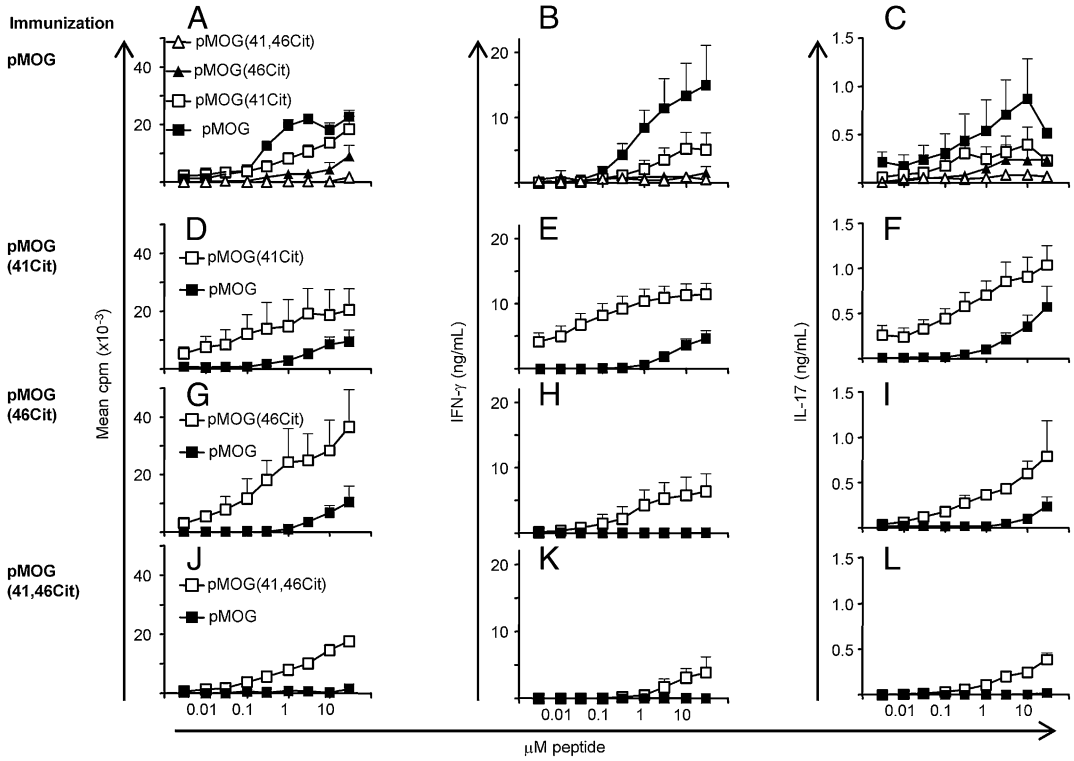


FIGURE 3. Immunogenic properties of citrullinated APL. Mice were immunized with pMOG (*A–C*), pMOG(41Cit) (*D–F*), pMOG(46Cit) (*G–I*), or pMOG(41,46Cit) (*J–L*) in CFA. After 10 d, splenocyte recall responses to pMOG and citrullinated APL were measured by proliferation (*A*, *D*, *G*, *J*) IFN-γ production (*B*, *E*, *H*, *K*), and IL-17 production (*C*, *F*, *I*, *L*). Data (mean ± SEM) are from one of three experiments giving consistent results.

MOG to allow T cells recognizing pMOG(41Cit) to contribute to ongoing pathology. In support of this possibility, analysis of recall responses of pMOG-immunized mice at the height of EAE had revealed that T cell reactivity to pMOG(41Cit) could be detected in the CNS, but not in the spleen (Fig. 5*B*). To test this, we induced active EAE in CD45.2 mice by immunization with pMOG and, once the disease was at its height, we transferred CD45.1$^+$ lymph node cells that had been immunized and reactivated with either pMOG or pMOG(41Cit) (Fig. 7*A*). Importantly, the donor mice providing the pMOG(41Cit)-primed cells had been previously tolerized against pMOG (as described previously) to remove responsiveness to noncitrullinated pMOG (Fig. 7*B*). Transfer of either pMOG-reactive or pMOG(41Cit)-reactive cells led to an exacerbation of disease commencing 4–6 d after transfer (Fig. 7*C*). This timing fits precisely with our experience of the time taken for transferred cells to become established in the CNS (SMA, unpublished). Indeed, either transferred population could be detected within the CNS of host mice (Fig. 7*D*). The restimulation protocol used to provide donor cells generates pathogenic Th1 populations that can be reanalyzed for Ag-reactivity, using IFN-γ production as a readout (37). Importantly, flow cytometric analysis of Ag-induced CD4$^+$CD45.1$^+$ cell production of IFN-γ showed no evidence that the transferred pMOG(41Cit)-reactive cells could respond to WT pMOG (Fig. 7*E*, 7*F*). Mice that received either no cell transfer, or that received OVA responsive cells, showed no evidence of relapse and therefore had lower cumulative EAE scores after the day of transfer (Fig. 7*G*, 7*H*).

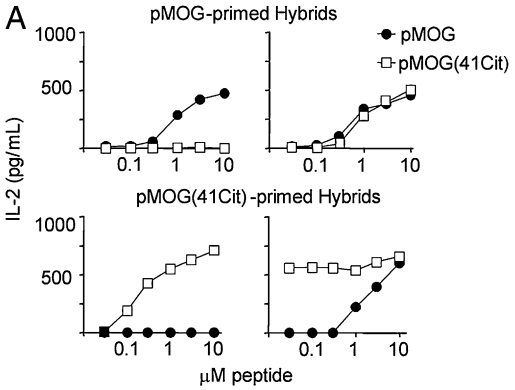


FIGURE 4. Analysis of pMOG and pMOG(41Cit) CD4$^+$ T cell hybridomas. *A*, Representative response profiles of four hybridomas. *B*, Frequency of hybridomas cross-reacting against both pMOG and pMOG (41Cit) and their TCRVβ usage, as assessed by flow cytometry.

Discussion

Our data indicate that pMOG(41Cit) can be highly immunogenic, in fact the data suggest that immunization with this citrullinated form triggers a larger population of T cells than is seen after immunization with WT pMOG (Fig. 5*B*). An extrapolation from this would be that the T cell repertoire is not purged of cells that can recognize pMOG(41Cit), either during thymic development or in the periphery, although this possibility requires further exploration. The model that emerges from our data is that the initiation of EAE requires the activity of T cells that can recognize the

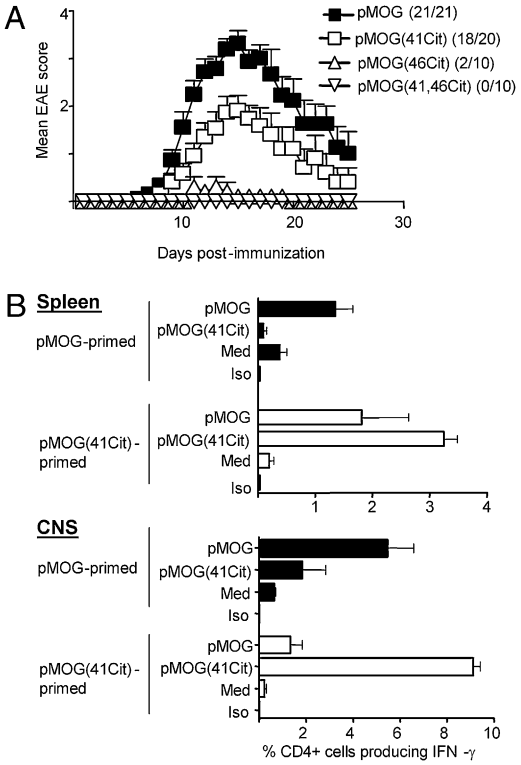


FIGURE 5. pMOG(41Cit) induces active EAE. *A*, Mice were immunized for EAE induction with the indicated peptide in CFA (disease incidence in brackets). pMOG(41Cit) provoked a significantly less severe EAE compared with native pMOG ($p < 0.05$, one-way ANOVA, followed by Tukey test). *B*, Spleen and CNS were collected at day 16–18 post-immunization for in vitro analysis of peptide-induced IFN-γ production by FACS. Data are mean ± SEM from eight individual mice. Iso, cells stained with the relevant isotype control Ab; Med, cells cultured in medium alone prior to staining.

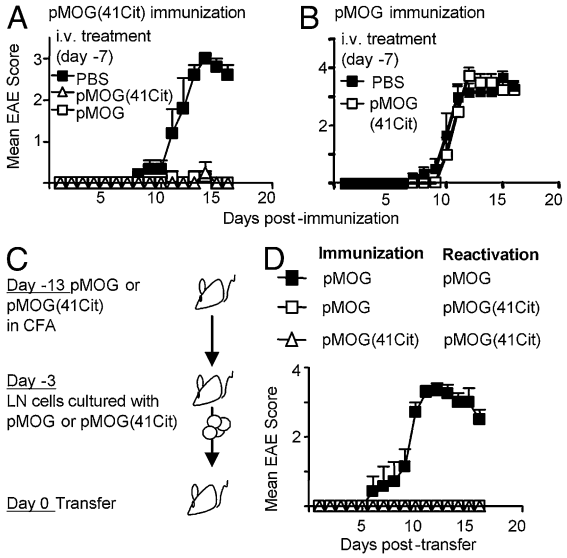


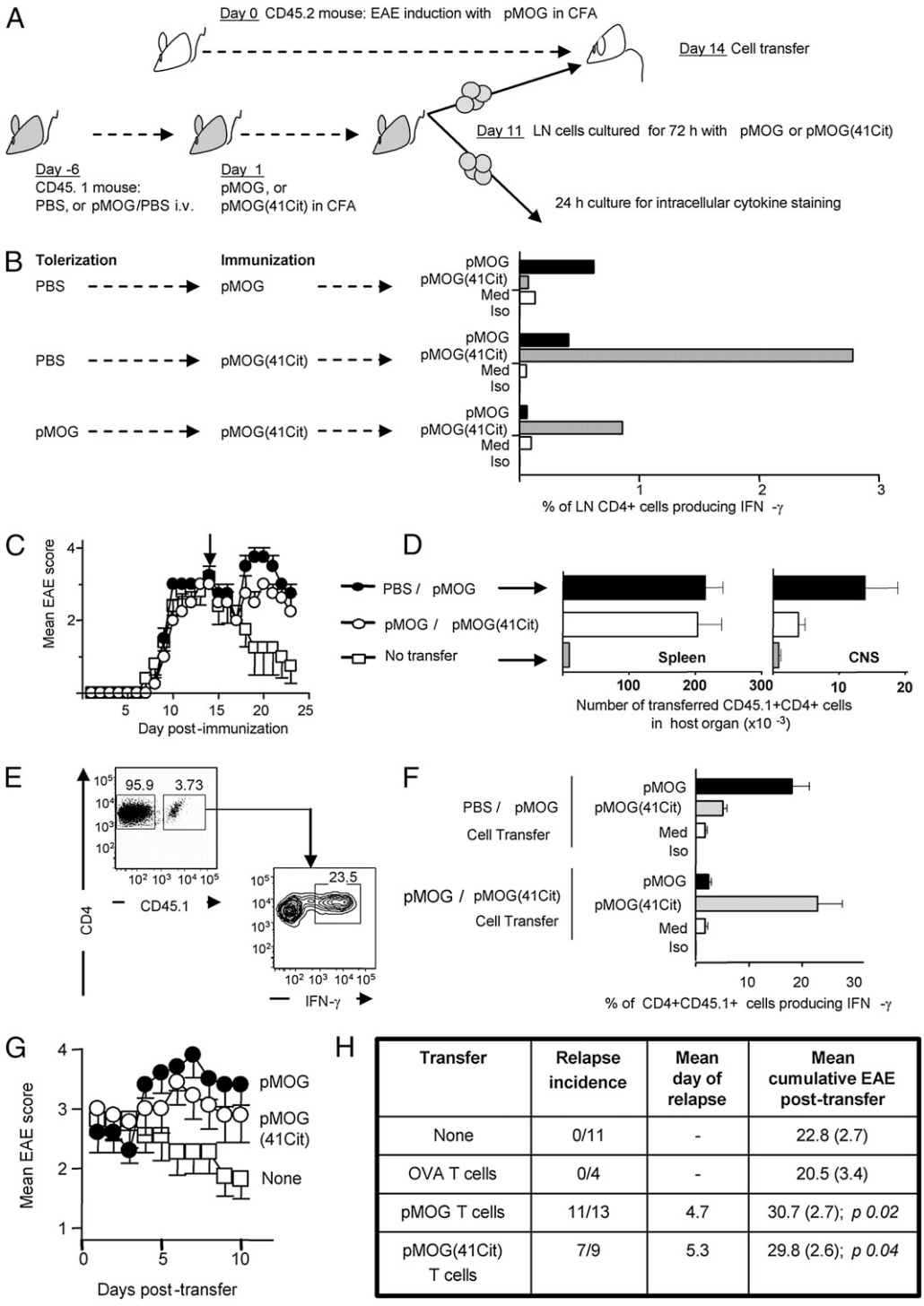
FIGURE 6. pMOG(41Cit)-induced EAE requires T cell recognition of native pMOG. Mice were either i.v. tolerized with pMOG or pMOG(41Cit) prior to EAE induction with pMOG(41Cit) (*A*), or tolerized with pMOG (41Cit) prior to EAE induction with pMOG (*B*) (seven mice per group, pooled from two experiments). *C* and *D*, Passive transfer of EAE using cells from mice immunized with either pMOG or pMOG(41Cit) and reactivated in vitro as indicated (four mice per group from one of two experiments).

noncitrullinated form of pMOG in the noninflamed CNS (those that specifically recognize only pMOG(41Cit) cannot transfer disease). Once inflammation in the CNS is established, PAD2 and PAD4 are upregulated, allowing for the citrullination of MOG and thereby providing an antigenic target for pMOG(41Cit)-reactive T cells that can exacerbate disease (Fig. 7*C*). Of note, as exemplified in Fig. 4, some T cells primed against pMOG(41Cit) showed particularly high sensitivity to this form of the epitope, indicating that they may be particularly damaging if able to respond to citrullinated MOG within the CNS.

The interest in the effects of citrullination of MOG on EAE had been tempered previously by the report that PAD2$^{-/-}$ mice were fully susceptible to pMOG induced EAE (34). That study did not address the role of PAD4, which we find to be also upregulated in the inflamed CNS. Nevertheless, our data are consistent with the PAD2$^{-/-}$ study, because pMOG-induced EAE can follow a normal course in mice that had been tolerized to pMOG(41Cit). Therefore, our conclusion is that, under normal conditions, T cells that can specifically recognize a citrullinated form of MOG are available but they cannot initiate disease. However, once EAE is underway, they can contribute to sustained pathology, dependent on PAD2 and/or PAD4 function within the CNS.

The upregulation of PAD2 during EAE was specifically seen in the CNS and was not mirrored in the lymphoid organs. If we envision a scenario in which T cells that can recognize citrullinated autoantigen(s) are primed as a consequence of ongoing inflammation it seems that this would most likely occur within the inflamed CNS itself. There is a precedent for this with recent reports that epitope spreading during EAE can be driven by Ag presentation by myeloid dendritic cells specifically within the inflamed CNS (38, 39).

Beyond functioning as a novel autoantigenic target, citrullination of TCR contacts within the MOG epitope could conceivably have had dramatically diverse consequences. There is a long literature on how exposure to APLs can trigger a variety of functional consequences within T cells both in vitro and in vivo (40). These range from weak agonist activity, through partial agonist (e.g., induction of cytokine release without concomitant proliferation) (41), to TCR antagonist activity (the ability to block TCR signaling even in the presence of the cognate peptide Ag) (42). We and others have reported on the use of such APLs to ameliorate EAE (43–45). Using pMOG-primed T cell lines, we tested our citrullinated APLs for possible partial agonist, or TCR antagonist activities in vitro and found no evidence for either (data not shown). Thus, we conclude that the 41Cit, 46Cit, and 41,46Cit modifications result in an agonist, a weak agonist, and a null peptide, respectively. A further possibility was that citrullination of MOG could act as a specific target for T cells with particular functional characteristics. In particular, we have reported on the remarkable expansion of Foxp3$^+$ regulatory T cells (Tregs) specifically within the inflamed CNS that triggers the recovery phase that we see in pMOG-induced EAE (28, 29). In passively transferred EAE it seems that these protective Tregs (that are all derived from the host T cell repertoire) have little if any reactivity to native MOG (29). Using BrdU incorporation as a measure of Treg activation (29), we found no evidence that these Tregs were able to specifically recognize either of the citullinated APLs (data not shown). Although our study did not support either of the possibilities described above, it remains entirely possible that in other pathologic scenarios autoantigen modification could have these effects. Although anti-pMOG Abs are generated after pMOG immunization (46), our experience is that the titers of these do not correlate with clinical severity of EAE (SMA, unpublished observations). Moreover, pMOG-induced EAE develops (and in fact is more



Transfer	Relapse incidence	Mean day of relapse	Mean cumulative EAE post-transfer
None	0/11	-	22.8 (2.7)
OVA T cells	0/4	-	20.5 (3.4)
pMOG T cells	11/13	4.7	30.7 (2.7); *p 0.02*
pMOG(41Cit) T cells	7/9	5.3	29.8 (2.6); *p 0.04*

FIGURE 7. pMOG(41Cit)-specific cells can exacerbate ongoing EAE. *A*, EAE was induced in CD45.2 mice by immunization with pMOG. On day 14, mice received CD45.1 donor cells that had been primed and reactivated with either pMOG, or pMOG(41Cit). The latter group were from donors previously tolerized to pMOG. Controls received no cell transfer (injection of PBS alone). *B*, Responsiveness of transferred cells to pMOG versus pMOG(41Cit) (assayed on day of lymph node harvesting from donor mice). *C*, Effects of cell transfer on EAE course. *D*, Numbers of donor cells in host spleen and CNS taken 10 d after cell transfer. *E*, Representative FACS plot illustrating IFN-γ production by donor cells sampled from spleen (pMOG(41Cit)-primed donor cells in response to pMOG(41Cit). *F*, Analysis of donor cell production of IFN-γ in response to pMOG and pMOG(41Cit). Data (mean ± SEM, four mice per group) are from one of four experiments giving consistent results. *G* and *H*, Cumulative data from four experiments showing the 10 d postcell transfer. Controls received no cell transfer (injection of PBS alone), or cells from lymph nodes of OVA-immunized CD45.2 mice that had been restimulated with OVA in vitro prior to transfer. *H*, Mean cumulative EAE refers to the total disease burden for the 10 d postcell transfer (i.e., the mean is taken from cumulative scores of individual mice for those 10 d). Figures in parentheses are SEM. *p* values refer to differences between the indicated group and the no transfer group (Kruskal-Wallis test). Iso, cells stained with the relevant isotype control Ab; Med, cells cultured in medium alone prior to staining.

severe) in B cell-deficient mice (47), excluding an obligate role for autoantibodies in this disease model. It would, however, be of interest to explore the pathogenic consequences of a humoral immune response specifically to MOG that has been citrullinated, either at residues 41 and/or 46, or at Arg residues outwith the 35–55 T cell epitope. This was beyond the scope of this study.

The evidence for a pathogenic role for citrullinaton of autoantigens in RA seems beyond doubt (48). Most work in this regard has focused on humoral immune responses, which are more straightforward to study. Most compelling is the recent demonstration of the ability to initiate arthritis with autoantibodies that specifically target a citrullinated form of CII (49). Some T cell studies have been undertaken using citrullinated Ags, however. Reminiscent of earlier observations in celiac disease showing that wheat gliadin peptides with Gln→Glu substitutions (mimicking the natural activity of tissue transglutaminase) had enhanced binding affinities for celiac disease-associated MHC molecules (50, 51), citrullinated vimentin peptides showed enhanced binding to the RA-associated DRB1*0401 molecule (16). However, elevated T cell responses to such peptides have not been reported in autoimmune arthritis (49). A recent report has described the induction of arthritis (characterized by synovial hyperplasia and ankylosis, but no gross inflammatory infiltrate) in DRB1*0401 transgenic mice after immunization with a citrullinated form of human fibrinogen, an autoantigen to which anti-Cit Abs are commonly found in RA patients (17). Of note, no arthritis was evident in response to either the noncitrullinated form of human fibrinogen, or citrullinated mouse fibrinogen. The disease was associated with the production of anti-Cit Abs and also showed T cell reactivity to α79–91(84Arg→Cit) peptide of human fibrinogen, but not to the native form of this peptide. Residue 84 was modeled to be a TCR contact residue, but that study did not provide unequivocal evidence that the T cell response to the citrullinated peptide was required for disease; immunization with the α79–91(84Arg→Cit) peptide was not arthritogenic (17). The effects of PTM on T cell reactivity to the autoantigen have been studied most extensively in CII-induced arthritis, where glycosylation is important (52). In mice expressing either A^q, or transgenic DR4, the key target for T cells is the 260–270 region. Galactosylation of residue 264 clearly leads to enhanced T cell reactivity and this seems to be also the case in RA (53–55). Using the oxidative environment of inflammation as a starting point, a previous study examined malondialdehyde-modification of recombinant rat MOG and found a more severe form of EAE when this form of the autoantigen was used in DBA/1 mice (56). The authors concluded that the most likely basis for this was enhanced uptake of the modified MOG by APC via scavenger receptors, rather than any direct effect on TCR recognition.

Others have reasoned that recognition of citrullinated MBP by T cells from MS patients might reflect an influence on pathogenesis (20). Intriguing evidence that peripheral blood T cell responses from MS patients are more robust when challenged with a citrullinated form of an immunodominant MBP peptide than the noncitrullinated form have lent some support to this view (57). Lewis rats have been reported to develop EAE after active immunization with a citrullinated form of MBP (21). The immunodominant epitope of MBP in this setting (72–85) includes an Arg at position 76 that acts as a TCR contact residue. Investigation of the effects of an Arg→Cit substitution at this residue concluded that this was sufficient to abrogate TCR signaling; a MBP (72–85)-reactive T cell clone and lymph node cells from MBP (72–85)-primed rats failed to respond to a 76Cit APL, and vice versa (58). Importantly, although that previous MBP study suggested the capacity to prime T cells that would respond only to the citrullinated form of the epitope, the ability of such cells to contribute to pathology was not determined. In this study, we have used precise modification of a TCR contact residue within the immunodominant epitope of MOG to provide proof of principle for this, but only as far as exacerbating established EAE. Nevertheless, it is conceivable that a pre-established T cell population capable of recognizing a citrullinated myelin autoantigen could initiate an autoimmune reaction, if PAD2 and/or PAD4 were upregulated in the CNS by another inflammatory stimulus. In accord with this thesis, a concise report has shown how citrullination is a general phenomenon in the inflamed tissues in several diseases of diverse cause (3). There are also a wide range of additional PTMs that might be either triggered or increased under inflammatory conditions (5). The most far-reaching implication of the data presented in this study is that current strategies aimed at identifying critical autoantigenic epitopes using the 20 naturally occurring amino acids may be missing a sizeable "hidden" repertoire focused on modified forms of the autoantigen. Identification of these represents a major challenge.

Disclosures

The authors have no financial conflicts of interest.

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