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

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

J Immunol 2013; 191:5489-5500; Prepublished online 30 October 2013;
doi: 10.4049/jimmunol.1301235
http://www.jimmunol.org/content/191/11/5489

Supplementary Material

http://www.jimmunol.org/content/suppl/2013/10/30/jimmunol.1301235.DC1

References

This article cites 49 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/191/11/5489.full#ref-list-1

Subscription

Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Regulatory T Cells Suppress the Late Phase of the Immune Response in Lymph Nodes through P-Selectin Glycoprotein Ligand-1

Stefano Angiari,*† Barbara Rossi,*† Laura Piccio,*‡ Bernd H. Zinselmeyer,* Simona Budui,* Elena Zenaro,* Vittorina Della Bianca,* Simone D. Bach,* Elio Scarpini,‡ Matteo Bolomini-Vittori,* Genni Piacentino,* Silvia Dusi,* Carlo Laudanna,*‡ 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.

Received for publication May 21, 2013. Accepted for publication October 1, 2013.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/$16.00

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)\textsubscript{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/6J female mice (6–8 wk old, obtained from The Jackson Laboratory) were used as wild type (WT) controls. Selplg\textsuperscript{−/−} 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), \(\alpha\) integrin (clone PS2), CD44 (clone IMB4), \(\alpha\)–selectin (clone Mel-14), and LFA-1 (clone Tibt-213) Abs were provided by E. C. Butcher (Stanford University). The rat anti-mouse VioBlue-conjugated CD4, PE-CD25, FITC-Foxp3, allophycocyanin-CTLA-4, FITC-CD3, PE-CD8, PE-CD19, PE-CD11c, PE-GITR, FITC-CD69, FITC-, PE- 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 ultraglutamine, 1 mM Na pyruvate, and 100 U/ml penicillin/streptomycin (all reagents from Lonza).

EAE induction

Mice were immunized s.c. in the flanks and tail base with 150 \(\mu\)g MOG\textsubscript{35–55} peptide (GenScript Corporation) in 200 \(\mu\)l emulsion consisting of equal volumes of PBS and CFA (Difco Laboratories), supplemented with 0.8 mg/ml mycobacterial \(H\textsubscript{3}7Ra\) (strain H37Ra; Becton-Dickinson). Mice received 20 ng of pertussis toxin (Alexis Biochemicals) i.v. at the time of immunization. Critical illness was defined as a paralysis score of 3 or dead animals.

To assess T cell proliferation in vivo, 10 \(\times\) 10\textsuperscript{6} CD4\textsuperscript{+} MOG\textsubscript{35–55}-primed T cells were labeled with CFSE and adoptively transferred into C57BL/6 recipient mice 7 d after MOG\textsubscript{35–55} immunization. In some experiments, 5 \(\times\) 10\textsuperscript{6} WT or Selplg\textsuperscript{−/−} Tregs were i.v. injected 18 h before MOG\textsubscript{35–55}-specific T cells. CSF\textsubscript{E}\textsuperscript{+} T cell proliferation was determined by flow cytometry analysis 4 d after T cell injection.

TPLSM: Sample preparation

Peripheral LNs and spleens were harvested from 8–10-wk-old C57BL/6J, or Selplg\textsuperscript{−/−} mice, dissociated mechanically, and washed twice after treatment with lysis buffer and antibiotic solution. CD4\textsuperscript{+}CD25\textsuperscript{+} T cells were isolated by magnetic cell sorting in a two-step procedure (Miltenyi Biotec), following the manufacturer’s instructions. Treg purity (typically 90–95%) was ascertained 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\textsuperscript{+}CD25\textsuperscript{+} cells were also Foxp3\textsuperscript{+}.

Isolation of CD4\textsuperscript{+}CD25\textsuperscript{−} Tregs

To assess T cell proliferation in vivo, 10 \(\times\) 10\textsuperscript{6} CD4\textsuperscript{+} MOG\textsubscript{35–55}-immunized mice or OT-II.2 OVA–immunized (Sigma-Aldrich) mice were killed by CO\textsubscript{2} asphyxiation. Cervical or inguinal LNs were removed and transferred into individual tubes. LNs were dissociated mechanically into a single cell suspension and washed twice after D-PBS containing 5 U/ml heparin. Nerve and spinal cord were removed, and the remaining tissue was minced and incubated in 0.125% Pronase for 1 h at 37˚C. Lymphocytes were isolated from the minced tissue by centrifugation. Lymphocytes were washed three times in D-PBS 1× and labeled with green CFSE (Invitrogen) following the manufacturer’s instructions.

Isolation of CD4\textsuperscript{+}CD25\textsuperscript{+} Tregs

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. 

The Jackson Laboratory. Mice were housed and used according to current European Community and National Institutes of Health rules. 

Isolation of CD4\textsuperscript{+}CD25\textsuperscript{+} Tregs

Peripheral LNs and spleens were harvested from 8–10-wk-old C57BL/6J, or Selplg\textsuperscript{−/−} mice, dissociated mechanically, and washed twice after treatment with lysis buffer and antibiotic solution. CD4\textsuperscript{+}CD25\textsuperscript{+} T cells were isolated by magnetic cell sorting in a two-step procedure (Miltenyi Biotec), following the manufacturer’s instructions. Treg purity (typically 90–95%) was ascertained 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\textsuperscript{+}CD25\textsuperscript{+} cells were also Foxp3\textsuperscript{+}.

In vivo migration assay with CFSE-labeled Tregs

Tregs from WT and Selplg\textsuperscript{−/−} mice were isolated as previously described and labeled with green CFSE (Invitrogen) following the manufacturer’s instructions. Tregs (5 \(\times\) 10\textsuperscript{5}) were injected i.v. in EAE mice 7 d post-immunization (dpi) with MOG\textsubscript{35–55} peptide. After 18 h, LNs from transferred mice were collected, washed, and mechanically dissociated. The number of CFSE\textsuperscript{+} Tregs was evaluated with flow cytometry analysis. Finally, the number of CFSE\textsuperscript{+} Tregs per LN was calculated.

Ex vivo T cell proliferation assay

Total cells were collected from the draining LNs of 2D2 MOG\textsubscript{35–55}–immunized mice or OT-II.2 OVA–immunized (Sigma-Aldrich) mice 7 d post-immunization (dpi). CD4\textsuperscript{+} cells were isolated by negative selection with magnetic cell sorting (Miltenyi Biotec). Cells were then labeled with CFSE and restimulated in vitro with 12 \(\mu\)g of MOG\textsubscript{35–55} or OVA respectively, in the presence of 1.0 \(\times\) 10\textsuperscript{6} 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 vivo T cell proliferation assay

To assess T cell proliferation in vivo, 10 \(\times\) 10\textsuperscript{6} CD4\textsuperscript{+} MOG\textsubscript{35–55}–specific T cells obtained from 2D2 MOG\textsubscript{35–55}–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 mice (18), 2 d after MOG\textsubscript{35–55} or OVA immunization. In some experiments, 5 \(\times\) 10\textsuperscript{5} WT or Selplg\textsuperscript{−/−} Tregs were i.v. injected 18 h before MOG\textsubscript{35–55}–specific T cells. CSF\textsubscript{E}\textsuperscript{+} T cell proliferation was determined by flow cytometry analysis 4 d after T cell injection.

Neuropathology

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 NovoRed 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 ImageJ 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.
CO₂-independent medium (Life Technologies) at room temperature. The LN 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 video-rate two-photon microscope. CMAC-labeled CD4⁺ T cells, CD11c-YFP cells, and T were excited using two Chameleon XR Ti:sapphire lasers (Coherent) and visualized with an Olympus XLUMPlanFI 20× objective (water immersed, numerical aperture, 0.95). For imaging, we used 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 emission from the three different fluorescent dyes was separated passing through 515- and 560-nm dichroic mirrors placed in series and detected at red (560–650 nm), green (490–560 nm), and blue (second harmonic) signal (<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 create time-lapse sequences, we scanned volumes of tissue in which each plane consists of an image of 100 μm spacing at 27–35-s intervals for up to 1 h. To increase signal contrast, we averaged 15 video frames for each z-slice. Multidimensional rendering was performed with Imaris (Bitplane).

**TSLSP: Data analysis**

Cells were detected based on fluorescence intensity and cell tracks obtained with Velocity (Improvison) software. One hundred to one hundred fifty cells per group were manually tracked over time from maximum-intensity, top-view image sequences. Tracks >3 min (≥12 time points) were included in the analysis. Instantaneous velocities were calculated from the distance moved between successive time points (∼27 s each). Cell velocities were reported as the median of instantaneous velocities in each cell track. Motility coefficients (mm²/min) were calculated for individual tracks by linear regression of displacement versus time point with T cell analysis software (18). The arrest coefficient is the proportion of time in which a cell does not move. It is calculated as the ratio between the time that a cell is not moving and the total time the cell is observed. Generally, it is high when T cells are engaged in stable contacts with other cells or they are swarming around a chemo-attracting microenvironment. T cell neighboring analysis was performed using a customized version of T cell analysis software (18). The arrest coefficient is the proportion of time in which a cell does not move. It is calculated as the ratio between the time that a cell is not moving and the total time the cell is observed. Generally, it is high when T cells are engaged in stable contacts with other cells or they are swarming around a chemo-attracting microenvironment. T cell neighboring analysis was performed using a customized version of T cell analysis software (18). The arrest coefficient is the proportion of time in which a cell does not move. It is calculated as the ratio between the time that a cell is not moving and the total time the cell is observed. Generally, it is high when T cells are engaged in stable contacts with other cells or they are swarming around a chemo-attracting microenvironment. T cell neighboring analysis was performed using a customized version of T cell analysis software (18). The arrest coefficient is the proportion of time in which a cell does not move. It is calculated as the ratio between the time that a cell is not moving and the total time the cell is observed. Generally, it is high when T cells are engaged in stable contacts with other cells or they are swarming around a chemo-attracting microenvironment.

**Results**

PSGL-1 deficiency increases the severity of EAE

PSGL-1 deficiency increases disease severity in several animal models of autoimmune diseases, although the mechanism is not understood (10, 12, 19). We tested the hypothesis that PSGL-1 regulates the activity of Tregs and first investigated the effect of PSGL-1 deficiency on the induction of EAE, a common animal model of autoimmune disease. High doses of pertussis toxin (PTX) can induce autoimmunity by reducing the number and activity of Tregs; therefore, we used low-to-moderate amounts of this adjuvant to avoid interferences (20, 21). We found that PSGL-1 knockout mice (Selplg−/−) immunized with the MOG35–55 peptide developed a more severe form of EAE than WT C57BL/6J mice as shown by significant increases in the mean maximum clinical score (2.6 ± 0.8 in WT mice compared with 3.6 ± 0.5 in Selplg−/− mice; p < 0.0003) and the cumulative disease score (73.9 ± 35.7 in WT mice compared with 111.7 ± 32.6 in Selplg−/− mice; p < 0.004; 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.
did (Fig. 1B). Because CD4+CD25+ cells are required to maintain immune system homeostasis and to inhibit the development of systemic autoimmunity (22, 23), these results could indicate that PSGL-1 is involved in Treg activity.

**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 pre-clinical 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 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 Treg-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](http://www.jimmunol.org/)

**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 mice 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, naive 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 MOG35–55 peptide were isolated from naive 2D2 mice and OVA-specific T cells (control cells) were obtained from naive OT-II.2 mice. These were injected i.v. into recipient CD11c-YFP mice 24 h after immunization with the MOG35–55 peptide (Fig. 3A). As expected, we observed a significant reduction in the speed and motility coefficient of MOG35–55-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 MOG35–55-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 MOG35–55-immunized mice resembled the movement of naive 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 MOG35–55-specific T cells in OVA-immunized mice were comparable to OVA-specific T cells in MOG35–55-immunized mice (Supplemental Fig. 2 A–C), suggesting no intrinsic differences in the ability of MOG35–55- and OVA-specific T cells to engage DCs in an Ag-dependent manner.

We next visualized the behavior of MOG35–55-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), MOG35–55-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 MOG35–55-specific CD4 T cells and DCs in the presence or absence of Tregs, specifying three categories: transient (>30 s, ∼500 s), intermediate (>500, ≤1800 s) and stable (>1800 s). The percentage of MOG35–55-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 MOG35–55-specific T cells and DCs was 981 s (Fig. 4F). Notably, there was no statistically significant difference in MOG35–55-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 naive T cells and changes in cell dynamics 1 dpi in draining LNs of MOG35–55-immunized mice. (A) MOG35–55-specific or OVA-specific T cells obtained from LNs and spleens of naive 2D2 mice or OT-II.2 mice were injected i.v. into CD11c-YFP mice 1 dpi with MOG35–55 peptide, and images were acquired after 6 h. (B) TPLSM representative tracks of MOG35–55- 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), (D) motility coefficient (**p < 0.0001) and (E) arrest coefficient (**p < 0.0001) of MOG35–55-specific T cells compared with OVA-specific T cells are shown. The results show that the motility of MOG35–55-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 1dpi, 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 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).
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.

**FIGURE 5.** Ag-dependent T cell activation and clustering 7 dpi. (A) MOG35–55-specific or OVA-specific T cells were obtained from 2D2 or OT-II mice immunized 7 d before with MOG35–55 or OVA peptide, respectively. Cells were injected i.v. into CD11c-YFP mice 7 dpi with MOG35–55 peptide, and images were acquired after 6 h. (B) TPLSM representative tracks of MOG35–55- and OVA-specific T cell (blue cells) movement in the draining LNs of recipient mice (green cells indicate DCs; Supplemental Videos 5 and 6). Cell tracks are as for Fig. 3. Original magnification ×20. (C) Mean velocity (**p < 0.0001), (D) motility coefficient (**p = 0.006), and (E) arrest coefficient (***p = 0.0033) of activated MOG35–55-specific T cells compared with activated OVA-specific T cells are shown. (F) Contact times between activated T cells and DCs in the draining LNs of recipient mice (*p < 0.05). (G) MOG35–55-specific and OVA-specific T cells were collected from the draining LNs of 2D2 and OT-II mice 7 dpi earlier with the MOG35–55 or OVA peptides, respectively. Cells were labeled with blue CMAC and injected i.v. into EAE mice 7 d after the injection of MOG35–55 peptide. LNs were collected and frozen 6 h later. Representative images were obtained after labeling B220+ cells (red cells) to visualize the three-dimensional structure of the LNs. Our results show that exogenous T cells localize mainly in the T cell area of draining LNs 6 h after transfer (upper panels). However, whereas OVA-specific T cells are randomly distributed inside the T cell area, MOG35–55-specific T cells are clustered (lower panels; white arrows indicate T cell clusters), suggesting that Ag-dependent T cell activation in draining LNs occurs during the late phase of the immune response. Scale bars, 100 μm (upper panels) and 50 μm (lower panels). (H) T cell distribution was analyzed on eight representative z-stacks, containing 12 cells each from three independent experiments. MOG35–55-specific T cells are mainly organized in clusters inside the LN. (I) CD4+ T cells from 2D2 MOG35–55-immunized mice or from OT-II OVA-immunized mice were labeled with CFSE and injected i.v. into C57BL/6 mice 7 d after injection with the MOG35–55 or OVA, respectively. In some experiments, CD4+ T cells from OT-II OVA-immunized mice were injected into MOG35–55-immunized mice. Four days after transfer, CFSE+ LN cells were assayed for proliferation by flow cytometry. The representative histogram plots of CFSE+ MOG35–55-specific or OVA-specific T cell proliferation suggest that Ag-specific T cell activation occurs in the draining LNs of immunized mice 7 dpi, because both the MOG35–55-specific and OVA-specific exogenous T cells proliferate efficiently in vivo in an Ag-dependent manner. Notably, OVA-specific T cells do not proliferate when transferred into MOG35–55-immunized mice, again suggesting Ag-dependent T cell activation in the draining LNs. The data represent five mice per group.
Next we sought to determine the role of Tregs and PSGL-1 on T cell activation during the late phase of the immune response. We adoptively transferred Tregs into CD11c-YFP recipient mice 7 dpi and analyzed T cell dynamics by TPLSM. As observed in naive nonimmunized mice, transferred Tregs preferentially migrated to the LNs of recipient mice, suggesting a significant increase in Treg number 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.

P-selectin is expressed on LN high endothelial venules under certain inflammatory conditions (34); therefore, we sought to exclude a possible defect in the migration of PSGL-1-deficient Tregs in the draining LNs of EAE mice 7 dpi. As expected, and supporting the data shown in Fig. 2C, we observed no differences in the migration of CFSE− or 3H-glycerol–labeled Tregs from WT and Selplg−/− mice 24 or 48 h after transfer, confirming that PSGL-1 does not regulate the accumulation of Tregs in draining LNs 7 dpi (Fig. 7E, 7F) (25).

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, 1-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⁻/⁻ 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⁻/⁻ 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 deficiency had no effect on exogenous Treg motility in the draining LNs of EAE mice 7 dpi, nor on the migration of Tregs to the same LNs. WT or Selplg⁻/⁻ Tregs were injected 18 h before MOG₃₅-₅₅-specific T-cell transfer into CD11c-YFP mice 7 dpi. No differences were observed in the mean velocity, motility coefficient, and arrest coefficient of WT and Selplg⁻/⁻ Tregs. Calculation of the relative distance of WT and Selplg⁻/⁻ Tregs form effector MOG₃₅-₅₅-specific T cells. PSGL-1 deficiency did not affect Treg proximity to T cells in draining LNs 7 dpi. WT or Selplg⁻/⁻ Tregs were isolated from the LNs of naive mice, labeled with green CFSE or radioactive labeled T cells. No differences in the migration of WT and Selplg⁻/⁻ Tregs were detected. Data in (E) and (F) represent the means of five mice per condition.

FIGURE 7. PSGL-1 deficiency has no effect on exogenous Treg motility in the draining LNs of EAE mice 7 dpi, nor on the migration of Tregs to the same LNs. WT or Selplg⁻/⁻ Tregs were injected 18 h before MOG₃₅-₅₅-specific T-cell transfer into CD11c-YFP mice 7 dpi. Twenty-four hours later, Tregs were analyzed to study the expression of the following markers: CD25, CD103, CD69, GITR, CTLA-4, and L-selectin. No differences were found between WT (black bar) and Selplg⁻/⁻ T (white bar) in terms of percentage of positive cells and mean fluorescence intensity. Data were obtained from one representative experiment from a series of three with similar results.

FIGURE 8. Flow cytometry analysis of activation markers on Tregs 7 dpi. WT (n = 4) or Selplg⁻/⁻ (n = 4) of CMAC⁺ Tregs (5 × 10⁶) were injected i.v. into C57BL/6-MOG₃₅-₅₅ immunized mice at 7 dpi. Twenty-four hours later, Tregs were isolated from the LNs of naive mice, labeled with green CFSE or radioactive labeled T cells. No differences in the migration of WT and Selplg⁻/⁻ Tregs were detected. Data in (E) and (F) represent the means of five mice per condition.
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, 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, compared with WT MOG35–55-primed T cells (blue cells; green cells indicate DCs). Cell tracks are the same as for Fig. 3. Original magnification ×20. (C) Mean velocity, (D) motility coefficient, (E) arrest coefficient, and (F) and clustering analysis of WT and Selplg<sup>−/−</sup> MOG35–55-primed T cells are shown. Results indicate that Selplg<sup>−/−</sup> MOG35–55-primed T cells have no defects in the motility behavior compared with WT MOG35–55-primed T cells (p < 0.05). MOG35–55-primed T cells also have the same motility behavior of MOG35–55-specific T cells obtained from 2D2 mice (data not shown).

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

---

**FIGURE 9.** Selplg<sup>−/−</sup> MOG35–55-primed T cells have normal motility. (A) MOG35–55-primed T cells were obtained by draining LNs of WT or Selplg<sup>−/−</sup> mice 7 dpi with MOG35–55 peptide. Cells were then injected i.v. into CD11c-YFP mice 7 d after immunization with MOG35–55, and images were collected 6 h later. (B) TPLSM representative tracks of WT or Selplg<sup>−/−</sup> MOG35–55-primed T cells (blue cells; green cells indicate DCs). Cell tracks are the same as for Fig. 3. Original magnification ×20. (C) Mean velocity, (D) motility coefficient, (E) arrest coefficient, and (F) and clustering analysis of WT and Selplg<sup>−/−</sup> MOG35–55-primed T cells are shown. Results indicate that Selplg<sup>−/−</sup> MOG35–55-primed T cells have no defects in the motility behavior compared with WT MOG35–55-primed T cells (p > 0.05). MOG35–55-primed T cells also have the same motility behavior of MOG35–55-specific T cells obtained from 2D2 mice (data not shown).
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 MOG15-55-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 recognition of MOG15-55-specific T cells, although their activation was comparable to that in WT cells (Fig. 8). They did not affect the cytokine balance in LNs (data not shown), suggesting that PSGL-1 is a key mediator Treg suppression during the later stages of the immune response. One hypothesis to explain the dominant role of PSGL-1 is that it can regulate DC activity during the late phase of the immune response by controlling contacts between Tregs and DCs as previously shown for CTLA-4 (6, 7, 24). PSGL-1 can also inhibit Ag-dependent chemokine secretion by DCs, the recruitment of inflammatory cells by LNs and the colocalization of Ag-bearing DCs and Ag-specific T cells (4, 45). The regulation of interactions between Tregs and DCs by PSGL-1 could modulate the local cytokine/chemokine profile within LNs, reducing the clustering and activation of Ag-specific T cells. The molecular basis of such interactions can involve direct interactions between PSGL-1 and chemokines such as CCL21 and CCL19 (9). PSGL-1 expressed on the surface of Tregs can sequester chemokines and prevent interactions between newly arrived CCR7+ T cells and APCs, favoring the formation of immune synapses (9, 47).

The production of cytokines and chemokines in the early stages of an immune response might compensate for the lower antigenic signal, thereby allowing efficient T cell priming during the late phase of the immune response. The presence of cytokines and chemokines is supported by the clustering of T cells, which indicates potential clonal expansion. Interactions between Tregs and DCs mediated by PSGL-1 could reduce the local pro-inflammatory cytokine/chemokine profile, disrupting Ag-specific T cell clustering and thus limiting the activation of Ag-specific T cells. In addition, PSGL-1 can bind directly to chemokines such as CCL21, which become scarce during the later phases of the immune response thus making the roles of Tregs and PSGL-1 more critical (48). Recent studies suggest that the inhibition of chemokine production in DCs by Tregs does not depend on the strength of the antigenic stimulus, whereas the suppression of T cell proliferation is more effective with lower antigenic stimulation as found during the late stages of an immune response (43) Treg activity might also be more relevant in the LNs following the expansion of the immune response, whereas at 1 dpi it may be more relevant in the periphery (49). Overall, PSGL-1 expression may be more critical for Treg-mediated suppression during the late phases of the immune responses. Although Tregs lacking PSGL-1 express key cellular markers for suppression and inhibit T cell priming soon after immunization, we cannot formally exclude a role for PSGL-1 in Treg lineage differentiation that might contribute to a functional defect during a later stage in the immune response.

In conclusion, our results show a previously unidentified role for PSGL-1 in the suppression of autoimmune by Tregs during the late stages of the immune response by attenuating sustained T cell activation in the LNs.

Disclosures
The authors have no financial conflicts of interest.

References


**Supplemental Table I.** Clinical and pathological features of WT and *Selplg*<sup>−/−</sup> mice actively induced with MOG<sub>35-55</sub> peptide.

<table>
<thead>
<tr>
<th>Active EAE</th>
<th>Disease onset (d.p.i.)</th>
<th>Mean maximum score</th>
<th>Mean cumulative score</th>
<th>Inflammatory area (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Demyelinated area (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Macrophage (cells/mm&lt;sup&gt;2&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; (cells/mm&lt;sup&gt;2&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>11,2±2,5</td>
<td>2,6±0,8</td>
<td>73,9±35,7</td>
<td>9,2±3,1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5,6±4,4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>89.0±5.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>91.5±20.3&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>PSGL-1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>11,0±2,8</td>
<td>3,6±0,5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>111,7±32,6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>24,2±15,2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>19,2±3,0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>107.7±9.6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>177.1±53.6&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percentage of the total spinal cord section.
<sup>b</sup>Number of positive cells per mm<sup>2</sup> of total spinal cord section.
<sup>c</sup><i>p</i>&lt;0.0003
<sup>d</sup><i>p</i>&lt;0.004
<sup>e</sup><i>p</i>&lt;0.01
Supplemental Table II. \textit{In vivo} proliferation of MOG\textsubscript{35-55}-specific T cells in dLNs of EAE mice in the presence or absence of exogenous WT or \textit{Selplg}^{-/-} T\textsubscript{Reg} cells.

<table>
<thead>
<tr>
<th></th>
<th>Division index</th>
<th>Proliferation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL (no T\textsubscript{Reg} cells)</td>
<td>0.30 ± 0.05</td>
<td>1.57 ± 0.08</td>
</tr>
<tr>
<td>WT T\textsubscript{Reg} cells</td>
<td>0.17 ± 0.00\textsuperscript{a}</td>
<td>1.43 ± 0.02\textsuperscript{a}</td>
</tr>
<tr>
<td>\textit{Selplg}^{-/-} T\textsubscript{Reg} cells</td>
<td>0.26 ± 0.08</td>
<td>1.52 ± 0.07</td>
</tr>
</tbody>
</table>
Regulatory T cells suppress the late phase of the immune response in lymph nodes through P-selectin glycoprotein ligand-1

Data supplement

Supplemental tables I-II

Supplemental figures 1-2

Supplemental videos 1-8
**Supplemental Table I.** Clinical and pathological features of EAE in WT and Selplg−/− mice actively induced with MOG35-55 peptide.

**Supplemental Table II.** *In vivo* proliferation of MOG35-55-specific T cells in dLNs of EAE mice in the presence or absence of exogenous WT or Selplg−/− TReg cells. Experimental details as for Fig. 2D. Division and proliferation indexes were calculated with the FlowJo software (Tree Star Inc.). aP < 0.02 compared to control condition (absence of TReg cells).

**Supplemental Figure 1.** TReg cells efficiently inhibit EAE development when transferred 7 dpi. (A) 2.5 x 10⁶ naïve TReg cells were injected into C57Bl/6J wild-type mice 1 day before active immunization with MOG35-55 peptide. TReg cell transplantation delayed the onset of disease compared to untreated mice (disease onset: day 15.8 ± 1.5 for control mice and day 19.0 ± 3.0 for transplanted mice; P < 0.05), but the effect was not sustained. (B) 2.5 x 10⁶ naïve TReg cells were injected into wild-type EAE mice 3 or 7 dpi with MOG35-55 peptide. TReg cell transfer 3 dpi had a mild effect on the disease, whereas administration 7 dpi significantly ameliorated the clinical course of the disease, reducing both the mean maximum score and the mean cumulative score in treated mice compared to control mice (mean maximum score: 3.1 ± 1.1 in control mice; 2.5 ± 1.5 in transplanted mice; P < 0.05. Mean cumulative score: 83.5 ± 28.1 in control mice; 56.0 ± 20.3 in transplanted mice; P < 0.05). (C) 2.5 x 10⁶ naïve TReg cells were injected into wild-type EAE mice 16 dpi with MOG35-55 peptide at disease peak. No significant effect was observed on disease course when compared to the control condition. The data are the mean ± standard error of the mean (S.E.M.) of 8-10 mice per group.

**Supplemental Figure 2.** Comparison of the dynamics between MOG35-55- and OVA-specific T cells in the draining LNs 1dpi and 7 dpi. MOG35-55-specific T cells were obtained from naïve and
MOG35-55-immunized 2D2 mice and injected intravenously 1dpi and 7 dpi in OVA-immunized C57BL/6-mice respectively ($n = 3$). OVA-specific T cells were obtained from naïve and OVA-immunized OT-II mice and injected intravenously 1 dpi and 7 dpi in MOG35-55-immunized C57BL/6-mice respectively ($n = 3$). Images were acquired 6 h later. (A) mean velocity, (B) motility coefficient and (C) arrest coefficient 1 dpi. (D) mean velocity, (E) motility coefficient and (F) arrest coefficient 7 dpi. Motility parameters of MOG35-55 T cells in OVA-immunized mice are comparable to OVA-specific T cells in MOG35-55-immunized mice, suggesting no intrinsic differences in the ability of MOG35-55-specific and OVA-specific T cells to engage DCs in an antigen-dependent manner. These results indicate OVA peptide as a correct negative control for MOG35-55-specific T cells.

**Supplemental Video 1.** This movie shows representative movement of OVA-specific T cells (blue cells) inside the draining lymph nodes of immunized mice 1 dpi (green cells: DCs). OVA-specific T cells move randomly tacking up all the space analyzed. In all movies, pseudocolor tracks are time mapped (see time colorbar on the right bottom) following the progression of T-cell movement from the beginning (blue) to the end (white). Scale bar: 20 μm.

**Supplemental Video 2.** This movie shows representative movement of MOG35-55-specific T cells (blue cells) inside the draining lymph nodes of immunized mice 1 dpi (green cells: DCs). The majority of MOG35-55-specific T cells are not moving because involved in stable physical contacts with DCs. Scale bar: 20 μm.

**Supplemental Video 3.** This movie shows representative movement of MOG35-55-specific T cells (blue cells) inside the draining lymph nodes of immunized mice 1 dpi in the presence of exogenous
wild-type $T_{\text{Reg}}$ cells (red cells) (green cells: DCs). Wild-type $T_{\text{Reg}}$ cells increase the movement of MOG$_{35-55}$-specific T cells, reducing contact time with DCs. Scale bar: 20 μm.

**Supplemental Video 4.** This movie shows representative movement of MOG$_{35-55}$-specific T cells (blue cells) inside the draining lymph nodes of immunized mice 1 dpi in the presence of exogenous $Selplg^{-/-}$ $T_{\text{Reg}}$ cells (red cells) (green cells: DCs). $Selplg^{-/-}$ $T_{\text{Reg}}$ cells are able to increase the motility of MOG$_{35-55}$-specific T cells to the same extent of wild-type $T_{\text{reg}}$ cells. Scale bar: 20 μm.

**Supplemental Video 5.** This movie shows representative movement of OVA-specific T cells (blue cells) inside the draining lymph nodes of immunized mice 7 dpi (green cells: DCs). OVA-specific T cells move randomly in all directions, with no apparent stable contact with DCs. Scale bar: 20 μm.

**Supplemental Video 6.** This movie shows representative movement of MOG$_{35-55}$-specific T cells (blue cells) inside the draining lymph nodes of immunized mice 7 dpi (green cells: DCs). MOG$_{35-55}$-specific T cells move slowly and are mainly organized in clusters. Some of the T cells are still involved in stable contacts with DCs. Scale bar: 20 μm.

**Supplemental Video 7.** This movie shows representative movement of MOG$_{35-55}$-specific T cells (blue cells) inside the draining lymph nodes of immunized mice 7 dpi in the presence of exogenous wild-type $T_{\text{Reg}}$ cells (red cells) (green cells: DCs). Wild-type $T_{\text{Reg}}$ cells interfere with cluster organization of MOG$_{35-55}$-specific T cells and induce an increase in T cell velocity and motility. Scale bar: 20 μm.
**Supplemental Video 8.** This movie shows representative movement of MOG$_{35-55}$-specific T cells (blue cells) inside the draining lymph nodes of immunized mice 7 dpi in the presence of exogenous Selplg$^{-/-}$ T$_{Reg}$ cells (red cells) (green cells: DCs). Selplg$^{-/-}$ T$_{Reg}$ cells fail to modulate MOG$_{35-55}$-specific T cells clustering and motility, compared to wild-type T$_{Reg}$ cells. Scale bar: 20 μm.