Scaffold protein Disc large homolog 1 is required for T-cell receptor-induced activation of regulatory T-cell function

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Edited* by Mark M. Davis, Stanford University School of Medicine, Stanford, CA, and approved December 22, 2011 (received for review June 22, 2011)

Foxp3

CD4

CD25

regulatory T cell (Treg) suppression of inflammation depends on T-cell receptor-mediated Nuclear Factor of Activated T cells1 (NFAT1) activation with reduced Akt activity. We investigated the role of the scaffold protein Disc large homolog 1 (Dlgh1) in linking the T-cell receptor to this unique signaling outcome. The Treg immunological synapse (IS) recruited fourfold more Dlgh1 than conventional CD4+ T-cell IS. Tregs isolated from patients with active rheumatoid arthritis, or treated with tumor necrosis factor-α, displayed reduced function and diminished Dlgh1 recruitment to the IS. Furthermore, Dlgh1 silencing abrogated Treg function, impaired NFAT1 activation, reduced phosphatase and tensin homolog levels, and increased Akt activation. Dlgh1 operates independently of the negative feedback pathway mediated by the related adapter protein Carma1 and thus presents an array of unique targets to selectively manipulate Treg function.

Results

To investigate the role of Dlgh1 in activation of in vitro Treg function, we first determined whether Dlgh1 is recruited to the IS. Human Tregs, CD4+CD25highCD127low cells, were isolated by flow cytometry (Fig. S1A), and 90% were Foxp3+ (Fig. S1B), or by positive selection by MACS (75–80% were Foxp3+; Fig. S1C). The Treg are 80% CD45RO+, suggesting that they have been previously activated by antigen in vivo (Fig. S1D) (24, 25). To compare IS of Tregs and CD4+CD25– T cells under identical conditions, we incubated the cells on planar bilayers containing mobile fluorescently labeled ICAM-1 and the stimulatory anti-CD3 antibodies to Dlgh1, and imaged by total internal reflection fluorescence microscopy (TIRFM). TIRFM only detects fluorescence within 200 nm of the interface between the T cells and the planar bilayers. There was a fourfold increase in the intensity of anti-Dlgh1 staining in FACS-sorted (Fig. L4), as well as MACS bead-purified (Fig. L8).


The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1110120109/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1110120109

PNAS | January 31, 2012 | vol. 109 | no. 5 | 1625–1630
Tregs compared with CD4+CD25− T cells under the same conditions, although there were no differences in total intracellular levels of the protein between the two cell populations (Fig. 1A and Fig. S2A).

Kinetic analysis demonstrated that the recruitment of Dlgh1 to IS in Tregs was slightly increased between 8 and 20 min (Fig. S2B), whereas Dlgh1 accumulation at IS in CD4+CD25− T cells was transient and peaked at 8 min (Fig. S2B). Introduction of the costimulatory signal CD80 into bilayers slightly increased the Dlgh1 recruitment to IS in Tregs and had no significant effect in CD4+CD25− T cells (Fig. S2C). Incubation of Tregs on bilayers containing anti-CD3 antibodies or ICAM-1 alone revealed that both TCR- and integrin-mediated signals are required for maximum recruitment of Dlgh1 (Fig. 1B). A critical role of LFA-1/ICAM-1 interaction is consistent with functional studies demonstrating the importance of LFA-1 for Treg function (26). By using ex vivo expanded human umbilical cord blood (UCB)-derived Tregs (27), we found the same pattern of increased enrichment of Dlgh1 at the IS compared with CD4+CD25− T cells expanded under the same conditions (Fig. 1C). Thus, Dlgh1 is strongly recruited to the Treg IS and is well positioned to play a unique role in Treg signaling.

Patients with RA have normal numbers of Tregs, but their suppressive function is decreased, based on in vitro assays (7, 28, 29). To investigate whether Dlgh1 recruitment to IS has functional consequences in Tregs, we performed imaging and flow cytometric analysis of samples from 10 RA patients with moderate to severe systemic inflammation [Disease Activity Score (DAS) between 4.32 and 6.63] and not treated with anti–TNF-α therapy (Fig. S3A) and found that, whereas total levels of Dlgh1 were not significantly different between freshly purified healthy and RA Tregs, the levels of Dlgh1 recruited to IS were significantly lower in RA Tregs compared with healthy controls (Fig. S3B and Fig. 2A). It has been reported that the proinflammatory mediator TNF-α inhibits Treg-suppressive activity in vitro, downregulates Foxp3, and is required for defective Treg activity in RA patients (7, 29). To evaluate the effects of TNF-α, we incubated Tregs purified from healthy donors with 50 ng/mL TNF-α overnight and then analyzed Dlgh1 recruitment to the IS as described above. We found that treatment with TNF-α significantly reduced levels of Dlgh1 at IS in Tregs (Fig. 2B). Thus, Dlgh1 enrichment at the IS strongly correlates with Treg-suppressive function.

To investigate whether Dlgh1 is important for Treg-suppressive function, we specifically silenced Dlgh1 gene expression using RNA interference (RNAi). Treatment with a mixture of four specific siRNAs for Dlgh1 resulted in an 89% reduction of Dlgh1 expression in freshly purified Tregs (Fig. 3A and Fig. S4). This reduction of Dlgh1 in human Tregs significantly impaired the ability to inhibit CD4+CD25− T-cell proliferation (Fig. 3B), as well as secretion of IFN-γ, IL-17, and IL-4 (Fig. 3C). Notably, reduction of Dlgh1 by siRNA resulted in a marked decrease of Foxp3 expression (Fig. 3 D and E). To exclude the possibility of off-target effects, we tested the impact of each siRNA duplex separately and found that the efficacy of those specific siRNAs to knock down Dlgh1 expression correlated with the ability to down-regulate Foxp3 expression and the suppressive function of Tregs (Fig. S5 A–C). Because each siRNA duplex would be expected to target distinct off-target mRNAs, the correlation across the four duplexes supports the conclusion that Dlgh1 is required for Treg Foxp3 expression and suppression of IFN-γ production by CD4+CD25− T cells. The same inhibition of Treg function by Dlgh1 suppression was observed in the antigen-presenting cell-dependent Treg assay (Fig. S5D).

Fig. 1. Dlgh1 is strongly recruited to IS in Tregs. Freshly FACS-sorted (A) and MACS bead (B) purified human blood CD4+CD25− (Treg) and CD4+CD25− T cells or expanded umbilical cord blood (UCB)-derived Treg and CD4+CD25− T cells (C) were introduced into bilayers containing both anti-CD3 (5 μg/mL) and ICAM-1 at 250 molecules per mm² (A and C) or anti-CD3 or ICAM-1 molecules alone (B), fixed at 8 min and permeabilized, stained with anti-Dlgh1 antibodies, and imaged by TIRFM. Shown are representative images. Dlgh1 staining was quantified by calculation of average fluorescence intensity in cells. Data are representative of three different experiments. P values were calculated by Mann–Whitney test.
We next investigated whether there is cross-talk between Dlg1 and the negative feedback pathway involving PKC-θ (29). Imaging analysis of Dlg1-silenced human Tregs revealed that Dlg1 is not required for PKC-θ localization in the distal pole during IS formation by Treg (Fig. 3F and Fig. S6). Moreover, treatment of Dlg1-silenced Treg with specific PKC-θ inhibitor C20 only partially restored Treg-suppressive function (Fig. 3G). These findings suggest that PKC-θ-mediated negative and Dlg1-mediated positive pathways regulate Treg function independently and that PKC-θ inhibition cannot fully compensate for the loss of Dlg1. Thus, defects in Dlg1 recruitment to the IS of Treg from patients with RA is one of the PKC-θ–independent mechanisms of Treg dysfunction in RA.

Dlg1 has been reported to have a positive or negative role in regulation of Teff function (30, 31). We again used siRNA to investigate the effect of Dlg1 down-regulation (Fig. S7A) on cytokine secretion and proliferation of human CD4⁺CD25⁻ T cells. Surprisingly, we found that silencing of Dlg1 did not affect the ability of CD4⁺CD25⁻ T cells to proliferate (Fig. S7B) or secrete IFN-γ (Fig. S7C) in response to TCR stimulation. Thus, Dlg1 is not required for TCR-induced CD4⁺CD25⁻ T-cell function under the conditions applied here.

NFATC1 activation is required for Treg-suppressive function (23). We initiated these studies with the model that Dlg1 augments NFATC1 activation through p38 as proposed by Miceli and colleagues (18). This model predicts that inhibition of p38 or silencing of Dlg1 would decrease p38 phosphorylation at Thr-180/Tyr-182 and decrease NFATC1 activation. Pretreatment of Tregs with a specific p38 inhibitor, SB203580, significantly down-regulated their ability to suppress IFN-γ secretion from CD4⁺CD25⁻ T cells (Fig. S8). Moreover, silencing of Dlg1 in Tregs inhibited p38 phosphorylation (Fig. 4A) and NFATC1 activation (Fig. 4B) in response to TCR stimulation. Thus, Dlg1 contributes to activation of p38 and NFATC1 in Tregs.

Optimal Treg function requires reduced Akt activation compared with Teff (22, 32). Akt membrane recruitment and activation depends upon phosphatidylinositol-3,4,5 trisphosphate, which is destroyed by PTEN (33). Dlg1 interacts with PTEN through a PDZ domain, stabilizes, and recruits it to the membrane where PTEN is active (19–21). We initiated our studies based on the model that Dlg1 could stabilize and recruit PTEN to suppress Akt activation in Treg, as it does in other cells. Indeed, communoprecipitation analysis confirmed Dlg1/PTEN interaction in Tregs (Fig. 4C). Furthermore, by using specific antibody against PTEN, we found that Dlg1 silencing reduced PTEN levels by 75% in both Tregs and CD4⁺CD25⁻ T cells (Fig. 4D and Fig. S9A). Moreover, Dlg1 silencing in Tregs and CD4⁺CD25⁻ T cells increased levels of Akt phosphorylation on Ser-473, a signature of membrane recruitment and activation, in response to TCR stimulation (Fig. 4E and Fig. S9B). Dlg1 silencing also resulted in increased NF-kB activation in Treg (Fig. 4B), which may be directly related to increased Akt activity because we demonstrated above that the PKC-θ pathway is not regulated by Dlg1. Thus, Dlg1 mediates p38-dependent activation of NFAT and inhibits Akt and NF-kB signaling pathways through PTEN stabilization in Tregs (Fig. 4F).

**Discussion**

TCR signals activate the opposing functional programs of Teffs and Tregs (1). TCR signaling pathways defined first in Teffs have been shown to behave differently in Tregs (34). We recently discovered that PKC-θ and Carma1, major signaling components in the NF-kB activating pathway, mediate negative feedback signaling for Treg function (29). Similarly, Akt signaling promotes Teff activation, but levels of activation observed in Teff are inhibitory to Treg function (22). Finally, ZAP-70 kinase activity is essential for Teff signaling, but not for Treg activation, although ZAP-70 plays a scaffolding role for LFA-1 activation in Treg (12). Here, we demonstrated that Dlg1 scaffolds two critical signaling pathways in Tregs that generate a distinct TCR signaling network, in which NFATC1 activation is high and Akt and NF-kB activation are low. These outcomes are associated with the ability of the Dlg1 scaffold to mediate p38 activation and PTEN stabilization.

Dlg1 selectively activates p38 in antigen-experienced T cells, whereas naïve T cells preferentially induce ERK phosphorylation in response to TCR activation (35). Human peripheral blood Tregs are highly skewed toward antigen-experienced cells

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**Fig. 2.** Dlg1 recruitment to IS correlates with Treg suppressive function. Freshly purified Tregs from healthy donors or RA patients were untreated (A) or TNF-α-treated (50 ng/mL for 24 h) (B), introduced to bilayers with anti-CD3 and ICAM-1, fixed, and imaged by TIRFM. Shown are representative images. Dlg1 staining was quantified by calculation of average fluorescence intensity in cells. Data are representative of seven (A) or three (B) different experiments. *P* values were calculated by Mann–Whitney test.
T cells at a 1:3 ratio and activated with T cells resulted in only 30% down-regulation of T-cell proliferation (22), suggesting that the PI3K/Akt signaling pathway negatively regulates human Tregs. Consistent with this finding, our results demonstrate that Dlgh1-mediated decreases of intracellular levels of PTEN lead to increased TCR-induced Akt activation and significant down-regulation of the suppressive Treg function. Studies from other cellular systems have demonstrated that Dlgh1 binds to PTEN through its C-terminal PDZ domain and specifically suppresses the PI3K pathway (40). In humans, Tregs are naïve (Fig. S1D), they rely on ERK-mediated signaling rather than on Dlgh1/p38 signaling pathways (35). Indeed, Dlgh1 silencing in CD4+CD25+ T cells resulted in only 30% down-regulation in TCR-induced p38 phosphorylation compared with 80% inhibition in Tregs (Fig. S9C). In addition, the fact that Dlgh1 specifically mediates activation of NFATc1, but not NF-κB

Fig. 3. Dlgh1 is required for Treg function. (A) Freshly purified human Tregs were transfectected with small interfering RNA (siRNA) targeting Dlgh1 or with control siRNA by AMAXA and plated in presence of IL-2 (300 IU/mL). After 48 h Dlgh1 expression was measured by Western blot analysis. (B) siRNA-transfected Tregs were mixed with CD4+CD25+ T cells at a 1:3 ratio and activated with anti-CD3/CD28 dynal beads. CD4+CD25+ T-cell proliferation was determined after 96 h by CFSE dilution. Representative experiment of three is shown. (C) The supernatants were analyzed for IFN-γ, IL-17, and IL-4 after 48 h. (D and E) Foxp3 expression was determined by flow cytometry 48 h after Treg transfection (D), and average of three different experiments is shown (E). (F and G) Some Tregs 48 h after transfection were introduced to biolayers with anti-CD3 and ICAM-1, fixed, stained for PKC-θ, and imaged by confocal microscopy (F) or treated with PKC-θ inhibitor, C-20 (1 μM, 30 min), washed, and then mixed with untreated CD4+CD25+ T cells at ratio 1:3 (G). Average of three (E–G) or four (A and C) different experiments are shown. P values were calculated by t test. *P < 0.05.

(25). Naïve Tregs are a minor population in humans, and it is not clear whether this population also uses Dlgh1 because the contribution of these cells may be obscured by the memory cells. Our data are fully consistent with earlier models showing that Dlgh1 forms a scaffold for ZAP-70 and Lck to recruit and activate p38 (18). ZAP-70 catalytic activity is not needed for mouse Treg function (12), but further work is needed to determine whether Dlgh1 is required for Treg function. (Zanin-Zhorov et al.)
controls, leading to a delay in tyrosine–protein phosphatase nonreceptor type 6 (SHP-1) recruitment to the IS and sustained TCR-induced ZAP-70 and NF-κB signaling (44). Interestingly, Dlgh1 can interact with ezrin and contribute to the negative regulation of ERK signaling pathway (45). Collectively, our results together with published data suggest that the activation of signaling pathways is altered in RA patients, and further analysis is required to evaluate the prognostic value of this knowledge.

In summary, Dlgh1 mediates a key activating signal downstream of the TCRs that operate in opposition to the PKC-θ/Carma1-mediated negative feedback pathway that we recently identified in Tregs (29). Unlike the PKC-θ/Carma1 system, which is reciprocally an activating pathway for T effector functions, our results do not support a reciprocal role of Dlgh1 in inhibition of CD4+CD25+ T-cell functions. Although Dlgh1 has no enzymatic activity that could be targeted by a small molecule, it is conceivable that it could still be targeted therapeutically because an RNAi-mediated suppression generates significant impairment of Treg function, which could be useful in vaccination, in combating chronic infection, and in immunotherapy for cancer (46). This therapy would need to be carefully targeted because of potential off-target effects and the diverse roles of Dlgh1 in cell–cell communication in many organ systems (47).

**Materials and Methods**

**Cell Purification.** CD4+CD25hi, CD4+CD25+ and CD4+CD25− T cells were purified from the peripheral blood of healthy human donors between the ages of 16 and 75 years (29) (New York Blood Center) or from 10 patients with RA in different stages (according to DAS; Fig. 3A) as described (48). The New York University Institutional Review Board has reviewed the use of human specimens for this study. UCB CD25+ and CD25− T cells were isolated from frozen UCB units (National Placental Blood Program, New York Blood Center) by positive selection using directly conjugated anti-CD25 magnetic microbeads and expanded as described (27).

**Planar Lipid Bilayers.** Planar lipid bilayers containing anti-CD3 antibodies (5 μg/ml) and ICAM-1 (250 molecules per nm²) were prepared in parallel-plate flow cells as described (29). The flow cell containing the bilayers was warmed up to 37°C; cells were injected in 500 μl of Hepes-buffered saline containing 1% human serum albumin; and images were collected on a custom automated Nikon inverted fluorescence microscope.

**Microscopy.** All TIRF imaging was performed on the custom automated Nikon inverted fluorescence microscope using the 100x/1.45 N.A. TIRF objective from Nikon. TIRF illumination was set up and aligned according to the manufacturer’s instructions as described (49). Briefly, cells interacted with the bilayers for 8 min at 37°C and were fixed with 2% PFA; permeabilized with 0.05% Triton X-100; blocked and stained with rabbit polyclonal antibodies to Dlgh1 (H-60; sc-25661), PTEN (sc-212), or Carma1 (Card 11, C-12) from Santa Cruz Biotech for 20 min; and then incubated with fluorescently tagged goat anti-rabbit Fab (Invitrogen). Controls included the use of nonimmune species-matched IgG. Measurement of signaling was done as described (29). Confocal microscopy was carried out on a Zeiss LSM 510 Meta imaging system (63×1.4 NA; Zeiss) using appropriate factory-set filters and dichroics for different fluorophores as described (29).

**In Vitro Suppression Assays.** CD4+CD25− T cells were treated or not washed, and added at a ratio of 1:3 (1.25 × 10^5; 5 × 10^5) to CD4+CD25+ T cells at final concentration of 2 × 10^6 per ml (cytokine secretion) or 2 × 10^5 per ml (proliferation). The cells were cocultured on anti-CD3 mAb (5 μg/ml) precoated 24-well plates for 24–48 h (cytokine secretion) or 96 h (proliferation). Human TNF-α (210-TA) was purchased from R&D Systems and added to cocultures where indicated. The PKC-θ inhibitor, compound 20, was provided by Boehringer-Ingelheim Pharmaceuticals and dissolved in DMSO (50). T cells were pretreated for 30 min at a concentration of 1 μM at 37°C and washed three times. Cytokine secretion was determined by ELISA as described (29), using human IFN-γ Cytoset (Biosource) and IL-17 and -4 (Invitrogen). Proliferation was assessed by 5-bromo-2′-deoxyuridine (BrdU) incorporation. TCR/CD3 complex formation was determined by immobilized anti-CD3 antibodies (5 μg/ml) from Nikon. TIRF illumination was set up and aligned according to the manufacturer’s instructions as described (49). Briefly, cells interacted with the bilayers for 8 min at 37°C and were fixed with 2% PFA; permeabilized with 0.05% Triton X-100; blocked and stained with rabbit polyclonal antibodies to Dlgh1 (H-60; sc-25661), PTEN (sc-212), or Carma1 (Card 11, C-12) from Santa Cruz Biotech for 20 min; and then incubated with fluorescently tagged goat anti-rabbit Fab (Invitrogen). Controls included the use of nonimmune species-matched IgG. Measurement of signaling was done as described (29). Confocal microscopy was carried out on a Zeiss LSM 510 Meta imaging system (63×1.4 NA; Zeiss) using appropriate factory-set filters and dichroics for different fluorophores as described (29).

**Flow Cytometry.** Indicated populations of T cells were stained (30 min, 4°C) with PE-labeled anti-CD25 (Miltenyi Biotec) and FITC-labeled anti-CD127
siRNA duplexes (siRNAs) were synthesized and purified by Qiagen as described (51). A mixture of four Dlgh1-specific siRNAs was used by Qiagen as catalogue nos. S100005584 (Dlgh1-1), S102632518 (Dlgh1-2), S103046099 (Dlgh1-8), and S103102799 (Dlgh1-9). Control siRNA was purchased from Qiagen (1027281). Transfections of freshly purified T cells were performed by using the human T-cell Nucleofector kit (Amaxa Biosystems, Lonza) as described (29).

Western blot and immunoprecipitation. We lysed cells in radioimmuno precipitation assay buffer (pH 8) supplemented with protease and phosphatase inhibitors. After 20 min of centrifugation at 10,000 × g at 4 °C, Dlgh1 and PTEN were immunoprecipitated by incubation for 1 h at 4 °C with 2 μg of anti-Dlgh1 antibody (610875; BD Transduction Laboratories), anti-PTEN (B-1; sc-133197; Santa Cruz Biotech), or normal mouse IgG (sc-2025; Santa Cruz Biotech) followed by overnight incubation with protein A/G PLUS-Agarose beads (sc-2003; Santa Cruz Biotech). The immunoprecipitates were washed five times with cold PBS, loaded on an SDS/PAGE gel, and transferred to nitrocellulose membrane. The membranes were blocked, probed, washed, and stained with secondary antibodies from LI-Cor. Immunoreactive protein bands were visualized by using an Odyssey Infrared Imaging system. Anti-α-tubulin antibodies were used as loading controls.

NFTac1 and NF-κB/p50 Activation Assays. Cells were activated on anti-CD3 mAb (5 μg/mL) and lysed, and NFTac1-activation and p50-specific binding to NF-κB consensus sequence were tested by TransFactor NFTac1 Chemiluminescent Kit and TransFactor NF-κB p50 Colorimetric Kit, respectively (nos. 6311916 and 6311955; Clontech Laboratories), according to manufacturer’s instructions.

Statistics. We determined P values by Mann–Whitney or two-tailed t test by using the GraphPad Prism software.

ACKNOWLEDGMENTS. This work was supported by National Institutes of Health Grants R37AI43542 (to M.L.D.), P20Y01696 (to M.L.D.), R01AI14647 (to J.J.L.), R56AI8553 (to J.J.L.), Z01CA067693 (to B.R.B.), Leukemia and Lymphoma Translational Research Grants R029-07 (to B.R.B.), Osaka University Immunology Frontier Research Center (to S.K.), and the The Leona M. and Harry B. Helmsley Charitable Trust (to J.J.L.).

(eBioscience) antibodies and washed with PBS (containing 0.05% BSA and 0.05% sodium azide). For intracellular staining, cells were fixed and permeabilized with the phospho-specific Foxp3 (PCH101) or Dlgh1. Then, the cells were incubated (30 min, 4 °C) with FITC-conjugated secondary antibodies (Jackson Immunoresearch). We analyzed samples in a FACS calibur machine (BD).

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