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Regulatory T Cells Suppress the Late Phase of the Immune Response in Lymph Nodes through P-Selectin Glycoprotein Ligand-1

Stefano Angiari,*1 Barbara Rossi,*1 Laura Piccio,*1 Bernd H. Zinselmeyer,* Simona Budui,* Elena Zenaro,* Vittorina Della Bianca,* Simone D. Bach,* Elio Scarpini,*1 Matteo Bolomini-Vittori,* Gennj Piacentino,* Silvia Dusi,* Carlo Laudanna,*1,5 Anne H. Cross,* Mark J. Miller,‖ and Gabriela Constantin*

Regulatory T cells (Tregs) maintain tolerance toward self-antigens and suppress autoimmune diseases, although the underlying molecular mechanisms are unclear. In this study, we show that mice deficient for P-selectin glycoprotein ligand-1 (PSGL-1) develop a more severe form of experimental autoimmune encephalomyelitis than wild type animals do, suggesting that PSGL-1 has a role in the negative regulation of autoimmunity. We found that Tregs lacking PSGL-1 were unable to suppress experimental autoimmune encephalomyelitis and failed to inhibit T cell proliferation in vivo in the lymph nodes. Using two-photon laser-scanning microscopy in the lymph node, we found that PSGL-1 expression on Tregs had no role in the suppression of early T cell priming after immunization with Ag. Instead, PSGL-1-deficient Tregs lost the ability to modulate T cell movement and failed to inhibit the T cell–dendritic cell contacts and T cell clustering essential for sustained T cell activation during the late phase of the immune response. Notably, PSGL-1 expression on myelin-specific effector T cells had no role in T cell locomotion in the lymph node. Our data show that PSGL-1 represents a previously unknown, phase-specific mechanism for Treg-mediated suppression of the persistence of immune responses and autoimmunity induction. The Journal of Immunology, 2013, 191: 5489–5500.

R egulatory T cells (Tregs) are required to maintain immune system homeostasis by suppressing autoimmunity and moderating peripheral inflammation induced by pathogens and environmental insults (1, 2). Naturally occurring Tregs develop in the normal thymus, but induced Tregs can also be generated from naive T cells in the periphery (2). In mice, the transcription factor forkhead box P3 (Foxp3/scurfyn) controls both the development and activity of Tregs (3). Tregs suppress the activation and expansion of naïve T cell populations and their differentiation into effector T cells (including the T helper cells TH1, TH2, and TH17), thus regulating many diverse physiologic and pathologic immune responses (1, 2).

Previous studies have shown that one of the main suppressive mechanisms used by Tregs is the modulation of dendritic cell (DC) function (2, 4, 5). Indeed, elegant studies using two-photon laser scanning microscopy (TPLSM) have shown that Tregs can suppress early Ag presentation in the lymph nodes (LNs) shortly after Ag challenge, by directly establishing contacts with DCs and blocking the formation of stable conjugates between DCs and naïve T cells (6, 7). However, whether Tregs exert their influence on T cell–DC contacts during later phases of the immune response is not yet understood. Moreover, the molecular mechanisms mediating the suppression of T cell–DC contacts by Tregs are presently unknown.

The mucin P-selectin glycoprotein ligand-1 (PSGL-1) is a rolling receptor for P, L, and E selectins and is therefore a key mediator of adhesion for leukocyte trafficking at inflamed sites (8). PSGL-1 is also required for T cell homing to secondary lymphoid organs, reflecting its ability to bind specific chemokines such as CCL21 and CCL19 and thus increase T cell chemotaxis (9). In addition to its roles in cell trafficking, PSGL-1 expression on effector T cells has been shown to suppress T cell proliferation (10), and the cross-linking of PSGL-1 appears to induce the caspase-independent death of activated T cells (11). Moreover, PSGL-1 deficiency increases the severity of several animal models of autoimmune diseases, including lupus and inflammatory bowel disease, but the mechanisms responsible for this immune dysregulation are not understood (10, 12).

Tregs have been shown to suppress autoimmune diseases in numerous experimental models with experimental autoimmune encephalomyelitis (EAE) in mice. However, the mechanism underlying this suppression is not fully understood. In this study, we show that mice deficient for P-selectin glycoprotein ligand-1 (PSGL-1) develop a more severe form of experimental autoimmune encephalomyelitis than wild type animals do, suggesting that PSGL-1 has a role in the negative regulation of autoimmunity. We found that Tregs lacking PSGL-1 were unable to suppress experimental autoimmune encephalomyelitis and failed to inhibit T cell proliferation in vivo in the lymph nodes. Using two-photon laser-scanning microscopy in the lymph node, we found that PSGL-1 expression on Tregs had no role in the suppression of early T cell priming after immunization with Ag. Instead, PSGL-1-deficient Tregs lost the ability to modulate T cell movement and failed to inhibit the T cell–dendritic cell contacts and T cell clustering essential for sustained T cell activation during the late phase of the immune response. Notably, PSGL-1 expression on myelin-specific effector T cells had no role in T cell locomotion in the lymph node. Our data show that PSGL-1 represents a previously unknown, phase-specific mechanism for Treg-mediated suppression of the persistence of immune responses and autoimmunity induction.

1S.A. and B.R. contributed equally to this work.

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The online version of this article contains supplemental material.

Abbreviations used in this article: CMAC, 7-amino-4-chloromethylcoumarin; CMTMR, 5-(and-6)-((4-chloromethyl)benzoyl)amino)tetramethylrhodamine; DC, dendritic cell; dpi, days postimmunization; EAE, experimental autoimmune encephalomyelitis; LN, lymph node; MOG, myelin-oligodendrocyte glycoprotein; PSGL-1, P-selectin glycoprotein ligand-1; PTX, pertussis toxin; T cell, T effector cell; Tregs, regulatory T cells; T helper cells TH1, TH2, and TH17, thus regulating many diverse physiologic and pathologic immune responses (1, 2).

Previous studies have shown that one of the main suppressive mechanisms used by Tregs is the modulation of dendritic cell (DC) function (2, 4, 5). Indeed, elegant studies using two-photon laser scanning microscopy (TPLSM) have shown that Tregs can suppress early Ag presentation in the lymph nodes (LNs) shortly after Ag challenge, by directly establishing contacts with DCs and blocking the formation of stable conjugates between DCs and naïve T cells (6, 7). However, whether Tregs exert their influence on T cell–DC contacts during later phases of the immune response is not yet understood. Moreover, the molecular mechanisms mediating the suppression of T cell–DC contacts by Tregs are presently unknown.

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Tregs have been shown to suppress autoimmune diseases in numerous experimental models with experimental autoimmune encephalomyelitis (EAE) in mice. However, the mechanism underlying this suppression is not fully understood. In this study, we show that mice deficient for P-selectin glycoprotein ligand-1 (PSGL-1) develop a more severe form of experimental autoimmune encephalomyelitis than wild type animals do, suggesting that PSGL-1 has a role in the negative regulation of autoimmunity. We found that Tregs lacking PSGL-1 were unable to suppress experimental autoimmune encephalomyelitis and failed to inhibit T cell proliferation in vivo in the lymph nodes. Using two-photon laser-scanning microscopy in the lymph node, we found that PSGL-1 expression on Tregs had no role in the suppression of early T cell priming after immunization with Ag. Instead, PSGL-1-deficient Tregs lost the ability to modulate T cell movement and failed to inhibit the T cell–dendritic cell contacts and T cell clustering essential for sustained T cell activation during the late phase of the immune response. Notably, PSGL-1 expression on myelin-specific effector T cells had no role in T cell locomotion in the lymph node. Our data show that PSGL-1 represents a previously unknown, phase-specific mechanism for Treg-mediated suppression of the persistence of immune responses and autoimmunity induction.


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mune encephalomyelitis (EAE) (13), but little is known of the underlying mechanisms. In this study, we show that Tregs lacking PSGL-1 cannot suppress autoimmunity in a common EAE model induced with the MOG (myelin-oligodendrocyte glycoprotein)35–55 peptide. TPLSM experiments performed in explanted intact LNs showed that PSGL-1–deficient Tregs are unable to modulate T cell locomotion and fail to inhibit the formation of T cell–DC conjugates during the late phase of the immune response, which is characterized by sustained Ag-dependent T cell activation. Interestingly, PSGL-1–deficient Tregs preserved the capacity to suppress early T cell priming shortly after Ag challenge, suggesting that Tregs use phase-specific mechanisms to suppress the immune responses. Our results unveil a novel mechanism of immune system control and show that PSGL-1 expression on Tregs is responsible for the attenuation of persistent T cell activation in the LN essential for autoimmunity induction.

**Materials and Methods**

**Mice**

C57BL/6 female mice (6–8 wk old, obtained from The Jackson Laboratory) were used as wild type (WT) controls. Selplg−/− mice were provided by R. McEver from the Oklahoma University (14), and CD11c–yellow fluorescent protein (YFP) mice were provided by M. Nussenzweig from Rockefeller University (15). 2D2 and OT-II.2 mice were obtained from The Jackson Laboratory. Mice were housed and used according to current European Community and National Institutes of Health rules.

**FACS reagents and culture medium**

The rat anti-mouse PSGL-1 (clone 4RA10), α-integrin (clone PS2), CD44 (clone MM64), 150-5, t-selectin (clone Mel-14), and LFA-1 (clone Tib-213) Abs were provided by E. C. Butcher (Stanford University). The rat anti-mouse VioBlue-conjugated CD4, PE-CD25, FITC-Foxp3, allelophycocyanin-CTLA-4, FITC-CD3, PE-CD8, PE-CD19, PE-CD11c, FITC-CD103, PEGITR, FITC-CD69, FTCE- and allophycocyanin-isotype controls and PE- and FITC-secondary Abs were all obtained from eBioscience or Miltenyi Biotec. Flow cytometry acquisition was performed with FACSscan (Becton Dickinson) or MACSQuant Analyzer (Miltenyi Biotec). Analysis was performed with the FlowJo software (Tree Star). For all cell cultures, we used RPMI 1640 containing 10% v/v FBS, 1 mM Hepes, 1 mM Na pyruvate, and 100 U/ml penicillin/streptomycin (all reagents from Lonza).

**EAЕ induction**

Mice were immunized s.c. in the flanks and tail base with 150 μg MOG35–55 peptide (GenScript Corporation) in 200 μl emulsion consisting of equal volumes of PBS and CFA (Difco Laboratories), supplemented with 0.8 mg/ml mycobacterial cell wall (Mycobacterium tuberculosis) H37Ra (Becton-Dickinson). Mice received 20 ng of pertussis toxin (Alexis Biochemicals) i.v. at the time of immunization and 48 h later. Clinical scores were recorded daily for autoimmunity induction.

**Neuropathology**

EAЕ mice were sacrificed at the disease peak, and spinal cords were collected and frozen in liquid nitrogen; 10-μm sections were analyzed for histologic examination using H&E for detection of inflammatory infiltrates and Sp100/Spc-110 staining for coloration for myelin. Spinal cord demyelination was identified on individual images, traced manually on the composite images and surface areas measured using ImageJ software and expressed as a percentage of the total surface area. To quantify inflammatory activity in the spinal cord, we performed immunohistochemical analysis with a purified rat anti-mouse CD4 (L3T4) Ab for T cells (BD Pharmingen) and purified rat anti-mouse F4/80 Ab-pan (eBiosciences) for macrophages. All primary Abs were labeled with biotinylated secondary anti-rat Abs (Vector Lab) followed by Novared detection according to standard protocols. Quantification was performed on every fifth section. Images were captured at 40× magnification with a digital camera and analyzed using Image software. The images were quantified by determination of the total number of positive elements exclusively in the white matter and reported as the number of positive elements per square millimeter.

**Isolation of CD4+CD25+ Tregs**

Peripheral LNs and spleens were harvested from 8–10-wk-old C57BL/6, or Selplg−/− mice, dissociated mechanically, and washed twice after treatment with lysis buffer and antibiotic solution. CD4+CD25+ Tregs were isolated by magnetic cell sorting in a two-step procedure (Miltenyi Biotec), following the manufacturer’s instructions. Treg purity (typically 90–95%) was evaluated by labeling isolated cells with VioBlue-conjugated CD4 and PE-conjugated CD25 Abs. Foxp3 expression was determined using a rat anti-mouse Foxp3 Ab (eBioscience). All CD4+CD25+ cells were also Foxp3+.

**In vivo migration assay with CFSE-labeled Tregs**

Tregs from WT and Selplg−/− mice were isolated as previously described and labeled with green CFSE (Invitrogen) following the manufacturer’s instructions. Tregs (5 × 106) were injected i.v. in EAE mice 7 d post-immunization (dpi) with MOG35–55 peptide. After 18 h, LNs from transferred mice were collected, washed, and mechanically dissociated. The number of CFSE+ Tregs was evaluated with flow cytometry analysis. Finally, the number of CFSE+ Tregs per LN was calculated.

**In vivo Treg migration assay with [3H]-glycerol**

We cultured 4 × 106 Tregs (isolated from WT and Selplg−/− mice as described above) in a 48-well plate, in 1 ml of medium containing 25 μCi/ml [3H]-glycerol (GE Healthcare). The cells were collected after 18 h, washed three times with 1× D-PBS, resuspended in 500 μl of the same solution, and injected in mice 7 dpi with the MOG35–55 peptide. The mice were sacrificed after 24 h and perfused with 1× D-PBS including 5 U/ml heparin. Several organs were collected, weighed, and sonicated in water with Sonopuls (Bandelin Electronic). All samples were supplemented with 17 ml scintillation fluid (Ultima Gold from Perkin-Elmer), and the radioactivity was measured with a β-counter (Perkin-Elmer). The data were presented as the number of migrating cells per gram of tissue (16).

**Ex vivo T cell proliferation assay**

Total cells were collected from the draining LNs of 2D2 MOG35–55-immunized mice or OT-II.2 OVA–immunized (Sigma-Aldrich) mice 7 dpi. CD4+ cells were isolated by negative selection with magnetic cell sorting (Miltenyi Biotec). Cells were then labeled with CFSE and restimulated in vitro with 12 μM of MOG35-55 or OVA respectively, in the presence of 1.0 × 105 APCs (irradiated splenocytes). T cell proliferation was analyzed after 3 d by flow cytometry analysis (17). To compare the different conditions, a division index (the average number of cell divisions that a cell in the original population has undergone) and a proliferation index (the total number of divisions divided by the number of cells that went into division) were calculated with FlowJo software.

**In vitro T cell proliferation assay**

To assess T cell proliferation in vivo, 10 × 104 CD4+ MOG35-55–specific T cells obtained from 2D2 MOG35–55-immunized mice or OVA–specific T cells obtained from OT-II.2 OVA–immunized mice were labeled with CFSE and adoptively transferred into C57BL/6 J recipient mice 7 d after MOG35–55 or OVA immunization. In some experiments, 5 × 107 WT or Selplg−/− Tregs were i.v. injected 18 h before MOG35–55–specific T cells. CFSE+ T cell proliferation was determined by flow cytometry analysis 4 d after T cell injection.

**TPLSM: Sample preparation**

CD4+ T cells and Tregs were labeled for 30 min at 37°C with 40 mM 7-amino-4-chloromethylcoumarin (CMAC, Invitrogen) or 10 mM 5-(and-6)-((4-chloromethyl)benzoyl)amino) tetramethylrhodamine (CMTMR), respectively. CD4+ T cells (105) and 5 × 106 Tregs were transferred intraperitoneally to a CD11c–YFP recipient mouse at different time-points as shown. Six hours after the CD4+ T cell transfer, CD11c–YFP mice were killed by CO2 asphyxiation. Cervical or inguinal LNs were removed and transferred into
CO₂-independent medium (Life Technologies) at room temperature. The LNs were bound with a tissue-specific glue (Vetbond; 3M) on a coverslip, adhered with grease to the bottom of the imaging-flow chamber, and continuously maintained at 37°C by perfusion with warm medium (RPMI 1640 with 25 mM HEPES; Life Technologies) bubbled with a mixture of 95% O₂ and 5% CO₂ before and throughout the period of microscopy.

**TPLSM: Data acquisition**

Time-lapse imaging was performed with a custom-built dual-laser videorate two-photon microscope. CMAC-labeled CD4⁺ T cells, CD11c-YFP cells, and Treg cells in WT LNs were excited using two different T-cell laser tuned to 780–820 nm. Fluorescence emission from the three different fluorescent dyes was separated passing through 515- and 560-nm dichroic mirrors placed in series and detected as red (560–650 nm), green (490–560 nm), and blue (second harmonic) signal (K,458 nm) channels by three head-on Bialkali photomultiplier tubes (PMTs). A customized version of ImageWarp (A&B Software) was used during real-time acquisition to process and archive the image data. To reduce simultaneous laser excitation, CD11c-YFP positive cells were excited by a laser tuned to 890–900 nm, CMAC and CMTMR dyes were excited using a second laser tuned to 780–820 nm. Fluorescence intensity was measured by taking 21 sequential steps at 2.5 µm spacing during real-time acquisition to process and archive the image data. The image analysis showed larger areas of inflammation and demyelination in the spinal cords of Selplg⁻/⁻ mice (p < 0.0004; Fig. 1A and Supplemental Table I). Neuropathologic analysis showed larger areas of inflammation and demyelination in the spinal cords of Selplg⁻/⁻ mice and more infiltrated macrophages and CD4⁺ cells in the CNS (Fig. 1C, 1D, and Supplemental Table I). It was previously shown that T cell trafficking is reduced in the LNs of Selplg⁻/⁻ mice, suggesting that the immune response in the LNs is reduced (9). However, in contrast with this expectation, the increased severity of the disease in Selplg⁻/⁻ mice indicates a profound defect in the regulation of autoimmunity because of the absence of PSGL-1. Furthermore, when we transferred total LN cells isolated from WT and Selplg⁻/⁻ mice immunized with MOG35–55 peptide, we found that T cells lacking PSGL-1 induced a more severe form of the disease than WT cells.
Tregs deficient in PSGL-1 fail to suppress autoimmunity in vivo

We next addressed the ability of Tregs lacking PSGL-1 to ameliorate the clinical course of EAE in C57BL/6J mice immunized with the MOG35–55 peptide. We established that Tregs from WT mice limit the development of EAE when transferred in the preclinical disease phase, whereas they did not reduce the severity of EAE when injected at the disease peak (Supplemental Fig. 1). In particular, we found that WT Tregs transferred 7 d dpi had a stronger inhibitory effect (sustained over several weeks after the onset of disease) than Tregs administered before or shortly after immunization (Supplemental Fig. 1). However, we found that Tregs without PSGL-1 were completely unable to suppress the disease when administered 7 dpi (Fig. 2A). Expression of Foxp3, a master regulator of mouse Treg function, as well as cytotoxic T lymphocyte Ag (CTLA)-4 and adhesion molecules such as α4-integrin, lymphocyte function-associated Ag (LFA)-1, CD44 and l-selectin were not altered in Tregs in PSGL-1–deficient mice when compared with WT mice, suggesting that these molecules were not directly involved (Fig. 2B and data not shown) (2, 3, 24).

We also found no differences in the percentage of CD4+CD25+Foxp3+ cells in LNs when we compared WT and Selplg−/− mice with or without induced EAE, suggesting that PSGL-1 is not required for Treg migration in the high endothelial venules (Fig. 2C) (25). Notably, WT Tregs inhibited T cell proliferation in vivo, whereas Selplg−/− Tregs failed to modulate the proliferation of effector T cells 7 dpi, supporting a role for PSGL-1 in the control of T cell activation (Fig. 2D and Supplemental Table II). These data suggest that PSGL-1 is required for T-reg-mediated suppression of autoimmunity by modulating T cell proliferation in the LNs.

PSGL-1 expression on Tregs has no role in the suppression of early T cell priming

T cell activation requires contacts between naive T cells and DCs, which are established in the LNs soon after Ag challenge to initiate cell-mediated adaptive immune responses (18, 26, 27). TPLSM studies have shown that the recognition of peptide the number of CFSE+ Tregs/LN MHC (pMHC) by naive T cells leads rapidly to decreased T cell migration velocity and increased contact durations with DCs (28, 29). Therefore, the quantitative analysis of T cell dynamics has been used widely to detect Ag recognition.

**FIGURE 2.** Selplg−/− Tregs fail to suppress EAE and T cell proliferation. (A) Active EAE was induced in WT mice by immunization with MOG35–55 peptide, and 2.5 × 10⁶ naive WT (n = 8) or Selplg−/− (n = 8) Tregs were transplanted 7 dpi (red arrow). Data are from a representative experiment from three with similar results. (B) Total cells were collected from the LNs of naive WT and Selplg−/− mice. The expression of Foxp3 and CTLA-4 in WT (blue line) and Selplg−/− (green line) CD4+CD25+ cells was analyzed by flow cytometry (red line = isotype control). Data reflect one representative experiment from three with similar results. (C) Draining LNs from naive and actively EAE-immunized WT (n = 9) or Selplg−/− (n = 9) mice at 1 and 7 dpi were collected, and the percentage of CD25+Foxp3+ Tregs was determined with flow cytometry. No differences were found between WT and Selplg−/− mice. (D) CD4+ T cells from 2D2 MOG35–55-immunized mice were labeled with CFSE and injected i.v. into C57BL/6 mouse 7 dpi with MOG35–55 peptide. After 4 d, cells were assayed for cellular proliferation by flow cytometry. In some experiments, naive Tregs from WT or Selplg−/− mice were injected into host mice 18 h before CD4+ T cell transfer. Shown are representative histogram plots of CFSE+ MOG35–55-specific T cells proliferating in the absence (red line) or presence (blue line) of Tregs (see also Supplemental Table II). Results are representative of seven to eight mice per group.
in vivo. Previous studies have shown that Treg-mediated inhibition of T cell activation was associated with increased T cell motility and reduced contact time between Ag-specific T cells and DCs during the early phase of the immune response; this has been established in EAE induced by myelin basic protein (MBP) and in a model of autoimmune diabetes (6, 7). We therefore used TPLSM to investigate the effect of PSGL-1 deficiency on Ag-dependent T cell activation in explanted draining LNs, which recapitulate the T cell priming dynamics observed by intravital microscopy in surgically exposed LNs (18, 26, 28, 30, 31). We confirmed that, in our system and in the absence of Ag, naïve T cells moved at a velocity of ~10 μm/min, in a stop-and-go manner, and motility analysis indicated that this approximated a random-walk pattern (data not shown). These results agreed with data from earlier studies of explanted LNs (32, 33) and from in vivo studies of LN preparations (28, 30). T cells specific for the MOG_{35–55} peptide were isolated from naïve 2D2 mice and OVA-specific T cells (control cells) were obtained from naïve OT-II.2 mice. These were injected i.v. into recipient CD11c-YFP mice 24 h after immunization with the MOG_{35–55} peptide (Fig. 3A). As expected, we observed a significant reduction in the speed and motility coefficient of MOG_{35–55}-specific T cells in LNs 6 h after injection compared with OVA-specific T cells, consistent with robust Ag recognition (Fig. 3B–D; Supplemental Videos 1 and 2) (18, 29, 32). In addition, the arrest coefficient of MOG_{35–55}-specific T cells was significantly higher, suggesting there were prolonged contacts with Ag-presenting DCs (Fig. 3E) (26, 27, 29). As expected, the motility behavior of OVA-specific T cells in MOG_{35–55}-immunized mice resembled the movement of naïve T cells in immunized or control mice (data not shown), confirming that, in our system, T cell behavior is Ag dependent. Importantly, the velocity, motility coefficient, and arrest coefficient of MOG_{35–55}-specific T cells in OVA-immunized mice were comparable to OVA-specific T cells in MOG_{35–55}-immunized mice (Supplemental Fig. 2 A–C), suggesting no intrinsic differences in the ability of MOG_{35–55} and OVA-specific T cells to engage DCs in an Ag-dependent manner.

We next visualized the behavior of MOG_{35–55}-specific T cells and their contacts with DCs in the presence or absence of exogenous WT and PSGL-1–deficient Tregs. Tregs were transferred by tail injection 18 h before the induction of EAE to allow migration to the LNs and interactions with DCs (Fig. 4A). As expected and reported previously (7), MOG_{35–55}-specific T cells showed a significant increase in both speed and motility coefficients 6 h after transfer, as well as a lower arrest coefficient in the presence of WT Tregs (Fig. 4B–E; Supplemental Video 3), suggesting that Tregs attenuate the establishment of stable contacts during T cell priming by DCs (6, 7). We also determined the contact duration between MOG_{35–55}-specific CD4 T cells and DCs in the presence or absence of Tregs, specifying three categories: transient (>30, ≤500 s), intermediate (>500, ≤1800 s) and stable (>1800 s). The percentage of MOG_{35–55}-specific T cells making contacts with DCs during this early phase of the immune response was 63% of all analyzed cells. Of all analyzed cells, 13% were stable, 36% transient, and 51% intermediate. The mean contact time between MOG_{35–55}-specific T cells and DCs was 981 s (Fig. 4F). Notably, there was no statistically significant difference in MOG_{35–55}-specific T cell speed or motility coefficient induced by WT and Selplg^{−/−} Tregs (Fig. 4B–E; Supplemental Videos 3 and 4) and both types of Tregs also reduced the arrest coefficient and the mean contact time in a similar manner (Fig. 4E, 4F). These data suggest that PSGL-1 is not required for Tregs to suppress the initiation of the adaptive immune response.

**FIGURE 3.** Ag recognition by naïve T cells and changes in cell dynamics 1 dpi in draining LNs of MOG_{35–55}-immunized mice. (A) MOG_{35–55}-specific or OVA-specific T cells obtained from LNs and spleens of naïve 2D2 mice or OT-II.2 mice were injected i.v. into CD11c-YFP mice 1 dpi with MOG_{35–55} peptide, and images were acquired after 6 h. (B) TPLSM representative tracks of MOG_{35–55}- and OVA-specific T cells (blue cells) inside the draining LNs of immunized mice (green cells indicate DCs; Supplemental videos 1 and 2). Pseudo-color cell tracks are shown, indicating the time progression of T cell movement from the beginning (blue) to the end (yellow) of the imaging period. Original magnification ×20. (C) Mean velocity (**p < 0.0001) and (D) motility coefficient (**p < 0.0001) and (E) arrest coefficient (**p < 0.0001) of MOG_{35–55}-specific T cells compared with OVA-specific T cells are shown. The results show that the motility of MOG_{35–55}-specific T cells was strongly reduced after Ag challenge compared with non-antigen-specific (OVA) T cells.
Ag-dependent T cell activation persists during the late phase of the immune response

Having already shown that Tregs lacking PSGL-1 do not inhibit T cell proliferation when injected 7 dpi (Fig. 2D), we investigated the role of PSGL-1 in the advanced phase of the immune response. MOG35–55 and OVA-specific T cells were obtained 7 dpi as described above and were injected into CD11c-YFP mice 7 dpi with the MOG35–55 peptide (Fig. 5A). As shown for the initiation of the immune response, MOG35–55-specific T cells moved more slowly and had a lower motility coefficient and a higher arrest coefficient than OVA-specific T cells, suggesting that Ag recognition by T cells occurs during the late phase of the immune response (Fig. 5B–E; Supplemental Videos 5 and 6). As was the case at 1 dpi, the motility parameters of MOG35–55-specific T cells in OVA-immunized mice were comparable to OVA-specific T cells in MOG35–55-immunized mice (Supplemental Fig. 2D–F). However, MOG35–55-specific T cells had a greater speed and motility coefficient and a lower arrest coefficient 7 dpi compared with 1 dpi, suggesting that T cell activation-induced stop signals were less potent at later time points. We found that 49% of MOG35–55-specific T cells were in contact with DCs 7 dpi and that the mean duration of interactions was 570 s (Fig. 5F), showing a significant reduction in contact time compared with day 1 (Fig. 4F). A large proportion of T cell–DC contacts were transient (69% of the total conjugates), whereas the percentage of the intermediate contacts fell from 53% at the early phase to 11% at the late phase. Surprisingly, the number of stable contacts remained constant at ~20%, suggesting a selective contact dynamics profile with a preference for either stable or transient contacts during the late phase of the immune response. As expected, only transient contacts were observed between OVA-specific T cells and DCs in MOG35–55-immunized CD11c-YFP mice (Fig. 5F).

Interestingly, we found that MOG35–55-specific T cells tended to cluster together, presumably around unlabeled DCs (Fig. 5G and Supplemental Video 5). In contrast, activated OVA-specific T cells were distributed randomly (Fig. 5G). We investigated this distribution in more detail using the T cell analysis software (18). Whereas OVA-specific T cells were distributed randomly and did not form clusters, MOG35–55-specific T cells were associated with up to seven neighbors within 25 μm (Fig. 5H). This peculiar spatial distribution of the MOG35–55-specific T cells 7 dpi supports the likelihood that Ag-dependent events occur in the LNs during the late phase of the immune response.

We investigated the correlation between the Ag-dependent behavior observed by TPLSM and the T cell activation in LNs by measuring the proliferation of adoptively transferred CFSE-labeled T cells in EAE mice 7 d after they were injected with MOG. MOG35–55-specific T cells were able to proliferate efficiently, whereas OVA-specific control T cells did not, indicating that T cell activation during the late phase of the immune response is Ag dependent and not merely a result of the inflammatory microenvironment in the draining LN (Fig. 5I). The in vitro proliferative capacity of MOG35–55-specific and OVA-specific T cells...
were similar (data not shown), and there was no detectable difference in the cytokine profile of OVA- and MOG35–55-specific T cells in OT-II and 2D2 mice 7 dpi (data not shown). Overall, these results suggest that Ag-dependent T cell activation is maintained during the late phase of the immune response and may be essential for the induction of autoimmunity.
PSGL-1 expression on Tregs inhibits sustained T cell activation during the late phase of the immune response

Next we sought to determine the role of Tregs and PSGL-1 on T cell activation 7 dpi. We adoptively transferred Tregs into CD11c-YFP recipient mice 7 dpi, and images were acquired after 6 h. (Fig. 6A) We observed no difference in the draining LNs of recipient animals (data not shown). MOG35–55-specific T cells had a much higher speed and motility coefficient and a lower arrest coefficient in the presence of WT Tregs (Fig. 6B–E; Supplemental Video 7). In addition, WT Tregs inhibited the formation of stable conjugates between MOG35–55-specific T cells and DCs, reducing the mean contact time to 53% (Fig. 6F). Tregs also disrupted the clustering of MOG35–55-specific T cells (Fig. 6G), suggesting that Tregs regulate Ag recognition by activated T cells during the late phase of the immune response. Importantly, we found that Tregs lacking PSGL-1 failed to modulate the movement dynamics of MOG35–55-specific T cells (Fig. 6B–E; Supplemental Video 8), the duration of conjugates with DCs (Fig. 6F), and their spatial clustering (Fig. 6G). These results suggest that PSGL-1 is required for the modulation of sustained T cell activation by Tregs during the late phase of the immune response. These TPLSM data correlated with the functional data showing that WT Tregs inhibited T cell proliferation at 7 dpi, whereas in contrast, PSGL-1 deficient Tregs (Selplg−/−) failed to inhibit the proliferation of MOG35–55-specific T cells, further supporting a role for PSGL-1 in the control of ongoing T cell activation (Fig. 2D). Transferred exogenous Tregs displayed high mean velocity and motility coefficient and low arrest coefficient values, similar to naive polyclonal effector T (Teff) cells (Fig. 7A–C), suggesting stochastic movement inside the LN during the acquisition time. Importantly, no differences were found between WT and Selplg−/− Treg behavior (Fig. 7A–C). We also found that WT and PSGL-1-deficient Tregs displayed the same proximity to Teff cells (Fig. 7D). These data suggest that the reduced suppressive capacity of PSGL-1-deficient Tregs did not reflect the defective intranodal migration pattern or distribution around Teff cells.

To explain why polyclonal naive Tregs are able to influence MOG35–55-specific T cell behavior in the draining LNs of immunized mice, we evaluated the Treg surface activation markers GITR, CTLA-4, L-selectin, CD25, CD69, CD103, and CD44 after congenic transfer in recipient mice. We found that the expression of such markers did not differ between WT and PSGL-1-deficient Tregs (Fig. 8A, 8B), suggesting their comparable activation in the draining LNs of recipient mice.

We finally used TPLSM to examine the effect of PSGL-1 deficiency on the priming behavior of MOG35–55-specific T cells in the absence of exogenous Tregs 7 dpi. No significant differences were observed in the motility behavior or clustering of MOG35–55-prime T cells purified from MOG35–55-immunized WT and...
Selplg<sup>−/−</sup> mice (Fig. 9). This observation suggests that a selective reduction of Treg activity is the functional defect leading to the increased severity of EAE in Selplg<sup>−/−</sup> mice. Thus, our results show that PSGL-1 expressed selectively on Tregs is involved in the suppression of persistent T cell activation in the LNs required for the induction of autoimmunity.

**Discussion**

We have evaluated the effect of PSGL-1 deficiency on the activity of Tregs in EAE, a common animal model of autoimmune disease. Our results showed that the loss of PSGL-1 eliminates the ability of Tregs to suppress T cell proliferation in vivo and inhibit the disease. We used TPLSM in explanted LNs, a model equivalent to in vivo imaging in live animals (18, 26, 28, 30, 31), and found that PSGL-1 was critical for the suppression of sustained T cell activation by Tregs during the late phase of the immune response, correlating with changes in the movement of MOG<sub>35–55</sub>-specific T cells and their ability to inhibit the formation of stable contacts with DCs. However, PSGL-1 had no role in the suppression of T cell priming by Tregs during the early phase of the immune response, suggesting that Tregs use phase-specific mechanisms to suppress immune responses.

PSGL-1 is involved in the trafficking of several immune cell populations under both physiologic and pathologic conditions (8, 35), as well as the suppression of immune responses, by controlling T cell proliferation and apoptosis and by inducing a DC tolerogenic phenotype (10, 11, 25). Importantly, recent studies have shown that the loss of PSGL-1 increases disease severity in models of dextran sulfate sodium–induced colitis and autoimmune diseases such as lupus and inflammatory bowel disease, although the mechanism was unclear (10, 12). In agreement with previous reports, we found that the absence of PSGL-1 increased the severity of EAE in mice actively immunized against this disease (36). Given that T cell homing in LNs is also reduced in PSGL-1–deficient mice (9), our results suggest that the severe form of EAE observed in Selplg<sup>−/−</sup> mice reflects the disruption of normal autoimmune regulatory mechanisms. This idea is also supported by our results showing that the injection of PSGL-1–deficient T cells into WT mice induces a more severe disease than the injection of WT T cells (22).
Our EAE model data appear to contrast with previous results, suggesting that PSGL-1 deficiency has no significant effect on the development of EAE (37, 38). This discrepancy could reflect the use of adjuvants such as PTX, which in large amounts can selectively reduce the absolute number and activity of Tregs in vivo by inducing the secretion of IL-6, a cytokine that inhibits the generation of Foxp3⁺ Tregs and favors the differentiation of pathogenic Th17 cells (2, 20, 21). The larger amounts of PTX used in other studies may have suppressed the Treg compartment and overshadowed the functional defects associated with PSGL-1 deficiency.

The mechanisms by which Tregs regulate the immune response are complex and incompletely understood, but there is a consensus that DCs are the major target of Tregs in lymphoid organs (2, 4, 6, 7). The regulation of contact dynamics between T cells and DCs is an essential component of the T cell activation process (18, 26, 27). Our TPLSM data from LNs 1 dpi showed that the mobility of MOG35–55-specific T cells is reduced in the presence of Ag, resulting in the formation of stable contacts with DCs in draining LNs (26). The transfer of exogenous polyclonal WT Tregs increased the motility of MOG35–55-specific T cells and reduced their contact time with DCs, in agreement with previous studies showing that Tregs affect early immune responses by attenuating naive T cell priming (6, 7). In agreement with previous studies, the polyclonal, naturally occurring naive Tregs we injected had a strong effect on the motility of Teff cells, regardless of their Ag specificity (6, 7), possibly because they preferentially migrate to secondary lymphoid organs to inhibit APCs (39). Our results also showed that PSGL-1 on the surface of Tregs is not required for this early-stage repression, supporting our data showing that Tregs have a less significant effect on the severity of EAE when injected immediately before or after immunization. This suggests that PSGL-1–dependent regulatory mechanisms are dormant at this stage and need to be activated later during the immune response.

Although T cell activation during the initial phase of the immune response has been studied in detail, nothing is known about the behavior of T cells and the role of Tregs during later phases. Our TPLSM analysis showed that effector T cells are more motile, have a lower arrest coefficient, and have a shorter contact time with DCs 7 dpi compared with 1 dpi, suggesting that Ag-dependent activation is apparently less effective. However, the number of stable contacts between T cells and DCs was greater 7 dpi than 1 dpi, suggesting that efficient Ag-dependent priming is also maintained during later phases of the immune response. Interestingly, the percentage of intermediate contacts was strongly reduced by 7 dpi and the immune response was more selective, with most T cells establishing transient or prolonged contacts with DCs. Interestingly, MOG35–55–specific T cells tended to cluster, suggesting that efficient proliferation and therefore clustering are prerequisite for the clonal expansion and persistence of an immune response. However, it is unclear whether in our model T cells undergo homotypic aggregation and form multifocal synapses for cytokine delivery, which is crucial for full T cell activation and differentiation (40, 41). Control OVA-specific T cells were more motile and did not form clusters like the MOG35–55–specific T cells, clearly indicating that the behavior of myelin-specific T cells 7 dpi was Ag dependent. Sustained T cell activation and proliferation requires persistent Ag availability and the presentation of the Ag by migratory DCs (42). The slower migration and higher arrest coefficient of OVA-specific T cells at 7 dpi compared with 1 dpi may suggest the presence of a more inflammatory milieu. Our observations suggest that Ag-dependent T cell activation persists during the late phase of the immune response and is required, together with a more inflammatory milieu, to sustain the immune response and induce autoimmunity successfully.

PSGL-1 expression on the surface of Tregs is not relevant at 1 dpi, when strong antigenic stimulation can limit the overall suppression of T cell activation (43). Although antigenic stimulation...
in the LN is less efficient during the late phase of the immune response, we found that T cells continue to proliferate and that a consistent proportion remains in stable contact with DCs. Sustained T cell activation and proliferation requires persistent Ag availability and the presentation of Ag by migratory DCs (42, 44). During the late stage of the immune response, we found that WT Tregs were able to increase the motility of MOG35–55-specific T cells, reduce the contact time between T cells and DCs and disrupt T cell clustering, thus reducing T cell proliferation in vivo. Interestingly, our preliminary results indicate that the levels of IFN-γ, but not IL-17, were reduced in LNs collected from mice containing transferred Tregs (compared with control animals), suggesting that transferred Tregs preferentially influence responses mediated by Th1 but not Th17 cells (data now shown).

However, Tregs lacking PSGL-1 were unable to attenuate Ag responses mediated by Th1 but not Th17 cells (data now shown). Interestingly, our preliminary results indicate that the levels of IFN-γ, but not IL-17, were reduced in LNs collected from mice containing transferred Tregs (compared with control animals), suggesting that transferred Tregs preferentially influence responses mediated by Th1 but not Th17 cells (data now shown).

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