Autoimmunity, June 2013; 46(4): 269–278

ISSN 0891-6934 print/1607-842X online
DOI: 10.3109/08916934.2013.767893

informa
healthcare

A modified superantigen rescues Ly6G$^-$CD11b$^+$ blood monocyte suppressor function and suppresses antigen-specific inflammation in EAE

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(*Submitted 4 November 2012; revised 5 January 2013; accepted 14 January 2013*)

Abstract
In a previous study, we showed that the Ly6G$^-$CD11b$^+$ blood monocytes residing in naïve mice are intrinsically immunosuppressive and that loss of this suppressive function may contribute to the development of autoimmunity in experimental autoimmune encephalomyelitis (EAE), a murine model of human multiple sclerosis. Here we report that mice treated with a modified superantigen coupled to myelin oligodendrocyte glycoprotein 35–55 (MOG_{35-55}) peptide (DM-MOG_{35-55}) suppressed the development of EAE. The treatment was associated with impaired MOG_{35-55}-specific T cell proliferation and a decrease in IL-17 and IFNγ production in the draining lymph nodes. Analysis of circulating blood immune cells showed that the suppressor function of Ly6G$^-$CD11b$^+$ blood monocytes was reduced in EAE mice, but was restored in mice treated with DM-MOG_{35-55}. Importantly, adoptive transfer of blood CD11b$^+$Ly6G$^-$ cells isolated from DM-MOG_{35-55}-treated mice protected recipient mice from developing EAE. Together, these results show that DM coupled to the auto-antigen MOG_{35-55}: 1) suppresses EAE via antigen-specific suppression of T cell responses, and 2) re-establishes suppressor function of Ly6G$^-$CD11b$^+$ blood monocytes. Auto-antigens coupled to DM could therefore represent a new therapeutic approach for controlling inappropriate inflammation in autoimmune diseases such as multiple sclerosis by inducing antigen-specific T cell suppression.

Keywords: *Autoimmunity, EAE, monocyte, superantigen, T cell, antigen-specific suppression*

Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease whereby an autoimmune response is directed against the myelin sheath that protects axons of the central nervous system (CNS). The subsequent destruction of the myelin sheath interferes with motor and sensory nerve function resulting in significant disability. Currently, there is no cure for MS [1,2]. There are a number of therapies in clinical use that provide medium-term therapeutic benefit, including anti-inflammatory interferons (IFNs), mitoxantrone [3], natalizumab [4,5] and Copaxone [6]. However, the action of these treatments can also lead to a number of undesirable side effects, including multifocal leukoencephalopathy, infection and cardiotoxicity [3,7–9].

The ideal treatment for MS and other autoimmune diseases is one that blocks the inappropriate antigen-specific inflammatory responses that are the underlying cause of disease. Only a small number of experimental antigen-specific therapies have made it to clinical trial to date. Oral administration of both myelin basic protein (MBP) and MBP-derived altered peptide ligands has been shown to suppress experimental autoimmune encephalomyelitis (EAE) in mice [10–12]. Unfortunately, these treatments have not exhibited any therapeutic benefit for MS patients,

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either through lack of efficacy [13], or as a result of unexpected disease exacerbation and hypersensitivity [14,15]. Nevertheless, these studies do provide evidence that autoantigen-specific immune responses can be raised in MS patients.

An alternative approach to regulating antigen-specific immune responses is to couple immunoregulatory agents to disease-specific antigens [16]. Bacterial superantigens, produced by *Staphylococcus aureus, Streptococcus pyogenes* and *Streptococcus equi*, are widely recognised as powerful modulators of the immune system capable of driving non-specific activation of a large number of T cells [17]. The phenomenal T cell responses triggered by native superantigens generally precludes their use *in vivo* however mutated superantigens have been investigated as novel agents for treating haematopoietic tumours [18].

The immune response induced by superantigens is related to their ability to simultaneously engage Major Histocompatibility Complex II (MHCII) on antigen presenting cells (APCs) and the T cell receptor (TCR) on T cells [17,19]. A variant of a Streptococcal mitogenic exotoxin Z variant 2 (SMEZ-1) protein with a mutated TCR binding domain has been used to specifically target $MHCII^+$ antigen presenting cells. Antigens coupled to this TCR-mutated SMEZ-1 and injected into mice resulted in efficient antigen presentation and a potent antigen-specific T cell response [20]. This provides evidence that mutated superantigens can be used to develop targeted, antigen-specific immune regulation.

We have shown previously that $Ly6G^-CD11b^+$ blood monocytes are intrinsically suppressive and lose the ability to suppress T cell responses in mice with EAE [21,22]. The ability to re-establish this suppressive function may therefore provide a mechanism for protecting against the development of autoimmune diseases such as MS.

SMEZ-2 (DM) is a modified superantigen with specific mutations designed to eliminate the TCR and MHCII interactions responsible for classical superantigen activity. This study investigated the antigen-specific immunoregulatory effect of DM coupled to the myelin peptide MOG_{35-55} (DM-MOG_{35-55}) in a murine model of CFA/MOG_{35-55} induced EAE, including the ability of DM-MOG_{35-55} to modify $Ly6G^-CD11b^+$ blood monocyte suppressor function.

Materials and methods

Mice and reagents

C57BL/6J mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were bred and maintained in the Biomedical Research Unit of the Malaghan Institute of Medical Research. All animal studies were approved by the Victoria University of Wellington Animal Ethics Committee and performed in accordance with the guidelines of the Victoria University of Wellington Animal Ethics Committee, New Zealand.

MOG_{35-55} and Ovalbumin peptide 323–339 ($OVA_{323-339}$) were synthesised by Mimotopes (Clayton, Victoria, Australia) with a purity of >97%. Modified SMEZ-2-DM was provided by Professor John Fraser (The University of Auckland, New Zealand). Expander beads and NuPage gel were from Invitrogen (Carlsbad, CA, USA). Lympholyte®-Mammal cell separation medium was from Cedarlane Laboratories Limited, Canada. N^Gmonomethyl-L-arginine (L-NMMA) was from SigmaAldrich (St. Louis, MO, USA). N^{ω}hydroxy-nor-L-arginine (nor-NOHA) was from Calbiochem, Germany. Anti-PE Automacs beads and Automacs buffer were from Miltenyi Biotec (Bergisch Gladbach, Germany).

Immunohistochemistry zinc fixative was from BD Biosciences (Sparks, MD, USA). [^{3}H]-thymidine was from GE Healthcare, UK. Vivaspin column 500 was from Sartorius (Göttingen, Germany). Spin-X polypropylene microcentrifuge tube (0.22 μm pore cellulose acetate membrane filter) was from Corning Inc. (Lowell, MA, USA). Complete Freund's adjuvant (CFA) and *Mycobacterium tuberculosis* strain H37RA was from Difco Laboratories (Sparks, MD, USA). Pertussis toxin was from List Biological (Campbell, CA, USA). [^{3}H]thymidine was from GE Healthcare (Piscataway, NJ, USA)

EAE induction and clinical evaluation

EAE was induced in 8–12-week old C57BL/6J mice by subcutaneous (s.c.) immunisation into hind limb flanks with 50 μg MOG_{35-55} peptide and 500 μg heat-killed *Mycobacterium tuberculosis* strain H37RA emulsified in CFA (0.2 ml, day 0), followed by an intraperitoneal injection of 250 ng pertussis toxin in PBS on day 1.

Mice were monitored daily after immunisation and the development of EAE was scored using the following scale: 0, unaffected; 1, loss of tail tonicity; 2, flaccid tail; 3, flaccid tail and affected hind leg(s); 4, both hind legs paralysed; 5, hind body paresis, moribund state. Moribund mice were sacrificed.

Histology

On day 40, mice were sacrificed by asphyxiation with carbon dioxide (CO_2). The spinal cords were removed by flushing the spinal column with sterile phosphate buffered saline (PBS). The spinal cords were fixed in zinc fixative, embedded in paraffin, sectioned longitudinally (6 μm), mounted on slides and stained with hematoxylin and eosin (H&E). Sections were analysed with an Olympus BX51 microscope (400× magnification) and Olympus DP70 digital camera and analysis software (Olympus, Center Valley, PA, USA).

Confocal microscopy

Fluorescently labelled cells (DM-Alexa 488, CD11b-Alexa555, DAPI) were visualised using a Leica TCSSP2 confocal microscope (Leica Microsystems, Germany). Images were acquired at 22°C using Leica Confocal software. Acquired images were processed using NIH ImageJ image software (NIH, Maryland, USA).

Conjugation of the MOG_{35-55} peptide to superantigen SMEZ-2-DM. SMEZ-2-DM

Double mutant SMEZ-2 was provided by Professor John Fraser (University of Auckland, New Zealand). SMEZ-2-DM (DM) is defective in both TCR and MHCII binding, and contains the mutations Y18A, D42C, H202A and D204A. The D42C mutation introduces an exposed cysteine into the former TCR binding site for direct conjugation of peptides.

Conjugation. SMEZ-2-DM (0.2 mM) was incubated with MOG_{35-55} peptide (2 mM in 0.1% acetic acid, overnight, room temperature) then the uncoupled MOG_{35-55} peptide was removed (10 KDa cut-off Vivaspin column 500). The DM-MOG_{35-55} conjugate solution was spun through a Spin-X polypropylene microcentrifuge tube (0.22 μm pore cellulose acetate membrane filter) and stored at 4°C. Formation of the DM-MOG_{35-55} conjugate was confirmed by NuPage gel (S1), run as per manufacturer's instructions [19].

DM, DM-MOG_{35-55} administration. DM or DM-MOG_{35-55} was either co-administered as part of the EAE-inducing emulsion (as above) or prepared in CFA (Figure 1B), IFA (Figure 1B) or PBS (Figure 1C) and administered subcutaneously in the neck as indicated.

Purification of blood $CD11b^{+}Ly6G^{-}$ cells. Blood was isolated from mice via cardiac puncture and centrifuged over a Lympholyte®-Mammal density gradient as per manufacturer's instructions. The

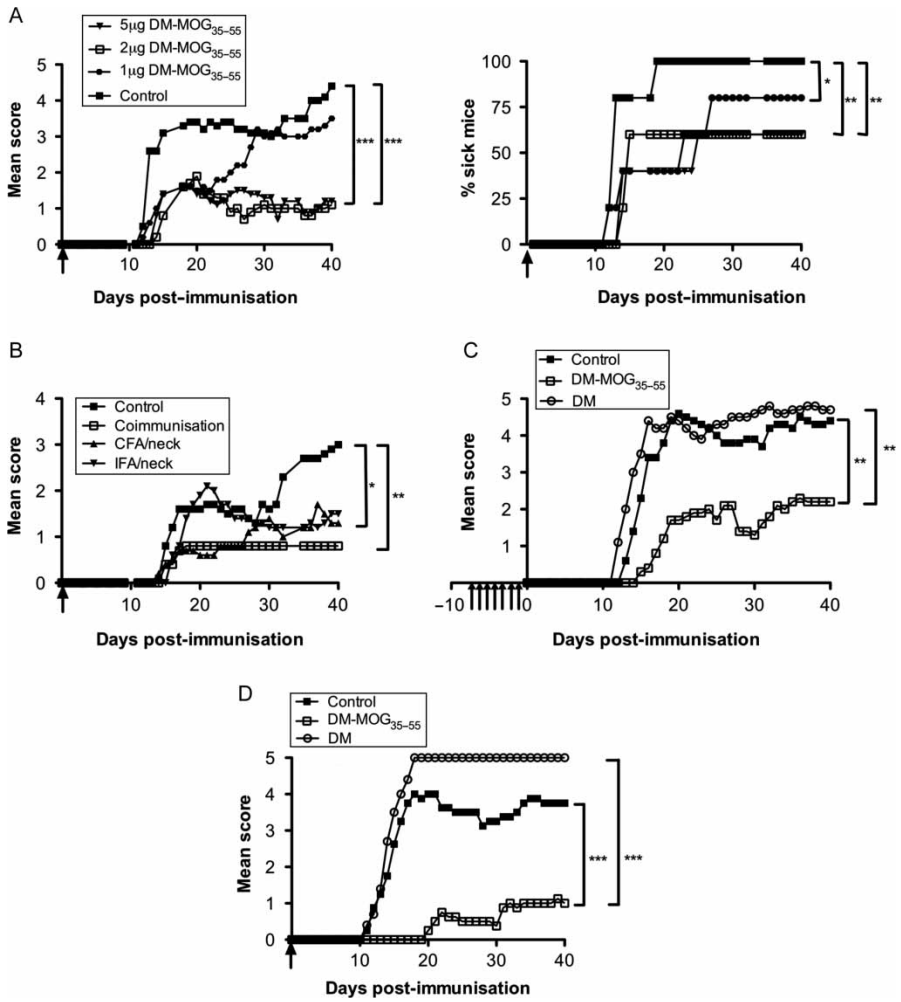

Figure 1. DM-MOG_{35-55} suppressed EAE in C57BL/6J mice. C57BL/6J mice were injected subcutaneously in the flank with MOG_{35-55} / CFA A. Co-administration of DM-MOG_{35-55} in the flank B. Single dose of 2 μg DM-MOG_{35-55}/IFA was injected subcutaneously in the neck C. Multiple doses of 2 μg DM-MOG_{35-55}/PBS subcutaneously in the neck. D Co-administration of 2 μg DM-MOG_{35-55} or DM alone in the flank. $n = 5$ mice per group. Statistical analysis was performed using a one-tailed Mann–Whitney U-test. $***p < 0.0001$, $**p < 0.001$, $*p < 0.01$. Arrow = DM-MOG_{35-55} administration.

interface containing the blood $CD11b^+Ly6G^-$ cells was removed, washed in complete Iscoves modified Dulbecco's medium and resuspended in AutoMACS buffer (1×10^6 cells/ml, 2 mM EDTA, 1% fetal calf serum, PBS). Dead cells and cells with non-specific binding properties were removed by a single pass through an AutoMACS column. The cells were resuspended in AutoMACS buffer (5×10^8 cells/ml) and incubated with CD11b-PE antibody (15 min, ice). The cells were washed, resuspended in AutoMACS buffer (5×10^8 cells/ml) and incubated under rotation with anti-PE beads (10 μl/5×10^7 cells, 30 min, 4°C). Cells were then washed and resuspended in AutoMACS buffer (1×10^6 cells/ml) and passed through a MACS column to positively select for $CD11b^+$ cells.

Proliferation assay. Responder cells from the draining lymph nodes (DLN) of C57BL/6J mice ($0.2–1 \times 10^6$ cells/ml, cIMDM) were cultured for 72 h in the presence of peptide or antibody as indicated. After 72 h, 0.25 μCi [^{3}H]thymidine was added and the cells incubated for 8–16 h. The cells were harvested to filtermats using an automated cell harvester and radioactive counts measured on a Topcount Microplate scintillation counter (Wallac, Woodbridge, ON, USA). Alternatively, after 72 h culture supernatants were collected for cytokine analysis using the Bio-Plex cytokine detection system (Bio-Rad, Hercules, CA, USA).

Monocyte suppression assay. Responder DLN cells from C57BL/6J mice ($0.2–1 \times 10^6$ cells/ml, cIMDM, 37°C) were activated with peptide or antibody and incubated in the presence of different ratios of purified blood $CD11b^+Ly6G^-$ cells as indicated. The cells were harvested and radioactive counts measured as above.

% Suppression was calculated using the following formula:

$$[1 - \text{cpm (target cells with suppressor cells)} / \text{cpm (target cells alone)}] \times 100\%$$

NOS and Arginase-1 Inhibition. N^Gmonomethyl-L-arginine (L-NMMA, 500 μM) and/or N^ωhydroxy-nor-L-arginine (nor-NOHA, 500 μM) were added to the *in vitro* suppression assay to block the action of nitric oxide synthase (NOS) and arginase 1, respectively [23].

Results

DM-MOG_{35-55} suppresses EAE in C57Bl/6J mice

Superantigen augments adaptive immune responses via simultaneous binding of MHCII and the TCR. To remove this adjuvant capability, we generated the superantigen variant SMEZ-2(DM), with mutations in two different domains that eliminates the binding to the TCR and MHCII. To investigate if DM-autoantigen conjugate could modulate immune responses *in vivo*, MOG_{35-55} was coupled to DM (Supplementary Figure 1) and DM-MOG_{35-55} was tested for the ability to suppress MOG_{35-55} induced EAE.

Subcutaneous DM-MOG_{35-55} treatment resulted in a dose-dependent decrease in both the severity and incidence of disease (Figure 1A and Table 1). To determine whether DM-MOG_{35-55} was acting systemically or locally, CFA/DM-MOG_{35-55}, IFA/DM-MOG_{35-55} or multiple doses of PBS/ DM-MOG_{35-55} were administered subcutaneously in the neck, with EAE immunisation in the flank. All these treatments suppressed EAE to some extent (Figure 1B,C), confirming that DM-MOG_{35-55} was acting systemically and that suppression was not dependent on the delivery vehicle. DM treatment alone did not suppress EAE (Figure 1C,D). With DM treatment alone, both DM and MOG_{35-55} are present in the EAE-inducing emulsion but are unconjugated. Therefore conjugation of DM to the MOG_{35-55} peptide appears to be necessary for EAE suppression.

A key feature of MS and EAE is the infiltration of inflammatory cells into the CNS [24,25]. In this study, the spinal cords isolated from EAE control mice showed extensive cellular infiltration into the foci of the spinal cords and loss of tissue structure (Figure 2). Analysis of the individual clinical scores of EAE mice treated with 2 μg DM-MOG_{35-55} showed that they

Table 1. Reproducibility of the treatment using DM-MOG_{35-55}.

Experimental Number	Treated mice (Sick/total)	% Sick of treated mice	Control mice (Sick/total)	% Sick of control mice
1	2/4	50%	5/5	100%
2	2/5	40%	5/5	100%
3	1/5	20%	5/5	100%
Total	5/14	35.71%	15/15	100%

C57BL/6J mice were treated with EAE inducing emulsion plus 2 μg of DM-MOG_{35-55}. Age- and sex-matched mice left untreated were employed as the experimental controls.

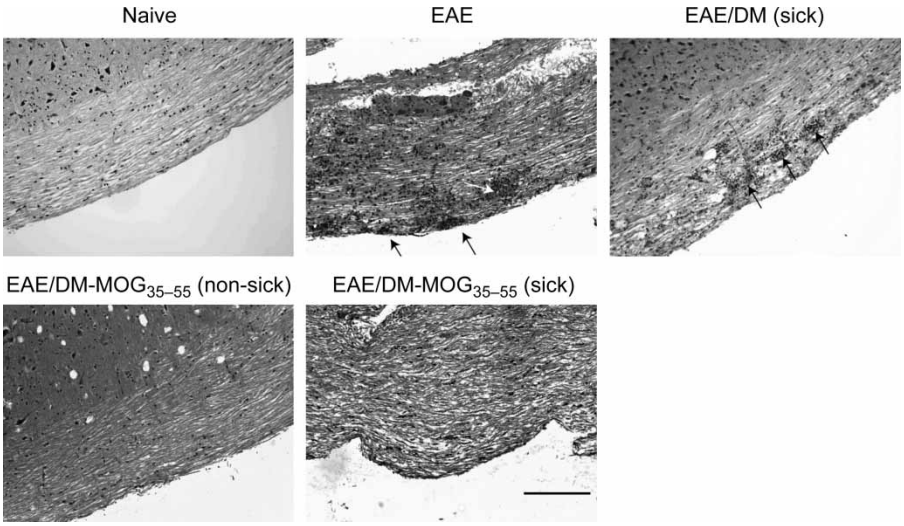

Figure 2. DM-MOG_{35-55} treatment decreases disease pathology in EAE in mice. C57BL/6J mice were immunised subcutaneously in the flank with MOG_{35-55} / CFA ± 2 μg DM-MOG_{35-55} or DM. On day 40, H&E stained spinal cord sections were prepared from naïve, EAE control, EAE/DM treated (sick) and EAE/DM-MOG_{35-55} treated (non-sick and sick) mice. A representative slide from each group ($n = 5$ per group) is shown. Arrow = infiltrating lymphocytes. Scale bar = 200 μm.

could be separated into two sub groups; 1) 'sick' — mice exhibiting decreased disease score compared to EAE controls and 2) 'non-sick — completely protected mice that exhibited no physical signs of disease (Table 2). Consistent with the pattern of individual disease incidence and severity, spinal cords from 'sick' mice treated with DM-MOG_{35-55} showed reduced cell infiltration and tissue damage compared to naïve controls. Spinal cords from DM-MOG_{35-55}-treated mice that did not develop EAE (non-sick) showed comparable pathology to naïve controls. Thus, DM-MOG_{35-55} treatment decreased the incidence and severity of disease, and this correlated with decreased inflammation and cellular infiltration into the CNS.

DM-MOG_{35-55} mediated suppression of EAE is antigen- specific

To minimise side effects, the optimal therapy for MS is one that targets antigen-specific autoimmune responses. To determine whether DM-MOG_{35-55} mediated suppression of antigen-specific autoimmune T cells, draining lymph node (DLN) cells harvested from all of the different treatment groups were restimulated *ex vivo* with MOG_{35-55}, the mycobacterium-derived purified protein derivative (PPD) present in the CFA immunisation emulsion or anti-CD3 antibody, and T cell proliferation and cytokine production was measured.

As shown in Figure 3A, DLN cells from DM-MOG_{35-55}-treated EAE mice exhibited reduced responses to MOG_{35-55} restimulation *ex vivo* compared to control EAE mice. Importantly, DM-MOG_{35-55} treatment did not affect cell proliferation in response to PPD or anti-CD3 antibody stimulation. This provided evidence that DM-MOG_{35-55} was inducing MOG-specific T cell suppression. As expected, the DLN cells did not proliferate in response to the control peptide $OVA_{323-339}$. Furthermore, *in vivo* administration of DM-$OVA_{323-339}$ failed to suppress MOG_{35-55}-induced EAE (Supplementary Figure 2) providing additional evidence that DM-MOG_{35-55}-induced suppression was MOG_{35-55}-specific.

The production of IL17 [24,25] and IFNγ [26] are both linked with inflammation in MS and EAE. In this study, DLN cells from non-sick DM-MOG_{35-55}

Table 2. DM-MOG_{35-55} treatment-suppressed EAE.

Treatment	Incidence of EAE (%)	Mortality (%)	Mean maximum severity of sick mice	Mean day of onset of sick mice
Control	15/15 (100%)	5/15 (33.33%)	4.04 ± 0.74	16.57 ± 6.03
DM-MOG_{35-55}	5/14 (35.71%)	0/14 (0%)	3.25 ± 0.61^{ns}	18.33 ± 6.56^{ns}

C57BL/6J mice were treated with EAE inducing emulsion plus 2 μg of DM-MOG_{35-55}. Age- and sexmatched mice left untreated were employed as the experimental controls. The incidence of EAE, mortality, mean maximum score of each group ± SD and mean day of onset ± SD are shown. Significant differences are indicated, ns, not significant ($p > 0.05$). Analysis of statistical significance was performed using a one-tailed Mann–Whitney U-test. $**p < 0.001$, $*p < 0.01$.

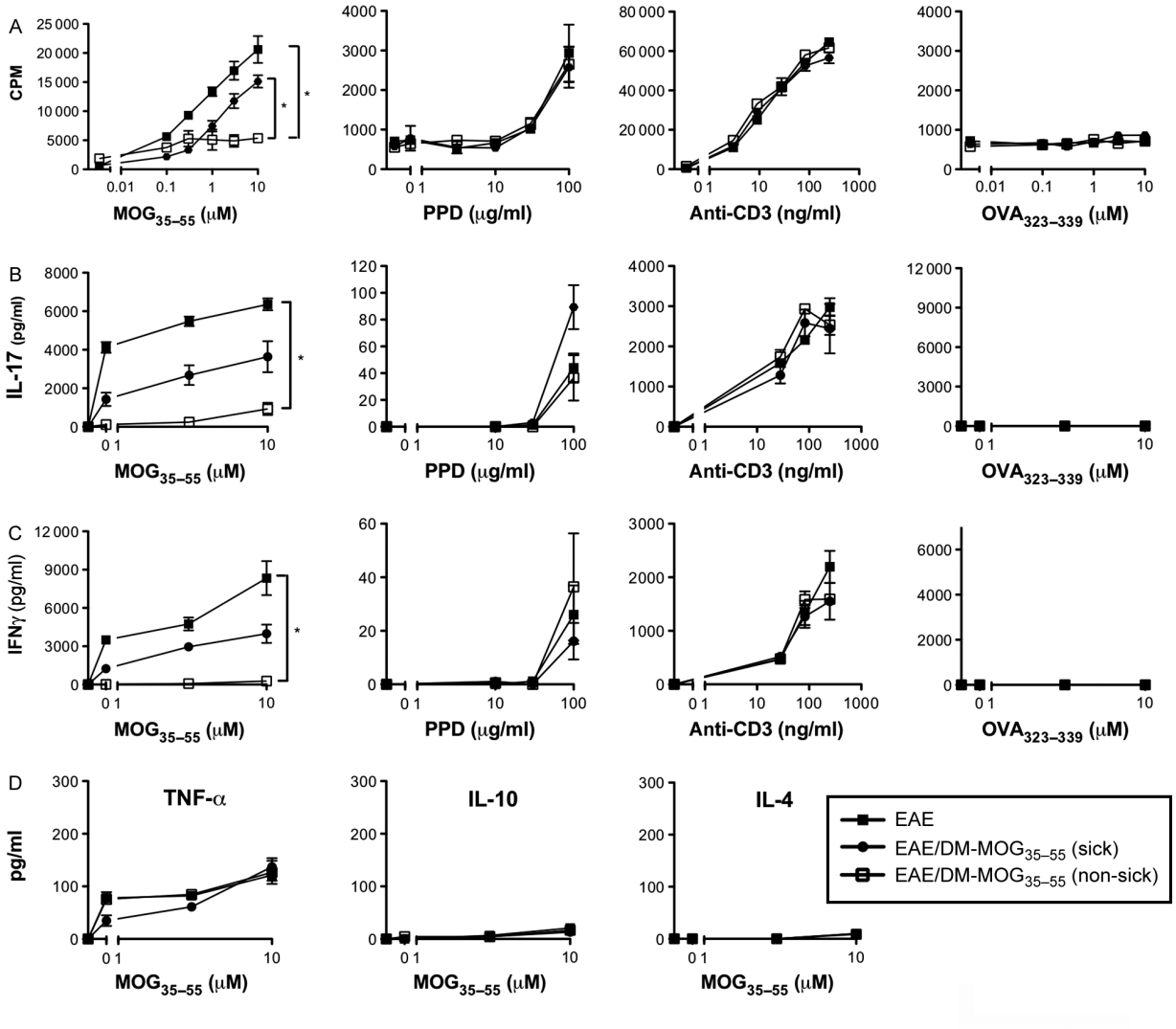

Figure 3. DM-MOG_{35-55} treatment induces antigen-specific inhibition of T cell activation. C57BL/6J mice were immunised subcutaneously in the flank with MOG_{35-55} / CFA ±2 μg DM-MOG_{35-55}. On day 40 inguinal lymph nodes were collected and single cell suspension was prepared. The cells from the lymph nodes were restimulated *ex vivo* with MOG_{35-55}, PPD, CD3 antibody or $OVA_{323-339}$ for 72 h. The C57BL/6J mice induced with EAE without treatment were used as the experimental control. A. Cell proliferation was measured by the incorporation of [^{3}H]-thymidine B, C, D. Supernatant was collected at 72 h, and cytokine levels were measured by Bio-Plex cytokine assay. Data represent one of 2 replicate experiments, n = 5 mice per group. Statistical analysis was performed using a one-tailed Mann–Whitney U-test. $\star p < 0.01$. Error bars are S.E.M.

treated mice produced significantly less IL17 and IFNγ compared to cells from the EAE control mice (Figure 3B,C). DM-MOG_{35-55} treated mice that exhibited reduced disease (sick mice) also produced lower levels of both IFNγ and IL17 in response to MOG_{35-55} restimulation. No significant differences in cytokine production by DLN cells were observed in response to PPD and antiCD3 antibody restimulation *ex vivo*, illustrating that the reduction in IL17 and IFNγ production was restricted to MOG_{35-55}-specific T cell responses. In this study, T cells from DM-MOG_{35-55} treated mice did not produce IL-4 or IL-10 indicating that DM-MOG_{35-55} was not skewing the responding T cells from a Th1 to a Th2 phenotype (Figure 3D).

DM-SMEZ2 targets blood CD11b$^+$Ly6G$^-$ monocytes and enhances blood monocyte suppressor function

DM carries mutations that disrupt MHCII binding thereby removing preferential MHC-dependent targeting of superantigen to MHCII^{hi} antigen-presenting cells (APC). As such DM has the potential to associate with alternative cell types to induce suppression of EAE. To identify the cell population(s) being targeted by DM, mice were injected i.v. with Alexa488-labeled DM, and blood and tissues analysed by flow cytometry for the presence of DM-Alexa488 positive cells.

As shown in Figure 4A, DM$^+$ cells were found to bind MHC$^-$ cells in the blood. These DM$^+$ cells were also identified in MHC$^{-/-}$ mice, confirming that the

association of the DM with the cells occurred independent of MHC II molecules (Figure 4B). Surface marker analysis of the DM^+ cells showed that the DM^+ cells were $CD11b^+Ly6G^-$ $F4/80^{int}$ $CD11c^-$ and predominantly Ly6C positive (Figure 4C). DM^+ cells isolated from the blood exhibited myeloid-like monocyte morphology with a large kidney-shaped nucleus (Figure 4D), consistent with a blood monocyte phenotype [22,27]. Confocal microscopy of DM^+ monocytes revealed that DM was taken up into intracellular compartments by the cells, rather than remaining bound on the cell surface (Figure 4D).

We showed previously that naïve circulating monocytes exhibited myeloid-derived suppressor cell-like suppressor function via an iNOS-dependent mechanism that is switched off in EAE [22]. As DM-MOG_{35-55} treatment was not associated with Th1/Th2 switching we asked whether DM could be suppressing EAE by reversing EAE-associated loss of blood monocyte suppressor function. DM^+ blood monocytes tested for their ability to suppress T cell function *ex vivo* exhibited enhanced suppressive activity against T cell proliferation, compared to naïve blood $CD11b^+Ly6G^-$ suppressor cells

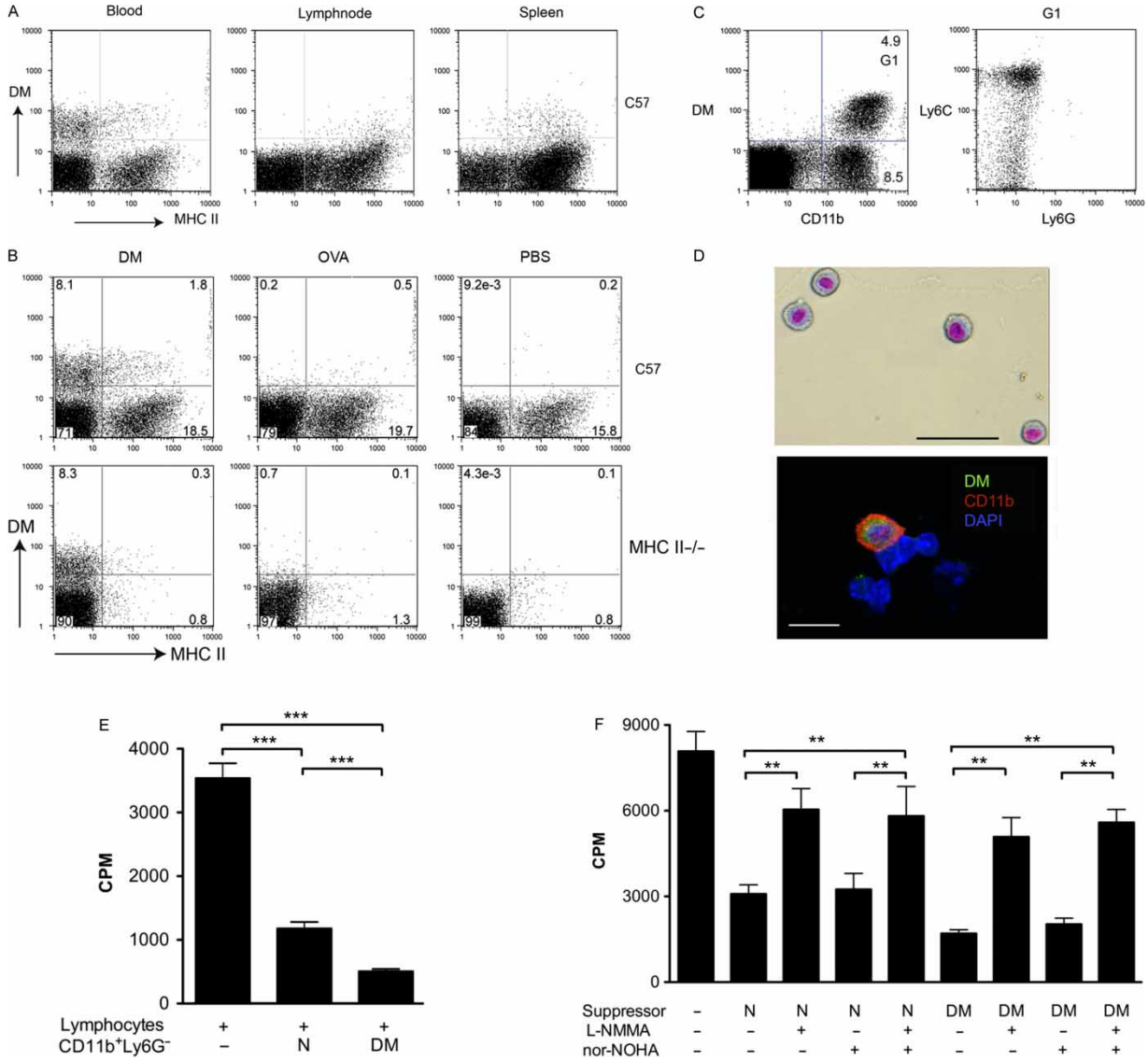

Figure 4. DM binds blood $CD11b^+Ly6G^-$ monocytes and enhances monocyte suppressor function. A-C. C57BL/6J or $MHCII^{-/-}$ mice were injected i.v. with 50 μg DM-Alexa 488, 75 μg OVAAlexa 488 or PBS. After 3 h, cells from the blood, spleens and lymph nodes were collected and DM^+ cells analysed by flow cytometry. D. Blood DM-Alexa 488^+ cells were sorted by FACS and stained with Diff-Quik (upper image, scale bar 50 μm). Whole blood cells were fluorescently labelled with CD11b, and DAPI and analysed by confocal microscopy (lower image, scale bar 10 μm). E. $CD11b^+Ly6G^-$ blood cells from naïve or DM-Alexa 488 injected mice were co-cultured with naïve C57BL/6J splenocytes (1:1 ratio) activated with anti-CD3 (2 μg/ml) and anti-CD28 (3 μg/ml) antibodies. Cell proliferation was measured by $[^3H]$ thymidine incorporation. Combined data from two separate experiments shown. F. Suppression of cell proliferation by naïve and DM^+ monocytes was measured in the presence of 500 μM of the iNOS inhibitor LNMMA and/or 500 μM of the arginase inhibitor nor-NOHA. N = naïve. Data represent one of 3 replicate experiments, $n = 5$ mice per group. Statistical analysis was performed using one-way ANOVA followed by Bonferroni tests $***p < 0.0001$, $**p < 0.001$. Error bars are S.E.M.

(Figure 4E). Whereas inhibition of nitric oxide production with the NOS inhibitor L-NMMA resulted in partial abrogation of the DM-dependent suppressor function, blockade of arginase-1 had no effect on T cell proliferation (Figure 4F). Together these results indicated that DM-MOG_{35-55} was ameliorating EAE by re-engaging iNOS-dependent, antigen-specific blood monocyte suppressor function.

Blood $CD11b^+Ly6G^-$ cells play a key role in DM-MOG_{35-55}mediated suppression of EAE

Next we investigated whether DM-MOG_{35-55} treatment altered blood $CD11b^+Ly6G^-$ cells suppressive capability in EAE. Blood $CD11b^+Ly6G^-$ cells isolated from untreated EAE mice and EAE mice treated with DM-MOG_{35-55}, were tested for their ability to suppress T cell proliferation. Consistent with earlier findings [22] the blood monocytes isolated from control EAE mice exhibited reduced suppression of T cell proliferation *ex vivo*, compared to blood monocytes from naïve mice (Figure 5A).

Treatment of EAE mice with DM-MOG_{35-55} restored blood monocyte suppressor function (Figure 5A) and adoptive transfer of these cells conferred protection to the recipients, observed as a decrease in both the incidence and severity of disease (Figure 5B). Protection from EAE was not observed in mice receiving blood $CD11b^+Ly6G^-$ suppressor cells from either naïve donor mice or donor mice treated with DM alone. Together, these results confirmed that DM-MOG_{35-55} treatment induced robust suppressive activity by blood monocytes in EAE mice and that these cells could induce suppression of MOG_{35-55}-induced EAE in recipient animals.

Discussion

In this study we report that subcutaneous administration of DM-MOG_{35-55} suppresses EAE. Interestingly, the suppression of EAE involves the induction of MOG_{35-55} specific suppressor function and occurs without affecting T cell responses to alternative immune stimuli. Since most of the treatments available for human MS and other autoimmune diseases are antigen nonspecific [28–31], DM-MOG_{35-55} represents a potential therapeutic capable of inducing antigen-specific suppression for the treatment of MS.

A key feature of EAE and MS is the infiltration of inflammatory cells into the CNS and the production of IL17 [24,25] and IFNγ [9,32,33]. In this study DM-MOG_{35-55} treatment was associated with decreased pathology in the CNS. Importantly, *ex vivo* assays indicated that a decrease in the incidence and severity of disease in DM-MOG_{35-55}-treated mice was associated with less antigen-specific T cell proliferation as well as decreased production of IFNγ

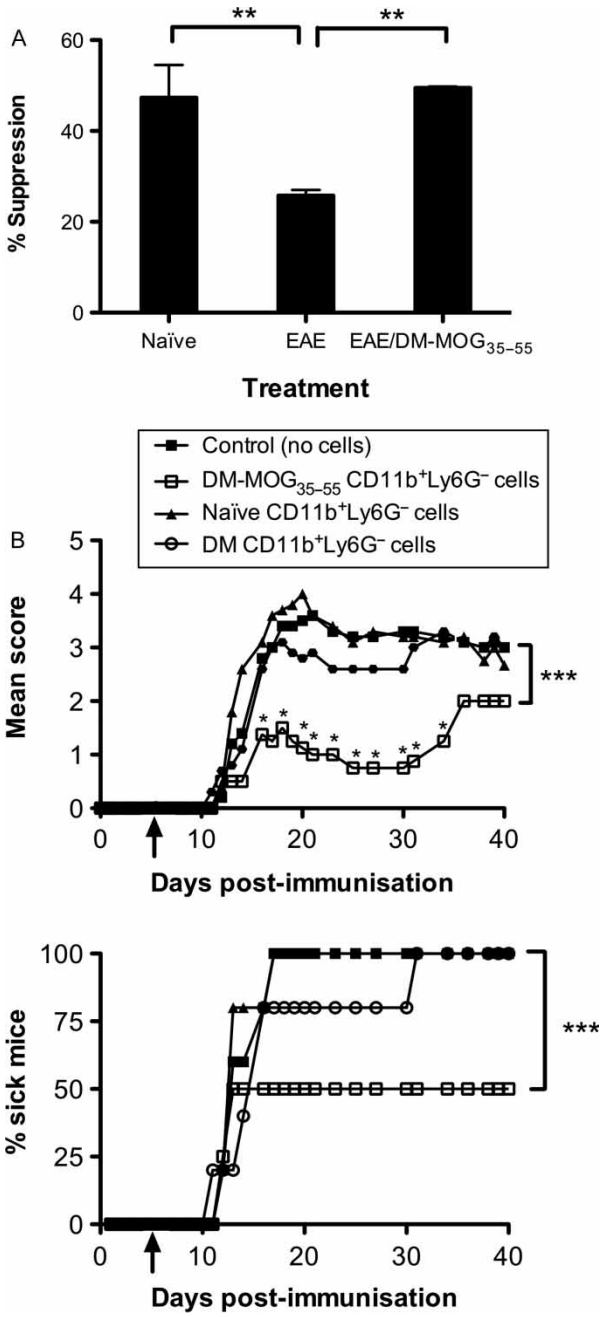

Figure 5. Adoptive transfer of DM-MOG_{35-55}-induced blood monocytes confers protection from EAE. C57BL/6J mice were immunised with EAE emulsion (CFA/MOG_{35-55}) ± DM-MOG_{35-55} (2 μg) subcutaneously and blood monocytes purified on day 5 post-immunisation. A. Purified blood monocytes were incubated with naïve lymph node cells (1:1 ratio) activated by antiCD3/antiCD28 expander beads. At 24 h, [^{3}H]thymidine was added to the cell culture and incubated for a further 16 h. B. Purified $CD11b^+Ly6G^-$ blood monocytes from naïve, DM- or DM-MOG-treated mice were adoptively transferred (2×10^5 blood monocytes, i.v.) into recipient mice 5 days after administration of EAE emulsion. Data represent one of two replicate experiments. n = 5 mice per group. Statistical analysis was performed using a one-tailed Mann–Whitney U-test. $^{**}p < 0.001$, $^{*}p < 0.01$. Arrow = adoptive transfer. Error bars are S.E.M. Data for Figure 5B were compared with the control group at each time point and significance is indicated.

and IL-17 by DLN cells. The lack of inhibitory effects on DLN cells exposed to alternative stimuli provided further evidence that the observed suppression of T cell responses following DM-MOG_{35-55} was MOG_{3555} specific.

Previous studies investigating MS therapies report that successful treatments induce a switch in the cytokine response from Th1 and IFNγ production towards Th2 and IL-4/IL-10 production [34,35]. The MS treatment, GA, has been reported to generate type II monocytes capable of inducing Th2 cell differentiation and expansion of T_{reg} with antigen non-specific suppressor function [34]. In this study, DM-MOG_{35-55}-treated monocytes exhibited antigen-specific suppressor function independent of either IL-4 or IL-10 production.

Earlier antigen-specific therapies for MS have looked to induce tolerance to MS-related antigens through exposure to antigen alone [12,36,37]. Although it is yet to be proven, the absence of antigen targeting in these approaches may be a contributing factor towards the side effects observed in clinical trials. The antigen-specific suppression observed with DM-MOG_{35-55} treatment therefore has the potential to avoid the common side effects associated with current non-specific MS therapies.

The ability of DM to target monocytes and enhance their suppressor function (Figure 4E) may also play a key role in the ability of blood $CD11b^+Ly6G^-$ monocytes from DM-MOG_{35-55} mice to suppress EAE. We have shown previously that naïve circulating monocytes suppress T cell activation via an iNOS-dependent mechanism and that this suppressor function is switched off upon induction of EAE [21]. In this study, DM-MOG_{35-55} treatment not only re-established iNOS-dependent monocyte suppressor function but also induced antigen-specific protection against the development of EAE. The MS therapeutic agent Copaxone (Glatiramer Acetate/GA) also targets blood $CD11b^+Ly6G^-$ monocytes resulting in increased monocyte suppressor function and protection from the development of EAE [22]. The fact that DM-MOG_{35-55} induces a similar suppressive mechanism is encouraging for the development of DM-antigen therapy for the treatment of relapsing/remitting MS.

This study provides strong evidence that blood $CD11b^+Ly6G^-$ monocytes are key players in the suppressive effect of DM-MOG_{35-55} treatment in EAE. However, blood monocytes are precursors of various APCs, such as macrophages and dendritic cells. Further studies are warranted to determine if the DM-MOG_{35-55}-primed monocytes also contribute to suppression of EAE by differentiating into suppressor APCs capable of inhibiting the MOG_{35-55}-specific T cell response. The possibility that DM-MOG treatment may also enhance the suppressor function of the splenic $CD11b^+Ly6C^+$ myeloid-derived suppressor cells observed in EAE mice [38] is also worthy of future investigation.

We have shown in this article that the DM-MOG_{35-55} treatment restored the blood $CD11b^+Ly6G^-$ monocyte suppressive capability five days after the treatment (before the EAE symptoms) and adoptive transfer of these cells effectively protected the recipient mice against the disease. However, it remains unclear why some mice were not protected by the treatment and whether the blood $CD11b^+Ly6G^-$ monocytes suppressive capabilities differ between the DM-MOG_{35-55} treated "sick" and "non-sick" mice.

It should be noted that this study investigated the prophylactic effects of DM-MOG_{35-55} treatment on EAE. Although this prophylactic suppression of EAE by DM-MOG_{35-55} identifies the potential for using DM-autoantigen conjugates to induce antigen-specific suppression of autoimmunity, future studies will be required to assess the therapeutic capacity of the DM-MOG_{35-55} platform in established disease.

In summary, we report that DM-MOG_{35-55} treatment can induce systemic antigen-specific suppression of EAE and identify blood $CD11b^+Ly6G^-$ suppressor cells as mediating DM-MOG_{35-55}-induced immunosuppression *in vivo*. DM-antigen therapy therefore represents a potentially new therapeutic approach for establishing antigen-specific immune tolerance to disease-causing antigens in human MS and other autoimmune conditions.

Acknowledgements

We would thank to Kylie Price and Evelyn Spittle for their technical support. This research was supported by: the Lottery Health Research Committee (NZ), the Health Research Council (NZ) and the Wellington Medical Research Foundation (NZ).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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