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J Immunol 2015; 194:4362-4370; Prepublished online 27 March 2015; doi: 10.4049/jimmunol.1402384
http://www.jimmunol.org/content/194/9/4362

Supplementary Material
http://www.jimmunol.org/content/suppl/2015/03/27/jimmunol.1402384.DCSupplemental

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Regulatory T Cells Require TCR Signaling for Their Suppressive Function

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Regulatory T cells (Tregs) are a subset of CD4+ T cells that maintain immune tolerance in part by their ability to inhibit the proliferation of conventional CD4+ T cells (Tconvs). The role of the TCR and the downstream signaling pathways required for this suppressive function of Tregs is not fully understood. To yield insight into how TCR-mediated signals influence Treg suppressive function, we assessed the ability of Tregs with altered TCR-mediated signaling capacity to inhibit Tconv proliferation. Mature Tregs deficient in Src homology 2 domain containing leukocyte protein of 76 kDa (SLP-76), an adaptor protein that nucleates the proximal signaling complex downstream of the TCR, were unable to inhibit Tconv proliferation, suggesting that TCR signaling is required for Treg suppressive function. Moreover, Tregs with defective phospholipase Cγ (PLCγ) activation due to a Y145F mutation of SLP-76 were also defective in their suppressive function. Conversely, enhancement of diacylglycerol-mediated signaling downstream of PLCγ by genetic ablation of a negative regulator of diacylglycerol kinaseζ increased the suppressive ability of Tregs. Because SLP-76 is also important for integrin activation and signaling, we tested the role of integrin activation in Treg-mediated suppression. Tregs lacking the adaptor proteins adhesion and degranulation promoting adapter protein or CT10 regulator of kinase/C2111 regulator of kinase–like, which are required for TCR-mediated integrin activation, inhibited Tconv proliferation to a similar extent as wild-type Tregs. Together, these data suggest that TCR-mediated PLCγ activation, but not integrin activation, is required for Tregs to inhibit Tconv proliferation.

has been suggested to act as an “IL-2 sink,” binding up IL-2 released during an immune response to prevent IL-2–mediated division and survival of nearby conventional CD4+ T cells (Tconv) (22–24).

More recently, it has been shown that the catalytic function of ZAP70 is not requisite for Treg suppressive function, as long as scaffolding aspects of the protein remain intact (25). This is somewhat surprising, because the ability of ZAP70 to phosphorylate LAT and SLP-76 has long been considered an absolute requirement for TCR-mediated signaling, and indeed, ZAP70 catalytic function was necessary for several other TCR-mediated functions assessed in the study (10, 25). Interestingly, downstream activation of integrins remained intact in cells harboring catalytically inactive ZAP70, and adhesion to ICAM-1 was instead dependent upon the phosphorylation of ZAP70 at sites unrelated to its catalytic function. In addition, mutation of these same phosphorylation sites abrogated Treg suppressive function (25). These findings suggest ZAP70 to have scaffolding properties that are both required and sufficient for integrin activation and Treg suppressor function. Because disruption of surface integrin expression has also been shown to impede Treg function (26, 27), it seems that TCR-driven suppression may occur at least partly through TCR-driven activation of integrins that facilitate interactions with APCs.

In this study, we assessed the role of TCR-mediated signal transduction in the suppressive function of Tregs. Our data suggest that TCR-mediated signals downstream of PLCγ1, but not those leading to integrin activation, are required for the suppressive function of Tregs.

Materials and Methods

Mice

C57BL/6 (B6), B6.SJL (CD45.1+), TCRβ/β double-knockout (DKO), and B6 Foxp3.GFP reporter mice were purchased from The Jackson Laboratory, B6.SJL Foxp3.GFP reporter mice were generated by crossing B6.SJL mice to B6 Foxp3.GFP reporter mice. UBC-CreERT2 was used for acute deletion has been described previously (28). SLP-76 conditional heterozygous (cHet), SLP-76 conditional knockout (cKO), and SLP-76 Y145F mice have been previously described (29, 30). Bcl-xL transgenic mice were a gift from Dr. Craig Thompson (31). SLP-76 cKO Bcl-xL mice were generated by crossing Bcl-xL transgenic mice to SLP-76 cKO mice. SLP-76 conditional Y145F (cY145F) mice were generated by crossing SLP Y145F mice to SLP-76 cKO mice. DGKζ knockout (KO) mice were a gift from Drs. Gary Koretsky and Xiao-ping Zhong (32). Adhesion and degranulation promoting adapter protein (ADAP) KO mice were graciously provided by Dr. Gary Koretsky (33).

Conditional DGKζ KO (cDGKζ KO) mice were created by inGenious Targeting Laboratory (Ronkonkoma, NY). In brief, DGKζ genomic DNA was replaced with a construct containing an intronic 5′ lox site flanking a neomycin cassette inserted between exons 6 and 7, and a 3′ lox site inserted between exons 11 and 12. Upon expression of cre recombinase, a truncated form of DGKζ is expressed that contains the first 187 aa of DGKζ followed by 8 unrelated amino acids expressed from fraa.mshhifted codons in exon 12, followed by a stop codon in exon 12. The expressed region of DGKζ contains one of the DAG-binding C1 domains and a portion of the second C1 domains, but no other functional or enzymatic components. For the inducible deletion of DGKζ, mice were subsequently bred to B6 mice that express Erα-Cre recombinase fusion protein, such that Cre activity was induced in cells exposed to tamoxifen. CT10 regulator of kinase (Crk)/Crk-like (Crkl) loxP-flanked (floxed) × CD4-cre mice (Crk/Crkl DKO) and CD4-cre–littermate controls on a mixed B6 × 129 genetic background were generated by breeding Crk floxed/floxed/Crkl floxed/floxed mice to CD4-cre mice. All mice were at least 6 wk of age at time of use and were housed in pathogen-free conditions and treated in strict compliance with Institutional Animal Care and Use Committee regulations of the University of Pennsylvania and the Children’s Hospital of Philadelphia.

Reagents and Abs

Tamoxifen was purchased from Sigma-Aldrich (St. Louis, MO), CFSE and LIVE/DEAD Fixable Dead Cell stain was purchased from Molecular Probes, Invitrogen (Carlsbad, CA). Abs were purchased from either BD PharMingen (San Diego, CA); anti-CD4 (RM4-5), anti-CD25 (PC61), anti-CD3 (2C11), and anti-CD28 (37.51); Biolegend (San Diego, CA); anti-CD45.2 (104), anti-CD45.1 (A20), and anti-CD4 (GK1.5); or eBioScience (San Diego, CA); anti-Foxp3 (FJK-16s), anti-CD69 (HI.2F3), anti-glucocorticoid-induced TNFR family–related gene (GITR) (DTA-1), and anti-CTLA-4 (UC10-4B9).

Flow cytometry, cell sorting, and data analysis

For flow-cytometric analyses, cells were stained with Abs against surface Ags at 4°C for 20 min in PBS. Intracellular Foxp3 staining was performed with the Foxp3 Staining Set (eBioscience) according to the manufacturer’s protocol. Flow cytometry was performed with an LSR II or FACSCount (BD Biosciences). For cell sorting, T cells were purified with either CD4 or CD90.2 magnetic beads using MACS columns (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol before cell-surface staining. FACS was performed with a FACS Aria cell sorter (BD Biosciences). FACS-sorted populations were typically 95–99% purity. Data were analyzed with FlowJo software (TreeStar, Ashland, OR). Cell division data from Treg CFSE dilution profiles, gated on live CD4+Foxp3+ cells, were transformed into “Division Index” data using FlowJo’s “Proliferation” function. The “Division Index” measures the average number of division per cell. Suppressive ability of Tregs was normalized for each assay by calculating the “percent suppression” at each Treg dilution. Because baseline Tconv division (no cocultured Tregs, 0% suppression) can vary between experimental repeats, this calculation allowed for comparison of multiple assays. The division index of Tclosvuls cultured at a particular Tconv/Treg ratio was divided by baseline Tconv division for the same assay. This fraction was multiplied by 100 and subtracted from 100 to achieve the “% Suppression of Tconv Division” for the particular Treg genotype across all experimental repeats.

Tamoxifen treatment

Tamoxifen was resuspended in ethanol (1 g/ml), diluted in corn oil to a final concentration of 20 mg/ml, and heated on a 37°C cell shaker until dissolved. Mice were weighed on the first day of tamoxifen administration and were treated with 200 μg tamoxifen per gram body weight by oral gavage. Tamoxifen administration was repeated each day for 5 consecutive days.

Western blot analysis

YFP+CD4+ T cells were sorted from tamoxifen-treated T2-Cre+ wild-type (WT) or cDGKζ KO mice (with a YFP-based Cre reporter) by flow cytometry. The cells were then lysed in 1% Igepal in TBS with protease/phosphatase inhibitors (protease inhibitor mixture solution [Roche, Sigma-Aldrich], 1 mM sodium orthovanadate, 50 mM sodium fluoride, 50 mM sodium pyrophosphate, 0.2 mM dithiothreitol, and 1 mM benzamidine), and the proteins were resolved by SDS-PAGE (Bio-Rad Laboratories, Hercules, CA). Total DGKζ and β-actin were analyzed by Western blot. The Abs against β-actin and DGKζ were obtained from Santa Cruz Biotechnology (Dallas, TX).

Treg suppression assays

MACs-enriched CD90.2+ or CD4+ T cells were FACS-sorted for CD4+ CD25+ Tregs (CD45.2+) or were FACS-sorted for CD4+Foxp3+ Tclosvuls (CD45.1+) from WT B6.SJL Foxp3.GFP reporter mice. The Tclosvuls were CFSE-labeled and cultured at various ratios with Tregs in the presence of irradiated T cell–depleted CD45.2+ feeder cells and soluble anti-CD3 (0.5–1 μg/ml). CFSE labeling was performed by resuspending cells with PBS containing CFSE (5 mM) at 37°C followed by continuous shaking for 9 min. The reaction was then immediately quenched with 100% PBS, and the cells were washed before culture. CFSE dilution of Tclosvuls (CD4+ CD45.1+) was assessed by flow cytometry after 4 d in culture. For in vivo suppression assays, T cell–deficient (TCRβ/βDKO) mice were adoptively transferred with 7.5 × 105 Tclosvuls (CD4+CD25+ GITR+ CD44+) from B6.SJL (CD45.1+) mice with or without 2.5 × 105 Tregs (CD4+CD25+GITR+) or B6 or Y145F KI mice (CD45.2+). Seven days later, peripheral lymph nodes were harvested and the absolute number of CD45.1+ Tclosvuls was determined by flow cytometry.

Generation of mixed bone marrow chimeras

Donor bone marrow (BM) was depleted of T cells by CD4 and CD8 magnetic bead depletion (Miltenyi). T cell–depleted BM from CD45.1+ CD45.2+ WT donor (competitor) mice were mixed at a 1:1 ratio with CD45.2+ donor BM (WT or DGKζ KO), and a total of 3–4 × 106 BM cells
were injected i.v. into lethally irradiated (11 Gy) CD45.1+ recipient mice. Splenic Tregs were FACS-sorted from mixed BM chimeric mice 9–11 wk after BM transplant.

Statistical analyses
All values were graphed and analyzed for statistical significance using Prism software. Paired or unpaired two-tailed Student t test or the Mann–Whitney U test was used to calculate each p value as indicated in the figure legends. The p values < 0.05 were considered statistically significant.

Results
Tregs require TCR signaling through SLP-76 to mediate suppressive function
To test the role of TCR signaling in Treg function, we assessed the in vitro suppressive function of Tregs lacking SLP-76. SLP-76–deficient T cells exhibit almost no measurable activation of signal transduction pathways emanating from the TCR (34). Because SLP-76 is necessary for T cell development, we used a previously described Cre-lox conditional deletion strategy (SLP-76 cKO) to delete a floxed copy of SLP-76 in mature mouse Tregs using a tamoxifen-inducible system with a YFP-based Cre reporter (30). SLP-76 cKO and SLP-76 cHet littermates (SLP-76 cHet mice) were treated with tamoxifen for 5 d, followed by a 7-d rest period (Supplemental Fig. 1A), after which residual SLP-76 protein can no longer be detected in T cells (30). TCR-mediated CD69 up-regulation was seen on peripheral blood YFP+CD4+ T cells taken from SLP-76 cHet, but not SLP-76 cKO mice, suggesting that TCR signaling was indeed ablated in CD4+ T cells from SLP-76 cKO mice (Supplemental Fig. 1B). To assess the suppressive ability of mature Tregs lacking SLP-76, we sorted splenic YFP+CD4+CD25+ Tregs from tamoxifen-treated SLP-76 cHet, SLP-76 cKO, or SLP-76 cKO/Bcl-xL mice for 4 d in the presence of irradiated T cell–depleted splenocytes and anti-CD3. Representative CFSE plots from cultures lacking SLP-76, we sorted splenic YFP+CD25+CD4+ T cells from either SLP-76 cKO or SLP-76 cHet mice by flow cytometry. FACS-sorted Tregs were cocultured with CFSE-labeled CD4+ Tconvs and irradiated splenocytes in the presence of anti-CD3 stimulation. After 4 d of coculture, SLP-76 cHet Tregs, but not SLP-76 cKO Tregs, suppressed anti-CD3–mediated division of Tconvs, indicating that SLP-76 is required for Treg-mediated inhibition of Tconv proliferation (Fig. 1A and 1B).

Because SLP-76 cKO naive and effector T cells display a survival defect (35, 36), we tested whether a lack of fitness was responsible for the functional defect observed in SLP-76 cKO Tregs. The number of Tregs recovered from the suppression assays was significantly decreased in cultures with SLP-76 cKO compared with SLP-76 cHet Tregs (Fig. 1C). Moreover, the proportion of LIVE/DEAD stain+ SLP-76 cKO Tregs was significantly elevated compared with SLP-76 cHet Tregs (Fig. 1D). To enhance the survival of SLP-76 cKO Tregs, we bred SLP-76 cKO mice to mice transgenically expressing the prosurvival protein Bcl-xL in all T cells (31). Although forced Bcl-xL expression increased the in vitro survival of SLP-76 cKO Tregs (Fig. 1C and 1D), the suppressive function of SLP-76 cKO Bcl-xL Tregs was similar to SLP-76 cKO Tregs, which was significantly diminished compared with SLP-76 cHet Tregs (Fig. 1A, 1B, and 1E).

It was recently shown that the acute deletion of the TCR leads to various phenotypic changes in the effector/differentiation state of Tregs in vivo (37, 38). To test the impact of SLP-76 ablation on the phenotype of Tregs used in our study, we measured the expression of various costimulatory molecules and effector/differentiation markers on SLP-76 cKO Tregs. The expression of CD62L, GITR, CTLA4, CD25, ICOS, CD39, CD103, and CXCR3 were similar between SLP-76 cHet and cKO Tregs, whereas, of the markers examined, CD69 and KLRG1 were decreased in SLP-76 cKO Tregs (Fig. 1A, 1B, and 1E). Representative CFSE plots from cultures lacking Tregs or containing a 1:1, 2:1, or 4:1 Tconv/Treg ratio are shown, and the division index of Tconv/Treg ratios is shown. (C) The absolute number of live and (D) the division index of Tconvs at various Tconv/Treg ratios is shown. (C) The absolute number of live and (D) the division index of Tconvs at various Tconv/Treg ratios is shown. (E) The absolute number of live and (D) the division index of Tconv/Treg ratios is shown. (E) The absolute number of live and (D) the division index of Tconv/Treg ratios is shown. (E) The absolute number of live and (D) the division index of Tconv/Treg ratios is shown.
markers. Moreover, compared with SLP-76+/+ controls, the loss of one allele of SLP-76 (SLP-76 cHet) was sufficient to cause Tregs to variable extents display reduced expression of GITR, CTLA4, ICOS, KLRG1, CXCR3, and CD103 (Supplemental Fig. 2). Of the markers tested, a reduction in ICOS and KLRG1 was most consistently observed. This suggests that even partial attenuation of TCR signaling caused by the acute loss of one allele of SLP-76 can impact effector/differentiation markers on Tregs.

**Y145 of SLP-76 is required for optimal suppressive function of Tregs**

TCR-induced activation of PLCγ1 is dependent on the phosphorylation of SLP-76 at Y145. To more selectively investigate the role of TCR-mediated PLCγ1 activation in Treg function, we used mice expressing a Y→F mutation at Y145 of SLP-76 (SLP-76 Y145F), which exhibit greatly diminished TCR-mediated activation of PLCγ1 (29). Similar to SLP-76 cKO Tregs, SLP-76 Y145F Tregs were significantly impaired in their ability to suppress Tconv division (Fig. 2A and 2B). The viability of SLP-76 Y145F Tregs was similar to that of WT Tregs (Fig. 2C), suggesting that the defect in suppression was not due to decreased number of Tregs in the cultures. SLP-76 Y145F Tregs displayed some suppressive capacity at Treg/Tconv ratios ≥2:1, suggesting that they were slightly more functional than SLP-76 cKO Tregs (data not shown).

We next tested the in vivo function of SLP-76 Y145F Tregs. T cell–deficient mice were adoptively transferred with Tconvs with or without Tregs from WT or SLP-76 Y145F mice, and 7 d later we assessed the number of T cells present in peripheral lymph nodes. We found that the absolute number of Tconvs was significantly decreased in mice receiving WT Tregs compared with no Tregs (Fig. 2D). In contrast, the absolute number of Tconvs was not significantly different between mice receiving SLP-76 Y145F Tregs and no Tregs (Fig. 2D). Thus, SLP-76 Y145F Tregs also display functional defects in suppressing Tconv proliferation in vivo.

The SLP-76 Y145F mutation alters T cell selection in the thymus (29), which could contribute to the defective Treg function that was observed. Thus, we additionally tested the function of Tregs that express a floxed WT copy of SLP-76 in addition to SLP-76 Y145F (SLP-76 cY145F mice), such that a WT SLP-76 copy was present during T cell development but could be inducibly deleted in the periphery through a tamoxifen-inducible Cre. Using a YFP-based cre reporter to detect cells with SLP-76 deletion, we FACS-sorted YFP+CD4+Foxp3+ T cells from SLP-76 cY145F mice and SLP-76 cHet mice that had been treated with tamoxifen for 5 d and rested for 7 d. Similar to SLP-76 Y145F Tregs, SLP-76 cY145F Tregs also exhibited an attenuated ability to suppress Tconv division (Fig. 2E). Together, these results demonstrate that phosphorylation of SLP-76 is critical for optimal Treg suppressive function and suggest that PLCγ1 is an important downstream mediator of this process.

**Enhancement of DAG signals increases the suppressive function of Tregs**

One major outcome of PLCγ1 activation is the generation of the potent second messenger, DAG. To determine whether the sele-
tive enhancement of DAG signaling downstream of PLCγ1 aug-
ments the suppressive capacity of Tregs, we examined the func-
tion of Tregs from mice lacking the DAG-metabolizing kinase
DGKζ, which exhibit prolonged DAG-mediated signaling down-
stream of PLCγ1 activation (32, 39, 40). To exclude cell-extrinsic
effects that could be potentially brought out by germline DGKζ
deficiency, we generated mixed BM chimeric mice (CD45.1+/WT
host) with WT BM (CD45.1+/CD45.2+) mixed at a 1:1 ratio with
either WT or DGKζ KO BM (CD45.2+). After reconstitution of
the T cell compartment (9–11 wk after BM transplant), splenic
Tregs (CD25+CD4+) or WT competitor (CD45.1+/CD45.2+) and
of WT or DGKζ KO experimental (CD45.2+) origin were sorted
by flow cytometry and their inhibitory activity against Tconv
proliferation was tested. As expected, the suppressive ability was
similar between WT competitor and WT experimental Treg
populations sorted from WT/WT mixed BM chimeras (Fig. 3A). In
contrast, Tregs of DGKζ KO BM origin exhibited significantly
increased suppressive capacity compared Tregs of WT competitor
origin from the same mouse (Fig. 3A). Because thymic selection
is impacted by DGKζ deficiency (41), we generated mice with two
floxed DGKζ alleles that were additionally crossed to a tamoxifen-
inducible Cre and YFP-based Cre reporter (cDGKζ KO mice).
Treatment of these mice with tamoxifen led to efficient deletion of
DGKζ in T cells (Fig. 3B). Similar to DGKζ KO Tregs, FACS-
sorted Tregs (YFP+/CD25+/CD4+) from DGKζ KO Tregs exhibited
an enhanced ability to limit Tconv proliferation in vitro (Fig. 3C
and 3D). Especially striking was that the inhibition of Tconv di-
vision was still apparent by cDGKζ KO Tregs at a 1:8 and 1:16
Treg/Tconv ratio, dilutions at which WT Tregs are consistently
unable to effectively mediate suppression (Fig. 3C and 3D). To-
gether, these data suggest that DAG pathways play a positive role
in the suppressive function of Tregs.

To test the impact of DGKζ ablation on the phenotype of Tregs
used in our study, we measured the expression of various co-
stimulatory molecules and effector/differentiation markers on
DGKζ cKO Tregs. The expression of CD62L, GITR, CTLA4,
CD25, ICOS, CD103, KLRG1, CD69, and CXCR3 were
similar between DGKζ cKO and DGKζ+/+ Tregs (Supplemental
Fig. 3). These data suggest that although the loss of TCR signaling
by SLP-76 deficiency impacts the expression of Treg effector/
differentiation markers, the specific increase in DAG-mediated signaling does not alter these markers on Tregs.

**Tregs that lack proteins involved in TCR-mediated inside-out integrin activation display intact suppressive function**

Signals from the TCR induce a conformational change whereby integrins achieve a high-affinity state in a process known as “inside-out” signaling (42). Because integrin activation is important for Treg function (26, 27), we assessed the requirement of TCR-mediated inside-out integrin signaling in the ability of Tregs to inhibit Tconv division. We first tested the role of ADAP in Treg function, because ADAP is required for TCR-driven inside-out integrin signaling and is recruited to the immunological synapse by phosphorylated SLP-76 (43). ADAP KO mice exhibited normal Treg frequencies and numbers in both the thymus and spleen (Fig. 4A). Moreover, the expression of Treg surface molecules associated with function, including CTLA-4, CD25, and GITR, appeared grossly normal upon flow cytometric analysis (Fig. 4). The function of FACS-sorted splenic ADAP KO and WT littermate control Tregs were compared. Surprisingly, in comparison with WT Tregs, ADAP KO Tregs exhibited normal ability to suppress Tconv division (Fig. 4C).

As a complementary approach, we additionally tested the role of the Crk family of proteins in Treg function. Crk and CrkL proteins associate with the guanine nucleotide exchange factor C3G to promote activation of the GTPase Rap1 downstream of TCR signaling (44–46). Because Rap1 activation is critical for TCR-mediated inside-out activation of integrins such as LFA-1, Crk/CrkL DKO CD4+ T cells exhibit a partial defect in cellular adhesion upon TCR-induced activation (J. Burkhardt and Y. Huang, unpublished observations). Flow-cytometric analyses revealed a mild increase in Treg frequencies within the Crk/CrkL DKO thymus, but not in the periphery (Fig. 5A). As observed for ADAP KO Tregs, the expression of CTLA-4, CD25, and GITR were similar between Crk/CrkL DKO Tregs and WT littermate control Tregs (Fig. 5B). Functional assessment of splenic Tregs (CD25+ CD4+) revealed Crk/CrkL DKO Tregs suppress Tconv division at least as well as their WT counterparts (Fig. 5C). Together, these findings suggest that ADAP- and Crk/CrkL-dependent integrin activation may not be absolutely required for the suppressive function of Tregs.

**Discussion**

The requirement of TCR stimulation and the downstream signaling pathways in the suppressive function of Tregs has been somewhat controversial. In this study, using Tregs with genetically altered TCR signaling capacities, we probed the requirement of TCR-mediated signaling in the ability of Tregs to inhibit Tconv division. Our data demonstrate that phosphorylation of SLP-76 Y145 is critical for Treg suppressive function, suggesting that PLCγ1 activation is important for Treg function. This notion was further supported by the augmented suppressive function observed by DGKγ KO Tregs, which exhibit selectively enhanced TCR-mediated DAG production. Surprisingly, however, Tregs that lack molecules involved in TCR-mediated integrin activation displayed intact suppressive function. Together, these data suggest that TCR-mediated PLCγ activation, but not ADAP- and Crk/CrkL-dependent integrin activation, is required for Tregs to inhibit Tconv proliferation in vitro.

**FIGURE 4.** ADAP KO mice harbor Tregs with normal development and suppressive function. (A) Representative flow-cytometric plots and (B) the fraction of Tregs (out of CD4+ T cells) from the thymus and spleen of WT and ADAP KO Tregs are shown. (C) The expression of CTLA-4, CD25, GITR, and CD44 on Tregs from WT and ADAP KO Tregs is shown. (D) CFSE-labeled splenic CD4+Foxp3+ Tconvs were cultured with either WT or ADAP KO Tregs for 4 d in the presence of irradiated T cell–depleted splenocytes and anti-CD3. Representative CFSE plots from 4-d cultures lacking Tregs or containing a 1:2 or 1:1 Tconv/Treg ratio of either WT or ADAP KO Tregs are shown (top panels). A plot of the division index of Tconvs at various Treg/Tconv ratios is shown (bottom panel). (E) The division index of Tconvs at Tconv/Treg ratios of 1:2 and 1:1 were normalized to the baseline Tconv division (no Tregs) for each experiment and represented as mean percentage suppression ± SEM (n = 3 mice total).
A requirement for continuous TCR expression in the homeostasis and function of Tregs was recently demonstrated (37, 38). Our data are consistent with these findings and provide insight as to the signaling pathways required for these processes. Although Tregs that lack DGKζ maintained a cell-surface phenotype similar to that of WT mice, our data demonstrate that SLP-76 is required to maintain CTLA4, KLRG1, CD103, CD69, and CXCR3 expression, suggesting that continued expression of these cell-surface markers requires SLP-76–mediated signals. These changes in cell-surface phenotype between SLP-76 cKO are similar to those observed in mice lacking TCR signaling in mature Tregs. However, SLP-76 cKO Tregs were also skewed toward a “naive” CD62Llo phenotype that differed from mice lacking TCR expression in Tregs (37). These differences may represent SLP-76 requirements in non–TCR-mediated signaling or may simply be the result of the different approaches to timed deletion: Treg specific (37), inflammatory (38), or ubiquitous (our data). Direct comparison using identical Cre deleter lines will be needed to differentiate among these possibilities.

Our finding that TCR-mediated PLCγ1 activation and DAG production are important for Treg function is consistent with a previous report showing that ablation of PLCγ1 in the T cell lineage results in a paucity of Tregs with reduced suppressive function (47). However, the underlying mechanism of DAG involvement in the suppressive ability of Tregs is unclear. DAG typically activates multiple molecules downstream of the TCR stimulus, including Ras-GRP and PKCθ, leading to induction of the MAPK and NF-κB signaling pathways, respectively. It is possible that transcriptional alterations driven by these pathways promote upregulation of surface molecules or soluble factors associated with suppression. Little has been published, however, about how Treg-intrinsic transcription is altered after TCR stimulation to achieve active suppression. In addition to activation of these signal transduction pathways, DAG has been reported to activate the guanine-nucleotide exchange factor CALDAG-GEF, which, along with C3G, is required for efficient Rap1 activation and inside-out signaling to integrins such as LFA-1 (48, 49). Indeed, T cells that lack PLCγ1, and thus DAG production, fail to adhere to ICAM-coated surfaces (50). Because Treg-intrinsic integrin activation is thought to facilitate interactions between Tregs and DCs that are required for suppression (26, 27), we reasoned that it was possible that DAG-mediated signaling could contribute to Treg function by promoting integrin activation.

To assess the importance of TCR-induced inside-out integrin activation during Treg-mediated suppression, we evaluated the suppressive abilities of Tregs that lack molecules critical to this process. Surprisingly, Tregs deficient in either ADAP or Crk/CrkL proteins exhibited no appreciable functional defects in vitro. Because Crk/CrkL family members drive efficient TCR-driven Rap1 activation, and ADAP is necessary for TCR-driven plasma membrane localization of Rap1 (43–46), our results suggest that TCR-driven inside-out signaling leading to integrin activation may not be required for the suppressive function of Tregs in vitro. These findings are in contrast with recent evidence suggesting TCR-driven integrin activation is sufficient to induce suppression in vitro (25). In addition, other reports have shown that disruption of surface LFA-1 molecules renders Tregs unable to suppress Tconv division (26, 27). Tregs have been shown to outcompete Tconvs to form aggregates around DCs, and in doing so, they downregulate CD80/CD86 costimulatory molecules on the surface of DCs to prevent Tconvs from being fully stimulated (26). The integrin LFA-1 was shown to be required for this process, because

**FIGURE 5.** Crk/CrkL DKO Tregs do not show decreased suppressive function compared with WT Tregs. (A) Representative flow-cytometric plots and (B) the fraction of Tregs (out of CD4+ T cells) from the thymus and spleen of WT and Crk/CrkL DKO Tregs are shown. (C) The expression of CTLA-4, CD25, GITR, and CD44 on Tregs from WT and Crk/CrkL DKO Tregs is shown. (D) CFSE-labeled splenic CD4+Foxp3+ Tconvs were cultured with either WT or Crk/CrkL DKO Tregs for 4 d in the presence of irradiated T cell–depleted splenocytes and anti-CD3. Representative CFSE plots from 4-d cultures lacking Tregs or containing a 1:2 or 1:1 Tconv/Treg ratio of either WT or Crk/CrkL DKO Tregs are shown (top panels). A plot of the division index of Tconvs at various Treg/Tconv ratios are shown (bottom panel). (E) The division index of Tconvs at Tconv/Treg ratios of 1:2 and 1:1 were normalized to the baseline Tconv division (no Tregs) for each experiment and represented as mean percentage suppression ± SEM (n = 4 mice total).
LFA-1–deficient Tregs were unable to form these aggregates and unable to suppress Tconv proliferation (26, 27). Because ADAP and Crk proteins are required for optimal TCR-driven inside-out activation of LFA-1, it is peculiar that ADAP or Crk/Crkl KO Tregs have no defect in in vitro suppressive function. It is possible that compensatory pathways can induce integrin activation in Tregs apart from those directly mediated by the TCR. For example, CTLA-4 engagement can mediate inside-out signaling to integrins as well, and it has been found to augment TCR-induced LFA-1 activation (51, 52). Because Tregs constitutively express CTLA-4 and require this molecule to mediate suppression (53, 54), perhaps CTLA-4 is sufficient to induce cellular adhesion in the ADAP KO and/or Crk/Crkl DKO setting. Indeed, C3G can promote Rap1-driven integrin activation downstream of CTLA-4 engagement in a manner that may not involve ADAP (55). Alternatively, the defect in integrin activation by ADAP or Crk/Crkl deficiency may not be complete. In response to TCR stimulation, ∼50% of integrin activation is lost in Crk/Crkl KO T cells (Y. Huang and J. Burkhardt, unpublished results), a level that may still be sufficient for Tregs to interact with DCs. However, alterations of peptide/MHC quantity or affinity of TCR to peptide/MHC complexes could potentially bring out defects in suppression mediated by Tregs that lack ADAP or Crk/Crkl.

In one of the aforementioned studies, Tregs were able to exert suppression even when the catalytic function of Zap70 was inhibited. This was proposed to be because Zap70 phosphorylation sites necessary for scaffolding interactions between Zap70 and Crk proteins remained intact and could mediate inside-out signaling to integrins (25). In support of this notion, mutation of these phosphorylation sites abrogated Treg suppressive ability; although it should be noted that other aspects of TCR signal transduction, including PLCγ1 activation, were also disrupted in the absence of these phosphorylation sites (25, 56). Because our data suggest that Crk proteins are not required for Treg suppressive function, it is possible that TCR-driven integrin activation represents a process that is sufficient to induce suppression but is not required under all circumstances. Furthermore, the ability of phosphorylated Zap70 to promote integrin activation by serving as a scaffold might have been augmented by the overexpression of Zap70 in these studies (25). It is also possible that the level of inhibitor used to abrogate the catalytic function of Zap70 was sufficient to prevent TCR-driven responses in Tconvs, but not potent enough to fully prevent Treg suppressive ability because Tregs have a much lower TCR activation threshold than Tconvs (57). It is clear that integrins play a role in Treg function, but further work is required to dissect the signaling pathway that supports this function.

In summary, using T cells with targeted quantitative and qualitative signaling deficits, we have demonstrated the importance of TCR-mediated PLCγ activation and downstream DAG signaling for the suppressive function of Tregs. Although integrins are important for the suppressive function of Tregs, TCR-mediated inside-out signaling through ADAP or Crk/Crkl to activate integrins was not required by Tregs to achieve suppression. Further studies will be required to reveal how these signaling pathways drive Treg-mediated suppression, and will provide additional insight into the mechanisms by which Tregs suppress Tconv division.

Acknowledgments

We thank the laboratories of Drs. Gary Koretzky, Avinash Bhandoola, Edward Behrens, and Paula Oliver for thoughtful discussions and advice. We thank Drs. Gary Koretzky, Xiao-ping Zhong, and Craig Thompson for providing valuable reagents.

Disclosures

The authors have no financial conflicts of interest.

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