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Antigen Targeting to Plasmacytoid Dendritic Cells via Siglec-H Inhibits Th Cell-Dependent Autoimmunity

Jakob Loschko,*,† Sylvia Heink, †,1 Daniela Hackl,*,‡ Diana Dudziak, †,1 Wolfgang Reindl,*,1 Thomas Korn, †,1 and Anne B. Krug* *,†

Plasmacytoid dendritic cells (PDCs) have been shown to present Ags and to contribute to peripheral immune tolerance and to Ag-specific adaptive immunity. However, modulation of adaptive immune responses by selective Ag targeting to PDCs with the aim of preventing autoimmunity has not been investigated. In the current study, we demonstrate that in vivo Ag delivery to murine PDCs via the specifically expressed surface molecule sialic acid binding Ig-like lectin H (Siglec-H) inhibits Th cell and Ab responses in the presence of strong immune stimulation in an Ag-specific manner. Correlating with sustained low-level MHC class II-restricted Ag presentation on PDCs, Siglec-H–mediated Ag delivery induced a hyporesponsive state in CD4+ T cells leading to reduced expansion and Th1/Th17 cell polarization without conversion to Foxp3+ regulatory T cells or deviation to Th2 or Tr1 cells. Siglec-H–mediated delivery of a T cell epitope derived from the autoantigen myelin oligodendrocyte glycoprotein to PDCs effectively delayed onset and reduced disease severity in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis by interfering with the priming phase without promoting the generation or expansion of myelin oligodendrocyte glycoprotein-specific Foxp3+ regulatory T cells. We conclude that Ag delivery to PDCs can be harnessed to inhibit Ag-specific immune responses and prevent Th cell-dependent autoimmunity. The Journal of Immunology, 2011, 187: 6346–6356.

Plasmacytoid dendritic cells (PDCs) are a well-defined subpopulation of dendritic cells (DCs) found mainly in blood and lymphoid organs but also in peripheral tissues. They secrete large amounts of type I IFN upon stimulation by viruses or autoimmune complexes bearing TLR7 and TLR9 ligands. PDCs thus contribute to the antiviral immune response and have been implicated in the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus and Sjögren’s syndrome where type I IFNs play a role (1). Their role in CNS autoimmunity is controversial (2, 3). In addition to producing type I IFN and other cytokines, PDCs can present Ags to CD4+ T cells and are capable of cross-presenting Ags to CD8+ T cells (4–6). However, it has been shown that human resting as well as activated PDCs are also able to induce CD4+ regulatory T cells (Tregs) in vitro via expression of inducible costimulator ligand (ICOS-L) or indolamine dioxygenase (7, 8). Indeed, PDCs have been shown to inhibit immune responses in several murine disease models including asthma, allogeneic transplant rejection, type 1 diabetes, systemic lupus erythematosus, rheumatoid arthritis, and experimental autoimmune encephalomyelitis (EAE) by promoting Tregs or by reducing pathogenic effector T cell responses (9–15). In addition, PDCs in the liver and mesenteric lymph nodes are required for oral tolerance induction (16). However, most of these studies have been performed by selective depletion or adoptive transfer of PDCs and therefore cannot easily distinguish between innate and adaptive functions of PDCs in these models. A recent study using mice lacking MHC class II (MHC II) expression selectively in PDCs revealed that PDCs actually present autoantigen on MHC II in vivo and reduce autoimmune CD4+ T cell responses in an Ag-specific manner involving expansion of natural Tregs thus regulating CNS autoimmunity in murine EAE (2).

In contrast to conventional dendritic cells (CDCs), PDCs have a high turnover of peptide–MHC II complexes and do not stabilize peptide–MHC II complexes on the cell surface upon activation (17). PDCs are therefore less efficient than CDCs in MHC II presentation of exogenous soluble Ag, which is available at high concentrations for a short period of time. They appear to be specialized to present either endogenous Ags or exogenous Ags, which are constantly available or which are internalized after binding to cell surface receptors (5, 6, 18, 19). The studies conducted to date reveal that PDCs present Ags under specific conditions in vivo, but whether the ensuing T cell responses confer tolerance or immunity appears to be dependent on the mode of Ag delivery and on environmental signals.

Ab-mediated Ag delivery to DC subpopulations in vivo is an attractive approach to either induce or inhibit Ag-specific immune

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Abbreviations used in this article: CDC, conventional dendritic cell; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; ICOS-L, inducible costimulator ligand; MHC II, MHC class II; MOG, myelin oligodendrocyte glycoprotein; PDC, plasmacytoid dendritic cell; pHEL, hen egg lysozyme-derived peptide; pMOG, myelin oligodendrocyte glycoprotein-derived peptide; poly(C), polyinosinic-polycytidylic acid; pOVA, OVA peptide; PTx, pertussis toxin; rMOG, recombinant myelin oligodendrocyte glycoprotein; Siglec-H, sialic acid binding Ig-like lectin H, Treg, regulatory T cell.
responses avoiding cumbersome ex vivo manipulation and adoptive transfer of these rare cell types (20–24). Our aim was therefore to target Ags specifically to PDCs to analyze further their Ag-presenting function and to use this strategy for modulating Ag-specific immune responses in vivo.

Ag delivery to murine PDCs in vivo via the specifically expressed surface molecule sialic acid binding Ig-like lectin H (Siglec-H) led to continuous low-level Ag presentation on MHC II sufficient for CD4+ T cell priming. However, this Ag-targeting strategy not only failed to induce but even inhibited Th cell-dependent immunity. This potent Ag-specific inhibitory effect was mediated by attenuation of CD4+ T cell expansion and subsequent Th cell differentiation. Consequently, Siglec-H–mediated delivery of myelin autoantigen to PDCs effectively inhibited CNS autoimmunity by interfering with the priming phase of EAE.

Materials and Methods

Mice

Specific pathogen-free, female C57BL/6, C3H, and BALB/c mice were purchased from Harlan Winkelmann (Borchen, Germany) or were bred in our animal facility and were used at an age of 6–8 wk. DO11.10/Rag2 (BALB/c background) and Fospx3gfp knockin (on C57BL/6 background) (25) and CD45.1 C57BL/6 congenic mice were bred under specific pathogen-free conditions in our animal facility. Experiments were performed in accordance with German animal care and ethics legislation and were approved by the local government authorities.

Generation of recombinant Abs

Recombinant anti–Siglec-H fused to OVA was generated as described for recombinant anti–BST2–OVA (5). In brief, the variable regions of the H and L chains of the anti–Siglec-H rat mAb (440c, Ref. 26) were cloned into previously described vectors encoding for a mouse IgG1 Ab (anti–DEC205) fused to full-length OVA (20, 27). Anti–Siglec–H–hen egg lysozyme–derived peptide (pHEL) and anti–Siglec–H–myelin oligodendrocyte glycoprotein–derived peptide (pMOG) Abs were generated by replacing OVA with pHEL (aa 48–62, DGSTDYGILQINSR) and pMOG (aa 35–55, MEVGYWYRPSFSRVEHLYRNGK), respectively. Recombinant Ab–Ag fusion proteins were expressed and purified as described (5). Because of a mutation in the C3γ region, these Abs do not bind to Fc receptors (20).

In vivo Ag presentation

C3H mice were injected i.p. with 20 μg of the Ab–pHEL fusion proteins with or without 10 μg CpG 1668 (MWG Biotech, Ebersberg, Germany) or 50 μg polyinosinic:polycytidylic acid (polyI:C) (Invivogen, San Diego, CA). At the indicated time points, splenocytes and lymph node cells were stained with fluorescently labeled PDC-specific Abs (440c, 120G8), anti–CCR9, and the Aw3.18 Ab recognizing the complex of pHEL and MHC II (pHEL/I-Ak) and were analyzed by flow cytometry. CD11c, anti-CCR9, and the Aw3.18 Ab were purchased from Harlan Winkelmann (Borchen, Germany) or were bred in our laboratories. Propidium iodide was added to exclude dead cells. For intracellular cytokine staining, cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% saponin for 30 min, and stained with fluorescently labeled Abs (anti–IFN-γ, anti–IL-2). Analysis was performed using FlowJo software (Tree Star, Ashland, OR).

T cell transfer experiments

CD4+ T cells were isolated from splenocytes of DO11.10/Rag2−/− mice after treatment with collagenase D and DNase I (Roche, Mannheim, Germany) by negative selection using the CD4+ T cell MACS isolation kit (purity >90%; Miltenyi Biotech, Bergisch Gladbach, Germany). CD4+ T cells were labeled with 5 μM CFSE (Invitrogen, Karlsruhe, Germany) and 2 × 105 to 4 × 106 cells/mouse were injected i.v. into BALB/c mice. Recipient mice were injected i.p. with the indicated doses of chimeric OVA–fused or pHEL–fused Abs with or without 10 μg CpG 1668 24 h later. Where indicated, 100 μg soluble OVA (Hyglos, Regensburg, Germany) was administered i.p. at the same time. After 4 d, cells were isolated and analyzed by flow cytometry for CFSE dilution (proliferation) and expression of the indicated molecules. Where indicated, cells were restimulated with 5 μg/ml OVA peptide (pOVA, aa 323–339; GenScript, Piscataway, NJ) or 5 ng/ml PMA and 500 ng/ml ionomycin (both Sigma Aldrich, St. Louis, MO) for 6 h. Intracellular staining for cytokines was performed as described earlier.

Immunization of C57BL/6 mice with Ab–OVA fusion proteins

C57BL/6 mice were immunized i.p. with 10 μg of the indicated Ab–OVA fusion proteins or 10 μg soluble OVA in combination with 50 μg polyI:C. Anti–OVA Ab titers were determined in the serum by ELISA 14 d after immunization. For analysis of Th cell activation, C57BL/6 mice were immunized s.c. in the base of tail with 100 μg pOVA in CFA (Mycobacterium tuberculosis H37Ra; Difco, Detroit, MI) and received 200 ng pertussis toxin (PTx; Flow, Karlsruhe, Germany) i.v. on the same day and 2 d after the immunization with pOVA/CFA. Mice received 10 μg anti–Siglec–H–OVA 1 d prior to the immunization. Ten days after the immunization with pOVA/CFA/PTx, splenocytes were restimulated with 5 μg/ml pOVA ex vivo and stained for cytokines intracellularly.

Detection of OVA-specific and myelin oligodendrocyte glycoprotein–specific Abs by ELISA

ELISA for OVA-specific and myelin oligodendrocyte glycoprotein (MOG)–specific IgG Abs was performed according to the general protocol provided by BD Biosciences. Maxisorb ELISA plates (Nunc, Roskilde, Denmark) were coated with 5 μg/ml OVA or 10 μg/ml recombinant myelin oligodendrocyte glycoprotein (rMOG, aa 1–117). After washing and blocking, the plates were incubated with serial dilutions of the collected sera. For detection, biotinylated anti-mouse IgG1, IgG2b, and IgG2c Abs (1 μg/ml; BD Biosciences) and streptavidin–HRP (1:3000; GE Healthcare) or HRP-conjugated anti-mouse IgG (Southern Biotech) were used. ABTS was used as substrate (Roche), and the reaction was stopped by adding 1% SDS.

Absorbance was measured at 405 nm. Titers represent the highest dilution of serum showing an OD405 >0.1.

Experimental autoimmune encephalomyelitis

To induce EAE, C57BL/6 mice were immunized s.c. in the base of tail with 200 μl of an emulsion containing 200 μg pMOG (aa 35–55, MEVGYWYRPSFSRVEHLYRNGK; Auspep, Tullamarine, Australia) or 50 μg rMOG (aa 1–117; synthesized by Claude Bernard and kindly provided by Martin Weber) (30) and 500 μg M. tuberculosis H37Ra in CFA and received 200 ng PTx i.v. on the same day and 2 d after the immunization as described (31). Where indicated, mice received 10 μg anti–Siglec–H–MOG or anti–Siglec–H–OVA (irrelevant Ag control) 1 d prior to immunization with pOVA/CFA/PTx. Disease progress and severity were assayed using a clinical score as previously described (31).

Analysis of CNS cellular infiltrates

CNS-infiltrating cells were isolated after perfusion through the left cardiac ventricle with PBS. Brain and spinal cord were dissected and digested with collagenase D (2.5 mg/ml) and DNase I (1 mg/ml) at 37°C for 45 min. After passing the tissue through a 70-μm cell strainer, cells were separated by discontinuous Percoll gradient (70%/37%) centrifugation. Mononuclear cells were isolated from the interfase, washed, and restimulated with PMA, ionomycin, and GolgiStop at 37°C for 4 h. Cells were stained with Live/Dead Fixable Aqua Dead Cell Stain (Invitrogen) and the surface markers CD3 and CD4 for flow cytometry. Subsequently, cells were fixed and permeabilized (Cytofix/Cytoperm and Perm/Wash Buffer; BD Biosciences) and stained for cytokines IL-17a (clone TC11-18H10.1; Bio-Legend, San Diego, CA), IFN-γ, and IL-2.

Statistical analysis

Data are presented as the mean ± SEM or as the mean ± SD, as indicated. Statistical analyses (nonparametric two-tailed Mann–Whitney U test) or
ANOVA with post hoc Bonferroni corrected for multiple testing were performed with GraphPad Prism 5.01. Where indicated, the unpaired two-tailed Student t test was used to determine significant differences.

Results

Specific MHC II Ag presentation by murine PDCs after Ab-mediated Ag delivery

To achieve PDC-specific Ag presentation in vivo, we generated recombinant anti–Siglec-H and anti-BST2 Abs fused to the model Ag OVA or pHEL (Supplemental Fig. 1A). Specific binding to PDCs and not CDCs or other non-PDCs in the spleen was confirmed 3 h after injection (Supplemental Fig. 1B). Thus, PDCs are specifically targeted using these Abs.

To demonstrate directly Ag presentation on MHC II ex vivo, anti–Siglec-H–pHEL, anti-BST2–pHEL, or an isotype–pHEL control Ab were injected into I-A^b–expressing C3H mice. After 16 h, the density of pHEL/I-A^b complexes on the surfaces of splenocytes and lymph node cells was quantified by Aw3.18 Ab staining. pHEL/I-A^b complexes were detected on PDCs but not CDCs or any other non-PDCs in spleen (Fig. 1A). Ag presentation on MHC II was restricted to CCR9^+ PDCs, which have been shown to have tolerogenic activity upon adoptive transfer (11). Specificity of Ag delivery to PDCs was maintained also at later time points (48 and 72 h) after injection and in combination with CpG or polyI:C (5). Thus, Ag delivery via Siglec-H and BST2 leads to specific and efficient presentation of Ag on MHC II in CCR9^+ PDCs.

After a single injection of anti–Siglec-H–pHEL, pHEL/I-A^b complexes were continuously detected at a low density on the surfaces of PDCs for 6 to 8 d. In contrast, administration of anti-BST2–pHEL led to a high density of pHEL/I-A^b complexes on the surfaces of PDCs, but the Ag was presented for a much shorter time period (Fig. 1B). Thus, anti–Siglec-H–mediated and anti-BST2–mediated Ag delivery differ substantially in the strength and duration of the antigenic signal that is achieved, although both Abs target the Ag to PDCs.

We did not detect differences in the expression levels of MHC II, CD40, B7.1/CD80, B7.2/CD86, B7-H3, B7-H4, CD69, and CCR9 on PDCs or the production of cytokines (IL-6, IL-12, IFN-α) by PDCs in vitro after incubation with recombinant anti–Siglec-H and anti-BST2 Abs with or without CpG stimulation (Supplemental Figs. 2 and 3). Thus, engagement of Siglec-H or BST2 on the surface of PDCs by recombinant Abs is unlikely to alter the phenotype or function of PDCs in an Ag-independent nonspecific manner.

Anti–Siglec-H–mediated Ag delivery to PDCs inhibits Ag-specific immune responses

In our previous study, we demonstrated that immunization with anti-BST2–OVA combined with polyI:C as adjuvant elicits high titers of OVA-specific Abs with a broad range of IgG isotypes (5). We therefore investigated whether administration of anti–Siglec-H–OVA in combination with polyI:C leads to a comparable anti-OVA Ab response. Side-by-side comparison of anti–Siglec-H–

![FIGURE 1. PDC-specific Ag presentation after Ag targeting to Siglec-H and BST2. A, C3H mice were injected (i.p.) with 20 μg anti–Siglec-H–pHEL, anti-BST2–pHEL, or an isotype–pHEL fusion proteins. After 16 h, HEL-peptide–MHC II complexes (pHEL/I-A^b) were detected on the surfaces of PDCs, CDCs, and non-PDCs within splenocytes. Filled histograms, isotype–pHEL; open histograms, anti–Siglec-H–pHEL or anti-BST2–pHEL. Numbers in histograms indicate the mean fluorescence intensity (gray, isotype–pHEL control; black, anti–Siglec-H–pHEL or anti-BST2–pHEL). Dot plots show the correlation of pHEL/I-A^b formation and CCR9 expression for PDCs. The percentages are indicated in the quadrants. Results of one representative of three independent experiments are shown. B, C3H mice were injected (i.p.) with 20 μg anti–Siglec-H–pHEL or anti-BST2–pHEL. Presence of pHEL/I-A^b complexes on the surfaces of splenic PDCs was detected at different time points after injection. Filled gray histograms, PBS; open histograms, anti–Siglec-H–pHEL (upper row) or anti-BST2–pHEL (lower row). Numbers in histograms indicate the mean fluorescence intensity (gray, PBS; black, anti–Siglec-H–pHEL or anti-BST2–pHEL). Results of one representative of three independent experiments are shown.](http://www.jimmunol.org/content/163/11/6348/F1.large.jpg)
OVA/polyI:C and anti-BST2–OV A/polyI:C revealed that only BST2-mediated but not Siglec-H-mediated delivery of OVA to PDCs was capable of inducing Ab responses, and these were even stronger than those elicited by immunization with soluble OVA and polyI:C (Fig. 2A). Thus, persistent low-level MHC II Ag presentation on PDCs observed after anti–Siglec-H–mediated Ag delivery did not induce Ag-specific immunity even in the presence of a strong immunostimulatory adjuvant, whereas BST2-mediated Ag delivery combined with polyI:C was highly efficient in inducing immune responses as reported (5).

Lack of Ab production in response to immunization with anti–Siglec-H–OVA/polyI:C may be due to insufficient and therefore abortive antigenic stimulation but could also reflect inhibition of Ag-specific immune responses. To test this hypothesis further, mice were immunized with a high dose of soluble OVA combined with polyI:C to elicit Ab responses and simultaneously received the recombinant anti–Siglec-H–OVA Ab. As shown in Fig. 2B, production of anti-OVA Abs (IgG1, IgG2b, and IgG2c) in response to OVA/polyI:C immunization was almost completely abolished by simultaneous treatment with anti–Siglec-H–OVA and differed significantly from anti-OVA Ab titers detected in mice immunized with soluble OVA/polyI:C. Thus, Ag delivery to PDCs via Siglec-H is an efficient strategy to inhibit Ag-specific immune responses even in the presence of strong Th1-skewing immune stimulation.

**Ag delivery to PDCs via Siglec-H primes naive T cells but fails to induce de novo generation of Foxp3* Tregs**

The lack of Ab responses to anti–Siglec-H–OVA immunization could be due to hyporesponsiveness or deletion of Th cells. However, the observation that Siglec-H–mediated Ag delivery furthermore inhibits Ab responses to a high dose of soluble Ag formulated with a strong adjuvant suggests that active suppression may be induced by PDCs. A potent mechanism for active suppression of Ag-specific immune responses is the extrathymic de novo generation of Foxp3-expressing Tregs from naive precursors. We therefore tested the ability of PDCs to convert naive Ag-specific CD4+ T cells into adaptive Tregs in vivo.

CFSE-labeled DO11.10/Rag2−/− T cells were transferred into BALB/c mice to measure proliferation and de novo induction of Foxp3 expression 4 d after immunization with anti–Siglec-H–OVA (Fig. 3A). Expansion of a preexisting Foxp3+ population upon transfer can be excluded in this experimental setting because the transferred T cells do not express Foxp3. Immunization was performed in the presence or absence of CpG, which directly stimulates PDCs through TLR9 and has been shown to support

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**FIGURE 2.** Influence of anti–Siglec-H–OVA administration on the generation of OVA-specific Ab responses. A, C57BL/6 mice were immunized (i.p.) with 10 μg anti–Siglec-H–OVA, 10 μg anti-BST2-OVA, or 10 μg soluble OVA in combination with 50 μg polyI:C. OVA-specific Abs were quantified in the serum by isotype-specific ELISAs 14 d later (mean ± SD, n = 3–4). Insets depict Ab titers of mice immunized with soluble OVA/polyI:C and anti–Siglec-H–OVA/polyI:C, respectively (mean ± SD, n = 3–4), *p < 0.05 (Student t test). B, C57BL/6 mice were immunized (i.p.) with 100 μg soluble OVA in combination with 50 μg polyI:C. Where indicated, mice simultaneously received 10 μg anti–Siglec-H–OVA (i.p.). OVA-specific Abs were quantified in the serum by isotype-specific ELISAs 14 d later (mean ± SD, n = 6–7 mice per group). Insets depict Ab titers of mice immunized with soluble OVA/polyI:C and soluble OVA/anti–Siglec-H–OVA/polyI:C, respectively (mean ± SD, n = 6–7). *p < 0.05 (Student t test).
were detected by flow cytometry in DO11.10-TCR+ T cells from pooled splenocytes and lymph node cells. Dot plots show results from one representative

proliferation of naive Ag-specific CD4+ T cells. However, in contrast to anti-DEC205 treatment, Siglec-H–mediated Ag targeting to PDCs via Siglec-H leads to priming and initial CD4+/Foxp3+ T cells produced IL-2 (Fig. 3C) with PMA/ionomycin 4 d after anti–Siglec-H–OV A immunization, enhanced by injection of CpG (Fig. 3B).

In contrast to anti-DEC205–OV A, anti–Siglec-H–OV A failed to induce Foxp3 expression at any of the doses used in the absence or presence of CpG (Fig. 3B). Upon in vitro restimulation with PMA/انونycin 4 d after anti–Siglec-H–OV A immunization, T cells produced IL-2 (Fig. 3C) but no IFN-γ or IL-10 (data not shown). These results show that the strength of the antigenic signal and the costimulation provided by PDCs after Ag delivery via Siglec-H is sufficient for CD4+ T cell priming.

In contrast to anti-DEC205–OV A, anti–Siglec-H–OV A failed to induce Foxp3 expression at any of the doses used in the absence or presence of CpG (Fig. 3B). In all conditions with Siglec-H–mediated PDC-specific Ag presentation, less than 3% of the undivided transferred T cells expressed Foxp3 (Fig. 3D). Foxp3 expression decreased progressively with the number of cell divisions as previously reported (21).

Anti-Siglec-H–OV A dose-dependently induced proliferation of transferred naive OV A-specific CD4+ T cells, which was further enhanced by injection of CpG (Fig. 3B). Upon in vitro restimulation with PMA/انونycin 4 d after anti–Siglec-H–OV A immunization, T cells produced IL-2 (Fig. 3C) but no IFN-γ or IL-10 (data not shown). These results show that the strength of the antigenic signal and the costimulation provided by PDCs after Ag delivery via Siglec-H is sufficient for CD4+ T cell priming.

In contrast to anti-DEC205–OV A, anti–Siglec-H–OV A failed to induce Foxp3 expression at any of the doses used in the absence or presence of CpG (Fig. 3B). In all conditions with Siglec-H–mediated PDC-specific Ag presentation, less than 3% of the undivided DO11.10/Rag2−/− T cells expressed Foxp3, confirming that Foxp3 induction was inefficient (Fig. 3B, 3D). We conclude that Ag targeting to PDCs via Siglec-H leads to priming and initial proliferation of naive Ag-specific CD4+ T cells. However, in contrast to anti-DEC205 treatment, Siglec-H–mediated Ag targeting to PDCs is unable to induce conversion of naive T cells into Ag-specific Foxp3+ Tregs.

Siglec-H–mediated Ag presentation by PDCs inhibits Th cell expansion and Th1/Th17 differentiation in response to soluble Ag in vivo

Because inhibition of OVA-specific Ab responses was observed after coadministration of anti–Siglec-H–OV A and soluble OVA with adjuvant (Fig. 2B), we asked if anti–Siglec-H–OV A impairs Th cell responses to soluble OVA, which is predominantly presented on MHC II by CDCs (17). Mice that had received CFSE-labeled DO11.10/Rag2−/− cells 24 h earlier were immunized with a high dose of soluble OVA in the absence or presence of CpG as stimulus and simultaneously received anti–Siglec-H–OV A. Although the majority of OVA-specific T cells had proliferated and produced IL-2 after 4 d, administration of anti–Siglec-H–OV A considerably slowed down proliferation of OVA-specific CD4+ T cells in response to soluble OVA. The majority of T cells in mice immunized with soluble OVA alone proliferated seven times or more, whereas significantly more T cells underwent only 1 to 4 cell divisions when soluble OVA was administered simultaneously with anti–Siglec-H–OV A (Fig. 4A). The decelerated proliferation resulted in a reduced expansion of OVA-specific CD4+ T cells in response to soluble OVA even in the presence of CpG (Fig. 4B). Anti–Siglec-H fused to pHEL as irrelevant Ag did not influence the T cell response to soluble OVA (Fig. 4C) demonstrating Ag specificity. T cell proliferation was also attenuated when anti–Siglec-H–OV A was administered 2 d before immunization with soluble OVA, but not at earlier time points (Fig. 4D). Thus, persistent low-level Ag presentation on PDCs achieved by Siglec-H–mediated Ag delivery slows down proliferation and inhibits Th cell expansion in response to a high dose of soluble Ag administered with or without adjuvant stimulation.

We next investigated if the activation of endogenous non-TCR-transgenic Th cells and their differentiation into cytokine-producing effector cells is also affected by Siglec-H–mediated Ag delivery to PDCs. C57BL/6 wild-type mice were treated with anti–Siglec-H–OV A i.p. and were then immunized s.c. with pOVA in CFA. In addition, PTX was administered i.v. further to enhance Th1 and Th17 differentiation (32). Splenocytes were restimulated in vitro with pOVA 10 d after the immunization. The proliferative response to restimulation with different doses of pOVA was significantly lower in splenocytes from mice that had received anti–Siglec-H–OV A than in splenocytes of untreated mice (Fig. 5A). In
addition, the frequency of splenic CD4+ T cells producing IL-2, IFN-γ, or IL-17 upon peptide restimulation was greatly reduced when mice had been pretreated with anti–Siglec-H–ova 1 d prior to immunization with pOVA in CFA (Fig. 5B, 5C). A shift to IL-4–producing Th2 or IL-10–producing Tr1 cells was not observed. These results demonstrate that PDC-specific Ag presentation after Siglec-H–mediated delivery is capable of inhibiting the generation of Ag-specific Th1 and Th17 cells induced by a high dose of antigenic peptide in the context of strong immune stimulation.

Targeting of pMOG autoantigen to PDCs via Siglec-H delays the onset and reduces the severity of EAE

The influence of Siglec-H–mediated delivery of autoantigen to PDCs on Th cell-dependent autoimmunity was tested in the pMOG-induced EAE model. CNS autoimmunity in this model is critically dependent on pathogenic Th1 and Th17 cells that migrate to the CNS where they are reactivated to produce IFN-γ and IL-17 leading to further recruitment of inflammatory cells that cause tissue inflammation and demyelination. C57BL/6 mice received a single injection of anti–Siglec-H–pMOG or anti–Siglec-H–ova (irrelevant Ag control) or were left untreated. EAE was induced by pMOG/CFA/ptx immunization on the following day. Both untreated mice and mice treated with the anti–Siglec-H–ova control Ab developed disease with similar kinetics and severity. In mice that had been pretreated with anti–Siglec-H–pMOG 1 d before EAE induction, disease onset was delayed, and the maximum EAE score was significantly reduced in comparison with that of the control groups (Fig. 6A, Table 1). Consistent with alleviated disease, the number of CD4+ T cells infiltrating the CNS was reduced at the peak of EAE (day 17) in mice that had received anti–Siglec-H–pMOG compared with that in mice that had been pretreated with anti–Siglec-H–ova or were untreated (Fig. 6B).
Accordingly, the absolute numbers of cytokine-producing CD4+ T cells were reduced in anti–Siglec-H–pMOG treated mice compared with those in control animals (Fig. 6C). The fraction of IL-2, IFN-γ, or IL-17 single-producing CD4+ T cells was not changed upon treatment with anti–Siglec-H–pMOG. Only the percentage of IFN-γ/IL-17 double-producing cells within the CNS-derived CD4+ T cell compartment was slightly diminished in anti–Siglec-H–pMOG treated mice compared with that in controls (Fig. 6C). We did not observe a significant increase in the absolute numbers or the percentage of Foxp3+ CD4+ T cells in the CNS upon treatment with anti–Siglec-H–pMOG (Fig. 6D).

Although no significant increase of Tregs in the CNS was observed, a higher number of MOG-specific Tregs in the draining lymph node during T cell priming could have contributed to the observed effect of anti–Siglec-H–pMOG. To test whether MOG-specific Tregs were selectively expanded in the peripheral immune compartment during the priming phase of EAE by targeting pMOG to PDCs, Foxp3 expression was analyzed in transferred MOG-specific TCR transgenic 2D2 T cells (25) in anti–Siglec-H–pMOG treated and control mice 4 d after s.c. immunization with pMOG/CFA. Treatment with anti–Siglec-H–pMOG did not induce an increased expansion of Ag-specific Foxp3+ Tregs after immunization with pMOG/CFA (Fig. 7). Thus, targeting of pMOG to PDCs via Siglec-H is unlikely to convert naive MOG-specific T cells into Foxp3+ Tregs or expand preexisting MOG-specific Foxp3+ Tregs in the peripheral immune compartment. Rather, the priming of pathogenic effector Th cells is directly attenuated by anti–Siglec-H–pMOG treatment in an Ag-specific manner.

To investigate the influence of anti–Siglec-H–pMOG treatment on the production of MOG-specific autoantibodies, EAE was induced by MOG protein. Mice received either no treatment or were pretreated with anti–Siglec-H–OVA or anti–Siglec-H–pMOG 1 d prior to EAE induction. Similarly to the disease course in the pMOG-induced EAE, disease onset was delayed and disease severity was reduced in MOG protein-immunized mice treated with anti–Siglec-H–pMOG compared with those of control animals (Fig. 8A). The concentrations of MOG-reactive IgG autoantibodies including IgG1, IgG2a, and IgG2c isotypes were found to be reduced at the time of disease onset (Fig. 8B, C). Thus, autoantigen delivery to PDCs is effective in attenuating pathogenic effector Th cell responses and autoantibody responses, which both contribute to the pathogenesis of CNS autoimmunity in MOG protein-induced EAE.

**Discussion**

Our study shows that Ag targeting to PDCs in vivo can be harnessed to inhibit Th cell and Ab responses in the presence of strong immune stimulation in an Ag-specific manner. Ag delivery to this DC subpopulation per se was not tolerogenic, because the inhibitory effect on Th cell-mediated immunity was only achieved with Siglec-H–mediated but not BST2-mediated targeting of Ag to murine PDCs. Consistent with sustained low-level MHC II-restricted Ag presentation on PDCs after Siglec-H–mediated Ag delivery, CD4+ T cell expansion in response to a strong antigenic and costimulatory signal was halted and further differentiation to cytokine producing effector cells prevented, although Ag-specific
Tregs were not induced. Accordingly, inhibition of pathogenic Th1 and Th17 cell responses by Ag targeting to PDCs via Siglec-H led to delayed onset and amelioration of disease in murine EAE induced by MOG autoantigen.

**FIGURE 6.** Influence of anti–Siglec-H–pMOG pretreatment on pMOG-induced EAE. C57BL/6 mice were left untreated or received 10 μg anti–Siglec-H–pMOG or anti–Siglec-H–OVA (irrelevant Ag control) i.p. On the following day, mice were immunized with pMOG/CFA/PTx for EAE induction. A, EAE severity was assessed by clinical scoring until day 30 after the immunization. Results of one representative of three individual experiments are shown (mean ± SEM, six to eight mice per group). *p < 0.05 (Mann–Whitney U test). B–D, Foxp3gfp knockin mice were treated and immunized as described in A, and cellular infiltrates in the CNS were analyzed on day 17 postimmunization, the peak of disease in the untreated control group. B, Mononuclear cells isolated from individual CNS were stained for surface markers CD3 and CD4 to quantify the absolute numbers of Th cells infiltrating the brain. C, Cells restimulated ex vivo with PMA/ionomycin were stained intracellularly for IL-2, IFN-γ, and IL-17A. Absolute numbers and percentages are shown. The absolute numbers and frequencies of cytokine-producing cells were analyzed for vital CD3+/CD4+ Foxp3− cells. D, To quantify the absolute numbers and frequencies of Foxp3-expressing CD3+/CD4+ T cells, enhanced GFP-reporter expression was measured. Data are shown as mean ± SD (n = 3). *p < 0.05 (ANOVA, post hoc Bonferroni).

Ag targeting to PDCs via Siglec-H was inefficient in inducing and even inhibited Ag-specific immune responses. We found no evidence for modulation of the costimulatory or cytokine signal in PDCs by engagement of Siglec-H, but Siglec-H–mediated Ag
delivery led to continuous low-level Ag presentation on MHC II. This weak and persistent antigenic signal was sufficient to induce initial proliferation and IL-2 production in naive TCR-transgenic CD4+ T cells but did not support further Th cell polarization. We can thus confirm that PDCs can present exogenous Ag to CD4+ T cells after receptor-mediated delivery as reported (5, 6, 19, 33)

even when the density of peptide–MHC II complexes is low. However, the T cell response elicited by PDCs after Siglec-H–mediated Ag delivery is not sufficiently sustained to support an effective adaptive immune response.

In experiments using immunization with Ag fused to Siglec-H simultaneously with or shortly before administration of a bolus of soluble Ag, which is predominantly presented by CDCs (17), the majority of naive CD4+ T cells entered the cell cycle and started to proliferate and produce IL-2. However, compared with the immunization with soluble Ag alone, cell division did not further progress leading to reduced numbers of expanded Ag-specific CD4+ T cells. Our results suggest that CD4+ T cells are not unresponsive initially and are not rapidly deleted. However, proliferation is considerably slowed down after a few divisions following contact with PDCs, which provide a weak but persistent antigenic signal. The continuous presence of Ag on PDCs after Siglec-H–mediated delivery may lead to exhaustion and hyporesponsiveness of the T cells after successful initial priming (34). Reduced responsiveness of CD4+ T cells involving impaired c-Jun phosphorylation has been described when Ag was transgenically expressed in Dcs in vivo for prolonged time periods after the priming phase (34). Similarly, long-lasting presentation of low levels of nuclear Ags by DCs was required for the induction of T cell hyporesponsiveness (35).

Continuous or repeated administration of subimmunogenic doses of antigenic peptides or DEC205-mediated delivery of Ag to CD8a+ Dcs at low doses in the absence of costimulation induces tolerance mediated by Foxp3+ Tregs (13, 21, 36). However, we could not detect de novo generation of Foxp3+ Tregs from naive T cells after PDC-specific Ag presentation following Siglec-H–mediated delivery in contrast to DEC205-mediated Ag delivery to CD8a+ Dcs. This is most likely due to lack of TGF-β production by PDCs in contrast to CD8a+ Dcs (24). Inefficient activation of the mTOR pathway in the presence of specific environmental cues and in the absence of TGF-β leads to induction of hyporesponsiveness or anergy in T cells, whereas similar signals in the presence of TGF-β lead to the induction of Foxp3+ Tregs (37). Despite the lack of Treg induction by Ag targeting to PDCs, a single injection of Ag fused to the anti–Siglec-H Ab was highly efficient in inhibiting Ag-specific immune responses even in the presence of polyIC or CFA, which provide strong adjuvant stimulation. In contrast, DEC205-mediated Ag delivery to CD8a+ Dcs combined with polyIC or CFA, which provide strong adjuvant stimulation.
The role of PDCs in CNS autoimmunity is still controversial. PDC depletion during murine EAE revealed a divergent role of this cell population during the priming and the effector phases of the disease. Whereas depletion shortly before EAE induction reduced disease severity (3), depletion during acute or relapsing EAE exacerbated disease (9). A recent study demonstrated that the selective lack of MHC II expression on PDCs enhances the development of pathogenic pMOG-specific T cells during the priming phase of EAE (2). Our study confirms that Ag presentation by PDCs during the priming phase of an autoimmune response can be protective. Ag targeting to PDCs via Siglec-H prevented sufficient activation and expansion of Th cells and thus reduced the frequency of Th1 and Th17 cells generated in the periphery upon immunization with Ag in CFA without shifting to IL-4-producing Th2 or IL-10-producing Tr1 cells. In line with lower numbers of cytokine-producing effector Th1 and Th17 cells in the CNS, disease severity was significantly reduced in the pMOG-induced EAE model after pretreatment with anti–Siglec-H–pMOG but not in mice pretreated with anti–Siglec-H–OVA. Disease activity was similarly reduced in EAE induced by MOG protein, in which B cell responses play a critical role (38). This model allowed assessment of MOG-specific autoantibody responses, which were also reduced by anti–Siglec-H–pMOG pretreatment. These results show that delivery of autoantigen to PDCs not only attenuates Th cell responses but also inhibits autoantibody production, which significantly contributes to CNS autoimmunity.

Irla et al. (2) have reported that interaction of pMOG-presenting PDCs with CD4+ T cells promotes the expansion of natural Tregs after immunization with pMOG in CFA suggesting that dominant tolerance conferred by Tregs is supported by PDCs. In our study, however, targeting of pMOG to PDCs did not lead to a significantly higher percentage of Foxp3+ Tregs in transferred MOG-specific 2D2 T cells in spleen and draining lymph nodes during the priming phase or in the CNS during the effector phase of EAE. In addition, the proliferative response of transferred OVA-specific T cells to soluble Ag was only inhibited when the Ag was delivered to PDCs via Siglec-H at the same time or shortly before the soluble Ag pulse. Thus, Ag persistence during the priming phase was required for the observed inhibitory effect. These observations speak against Treg-mediated dominant tolerance as a major mechanism of action for the anti–Siglec-H–pMOG treatment and favor the interpretation that Ag presentation by PDCs after Siglec-H–mediated delivery induces a hyporesponsive state in Ag-specific CD4+ T cells thus preventing sufficient activation and differentiation to pathogenic Th1 and Th17 cells. We conclude that Ag delivery to PDCs can be tailored to inhibit Ag-specific immune responses and prevent Th cell-dependent autoimmunity in a Treg-independent manner.

**FIGURE 8.** Influence of anti–Siglec-H–pMOG pretreatment on MOG protein-induced EAE and anti-MOG Ab production. C57BL/6 mice were left untreated or received 10 μg anti–Siglec-H–pMOG or anti–Siglec-H–OVA (irrelevant Ag control) i.p. On the following day, mice were immunized with 50 μg MOG protein combined with CFA and PTx for EAE induction. A, EAE severity was assessed by clinical scoring until day 32 after induction (mean ± SEM, n = 5). *p < 0.05 (Mann–Whitney U test). B and C, MOG-specific Abs in the serum on day 12 after EAE induction were determined by ELISA. Titers are shown for total IgG (B, mean ± SD, n = 5). OD values of serial serum dilutions are shown for IgG isotypes (C, mean ± SD, n = 5). *p < 0.05 (Student t test).
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