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PDE8 controls CD4+ T cell motility through the PDE8A-Raf-1 kinase signaling complex

Chaitali P. Basole1, Rebecca K. Nguyen1, Katie Lamothe1, Amanda Vang1,2, Robert Clark1, George S. Baillie3, Paul M. Epstein4, and Stefan Brocke1*

1Department of Immunology, UConn Health, 2The National Hospital of Faroe Islands, 3Institute of Cardiovascular and Medical Sciences, University of Glasgow, 4Department of Cell Biology, UConn Health

*Corresponding author: sbrocke@uchc.edu

Abstract
The levels of cAMP are regulated by phosphodiesterase enzymes (PDEs), which are targets for the treatment of inflammatory disorders. We have previously shown that PDE8 regulates T cell motility. Here, for the first time, we report that PDE8A exerts part of its control of T cell function through the V-raf-1 murine leukemia viral oncogene homolog 1 (Raf-1) kinase signaling pathway. To examine T cell motility under physiologic conditions, we analyzed T cell interactions with endothelial cells and ligands in flow assays. The highly PDE8-selective enzymatic inhibitor PF-04957325 suppresses adhesion of in vivo myelin oligodendrocyte glycoprotein (MOG) activated inflammatory CD4+ T effector (Teff) cells to brain endothelial cells under shear stress. Recently, PDE8A was shown to associate with Raf-1 creating a compartment of low cAMP levels around Raf-1 thereby protecting it from protein kinase A (PKA) mediated inhibitory phosphorylation. To test the function of this complex in Teff cells, we used a cell permeable peptide that selectively disrupts the PDE8A-Raf-1 interaction. The disruptor peptide inhibits the Teff–endothelial cell interaction more potently than the enzymatic inhibitor. Furthermore, the LFA-1/ICAM-1 interaction was identified as a target of disruptor peptide mediated reduction of adhesion, spreading and locomotion of Teff cells under flow. Mechanistically, we observed that disruption of the PDE8A-Raf-1 complex profoundly alters Raf-1 signaling in Teff cells. Collectively, our studies demonstrate that PDE8A inhibition by enzymatic inhibitors or PDE8A-Raf-1 kinase complex disruptors decreases Teff cell adhesion and migration under flow, and represents a novel approach to target T cells in inflammation.

Keywords:
PDE8
CD4+ T cells
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Autoimmunity
T cell motility,
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1. Introduction

Ligand binding to Gs-coupled receptors leads to the generation of the second messenger cAMP following activation of the enzyme adenyl cyclase. Stimulation of the T cell antigen receptor (TCR) also leads to elevation of cAMP which is known to inhibit T cell proximal signaling, IL-2 production and T cell proliferation [1, 2]. cAMP exerts these inhibitory effects in T cells through cAMP dependent protein kinase (PKA) which blocks the mitogen-activated protein kinase (MAPK) and nuclear factor of activated T cells (NFAT) dependent signaling pathways [3]. The inhibitory action of cAMP is eliminated through the action of phosphodiesterase (PDE) enzymes that hydrolyze cAMP. PDEs 3B, 4A, 4B, 4D, 7A1, 7A3 and 8A1 are the isoforms expressed in T cells [4-8]. V-raf-1 murine leukemia viral oncogene homolog 1 (Raf-1) is an upstream regulator of the MAPK-extracellular signal–regulated kinase (ERK)1/2 module, which controls many fundamental biological processes, including T cell proliferation, survival and adhesion [9-12]. In this pathway Raf-1 phosphorylates and activates MAP/ERK kinase (MEK)1/2, which in turn phosphorylate and activate ERK1/2. ERK has more than 150 known substrates [9, 12], which mediate many of the pleiotropic functions of this pathway [13, 14]. Raf-1 regulation is complex and is still insufficiently understood. Critical events are the dephosphorylation of an inhibitory site, S259, which allows Raf-1 binding to activated rat sarcoma viral oncogene (Ras) and is a prerequisite for further activation. S259 is a target for phosphorylation by PKA [15-17], a family of enzymes whose activity is dependent on local intracellular levels of cAMP. Thus, S259 is a primary target of a complex system of crosstalk between the cAMP and the Raf-1-ERK signaling pathways. A recent report demonstrated that Raf-1 kinase binds to PDE8A in a signaling complex, which acts to protect Raf-1 from inhibitory phosphorylation by PKA [18-20]. Our previous work has shown that PDE8 controls T cell and breast cancer cell motility [6, 21-23]. Our goal here was to delineate the mechanism by which PDE8 controls distinct categories of T cell motility and determine its selective effect on regulatory and effector components of the T cell immune response.

Our recent work shows that CD4+ T cells isolated from the hilar lymph nodes draining lung tissue have increased PDE8A expression during the acute inflammatory stage in an ovalbumin induced allergic airway disease mouse model [24]. This further supports the concept of targeting PDE8A in inflammation. Recently, there has been a surge of interest in the cAMP specific PDE8 family of enzymes. PDE8 is expressed widely in human tissue [25] with functions in testosterone production [26], lymphocyte adhesion and chemotaxis [6, 21-23]. T cell activation induces cAMP and PDEs, including PDE8A, a cAMP-specific PDE with 40-100-fold greater affinity for cAMP than PDE4 [2, 5, 22, 27]. This unique feature has led to the suggestion that PDE8 enzymes may have an important role in protecting any associated protein from being affected by fluctuations in basal cAMP concentrations [5]. Compartmentalization of PDE8A in the cell to Raf-1 can regulate Raf-1 phosphorylation on S259, and, in so doing, regulate the cross-talk node whereby cAMP exerts an inhibitory effect on Raf-1 signaling [18-20], potentially affecting T cell activation and function.
Based on these observations, we hypothesized that PDE8A exerts its regulation of T cell function through the PDE8A-Raf-1 signaling pathway. Little is known regarding the specific functions of PDE8A within the immune system, especially whether the control of T cell motility is mediated through different effectors of the canonical Raf-1-ERK signaling cascade. B-Raf has been demonstrated to regulate VLA-4 integrin mediated adhesion in human T cells under shear stress independently of ERK signaling [28]. Here, we probed the PDE8A-Raf-1 kinase signaling pathway in Teff and regulatory T (Treg) cells, using specific pharmacological tools including a PDE8 inhibitor and peptide disruptor of the PDE8A-Raf-1 complex in adhesion assays under flow to assess the molecular regulation of downstream effectors of the PDE8A-Raf-1 complex.

2. Materials and Methods

2.1 Animals
Female 6-8 weeks old C57BL/6 mice were obtained from Jackson laboratories, Bar Harbor, ME. Foxp3gfp.KI knock-in (Foxp3gfp.KI) mice were obtained as a gift from Dr. Kuchroo [29]. Experiments were performed according to approved protocols at UConn Health (IACUC Protocol number 100794-1216).

2.2 Chemicals and antibodies
Recombinant mouse VCAM-1/CD106 Fc chimera (643-VM), recombinant mouse ICAM-1/CD54 Fc chimera (796-IC) were purchased from R&D systems, Minneapolis, MN. Protein A was purchased from Sigma-Aldrich (P7837). The primary antibodies for phospho-p44/42 MAPK (Thr202/Tyr204) (9101; 1:1000), p44/42 MAPK (9102; 1:1000), Phospho-Raf-1 (pSer259) (9421; 1:1000) and Raf-1 (9422; 1:1000) were purchased from Cell Signaling Technology. An additional Raf-1 antibody was purchased from BD Biosciences (610152; 1:2000). Anti-GAPDH (ab75834; 1:100,000) and anti-PDE8A antibodies (1:1000) were purchased from Abcam and Scottish Biomedical, respectively. The anti-CD3 antibody was purchased from Biolegend (100331). The secondary antibodies goat anti-rabbit IgG-HRP (65-6120, 1:4000), chicken anti-rabbit IgG-HRP (sc-2963, 1:5000) and goat anti-mouse IgG-HRP (sc-2005, 1:5000) were purchased from Invitrogen and Santa Cruz Biotechnology. The PDE8A-Raf-1 disruptor peptide (PDE8A: R454–T465; RRLSGNEYVLST) and scrambled control peptide (SYTVRLLGERNS) were synthesized as described [19]. The PDE8 selective inhibitor PF-04957325 was synthesized by Pfizer Inc., Groton, CT [6].

2.3 Isolation of Teff cells, Treg cells and CD4+ T cells
CD4+CD25− Teff and CD4+CD25+ Treg cells were isolated from the spleens of C57BL/6 mice using the CD4+CD25+ Treg Isolation kit (130-091-041, Miltenyi Biotec) as published previously [23]. For the generation of activated pathogenic CD4+ T cells, C56BL/6 or Foxp3gfp.KI mice were immunized with myelin oligodendrocyte glycoprotein peptide 35-55 (MOG35-55) in 200 µg Complete Freund’s Adjuvant (CFA) (MOG35-55/CFA) s.c. in the footpads [30, 31]. On d 10
post-immunization, CD4+ T cells were isolated from the draining popliteal lymph nodes (PLNs) using the CD4+ T cell Isolation Kit (130-104-454, Miltenyi Biotec).

2.4 Cell culture and treatments
Murine brain endothelium derived cell line bEnd.3 (ATCC, Manassas, VA) was cultured in DMEM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2mM L-glutamine and 10% fetal bovine serum as described [6]. In the flow assays, endothelial cells and CD4+ T cells were treated with 1 μM of the PDE8 inhibitor PF-04957325 or the DMSO vehicle control for 1 h, or with 10 μM PDE8-Raf-1 disruptor peptide or control peptide for 4 h. For the flow assay with naive Teff cells or Treg cells, cells were cultured with plate-bound anti-CD3 (10 μg/ml) with or without IL-2 (NIH) (100 Units/ml) for 18 h. For integrin expression analysis, splenic CD4+ T cells isolated from Foxp3gfp.KI mice were cultured with plate-bound anti-CD3 (10 μg/ml) for 18 h followed by treatment with PF-04957325 or vehicle control for 1 h or disruptor or control peptide for 4 h. The cells were then pre- incubated with anti-CD16/32 (clone: 2.4 G2, BD Biosciences) for blocking Fcγ III/II receptors followed by surface staining with anti-CD4 eFluor 450 (clone: RM4-5, 48-0042, eBioscience), anti-CD11a BV510 (clone: M17/4, 563669, BD Horizon), anti-CD49d PerCp-eFluor710 (Clone: R1-2, 46-0492, eBioscience), anti-CD44 PE (clone:IM7, 553134, BD Pharmingen) antibodies. Events were acquired on LSR II (BD Biosciences). Flow cytometry data were processed on FlowJo software (Tree Star). For the Western blot experiments, CD4+ T cells were treated with 1 μM PF-04957325 or 10 μM disruptor peptide and respective vehicle or scrambled peptide controls for 1 or 4 h followed by plate-bound 15 min activation with 5 μg/ml plate-bound anti-CD3.

2.5 Shear stress (flow) assay
The bEnd.3 cells were stimulated with 1.25 μg/ml LPS (Sigma-Aldrich) for 18 h on d 4 after seeding. The parallel plate flow chamber (Glycotech) was assembled onto the endothelial cell plate and mounted onto the stage of an inverted phase contrast microscope (Nikon Eclipse Ti) [32]. The CD4+ T cells were treated as described above and then were washed with cation free HBSS and resuspended in binding buffer [HBSS with CaCl2 and MgCl2, 10 mM HEPES (Life Technologies) and 2 mg/ml BSA fraction V (Roche)] at a concentration of 10^6 cells/50 μl binding buffer. The CD4+ T cells were then infused over the endothelial cell layer at a constant shear flow rate using a programmable syringe pump (Harvard Apparatus). Once the cells accumulate on the bEnd.3 cells, the shear rate was increased to 5 dyn/cm^2 for the entire 15 min of the assay (migration phase) and a time-lapse video recording was done under phase contrast and GFP illumination settings using Nikon NIS imaging software. The cells and the binding buffer (entire system) were maintained at 37 °C throughout the assay. Motion analysis was done manually on all the T cells that were present in the microscopic field of view. Each T cell was individually tracked starting from the accumulation phase throughout the migration phase. Only the T cells that were present during the accumulation phase were analyzed. The distinct cell tethers that were examined are defined as follow: cells that detach
immediately after application of shear flow were considered to be detached; cells that roll on the endothelial cell surface were rolling; cells that firmly adhere to the endothelial cell surface and remain stationary for at least 1 min were defined as adhesion; cells that form non-stationary adhesion, spread and migrate along the endothelial cell surface were included in the adhesion and locomotion category, while cells that undergo transendothelial migration (TEM) were included in the adhesion and TEM category. The number of cells in each category were counted and expressed as percentage of initially accumulated cells during the accumulation phase. For flow assays with immobilized vascular ligands, recombinant VCAM-1 Fc (2 μg/ml) or ICAM-1 Fc (5 μg/ml) (in 20 mg /ml BSA in PBS) was overlaid on protein A pre-coated polystyrene plates overnight at 4 °C. Plates were then washed with PBS three times followed by blocking with 20 mg/ml BSA solution for 2 h at 37 °C. The immobilized vascular ligand coated plates were assembled as the lower wall of the flow chamber as mentioned above. The interactions of the CD4+ T cells with the adhesive substrates were manually tracked as mentioned above. The cell tethers examined were rolling, transient tether, firm tether, spreading, locomotion and detachment [33]. Transient tether was defined as cells that stay for less than 4 s after flow starts, firm tether was defined as cells that stay for more than 4 s after flow starts, rolling was defined as cells that persistently rolled for at least 2 s after the flow starts, adherent cells were defined as cells that stay throughout the 15 min shear flow period. Spreading cells were defined as cells that increase their area, perimeter and also undergo darkening of the edges [34]. Cells undergoing spreading and migration were included in the locomotion category.

2.6 Western blot:
CD4+ T cells were centrifuged at 2000 rpm for 5 min, washed twice with ice-cold PBS and lysed using RIPA buffer (Teknova) supplemented with protease inhibitor cocktail (1:100; Sigma-Aldrich) and phosphatase inhibitor cocktail (1:10; Roche). The lysates were then centrifuged at 10,000 rpm for 10 min to remove the cells debris. Protein concentration was determined using BCA Protein Assay kit (Pierce). Equal amounts of protein were loaded and run on 10% SDS-PAGE gel. Proteins were then transferred onto a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked for 1 h at room temperature with 5% non-fat dry milk in Tris- buffered saline (Bio-Rad) supplemented with 0.1% tween-20 (Sigma-Aldrich; TBS-T) before adding the primary antibodies in 5% BSA in TBS-T and incubated overnight at 4 °C. Membranes were washed three times with TBS-T, followed by incubation with horseradish-peroxidase-conjugated secondary antibody (HRP) in 5% non-fat dry milk in TBS-T at room temperature for 2 h and washed 3 more times. Proteins were visualized and quantitated with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) using Syngene G:Box with GeneSnap BiolImaging software. Probing with ERK1/2 and Raf-1 antibody was used to normalize phospho ERK1/2 and phospho Raf-1 expression.

2.7 Statistical analyses:
Experimental groups were compared by analyzing data with Student’s unpaired t-test using GraphPad Prism version 7.00 for Mac. Probability levels for statistically significant differences are indicated by the p value in the results and corresponding asterisks in the figures. (*P<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

3. Results
3.1 Differential motility of naive and activated Teff and Treg cells under flow.
Since PDEs and cAMP levels significantly differ between Teff and Treg cells [23, 35], we first tested CD4+CD25- Teff and CD4+CD25+ Treg cells under naive and activating conditions for their ability to interact with LPS-activated endothelial cells (ECs) under shear flow. Mouse brain ECs were cultured as a monolayer, stimulated with LPS overnight, and the subsequent adhesive interactions with ECs were measured in the flow chamber assay. Compared to naive Teff cells, naive Treg adhered over two times more potently (11.1 ± 3.1% vs. 28.9 ± 2.0%, **p=0.0031) (Fig. 1A, Movies S1 and S2). These findings are similar to previous reports indicating that naive Teff cells have lower adhesion potential compared to naive Treg cells [36]. No significant difference was observed between adhesion of Teff cells and Treg cells after activation via their T cell receptor (TCR)/CD3ε (Fig. 1B, Movies S3 and S4) or addition of IL-2 along with TCR activation (Fig. 1C, Movies S5 and S6). The differential migratory potential of naïve Teff cells and Treg cells prompted us to study the effect of PDE8 inhibition using the PDE8 inhibitor PF-04957325 on motility of Teff and Treg cells.

3.2 PDE8 inhibition at the catalytic moiety leads to decreased adhesion of Teff cells but not Treg cells under shear flow conditions.
We have previously shown that PDE8A is differentially expressed in naive Teff cells versus naive Treg cells [23]. We now examined whether different expression levels of PDE8A can also be detected in pathogenic T cell populations involved in autoimmune inflammation. T cells were isolated at day 10 from draining lymph nodes of MOG35-55 immunized mice which is used to induce experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis (MS) [37, 38]. CD4+CD25- Teff cells showed significantly higher expression of PDE8A1 and PDE8A2 proteins compared to CD4+CD25+ Treg cells (Fig. 2A, B). There was no statistically significant difference in Raf-1 expression between the two cell populations (not shown). Next, we used Foxp3gfp.KI mice immunized with MOG35-55 to determine the effect of PDE8 inhibition on activated, pathogenic T cells. This approach enabled us to visually distinguish Teff and Treg cells and simultaneously measure the effect of inhibitors on Teff and Treg cells within the same experiment. PDE8 inhibition using 1 μM PF-04957325 led to a significant decrease in adhesion (100.0% vs. 63.3 ± 6.9%, **p=0.0019), and an increased trend of detachment, but this trend did not reach statistical significance (p=0.0510) of CD4+Foxp3-GFP Teff cells compared to vehicle control treatment (Fig. 2C, Movies S7 and S8). No significant effect was observed on adhesion (100.0% vs. 108.6 ± 26.1%,
p=0.7536) and detachment (100.0% to 105.2± 9.1%, p=0.5850) of CD4⁺Foxp⁺GFP⁺ Treg cells after PDE8 inhibition (Fig. 2D). The observed difference in PDE8A abundance between Teff and Treg cells could explain the differential effect of PDE8 inhibition on Teff cells vs. Treg cells in the flow assay.

3.3 Disruption of PDE8A-Raf-1 kinase signaling complex suppresses Teff and Treg cell adhesion.

PDE8A has been shown to interact with Raf-1 kinase without the need for accessory proteins or lipids and protect it from PKA mediated inhibitory phosphorylation. The Raf family members have been known to regulate adhesion of T cells to vascular ligands [28]. We next used a cell permeable peptide that disrupts the PDE8A-Raf-1 interaction [19] to determine whether this complex regulates CD4⁺ T cell motility under flow conditions. CD4⁺ T cells isolated from draining lymph nodes of MOG35-55 immunized Foxp3gfpmice and endothelial cells were treated with cell permeable disruptor or control peptides for 4 h. Treatment with the disruptor peptide decreased adhesion of CD4⁺GFP Foxp3+ Teff cells by 43.6 ± 14.3%, (100.0% vs. 56.4 ± 14.3%, *p= 0.0221). We also observed an increased trend of detachment, but this trend did not reach statistical significance (p=0.1919) (Fig.3A, Movies S9 and S10) compared to control. We also observed a decreased trend in adhesion of CD4⁺GFP⁺Foxp3⁺ Treg cells, but this difference was not significant (p=0.1590) (Fig. 3B).

3.4 Adhesion, spreading and locomotion of Teff cells to ICAM-1 are significantly affected by disruption of the PDE8A-Raf-1 complex, but not by PDE8 inhibition at the catalytic moiety.

Next, we assessed whether PDE8 or the PDE8A-Raf-1 kinase complex regulate adhesion via LFA-1 integrin mediated cell tether formation with ICAM-1. For this purpose, we tested interaction of CD4⁺ T cells on plates coated with recombinant ICAM-1-Fc protein under shear flow conditions. Treatment of CD4⁺ T cells with PF-04957325 did not lead to significant differences in CD4⁺GFP Foxp3+ Teff cell mediated transient tethers (100.0% vs. 112.8 ± 53.0%, p=0.8213), firm tethers (100.0% vs. 99.8 ± 9.6%, p=0.9809), spreading (100.0% vs. 66.0 ± 28.8%, p=0.3038), locomotion (100.0% vs. 62.6 ± 29.6%, p=0.2744), detachment (100.0% vs. 103.6 ± 2.3%, p=0.1944) (Fig. S1A), or adherence (100.0% vs. 68.6 ± 15.4%, p=0.1120) when ICAM-1 was used as a substrate in flow assays (Fig. 4A). There was also no significant effect of PF-04957325 treatment on CD4⁺GFP⁺Foxp3⁺ Treg cell mediated firm tether formation (100.0% vs. 90.0 ± 13.2%, p=0.4916), adherence (100.0% vs. 191.5 ± 83.5%) or detachment (100.0% vs. 88.0 ± 6.7%, p=0.1498) (Fig. S1B). In contrast, treatment with the PDE8A-Raf-1 disruptor peptide led to a significant 52.6 ± 13.3% decrease in CD4⁺GFP Foxp3⁺ Teff cell adherence, (100.0% vs. 47.4 ± 13.3%, *p=0.0167), a significant 59.3 ± 4.3% decrease in spreading (100.0% vs. 40.7 ± 4.3%, *** p=0.0002) and a significant 72.7 ± 4.6% decrease in locomotion, (100.0% vs. 27.3 ± 4.6%, ****p < 0.0001) (Fig. 4B), but no significant effect was observed on transient tethers (100.0% vs. 150.8 ± 95.9%, p=0.6240), firm tethers (100.0% vs. 60.3 ± 23.3%, p=0.1634) and detachment (100.0% vs. 106.1 ± 3.6% p=0.1688)
of Teff cells when ICAM-1 was used as a substrate (Fig. S1C, Movies S11 and S12). Of note, there was also a significant 43.3 ± 13.1% decrease in adherent cell tethers of CD4+GFP+Foxp3+ Treg cells interacting with ICAM-1 (100.0% vs. 56.7 ± 13.1%, *p=0.0299) after treatment with the disruptor peptide (Fig. 4C). No significant effect on firm tether formation (100.0% vs. 65.5 ± 25.3%, p=0.2450) and detachment (100.0% vs. 112.9 ± 9.3%, p=0.2358) of Treg cells was observed (Fig. S1D). These data implicate the LFA-1-ICAM-1 interaction as a target of PDE8A-Raf-1 kinase complex disruption.

3.5 CD4+ T cell mediated firm cell tether formation to VCAM-1 is unaffected by disruption of PDE8A-Raf-1 complex and PDE8 inhibition at the catalytic moiety.

To further explore whether the effect of the PDE8 inhibitor or the PDE8A-Raf-1 complex disruptor peptide on adhesion of CD4+ T cells was dependent on the integrin VLA-4, we tested binding of cells to its selective ligand, VCAM-1 under flow [39]. No significant differences were observed in transient tethers, (100.0% vs. 196.1 ± 81.9%, p=0.2849), rolling (100.0% vs. 87.6 ± 23.8%, p=0.6204), firm tethers (100.0% vs. 134.7 ± 36.6%, p=0.3805), adherence (100.0% vs. 127.9 ± 48.8%, p=0.5881) and detachment (100.0% vs. 99.8 ± 0.6%, p=0.7070) in CD4+ T cell interaction with VCAM-1 after treatment with PDE8 inhibitor (Fig. S2A). Treatment with disruptor peptide did not have any significant effect on transient tethers (100.0% vs. 90.4 ± 22.7%, p=0.6860), firm tethers (100.0% vs. 81.2 ± 13.3%, p=0.2074), adherence (100.0% to 69.5 ± 23.4%, p=0.2402) and detachment (100.0% vs. 102.5 ± 1.7%, p=0.1967). However, there was a significant 56.4 ± 7.3% suppression of rolling (100.0% vs. 43.6 ± 7.3%, **p=0.0015) after treatment with the disruptor peptide, indicating a selective effect of the peptide on initial loose tethers of T cells to VCAM-1 (Fig. S2B).

3.6 Integrin surface expression in CD4+ T cells is not altered by PDE8 inhibition at the catalytic moiety and marginally reduced by disruption of the PDE8A-Raf-1 complex.

α4 and αL integrins as well as CD44 are known cell surface molecules regulating myelin antigen specific Teff cell migration into the central nervous system [30, 31] and are targets of drug therapies [40]. We tested the effect of the PDE8 inhibitor and the disruptor peptide on the surface expression of these adhesion molecules by flow cytometry. PF-04957325 treatment did not significantly alter the mean fluorescence intensity (MFI) of αL integrin (p=0.2608), α4 integrin (p=0.1053) or CD44 (p=0.3675) in CD4+GFP+Foxp3+ GFP- Teff cells (Fig. S3A) or CD4+GFP+Foxp3+ Treg cells (p=0.5396; p=0.0677; p=0.0899, respectively; Fig. S3B). In contrast, there was a small but significant 2830 ± 645.8 unit decrease in the MFI of αL integrin expression (30957 ± 350.1 vs. 28127 ± 542.7, *p=0.0119) and a 259.7 ± 65.19 units decrease in MFI of CD44 expression (3453 ± 55.98 vs. 3194 ± 33.39, *p=0.0164) but not in MFI of α4 integrin (p=0.3814) in CD4+GFP+Foxp3+ GFP- Teff cells after treatment with the disruptor peptide (Fig. S3A). Disruptor peptide treatment did not significantly alter MFI of αL integrin (p=0.1025), α4 integrin (p=0.8069) or
CD44 (p=0.0573) in CD4+GFP+Foxp3+ Treg cells. Of note, PF-04957325 and disruptor peptide treatments did not affect the viability of CD4+ T cells (Fig. S4).

3.7 PDE8 inhibition at the catalytic moiety significantly suppresses ERK phosphorylation in CD4+ T cells activated by anti-CD3.

To find out whether the effect of the PDE8 and PDE8A-Raf-1 complex inhibition on CD4+ T cell motility directly affects Raf-1 or ERK signaling, we performed assays to determine inhibitory phosphorylation of Raf-1 at S259 and activating phosphorylation of ERK1/2 after treatment and activation through the TCR. ERK1/2 phosphorylation (Thr202/Tyr204) and Raf-1 phosphorylation (S259) was analyzed by Western blot (Fig. 5). We observed no significant effect on Raf-1 phosphorylation (100.0% vs. 104.3 ± 7.2%, p=0.5937), but a significant 36.2 ± 6.8% decrease in ERK1 phosphorylation (100.0% vs. 63.8 ± 6.2%, ***p=0.0002) and a significant 27.9 ± 9.366% decrease in ERK2 phosphorylation (100.0% vs. 72.1 ± 8.6%, *p=0.0125) in CD4+ T cells after 1 h treatment with PF-04957325 compared to the vehicle control (Fig. 5A,B). These results indicate that the PDE8 enzyme regulates in part the ERK1/2 signaling pathway. We also report that inhibition of PDE8 enzymatic activity led to a 71.4 ± 25.8% compensatory increase in PDE8A protein expression, (100.0% vs. 171.4 ± 23.7%, *p=0.0183) (Fig. 5C-D). Augmentation of PDE7A, PDE3B, PDE4B and PDE4D expression after treatment with cAMP elevating agents or gene family specific PDE inhibitors has been reported before [41-43], and we show here that this occurs with PDE8A as well.

3.8 Disruption of the PDE8A-Raf-1 complex increases inhibitory Raf-1 phosphorylation and activating ERK1/2 phosphorylation in CD4+ T cells activated by anti-CD3.

Treatment with the disruptor peptide for 1 h followed by 15 min anti-CD3 stimulation led to a 32.17 ± 8% decrease of ERK1 phosphorylation (100% to 67.8 ± 7.3%, **p=0.0020) and a 23.6 ± 6.3% decrease in ERK2 phosphorylation, (100% to 76.4 ± 5.8%, ** p=0.0033), but had no significant effect on PKA mediated inhibitory Raf-1 phosphorylation (100% vs. 111 ± 11.5%, p=0.3572) compared to the control treatment (Fig. 6A-B). In a kinetic study beyond the 1 h time point (Fig. 6A,C-D), treatment with the PDE8A-Raf-1 disruptor peptide for 2 h and 4 h led to a 38.9 ± 22% (100% vs. 138.9 ± 22%, p=0.1283) and 100.4 ± 40.5% (100% vs. 200.4 ± 40.5%, *p=0.0264) increase in Raf-1 phosphorylation, respectively. Additionally, there was an 67.8 ± 20.4% increase at 2 h (100% vs. 167.9 ± 20.4%, *p=0.0158) and a 201.3 ± 53.3% increase at 4 h (100% vs. 301.3 ± 49.6%, **p=0.0023) in ERK1 phosphorylation and a 33.2 ± 13% increase at 2 h (100% vs. 133.2 ± 13%, *p=0.0438) and a 184.6 ± 49.5% increase at 4 h (100% vs. 284.6 ± 46.1%, *p=0.0025) in ERK2 phosphorylation in CD4+ T cells exposed to the disruptor peptide. Since in flow assays the CD4+ T cells were treated with the peptide for 4 h, our data indicate that the major effect of the PDE8A-Raf-1 complex is dependent on Raf-1 signaling independent of the downstream ERK-MAPK pathway. Despite this robust effect on inhibitory Raf-1 phosphorylation, treatment with the PDE8A-Raf-1 disruptor peptide for 4 h leads to an increase of
activating ERK1/2 phosphorylation that could be mediated by a large number of effectors acting through ERK during T cell activation [44].

4. Discussion
Previous work has shown that PDE8A is expressed in activated CD4+ T cells [5, 6, 22, 23]. Functionally, we have reported that PDE8 regulates T cell motility as inhibition of PDE8 is a non-redundant means to suppress lymphocyte chemotaxis and adhesion [6, 22, 24]. Moreover, we have recently shown that PDE8A is expressed in breast cancer cell lines and inhibition of PDE8 suppresses breast cancer cell migration [21]. These previously described roles of PDE8 in cell motility prompted us to mechanistically investigate the specific cell migration categories and molecular signaling complexes that are regulated by PDE8.

PDEs control intracellular cAMP gradients and are positioned in discrete signaling complexes. Much is known about spatial arrangements and specific functions of some PDE isoforms such as PDE4 [45-47]. Much of this knowledge has stemmed from the use of disruptor peptides that have no effect on global PDE activity but can discretely displace small pools of tethered PDE from precise microdomains. For example, PDE4D5 forms signaling complexes with signaling and adhesion molecules that regulate spreading of cancer cells [48] and endothelial inflammation [49]. A dearth of information, however, exists for complexes involving PDE8A. A recent study by Baillie and colleagues demonstrated the first such PDE8 specific signaling complex [19]. In this study, we addressed a major gap in the literature by delineating the parts of the mechanism by which PDE8 inhibition acts in T cells, and by investigating the function of the PDE8A-Raf-1 complex in T cells. To analyze T cell migration under physiological conditions, we did motility assays under shear flow conditions. To delineate the role of PDE8 in different T cell populations involved in pro- and antiinflammatory immune responses, we subjected polyclonal and antigen-activated Teff and Treg cells to physiological shear stress in flow chamber assays. Our results indicate the higher adhesion of naive Treg cells compared to naive Teff cells. Further activation of these cells with plate bound anti-CD3 led to an increase in migratory potential of Teff cells, comparable to the Treg cells. Our results indicate that enzymatic inhibition of PDE8 using the specific PDE8 inhibitor, PF-04957325, leads to significant reduction of Teff cell adhesion. In contrast, Treg motility is not significantly affected by PDE8 inhibition. This differential effect on both the cell populations is consistent with the observed differential expression of PDE8A in Teff cells versus Treg cells where it is considerably more highly expressed in Teff cells. Treatment with the PDE8A-Raf-1 disruptor peptide had a more potent effect on Teff cell adhesion, which suggested the specific involvement of the PDE8A-Raf-1 signaling complex in the control of CD4+ T cell motility beyond a role for the global pool of PDE8 in T cells. Although it was shown that the disruptor peptide clearly inhibits Raf-1 binding to PDE8, it remains a concern that the disruptor peptide might possibly also inhibit the binding of Raf-1 to other proteins. However, it is noteworthy that
overexpression of a catalytically inactive mutant form of PDE8A displayed a
dominant negative phenotype and recapitulated the effects of treatment of cells
with the disruptor peptide, as did mice and Drosophila genetically modified to
knock out PDE8A [19]. Inasmuch as these treatments mimicked the effects of
disruptor peptide treatment on ERK activation and sensitization to stress-induced
death, it at least suggests that the primary action of disruptor peptide is to disrupt
the binding of Raf-1 from PDE8A, rather than from an association with other
protein(s), which otherwise would not have been reproduced by dominant
negative PDE8A overexpression and knockout of PDE8A in the germline.

In order to dissect the molecular mechanism by which PDE8 and the PDE8A-
Raf-1 complex regulate adhesion, we further tested the effect of the enzymatic
inhibitor and signaling complex disruptor on interaction of activated CD4⁺ T cells
isolated from lymph nodes of mice immunized with MOG35-55/CFA with
endothelial ligands, VCAM-1 and ICAM-1, molecules critically involved in
inflammation [40]. Treatment with the disruptor peptide led to a significant
reduction in firm adhesion, spreading and locomotion of Teff cells as well as
reduction in firm adhesion of Treg cells while interacting with ICAM-1. Thus
PDE8A-Raf-1 signaling complex regulates LFA-1 integrin mediated tether
formation while interacting with ICAM-1 vascular adhesion molecules. The
expression of αL, α4 integrin and CD44 expression in CD4⁺Foxp3⁺GFP⁺ Teff cells
and CD4⁺Foxp3⁺GFP⁺ Treg cells after treatment with the PDE8 enzymatic
inhibitor or the complex disruptor peptide was marginally reduced or unaltered
suggesting that the effect on adhesion was dependent on adhesion molecule
activation or coupling to the cytoskeleton rather than cell surface expression (Fig.
S3A, B) [28, 32, 33]. Importantly, neither PF-04957325 nor disruptor peptide
treatment affected the viability of cells exposed to doses and for time periods
identical to those used in functional assays (Fig. S4).

While most observed effects of disruptor peptide were recapitulated by treatment
with the PDE8-selective inhibitor, the effects of disruptor peptide were more
pronounced on some, such as adhesion to ICAM-1. We are not certain why this
difference exists in the potency of disruptor peptide as compared to PF-
04957325, but this may simply be a reflection of the different ways in which these
two treatments are acting. Whereas disruptor peptide dissociates PDE8A from
the signalosome complex and alters its localization in the cell such that it can no
longer regulate the pool of cAMP associated with regulating phosphorylation of
Raf-1, the PDE8-selective inhibitor, PF-04957325, must act to inhibit the catalytic
activity of PDE8A. One can never be certain that treatment of intact cells with an
enzyme inhibitor can fully inhibit that enzyme’s activity, as the capacity to inhibit
the target enzyme can be greatly influenced by how much of the inhibitor gets
into the cell, and how it distributes in the cell. Further, since the PDE8A remains
bound to Raf-1 in the signalosome complex upon treatment with PF-04957325, it
is possible that PF-04957325 may not gain full access to the catalytic site of
PDE8A while it is complexed with these other proteins. Another explanation may
lie in the selectivity of disrupting a limited signaling complex containing just
PDE8A versus inhibiting a potentially large number of PDE8 molecules, including PDE8A and 8B distributed throughout the cell. Since cell motility is tightly regulated in opposing directions by specific signaling pathways, it is possible that PF-04957325 acts on PDE8 acting in both adhesion activating and inhibiting signaling complexes. The net effect of a small molecule inhibitor could thus be quite different from the effect of a specific complex disruptor.

It is noteworthy that our data suggest a Raf-1 dependent but ERK independent effect of PDE8 inhibition on T cell motility as disruption of the PDE8A-Raf-1 complex through the disruptor peptide led to inhibitory phosphorylation of Raf-1 while activating ERK1/2. Previous reports implicate Raf-1 and B-Raf in regulating migration by controlling Rho GTPase mediated downstream signaling which regulates actin cytoskeletal and focal adhesion dynamics [50, 51]. Our findings further confirm the role of Raf-1 in regulating this process and point towards existence of the novel signaling complex regulating CD4+ T cell motility. Raf-1 is the major downstream effector linking TCR mediated Ras activation to MEK1/2 and ERK1/2 [44]. However, B-Raf has been identified as the most efficient in interacting with Ras [52, 53] and in activating ERK [54-56]. Importantly, ERK activation and cell proliferation can proceed independently of Raf-1, while Raf-1 can regulate the Rho downstream signaling during cell migration independently of ERK signaling [51], highlighting the independent regulation and function of these MAPKs in different signaling pathways. Importantly, in T cells, TCR stimulation can also lead to Ras/Raf-1-independent activation of ERK [44]. Moreover, findings that PDE8 inhibition has little effect on T cell proliferation [6, 24] indicates an ERK independent action. In contrast, PDE4 inhibition profoundly inhibits cell proliferation and ERK1/2 signaling [57], but has little effect on T cell motility [24, 58]. Taken together, our findings that inhibition of PDE8 and the PDE8A-Raf-1 signaling complex leads to suppression of T cell motility in the absence of marked inhibition of proliferation indicates a novel role for a pool of PDE8A regulating Raf-1 kinase independently of downstream ERK1/2 signaling.

5. Conclusions
This study demonstrates for the first time that T cell motility under physiological shear stress conditions is profoundly modulated by a pool of PDE8A. This effect is mediated by the interaction between PDE8A and Raf-1 kinase and constitutes a novel signaling axis for the investigation of T cell adhesion and migration.
Acknowledgements
We thank Pfizer Inc. for providing PF-04957325 through the CTP. We are grateful to Dr. Frank Menniti for his advice and discussions. This investigation was supported by the National Multiple Sclerosis Society (grant RG 4544A1/1), the Smart Family Foundation and Lea’s Foundation for Leukemia Research Inc.
Figure Legends

Fig. 1: Differential motility of naive and in vitro activated CD4+ Teff and Treg cells while interacting with endothelial cell monolayers under shear stress conditions.
Adhesion of CD4+CD25- Teff cells and CD4+CD25+ Treg cells isolated from spleens assayed under shear stress conditions (5 dyn/cm²): (A) naive (for naive Teff and naive Treg cell flow assays: n=4, 2 independent experiments), (B) anti-CD3 stimulation for 18 h (for anti-CD3 activated Teff cell assays: n=5, 3 independent experiments; anti-CD3 activated Treg cell assays: n=7, 6 independent experiments) and (C) anti-CD3 + IL-2 for 18 h (anti-CD3 + IL-2 Teff cell assays: n=7, 3 independent experiments; anti-CD3 + IL-2 Treg cell assays: n=5, 3 independent experiments). Data (mean ± SEM) are expressed as percentage out of total cells present in the flow chamber before the start of flow.

Fig. 2: PDE8 inhibition at the catalytic moiety suppresses CD4+ Teff cell motility but not Treg cell motility.
(A) The immunoblot shows PDE8A1 and PDE8A2 expression of in vivo MOG35-55 activated CD4+CD25- Teff and CD4+CD25+ Treg cells isolated from the draining PLNs on day 10 post immunization. (B) Relative PDE8A1 and PDE8A2 expression normalized to GAPDH. Data are from T cells isolated from lymph nodes pooled from 5 mice. Data represent mean ± SEM from 3 independent experiments. (C, D) LPS activated endothelial cells and MOG35-55 primed CD4+ T cells were treated with either vehicle or 1 μM PF-04957325 for 1 h. Adhesion and detachment of CD4+Foxp3+GFP- Teff cells (C) and CD4+Foxp3+GFP+ Treg cells (D) were analyzed under shear stress. Data represent mean ± SEM results from 4 independent experiments and are expressed as percentage of total cells that accumulate in the flow chamber before the flow starts. Percentage of cells in each category is normalized to the vehicle condition set at 100 percent.

Fig. 3: Disruption of the PDE8A-Raf-1 complex suppresses both CD4+ Teff and Treg cell motility.
LPS activated endothelial cells and MOG35-55 primed CD4+ T cells isolated from the draining PLNs on day 10 post immunization were treated with either 10 μM scrambled (control) peptide or disruptor peptide for 4 h. Adhesion and detachment of CD4+Foxp3+GFP- Teff cells (A) and CD4+Foxp3+GFP+ Treg cells (B) were analyzed under shear stress. Data represent mean ± SEM results from 3-4 independent experiments and are expressed as percentage of total cells that accumulate in the flow chamber before the flow starts. Percentage of cells in each category is normalized to the vehicle condition set at 100 percent.

Fig. 4: Adhesiveness to ICAM-1 significantly affected by disruption of the PDE8A-Raf-1 complex, but not by PDE8 inhibition at the catalytic moiety.
MOG35-55 primed CD4+ T cells isolated from the draining PLNs on day 10 post immunization were treated with vehicle or 1 μM PDE8 inhibitor for 1 h (A) or 10 μM control peptide or disruptor peptide for 4 h (B, C). Spontaneous tethering
(adherent cells, spreading or locomotion) of the CD4+Foxp3+GFP+ Teff cells (A, B) and CD4+Foxp3+GFP+ Treg cells (C) while interacting with high density ICAM-1-Fc (5 μg/ml) were analyzed under flow. Data represent mean ± SEM results from 3 independent experiments and are expressed as percentage of total cells that accumulate in the flow chamber before the flow starts. Percentage of cells in each category is normalized to the vehicle condition set at 100 percent.

**Fig. 5: Inhibition of PDE8 in CD4+ T cells diminishes ERK1/2 phosphorylation induced by CD3.** MOG35-55 primed CD4+ T cells isolated from the draining PLNs on day 10 post-immunization were treated with vehicle or 1 μM PF-04957325 for 1 h followed by anti-CD3 stimulation for 15 min. Cell lysates were then probed for (A) phospho Raf-1 (S259), Raf-1, phospho ERK1/2, ERK1/2, (C) PDE8A1 and GAPDH by Western blot. (B, D) Bands were quantitated by densitometry and data are presented as phospho-Raf-1 (S259) relative to Raf-1, phospho-ERK1/2 relative to ERK1/2 and PDE8A1 expression relative to GAPDH. Data represent mean ± SEM results from 4 independent experiments (n=7).

**Fig. 6: Disruption of the PDE8A-Raf-1 complex in CD4+ T cells increases inhibitory Raf-1 phosphorylation at serine 259 and increases ERK1/2 phosphorylation induced by CD3.** MOG35-55 primed CD4+ T cells isolated from the draining PLNs on day 10 post-immunization were treated with 10 μM control peptide (CP) or disruptor peptide (DP) for 1 h (A, B), 2 h (A, C) and 4 h (A, D) followed by anti-CD3 stimulation for 15 min. Cell lysates were then probed for phospho Raf-1 (S259), Raf-1, phospho ERK1/2, ERK1/2 and GAPDH by Western blot. (B-D) Bands were quantitated by densitometry and data are presented as phospho-Raf-1 (S259) relative to Raf-1 and phospho-ERK1/2 relative to ERK1/2 at 1, 2 and 4 h. Data represent mean ± SEM from 2 independent experiments (n=5).

References

Supporting Information

Figure Legends

Fig. S1: Adhesiveness to ICAM-1 significantly affected by disruption of the PDE8A-Raf-1 complex, but not by PDE8 inhibition at the catalytic moiety.
MOG\textsubscript{35-55} primed CD4\textsuperscript{+} T cells isolated from the draining PLNs on day 10 post-immunization were treated with vehicle or 1 μM PDE8 inhibitor for 1 h (A, B) or 10 μM control peptide or disruptor peptide for 4 h (C, D). Spontaneous tethering (rolling, transient tether, firm tether, spreading, locomotion) and detachment of the CD4\textsuperscript{+}Foxp3\textsuperscript{-}GFP\textsuperscript{-} Teff cells (A, C) and CD4\textsuperscript{+}Foxp3\textsuperscript{*}GFP\textsuperscript{*} Treg cells (B,D) while interacting with high density ICAM-1-Fc (5 μg/ml) were analyzed under flow. Data represent mean ± SEM results from 3 independent experiments and are expressed as percentage of total cells that accumulate in the flow chamber before the flow starts. Percentage of cells in each category is normalized to the vehicle condition set at 100 percent.

Fig. S2: Firm adhesiveness of CD4\textsuperscript{+} T cells to VCAM-1 is not affected by PDE8 inhibition at the catalytic moiety or by disruption of the PDE8A-Raf-1 complex.
MOG\textsubscript{35-55} primed CD4\textsuperscript{+} T cells isolated from the draining PLNs on day 10 post-immunization were treated with either vehicle or 1 μM PF-04957325 for 1 h (A) or 10 μM control peptide or disruptor peptide for 4 h (B). Spontaneous tethering (rolling, transient tether, firm tether, adherent cells) and detachment of the CD4\textsuperscript{+} T cells while interacting with high density VCAM-1-Fc (2 μg/ml) were analyzed under shear stress of 5 dyn/cm\textsuperscript{2}. Data represent mean ± SEM results from 4 independent experiments and are expressed as percentage of total cells that accumulate in the flow chamber before the flow starts. Percentage of cells in each category is normalized to the vehicle condition set at 100 percent.

Fig. S3: Integrin surface expression is not altered by PDE8 inhibition at the catalytic moiety and marginally reduced by disruption of the PDE8A-Raf-1 complex.
CD4\textsuperscript{+} T cells isolated from spleens of Foxp3\textsuperscript{gfp.KI} mice were treated with plate-bound anti-CD3 (10 μg/ml) for 18 h followed by treatment with vehicle or 1 μM PF-04957325 for 1 h or 10 μM control peptide or PDE8A-Raf-1 disruptor peptide for 4 h. Expression of αL integrin, α4 integrin, and CD44 was evaluated by flow cytometry. Data show MFI of the αL\textsuperscript{+}, α4\textsuperscript{+} and CD44\textsuperscript{+} cells within the Foxp3\textsuperscript{*}GFP\textsuperscript{*} Teff cell (A) and Foxp3\textsuperscript{*}GFP\textsuperscript{*} Treg cell populations (B). Data represent mean ± SEM results from 1 experiment (n=3).

Fig. S4: Inhibition of PDE8 or disruption of the PDE8A-Raf-1 complex does not affect viability of CD4\textsuperscript{+} T cells.
CD4\textsuperscript{+} T cells isolated from spleens of Foxp3\textsuperscript{gfp.KI} mice were treated with plate-bound anti-CD3 (10 μg/ml) for 18 h followed by treatment with vehicle or 1 μM PF-04957325 for 1 h or 10 μM control peptide or PDE8A-Raf-1 disruptor peptide for 4 h.
The viability of the CD4⁺ T cells after treatment was assessed using the trypan blue assay. The graphs represent the percentage of live and dead cells within each treatment group. Data represent mean ± SEM results from 1 experiment (n=3).

Supplemental Movies:

Movie S1: Naive Teff cells isolated from spleens of wild type C57BL/6 mice interacting with LPS activated brain endothelial cells under 5 dyn/cm² shear stress. 152 Teff cells counted during the accumulation phase before the shear flow starts. Scale bar 100 µm.

Movie S2: Naive Treg cells isolated from spleens of wild type C57BL/6 mice interacting with LPS activated brain endothelial cells under 5 dyn/cm² shear stress. 130 Treg cells counted during the accumulation phase before the shear flow starts. Scale bar 100 µm.

Movie S3: Teff cells activated with anti-CD3 for 18 h followed by interaction with LPS activated brain endothelial cells under 5 dyn/cm² shear stress. 48 Teff cells counted during the accumulation phase before shear flow starts. Scale bar 100 µm.

Movie S4: Treg cells activated with anti-CD3 for 18 h followed by interaction with LPS activated brain endothelial cells under 5 dyn/cm² shear stress. 106 Treg cells counted during the accumulation phase before shear flow starts. Scale bar 100 µm.

Movie S5: Teff cells activated with anti-CD3+IL-2 for 18 h followed by interaction with LPS activated brain endothelial cells under 5 dyn/cm² shear stress. 120 Teff cells counted during the accumulation phase before the shear flow starts. Scale bar 100 µm.

Movie S6: Treg cells activated with anti-CD3+IL-2 for 18 h followed by interaction with LPS activated brain endothelial cells under 5 dyn/cm² shear stress. 39 Treg cells counted during the accumulation phase before the shear flow starts. Scale bar 100 µm.

Movie S7: MOG₃₅-₅₅ activated CD4⁺ T cells isolated from draining lymph nodes of Foxp3gfp.KI mice and LPS activated brain endothelial cells were treated with vehicle control for 45 min followed by interaction with LPS activated brain endothelial cells under 5 dyn/cm² shear stress. 140 Foxp3-GFP⁻ Teff cells and 27 Foxp3-GFP⁺ Treg cells counted during the accumulation phase before flow starts. Scale bar 100 µm.

Movie S8: MOG₃₅-₅₅ activated CD4⁺ T cells isolated from draining lymph nodes of Foxp3gfp.KI mice and LPS activated brain endothelial cells were treated with PF-04957325 for 45 min followed by interaction with LPS activated brain endothelial cells under 5 dyn/cm² shear stress. 124 Foxp3-GFP⁻ Teff cells and 16 Foxp3-GFP⁺ Treg cells counted during the accumulation phase before flow starts. Scale bar 100 µm.
**Movie S9:** MOG<sub>35-55</sub> activated CD4<sup>+</sup> T cells isolated from draining lymph nodes of *Foxp3gfp.KI* mice and LPS activated brain endothelial cells were treated with control peptide for 4 h followed by interaction with LPS activated brain endothelial cells under 5 dyn/cm<sup>2</sup> shear stress. 333 Foxp3<sup>+</sup>GFP<sup>-</sup> Teff cells and 22 Foxp3<sup>+</sup>GFP<sup>-</sup> Treg cells counted during the accumulation phase before flow starts. Scale bar 100 μm.

**Movie S10:** MOG<sub>35-55</sub> activated CD4<sup>+</sup> T cells isolated from draining lymph nodes of *Foxp3gfp.KI* mice and LPS activated brain endothelial cells were treated with disruptor peptide for 4 h followed by interaction with LPS activated brain endothelial cells under 5 dyn/cm<sup>2</sup> shear stress. 233 Foxp3<sup>+</sup>GFP<sup>-</sup> Teff cells and 20 Foxp3<sup>+</sup>GFP<sup>-</sup> Treg cells counted during the accumulation phase before flow starts. Scale bar 100 μm.

**Movie S11:** MOG<sub>35-55</sub> activated CD4<sup>+</sup> T cells isolated from draining lymph nodes of *Foxp3gfp.KI* mice were treated with control peptide for 4 h followed by interaction with recombinant ICAM-1-Fc coated plates under 5 dyn/cm<sup>2</sup> shear flow. 396 Foxp3<sup>+</sup>GFP<sup>-</sup> Teff cells and 23 Foxp3<sup>+</sup>GFP<sup>-</sup> Treg cells counted during the accumulation phase before the flow starts.

**Movie S12:** MOG<sub>35-55</sub> activated CD4<sup>+</sup> T cells isolated from draining lymph nodes of *Foxp3gfp.KI* mice were treated with disruptor peptide for 4 h followed by interaction with recombinant ICAM-1-Fc coated plates under 5 dyn/cm<sup>2</sup> shear flow. 304 Foxp3<sup>+</sup>GFP<sup>-</sup> Teff cells and 14 Foxp3<sup>+</sup>GFP<sup>-</sup> Treg cells counted during the accumulation phase before the flow starts.
Figure S1

A

Vehicle control PF-04957325

Transient tethers (%)

Vehicle control PF-04957325

Firm tethers (%)

Vehicle control PF-04957325

Spreading (%)

Vehicle control PF-04957325

Locomotion (%)

Vehicle control PF-04957325

Detachment (%)

Vehicle control PF-04957325
Figure S1

B

Vehicle control  PF-04957325
0 50 100 150

Firm tethers (%)  Detachment (%)

Vehicle control  PF-04957325
0 50 100 150
Figure S1

C

Transient tethers (%)

Control peptide  Disruptor peptide

Firm tethers (%)

Control peptide  Disruptor peptide

Detachment (%)

Control peptide  Disruptor peptide

D

Firm tethers (%)

Control peptide  Disruptor peptide

Detachment (%)

Control peptide  Disruptor peptide
Figure S2

B

**Rolling (%)**

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**Transient tethers (%)**

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**Firm tethers (%)**

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**Adherent cells (%)**

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**Detachment (%)**

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Figure S3

A

Vehicle control
PF-04957325
Control peptide
Disruptor peptide

αL integrin MFI

Vehicle control
PF-04957325
Control peptide
Disruptor peptide

α4 integrin MFI

Vehicle control
PF-04957325
Control peptide
Disruptor peptide

CD44 MFI

B

Vehicle control
PF-04957325
Control peptide
Disruptor peptide

αL integrin MFI

Vehicle control
PF-04957325
Control peptide
Disruptor peptide

α4 integrin MFI

Vehicle control
PF-04957325
Control peptide
Disruptor peptide

CD44 MFI

A

Vehicle control
PF-04957325
Control peptide
Disruptor peptide

αL integrin MFI

Vehicle control
PF-04957325
Control peptide
Disruptor peptide

α4 integrin MFI

Vehicle control
PF-04957325
Control peptide
Disruptor peptide

CD44 MFI

B

Vehicle control
PF-04957325
Control peptide
Disruptor peptide

αL integrin MFI

Vehicle control
PF-04957325
Control peptide
Disruptor peptide

α4 integrin MFI

Vehicle control
PF-04957325
Control peptide
Disruptor peptide

CD44 MFI

*
Supplemental Movies

Still images

Movie S1

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Movie S4

Movie S4: Treg cells activated with anti-CD3 for 18 h followed by interaction with LPS activated brain endothelial cells under 5 dyn/cm² shear stress. 106 Treg cells counted during the accumulation phase before shear flow starts.
Movie S5

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MOG

35-55 activated CD4⁺ T cells isolated from draining lymph nodes of Foxp3gfp.KI mice were treated with disruptor peptide for 4 h followed by interaction with recombinant ICAM-1-Fc coated plates under 5 dyn/cm² shear flow. 304 Foxp3⁻GFP⁻ Teff cells and 14 Foxp3⁺GFP⁺ Treg cells counted during the accumulation phase before the flow starts.