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Cutting Edge: IL-2 Signals Determine the Degree of TCR Signaling Necessary To Support Regulatory T Cell Proliferation In Vivo

Tao Zou,*† Atsushi Satake,*† Evann Corbo-Rodgers,‡ Amanda M. Schmidt,* Michael A. Farrar,‡ Jonathan S. Maltzman,†,1 and Taku Kambayashi*,†

To ensure immune tolerance, regulatory T cell (Treg) numbers must be maintained by cell division. This process has been thought to be strictly dependent on the Treg TCR interacting with MHC class II. In this study, we report that Treg division does not absolutely require cell-autonomous TCR signaling in vivo, depending on the degree of IL-2–mediated stimulation provided. At steady state IL-2 levels, Tregs require cell-autonomous TCR signaling to divide. However, when given exogenous IL-2 or when STAT5 is selectively activated in Tregs, Treg division can occur independently of MHC class II and TCR signaling. Thus, depending on the amount of IL-2R stimulation, a wide range of TCR signals supports Treg division, which may contribute to preservation of a diverse repertoire of Treg TCR specificities. These findings also have therapeutic implications, as TCR signaling by Tregs may not be required when using IL-2 to increase Treg numbers for treatment of inflammatory disorders. The Journal of Immunology, 2012, 189: 000–000.

S uppression of immune responses against both self and foreign Ags by CD4+Foxp3+ regulatory T cells (Tregs) is an essential mechanism of self-tolerance (1). To maintain immune tolerance, a sufficient number of Tregs must be maintained in the periphery of the host, in part by their continuous cell division in the steady state (2). IL-2 is critical for the proliferation and maintenance of Tregs, as the acute neutralization of IL-2 in adult mice disrupts Treg homeostasis (3, 4), whereas IL-2R agonists augment Treg division and proliferation (5). Tregs do not produce IL-2 themselves, but constitutively express CD25, the high-affinity α subunit of the IL-2R, which allows them to respond to low levels of IL-2 produced by conventional CD4+ T cells (Tconvs) (3, 4).

In addition to IL-2, current dogma asserts that Treg division is absolutely dependent on TCR signaling by Tregs, because adoptively transferred Tregs fail to divide in MHC class II (MHCII) knockout (KO) hosts and the deletion of TCR signaling proteins in T cells results in decreased Treg division and survival (6–9). However, in each of the experimental approaches used to date, the loss of TCR signaling is not confined to Tregs, but also occurs in Tconvs, which are the major source of IL-2 that supports Treg division. Thus, the failure of Tregs to divide in previous studies may result from the lack of IL-2 production by Tconvs rather than a cell-autonomous requirement for TCR stimulation in Tregs. We propose this notion based on in vitro studies demonstrating that Tregs do not need MHCII to divide if exogenous IL-2 and contact with MHCII KO DCs are provided (10, 11).

In this study, we examined the necessity of cell-autonomous TCR signaling in Tregs for their division in vivo. Through the complementary approaches of restricting the peptide repertoire presented on MHCII and disrupting TCR signal transduction specifically in Tregs, we find that Treg division is partially dependent on a diverse peptide repertoire and is completely dependent on TCR signaling at steady state IL-2 levels. However, exogenous IL-2R stimulation induced by IL-2 immune complexes (ICs) or selective activation of the IL-2–induced STAT5 pathway in Tregs partially restored Treg division in the absence of MHCII or TCR signaling, indicating that TCR signals are not absolutely necessary in every setting. To our knowledge, these data demonstrate for the first time that although steady state Treg division requires TCR signaling, targeted IL-2R stimulation can partially overcome...
this requirement to promote Treg division in vivo. We propose that the combination of TCR and IL-2R/STAT5 signaling together determines the extent of cell division individual Tregs undergo and ultimately controls the homeostasis of this population.

Materials and Methods

**Mice**

Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76) conditional KO (cKO), SLP-76flox/− heterozygous (Het), and SLP-76flox/+ conditional heterozygous (cHet) mice were generated, as described (12). Constitutively active STAT5b (STAT5b-Ca) transgenic mice were generated, as described (13), and were bred with SLP-76 cKO and SLP-76 cHet mice. H-2D^Mox KO mice were a gift of Dr. T. Laufer (University of Pennsylvania, Philadelphia, PA). All other mice were purchased from The Jackson Laboratory or from Taconic Farms. Mice were housed in pathogen-free conditions and treated in strict compliance with Institutional Animal Care and Use Committee regulations of the University of Pennsylvania.

**Flow cytometry, cell sorting, and data analysis**

Abs for flow cytometry were purchased from BD Pharmingen (San Diego, CA), BioXcell (San Diego, CA), or Molecular Probes, Invitrogen (Carlsbad, CA). Flow cytometry and FACS were performed with an LSR II, FACSCanto, or a FACSAria cell sorter (BD Biosciences). Data were analyzed with FlowJo software (Tree Star) and Prism (GraphPad).

**Mixed bone marrow chimeras and T amoxifen administration**

T cell-depleted bone marrow (BM) from CD90.1<sup>+</sup>CD45.2<sup>+</sup> wild-type (WT) donor mice was mixed at a 1:1 ratio with CD90.1<sup>+</sup>CD45.2<sup>+</sup> SLP-76<sup>flox</sup> HET, SLP-76<sup>flox</sup> Het, or SLP-76<sup>flox</sup> KO BM, and a total of 3–4 × 10<sup>6</sup> BM cells was injected i.v. into lethally irradiated (9.5 Gy) CD45<sup>1</sup>−1 mouse recipients. Mice were used after 8–10 wk. For deletion of the floxed SLP-76 allele, mice were injected for consecutive 3 d, and mice were sacrificed for analysis of CFSE-labeled WT and H-2D<sup>DM</sup> KO cells. To test whether a diverse peptide repertoire presented on MHCII was needed for Treg division, we adoptively transferred MHCII-dependent manner to support Treg division. We used mice in which the TCR signaling molecule Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76) could be inducibly deleted by a Tamoxifen-inducible cre recombinase (15). A YFP reporter was used to mark cells with a history of cre-mediated recombination and thus deletion of the floxed SLP-76 allele. To preserve sufficient numbers of Tconv to provide IL-2 but still delete SLP-76 from a fraction of Tregs, we employed a mixed BM chimera approach in which WT donor BM was mixed with BM from a SLP-76<sup>flox</sup>−/− (cKO), SLP-76<sup>flox</sup>−/− (Het), or SLP-76<sup>flox</sup>+/− (cHet) donor and transplanted into irradiated WT recipients. After 8–10 wk, Tamoxifen was administered to all BM chimeras to delete SLP-76 from the SLP-76<sup>flox</sup> BM-derived T cells and administered BrdU to assess the extent of Treg division. In each of the experiments, cell division was analyzed by BrdU incorporation. Results and Discussion

**TCR signaling is required for Treg division in the steady state.**

To test whether a diverse peptide repertoire present on MHCII was needed for Treg division, we adoptively transferred CFSE-labeled WT and H-2D<sup>Mox</sup> KO CD4<sup>+</sup> T cells into H-2D<sup>Mox</sup> KO recipients. H-2D<sup>Mox</sup> KO mice are defective in their ability to exchange peptides from maturing MHCII molecules (14), virtually making all surface MHCII molecules loaded with a single peptide, CLIP. We reasoned that WT Tregs that have been selected on a diverse array of peptides would fail to encounter their cognate Ag when adoptively transferred into H-2D<sup>Mox</sup> KO hosts. In contrast, Tregs that have developed in H-2D<sup>Mox</sup> KO mice would have TCR specificities that react most suitably with CLIP. One month after adoptive transfer, we found that the division (Fig. 1A, 1B) and absolute number (Supplemental Fig. 1A) of WT Tregs were significantly diminished compared with H-2D<sup>Mox</sup> KO Tregs. Similarly, WT Tconv divided significantly less compared with H-2D<sup>Mox</sup> KO Tconv (Supplemental Fig. 1B). These data suggest that cell-autonomous TCR interactions with their selecting Ag/MHCII complexes are important for optimal Treg division. However, some Tregs can still divide without these TCR signals.

To investigate further the requirement of TCR signaling in Treg division, we devised a strategy to acutely and inducibly abrogate TCR signaling specifically in Tregs. This approach was needed to dissociate the TCR signaling capacity of Tregs from that of Tconvs, because Tconvs produce IL-2 in a TCR/MHCII-dependent manner to support Treg division. We used mice in which the TCR signaling molecule Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76) could be inducibly deleted by a Tamoxifen-inducible cre recombinase (15). A YFP reporter was used to mark cells with a history of cre-mediated recombination and thus deletion of the floxed SLP-76 allele. To preserve sufficient numbers of Tconvs to provide IL-2 but still delete SLP-76 from a fraction of Tregs, we employed a mixed BM chimera approach in which WT donor BM was mixed with BM from a SLP-76<sup>flox</sup>−/− (cKO), SLP-76<sup>flox</sup>−/− (Het), or SLP-76<sup>flox</sup>+/− (cHet) donor and transplanted into irradiated WT recipients. After 8–10 wk, Tamoxifen was administered to all BM chimeras to delete SLP-76 from the SLP-76<sup>flox</sup> BM-derived T cells and administered BrdU to assess the extent of Treg division. In each of the experiments, cell division was analyzed by BrdU incorporation. Results and Discussion

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Exogenous IL-2 can partially restore Treg division in the absence of MHCII or SLP-76–mediated TCR signaling

To test the role of MHCII in Treg division, CFSE-labeled T cells were adoptively transferred into either WT or MHCII KO recipients. Some of the recipient mice were also given IL-2 ICs (5) because CD4+ T cells cannot produce the IL-2 necessary for Treg division in MHCII KO mice. As expected, adoptively transferred Tregs divided in WT, but not in MHCII KO recipients (Fig. 2). However, with IL-2 IC administration, donor Tregs divided in MHCII KO hosts, indicating that IL-2 IC-induced Treg division can occur independently of MHCII-mediated TCR signaling (Fig. 2). Moreover, the frequency and absolute number of Tregs were increased in IL-2 IC-treated MHCII KO recipients, although this increase was reduced compared with IL-2 IC-treated WT mice (Supplemental Fig. 2A, 2B). Treg proliferation was comparable in IL-2 IC-treated MHCII KO recipients at 1 and 3 wk after adoptive transfer (Supplemental Fig. 2C), suggesting that IL-2 signaling in the absence of MHCII can sustain Treg division for extended periods of time. Furthermore, the proliferating Tregs maintained a diverse TCR Vβ repertoire in IL-2 IC-treated MHCII KO recipients (Supplemental Fig. 2D), suggesting that the proliferation of the Tregs was unlikely to represent an oligoclonal outgrowth of Tregs with selective TCRs.

We next examined whether IL-2 ICs would allow Tregs to proliferate in the absence of SLP-76–mediated TCR signaling. Tanomoxifen-treated WT/SLP-76 cHet and WT/SLP-76 cKO mixed BM chimeras were injected with PBS or IL-2 ICs at the start of a BrdU pulse. Whereas PBS-treated YFP+ SLP-76 cKO Tregs did not incorporate BrdU, IL-2 IC-treated YFP+ SLP-76 cKO Tregs incorporated BrdU at a rate similar to PBS-treated YFP+ SLP-76 cHet Tregs (Fig. 3). However, BrdU incorporation by IL-2 IC-treated YFP+ SLP-76 cKO Tregs was significantly less than that observed in both WT and donor Tregs within the same mice and YFP+ SLP-76 cHet Tregs from IL-2 IC-treated mice (Fig. 3). Together, these data suggest that IL-2 IC treatment partially restores the ability of Tregs to divide in the absence of MHCII- or SLP-76–mediated TCR signaling.

Isolated STAT5 activation is sufficient to partially restore Treg division in the absence of MHCII or TCR signaling in vivo

The IL-2R transmits its signals intracellularly through a JAK3-STAT5 pathway and two Shc-dependent pathways that activate either PI3K/Akt or Ras/MAPK signaling (4). To provide a mechanism for the ability of IL-2 ICs to promote TCR-independent Treg division, we tested the involvement of the STAT5 pathway, because transgenic expression of a constitutively active form of STAT5b (STAT5b-CA) is sufficient to induce thymic Treg differentiation and maintain Treg homeostasis in the absence of IL-2Rβ (13). We adoptively transferred CFSE-labeled WT and STAT5b-CA T cells into either WT or MHCII KO hosts to examine whether transgenic expression of STAT5b-CA was sufficient for Tregs to proliferate in the absence of MHCII. This approach also allowed us to test whether IL-2 signals were acting directly or indirectly on Tregs for their proliferation, as IL-2 IC treatment could be affecting Tregs indirectly through other IL-2–responsive cell types. Two weeks later, we found that although WT Tregs did not divide at all in MHCII KO hosts, proliferation of Tregs was partially restored in MHCII KO hosts when the adoptively transferred Tregs expressed STAT5b-CA (Fig. 4A, 4B).

We next examined whether STAT5b-CA expression would allow Tregs to divide in the absence of SLP-76–mediated TCR signaling. To this end, we intercrossed STAT5b-CA transgenic mice with SLP-76 cHet and SLP-76 cKO mice, treated them with Tamoxifen, adoptively transferred their T cells into WT hosts, and administered BrdU. Although SLP-76 cHet Tregs incorporated BrdU after a 7-d BrdU labeling period, almost no YFP+ SLP-76 cKO Tregs could be detected in recipient mice, precluding BrdU analysis on these cells (Fig. 4C). However, when the SLP-76 cKO Tregs expressed STAT5b-CA, YFP+ Tregs that incorporated BrdU were detected (Fig. 4C). Similar to the Tregs transferred into MHCII KO mice, the rescue of Treg survival and proliferation by expression of STAT5b-CA was partial, given that SLP-76 cHet Tregs incorporated significantly more BrdU than SLP-76 cKO Tregs expressing STAT5b-CA (Fig. 4D). Together, these data suggest that IL-2 promotes Treg proliferation by acting in a cell-autonomous manner and that IL-2–induced STAT5 signaling is sufficient to partially restore Treg division in the absence of TCR signaling. Thus, although TCR signals are important for Treg proliferation, they are not absolutely required for the division of Tregs.

Collectively, to our knowledge, the experiments presented in this study demonstrate for the first time that depending on the nature of the IL-2R stimulation that is provided, TCR/MHCII contacts are partially dispensable for Treg division. We propose that progressively stronger IL-2R stimulation is required to offset the loss of cell-autonomous TCR signals in Tregs to sustain the division and homeostasis of this population. In limiting amounts of IL-2, Tregs that encounter their cognate Ags most likely divide preferentially over Tregs bearing TCR specificities for rare Ags. However, in settings where IL-2
concentrations are high, such as during inflammation, even Tregs experiencing weak or nonspecific TCR interactions may be able to divide. This model offers a mechanism for maintenance of Tregs bearing TCRs specific for rare or sequestered self-Ags. The preservation of these Treg populations may allow for the retention of a diverse Treg TCR repertoire that is important for self-tolerance (16).

The TCR-independent Treg division induced by exogenous IL-2R stimulation has important implications for treatment of autoimmunity, systemic inflammatory diseases, and the maintenance of transplant tolerance. In these clinical conditions, an imbalance exists between the activity of effector Tconvs and that of Tregs (1). Although IL-2R stimulation may enhance the function of effector Tconvs, mouse models of autoimmunity and human studies suggest that IL-2 therapy may preferentially expand Treg populations and alter this imbalance in favor of immune tolerance (17, 18). IL-2 treatment increases Tregs and provides benefit in mouse models of graft-versus-host disease and in humans with chronic graft-versus-host disease (19, 20). Based on our data, we speculate that IL-2 may expand Tregs even when inhibitors of TCR signaling are concurrently provided. Although further studies are needed, our preliminary data suggest that IL-2 in combination with the TCR signaling inhibitor cyclosporine A inhibits Ag-specific Tconv proliferation while expanding Tregs (A. Satake, unpublished observations). As Tregs clearly have remarkable therapeutic potential to modulate human disease, further understanding of the mechanisms that govern their division and homeostasis will be crucial for properly harnessing the immunosuppressive functions of Tregs.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. The absolute number of Tregs recovered and the % divided Tconvs of WT origin are significantly less than that of DMα KO origin after adoptive transfer into DMα KO mice. CFSE and congenically labeled CD4⁺ T cells from WT and H-2DMα KO mice were mixed and injected i.v. into H-2DMα KO mice. (A) One month later, CD4⁺Foxp³⁺ Tregs of WT or H-2DMα KO donor origin was quantified and represented as mean ± SD of n = 3 mice per group. (B) CFSE dilution of donor WT or H-2DMa KO (left plot) CD4⁺Foxp³⁻ Tconvs was determined and represented as mean % CFSE diluted ± SD of n = 3 mice per group. One representative of 2 independent experiments is shown. * p<0.01, by ANOVA with Tukey’s post test.
Supplemental Figure 2. Exogenous IL-2 induces Tregs to proliferate and expand but still maintain a diverse TCR Vβ repertoire in the absence of MHCII in vivo. CFSE-labeled WT T cells were adoptively transferred into WT or MHCII KO mice and treated with IL-2 ICs. (A) One week later, the frequency of donor Tregs among total donor CD4+ T cells and (B) the total number of donor Tregs from WT and MHCII KO recipient mice were determined and represented as mean ± SEM of n = 7-8 mice/group from two independent experiments. (C) IL-2 ICs were injected into adoptively transferred WT and MHCII KO mice for 3 consecutive days at the beginning of every week for 1 or 3 weeks. BrdU was administered the last 5 days before harvest. BrdU incorporation by donor Tregs was determined 1 or 3 weeks post adoptive transfer and represented as mean ± SEM of n = 5-6 mice/group from two independent experiments. (D) T cells were
pooled from the WT or MHCII KO recipient mice and the fraction of donor Tregs expressing the indicated TCR Vβ was determined at 3 weeks post adoptive transfer. One representative of 2 independent experiments is shown. * p<0.05, ** p<0.01, and ns = not significant by unpaired, two-tailed Student’s t test or one-way ANOVA with Tukey’s post test.