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A 33D1^{+} DC/auto-reactive CD4^{+} T cell circuit maintains Il-2-dependent T_r cells in the spleen

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Abstract

Phenotypically and functionally diverse regulatory T cell (T_r cell) subsets populate lymphoid and non-lymphoid tissues where their maintenance and function are governed by unique homeostatic signals. Whereas T_r cells resident in non-lymphoid tissues depend on continual TCR signaling for their survival and function, phenotypically naïve T_r cells occupying secondary lymphoid organs (SLOs) are largely supported by paracrine Il-2 signaling. Crucially, the absence of either of these distinct T_r cell subsets results in pathogenic autoimmunity, underscoring their non-redundant roles in the preservation of self-tolerance. However, the cellular and molecular factors precipitating Il-2 release and subsequent maintenance of SLO-resident T_r cells are still poorly understood. Here we report that Il-2-dependent T_r cells in the spleen compete for a limiting supply of paracrine Il-2 generated by auto-reactive CD4^{+} T cells in response to MHCII-restricted auto-antigen activation by 33D1^{+} CD11b^{int} DCs. Manipulating this cellular circuit culminating in Il-2 production could have clinical benefits in settings where diminished T_r cell abundance is desired.

Introduction

The adaptive immune system provides protection and immunologic memory to a diverse array of foreign antigens. This must be achieved while remaining non-responsive to self-antigens, innocuous environmental antigens, and components of the commensal microbiota that inhabit mucosal surfaces. The generation and selection of αβT cells which fit these criteria occurs in the thymus where T cells somatically recombine a series of germ line encoded gene segments to generate a unique T cell receptor (TCR) that is then evaluated on its ability to bind to major histocompatibility complexes (positive selection) without recognizing MHC bearing self-peptides (negative selection). Cells which fail to meet these conditions are eliminated within the thymus. Despite the culling of non- or auto-reactive cells during T cell development, a smaller number of auto-reactive cells escapes negative selection and egress from the thymus where they can clonally expand after recognizing cognate self-antigen. Therefore, scarce auto-reactive T cells have the potential to cause

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Disclosures

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devastating autoimmunity if left unregulated. However, a second non-deletional mechanism of T cell development has evolved by which a portion of $CD4^+$ T cells bearing self-reactive TCRs survive negative selection and seed the periphery as regulatory cells. These regulatory T cells (T_r cells) express the master transcription factor Foxp3 and suppress aberrant auto-reactive T cell responses through a variety of mechanisms including sequestration of key T cell growth factors and metabolites, production of anti-inflammatory cytokines, and modulation of dendritic cell (DC) function (1, 2). The critical importance of T_r cells is best exemplified in the fatal multi-organ lymphoproliferative disease which develops in their absence due to non-functional or hypomorphic alleles of the *Foxp3* gene (3, 4).

Like phenotypically and functionally diverse effector T cells, T_r cell subsets exist in different tissues with unique homeostatic maintenance requirements (5, 6). Most broadly, T_r cells can be subdivided based on localization within lymphoid or non-lymphoid tissues. Whereas pro-survival signals downstream of Il-2 engagement maintain T_r cells within T cell zones of secondary lymphoid organs (SLOs) (7, 8), maintenance of T_r cells resident in non-lymphoid tissues is largely Il-2-independent, and distinct signals including TCR signaling (9), ICOS-mediated co-stimulation (10, 11), and Il-7 (12, 13), can modulate their abundance and function. In addition to regulating their abundance, the ability of T_r cells to sequester Il-2 helps inhibit the priming of auto-reactive T cells in SLOs. However, T_r cells cannot produce Il-2 themselves due to transcriptional repression at the Il-2 locus by Foxp3 (14, 15), and are therefore dependent on paracrine sources of Il-2 for their survival. As such, the consumption of Il-2 by SLO-resident T_r cells is both indispensable for their survival and essential to their function. Il-2 production by conventional T cells requires their interaction with antigen-presenting cells (APC) bearing cognate antigen and appropriate co-stimulatory molecules. Therefore the maintenance of Il-2 dependent T_r cells requires a tripartite circuit consisting of an antigen-bearing APC, an antigen-specific T cell, and a proximally located T_r cell. To date, the cellular and molecular factors which comprise this circuit and how they operate to maintain Il-2 dependent T_r cells is SLOs under homeostatic conditions has not been fully elucidated. Here we show that T_r cells resident in the spleen are under continual competition for a limiting supply of Il-2 and that subtle changes in Il-2 availability can profoundly influence immune activation. Moreover, we find that due to their potent ability to induce Il-2 release from conventional $CD4^+$ $Foxp3^-$ T cells through the presentation of MHCII-restricted auto-antigens, $33D1^+$ $CD11b^{int}$ DCs are key cellular players in the homeostatic maintenance of Il-2-dependent T_r cells.

MATERIALS AND METHODS

Mice

C57BL/6 (B6), B6.$CD4^{-/-}$, B6.$RAG^{-/-}$, B6.Il-$2^{-/-}$, OT-II, Balb.c and D011.10 mice were purchased from The Jackson Laboratory. CD11c-DTR-Tg mice, B6.$Foxp3^{gfp}$ mice, $BATf3^{-/-}$, and sOVA mice were provided by the following: CD11c-DTR-Tg mice; S. Zeigler (Benaroya Research Institute, Seattle WA), B6.$Foxp3^{gfp}$ mice; A. Rudensky (MSKCC, New York NY), $BATf3^{-/-}$ mice; K. Urdahl (CIDR, Seattle WA), sOVA mice; A. Abbas (University of California, San Francisco, CA). M. Pepper (UW, Seattle WA) and D. Raulet (UC, Berkley CA) supplied $MHCII^{-/-}$ and $\beta2M^{-/-}$ bones for the generation of chimeric

mice, respectively. Bone marrow chimeras were generated by reconstituting irradiated recipient mice (2 x 600 RAD separated by > 4 hours) with ≥ $2x10^6$ RBC-depleted bone marrow cells of the appropriate genotype. Chimeric mice were rested ≥ 8–10 weeks before experiments unless otherwise indicated. All mice were bred and maintained at Benaroya Research Institute and experiments were pre-approved by the Office of Animal Care and use Committee of Benaroya Research Institute. Non-chimeric mice used in experiments were between 7–14 weeks of age at time of sacrifice.

Flow cytometry and cell-sorting

For DC isolations, minced whole spleens were digested in PBS supplemented with 25μg/ml (final) Blenzme (Roche Liberase TM) for 30 minutes at 37°C under agitation. Cell suspensions were then passed through a 70 μm cell strainer into PBS-EDTA (2μM). Erythrocytes were lysed in ACK lysis buffer (Gibco) and the remaining leukocytes were washed in PBS-EDTA. For cell sorting or when otherwise required, DCs were enriched using CD11c-microbeads (Miltenyi – positive selection) according to the manufacturers protocol. Cell surface staining for flow cytometry was performed in FACS buffer (PBS-1% FCS) using the following antibody clones: TCRβ (H57-597), MHCII (M5-114), CD11c (N418), CD11b (M1/70), DC marker (33D1), CD4 (RM4-5), CD8α (53-6.7), CD44 (IM7), CD62L (MEL-14), DO11.10 (KJ1-26), CD25 (PC61.5). Cells were incubated in the appropriate antibody cocktail for 15 minutes at 4°C, then washed in FACS buffer before collecting events on an LSR II (BD Biosciences). For intracellular staining, surface antigens were pre-labeled before permeabilization with eBioscience FixPerm buffer. Cells were then washed and stained with antibodies against Foxp3 (FJK-16s) and/or Ki67 (Sol15). Sorting was performed using a FACS Aria (BD Biosciences) when experiments necessitated pure cell populations. Flow cytometry data was analyzed using FlowJo software (Treestar).

Ex vivo staining

To assess pSTAT5 levels directly *ex vivo*, spleens were immediately disrupted between glass slides into BD Cytofix/Cytoperm buffer. Cells were then incubated for 20 minutes at room temperature, washed in FACS buffer, resuspended in 500 μl 90% methanol, and incubated on ice for ≥ 30 min. Cells were stained for surface and intracellular antigens including pSTAT5 (pY694; BD Biosciences), for 45 min at room temperature. Due to intra-experiment variability in *ex vivo* pSTAT5 staining (typically ranging from 7–11% $pSTAT5^+$ for control T_r cells), T_r cell pSTAT5 data has been normalized to un-manipulated control T_r cells (set to 1) within individual experiments where multiple data sets are pooled.

In vitro DC/T cell co-cultures

For autologous DC/T cell co-cultures, $7.5x10^4$ CD11c-enriched or sorted DC subsets were co-incubated with $3x10^5$ purified GFP^- $CD4^+$ or $CD8^+$ T cells from $FoxP3^{GFP}$ mice in ½ surface area 96 well plates (Costar) in a final volume of 100 μl. The media used for cell culture (RPMI, 1% HEPES, 1% L-Glut, 1% Penn/Strep, 1% Sodium Pyruvate, 0.5% Gentamycin, βME (1X of 1000X stock)) was supplemented with 10% autologous mouse serum sourced from $FoxP3^{GFP}$ donors. Serum was heat inactivated at 57°C for 30 minutes before adding to media. Following 72H cultures, cell-free supernatants were diluted 5X in RP-10 (RPMI-10% FCS) and added to an equal volume suspension of $5x10^5$ $CD4^+$ T cells

(Invitrogen – negative isolation) of which ~10–15% were FoxP3$^+$ T$_r$ cells. Control cells received an equal volume of RP-10. Following 30 minute incubations at 37°C, cells were resuspended in BD Cytofix/Cytoperm buffer as previously described. pSTAT5 was measured amongst CD4$^+$ FoxP3$^+$ CD25^{hi} cells as a surrogate measurement of Il-2 production within each DC/T cell co-culture. Il-2$^{-/-}$ CD4$^+$ T cells were used as the T cell source for initial co-cultures where indicated. In some experiments, 5 μl of αMHCII antibody (M5-114) was added to culture media to block MHCII-mediated antigen presentation. For *in vitro* CD4$^+$ T cell activation and proliferation assays using model antigen, sorted DC subsets from Balb.c or sOVA were incubated in flat bottom 96 well tissue-culture plates (Cellstar) for 20H (early activation) or 72H (proliferation) with CD4-enriched DO11.10 cells at the indicated T cell:DC ratios. For proliferation assays, DO11.10 cells were pre-labeled with the cell tracking dye CFSE prior to culture.

DC depletions and adoptive transfers

CD11c-DTR-Tg or CD11c-DTR-chimeric mice were given an initial I.P. injection of 200 ng diphtheria toxin (Calbiochem), then additional 200ng injections every other day until sacrifice. Where indicated, sorted 33D1$^+$ CD11b^{int} or 33D1$^-$ CD11b^{lo} DCs of the indicated genotype and frequency were adoptively transferred into CD11c-DTR-Tg mice retro-orbitally directly following the first DTx treatment.

Antibody treatments and pan-GPCR inhibition

For Il-2 blocking experiments, mice were given 300 μg of αIl-2 (S4B6-1) or an equivalent amount of rat IgG (Sigma) by retro-orbital injection at minutes 0, 30, 60, 120, and 240 prior to sacrifice. For CD80/86 blocking experiments, mice were given a single injection of 100 ug αCD80 (16-10A1), αCD86 (GL1), both, or an equivalent amount of rat IgG (Sigma) by retro-orbital injection 48 hours prior to sacrifice. For pan-GPCR inhibition, CD11c-enriched cells were incubated for 1 hour at 37°C in RP-10 with/without 1 ug/ml pertussis toxin (List Biologics). Cells were washed 2X before i.v. injection into DC-depleted recipient mice.

In vivo DC labeling

Five micrograms of PE conjugated anti-CD45.2 (104-2) was injected into mice retro-orbitally 5 minutes prior to sacrifice. Splenocytes were prepared for flow cytometry as described and localization of adoptively transferred cells was determined by degree of PE-CD45.2 staining amongst CD45.1$^+$ (A20) DCs within the MHCII^{hi} CD11c^{hi} gate.

Statistical analysis

All data are presented as the mean values ± SEM unless otherwise indicated. Comparisons between groups were analyzed using unpaired Student’s t tests or one-way ANOVA as indicated in the figure legends.

Supplementary Information

There is no supplementary information.

RESULTS

Homeostatic Il-2 limits the frequency and function of splenic T_r cells

Il-2$^{-/-}$, Il-2Rα$^{-/-}$ and Il-2Rβ$^{-/-}$ mice developed spontaneous lymphoproliferative autoimmune disease characterized by expansion and hyper-activation of T cells demonstrating that Il-2 is dispensable as a T cell growth factor but critical for the preservation of self-tolerance through its ability to support T_r cells (16–20). Il-2 activation in T_r cells leads to the phosphorylation of signal transducer and activator of transcription 5 (STAT5), culminating in expression of pro-survival genes including Bcl-2 and Mcl-1. This sustains lymphoid-resident T_r cells and when present in excess can drive T_r cell proliferation and population expansion (21), thereby ensuring that the number of T_r cells is calibrated to the amount of Il-2 produced by effector T cells. To understand the kinetics of STAT5-phosphorylation (pSTAT5) downstream of Il-2 activation in T_r cells, Il-2 blocking antibodies (S4B6-1) were administered into mice i.v. where we saw the disappearance of T_r cell pSTAT5 by 30 minutes post-treatment. Thus, pSTAT5 characterizes recent Il-2 activation in T_r cells (Fig. 1A). Similarly, we used Il-2$^{+/+}$, Il-2$^{+/-}$, and Il-2$^{-/-}$ mice to show that pSTAT5 correlates with the degree of Il-2 availability and is virtually absent in Il-2$^{-/-}$ mice (Fig. 1B, 1C). Thus, additional γC cytokines such as Il-7 and Il-15 which utilize STAT5 for signaling appear to have minimal roles in the homeostatic maintenance T_r cells within SLOs. Consistent with its requirement for the prevention of spontaneous autoimmunity, Il-2-deficient mice developed massive splenomegaly and displayed hyper-activation of conventional CD4$^+$ and CD8$^+$ T cells by four weeks of age. Additionally, although they do not display any overt immunopathology, CD4$^+$ T cells from age-matched Il-2$^{+/-}$ mice also displayed a hyper-activated phenotype (Fig. 1D, 1E) with diminished frequencies of splenic and circulating T_r cells (Fig. 1C). Collectively, these data suggest the homeostatic reservoir of T_r cell-supportive Il-2 is limiting, and that small perturbations in Il-2 availability can substantially influence T_r cell abundance and immune activation. This is consistent with the identification of polymorphisms in the genes encoding IL-2 and CD25, where subtle changes in the production or function of these proteins is nonetheless associated with an increased risk of developing multiple autoimmune diseases including type-1 diabetes (22, 23), multiple sclerosis (24, 25), juvenile idiopathic arthritis (26), and systemic lupus erythematosus (27).

CD4$^+$ T cell-derived Il-2 is critical for the maintenance of Il-2-dependent T_r cells and the prevention of spontaneous autoimmunity

Il-2 can be produced by activated CD4$^+$ and CD8$^+$ T cells. Therefore, to better understand the contribution of CD4$^+$ vs. CD8$^+$ T cell-derived Il-2 in the homeostatic maintenance of Il-2-dependent T_r cells, bulk CD11c-enriched cells (60% MHCII^{hi} CD11c^{hi}) were cultured with autologous polyclonal FoxP3$^-$ CD4$^+$ or CD8$^+$ T cells in the presence of 10% autologous mouse serum. After 3 days, aliquots of each supernatant were briefly added to freshly isolated CD4$^+$ T cells and STAT5 phosphorylation was measured in FoxP3$^+$ CD25$^+$ T_r cells as a surrogate for soluble Il-2 within each culture (Fig. 2A). Importantly, given that all cell isolations/enrichments were performed in buffer devoid of foreign protein (e.g. from bovine calf serum or bovine serum albumin), Il-2 produced under these conditions is likely the result of activation of self-reactive T cells. Supernatants from CD4$^+$ T cells co-cultured

with $CD11c^+$ cells in the presence of autologous serum led to robust phosphorylation of STAT5 amongst $FoxP3^+$ $CD25^+$ T_r cells, while supernatants from similar cultures containing $CD8^+$ T cells did not. Additionally, Il-2 generation in $CD11c^+/CD4^+$ T cell co-cultures was MHC-Class II (MHCII) and DC-dependent as provision of an α-MHCII antibody (M5-114) or cultures devoid of DCs abolished Il-2 production (Fig. 2B). Using this culture system, naïve ($CD44^{lo}$ $CD62L^{hi}$) $CD4^+$ T cells produced more Il-2 than activated ($CD44^{hi}$ $CD62L^{lo}$) $CD4^+$ T cells in response to autologous DCs, although this difference was subtle (Fig. 2B). Whereas DCs have been reported to produce Il-2 upon activation (28), supernatants from co-cultures containing wild-type DCs and $Il\text{-}2^{-/-}$ $CD4^+$ T cells failed to induce pSTAT5 in T_r cells, confirming that $CD4^+$ T cells and not DCs are the principal source of T_r cell-supportive Il-2. (Fig. 2B). To determine if $CD4^+$ T cell-derived Il-2 supports T_r cell development and maintenance *in vivo*, mixed bone marrow chimeras were generated by reconstituting $RAG^{-/-}$ mice with T-cell depleted bone marrow from WT:$Il\text{-}2^{-/-}$ or $CD4^{-/-}$:$Il\text{-}2^{-/-}$ donors. Thus, within our $CD4^{-/-}$:$Il\text{-}2^{-/-}$ mixed chimeras, all $CD4^+$ T cells (including T_r cells) develop from $Il\text{-}2^{-/-}$ donor cells whereas other T cell populations derived from the $CD4^{-/-}$ donor cells remained Il-2-sufficient. By contrast, WT:$Il\text{-}2^{-/-}$ mixed chimeras retained a population of $CD4^+$ T cells capable of Il-2 production (Fig. 2C). Around week 5 post-reconstitution, coinciding with the emergence of donor-derived T cells from the recipient thymus, we noticed a striking deterioration in the physical appearance of our $CD4^{-/-}$:$Il\text{-}2^{-/-}$ mixed chimeras while control chimeras remained healthy. Upon sacrifice, we observed varying degrees of splenomegaly, hyper-activation of conventional $CD4^+$ and $CD8^+$ T cells (Fig. 2D), and a dearth of T_r cells within $CD4^{-/-}$:$Il\text{-}2^{-/-}$ mixed chimeras compared to WT:$Il\text{-}2^{-/-}$ controls (Fig. 2E). Furthermore, those T_r cells recovered were absent of pSTAT5 to a degree reminiscent of $Il\text{-}2^{-/-}$ mice (Fig. 2E). Thus, these experiments demonstrate $CD4^+$ T cell-derived Il-2 is critical for the maintenance of Il-2-dependent T_r cells and the prevention of autoimmune-like disease, whereas $CD8^+$ T cells have a minimal contribution to T_r cell maintenance despite their ability to produce Il-2.

DCs are required for T_r cell Il-2 activation *in vivo*

DC abundance can be experimentally manipulated through provision or Ab-mediated depletion of DC growth and survival factors including GM-CSF and Flt3L (29, 30). While such experiments have revealed T_r cell frequencies are intimately linked to the size of the DC niche, whether DCs directly modulate T_r cell frequencies via antigen presentation/co-stimulation, indirectly through Il-2 production, or both remains unclear. Therefore we next wanted to address a general role for DCs in the maintenance of Il-2-dependent and -independent T_r cells. Utilizing mice expressing the diphtheria toxin receptor (DTR) downstream of the CD11c promoter and containing an additional GFP reporter cassette (CD11c-DTR-Tg mice) (31), we observed DTR expression predominantly in $MHCII^{hi}$ $CD11c^{hi}$ cDCs (Fig. 3A). Accordingly, following three days of DTx treatment in CD11c-DTR-Tg mice, we achieved rapid and highly specific elimination of $MHCII^{hi}$ $CD11c^{hi}$ cDCs with minimal impact on the frequencies of non-DC cell subsets (Fig. 3B, 3C). T_r cell pSTAT5 was markedly diminished upon DC depletion demonstrating DCs were required for Il-2 activation in T_r cells (Fig. 3D). We next wanted to observe how T_r cell Il-2 activation and proliferation, respective hallmarks of Il-2-dependent and -independent T_r cells (32), were influenced by prolonged DC depletion. However, due to expression of CD11c on cell

populations in the CNS, off-target DTx toxicity limits the duration of DTx exposure to < 96 hours in CD11c-DTR-Tg mice. Therefore we generated CD11c-DTR chimeric mice to restrict DTR expression to CD11c-expressing cells of hematopoietic origin. Following three or seven days of DTx treatment in chimeric mice, we observed a similar loss of T_r cell pSTAT5 following acute DC ablation which was sustained at our one week time-point (Fig. 3E). Additionally, the overall frequency, as well as the percentage of actively proliferating T_r cells were reduced upon DC depletion, although both occurred after loss of T_r cell pSTAT5. Together these experiments demonstrate in addition to stimulating T_r cells directly through antigen presentation/co-stimulation, DCs are crucially required for support of Il-2-dependent T_r cells via their ability to stimulate paracrine Il-2 production.

33D1$^+$ CD11b^{int} DCs are potent activators of auto-reactive CD4$^+$ T cells

cDCs encompass a phenotypic and functional heterogeneous group of antigen presenting cells which cannot be evaluated on an individual basis in pan-DC depletion experiments. Therefore, to identify the relevant DC subsets supporting Il-2-dependent T_r cells, we assessed the ability of various DC subsets to present self-antigens to conventional CD4$^+$ T cells *in vitro*. Although classically defined using the markers CD4 and CD8α (33, 34), we identified three distinct cDC subsets in the spleen using the myeloid marker CD11b and the monoclonal antibody 33D1 (which recognizes the putative inhibitory receptor DCIR2) (35). 33D1$^+$ CD11b^{int} DCs comprise the majority of cDCs in the spleen and correspond with canonical “CD4 DCs”. The second most abundant DC subset, 33D1$^-$ CD11b^{lo} DCs, contain a mixed population of CD8α$^+$ and CD8α$^-$ cells (Fig. 4A, 4B) and are BATf3- and Flt3L-dependent; collectively arguing these cells represent canonical “CD8α DCs” despite heterogeneity of CD8α expression (data not shown). A third population of 33D1$^-$ CD11b^{hi} CD4$^{lo/-}$ CD8α$^{lo/-}$ DCs (Fig. 4A, 4B) was excluded as the candidate DC subset supporting Il-2-dependent T_r cells after observing these DCs survived DTx-mediated killing despite a profound loss of T_r cell pSTAT5 in their presence (data not shown). In order to assess the ability of 33D1$^+$ CD11b^{int} and 33D1$^-$ CD11b^{lo} (CD8α) DCs to activate CD4$^+$ T cells in response to a model self-antigen, DCs were sorted from mice expressing soluble OVA (sOVA) and co-cultured overnight with OVA-specific TCR-transgenic CD4$^+$ T cells. Consistent with published reports using targeted DC antigen delivery (36), 33D1$^+$ CD11b^{int} DCs proved to be the most potent CD4$^+$ T cell activators as measured by upregulation of the early T cell activation markers CD25 and CD69, whereas 33D1$^-$ CD11b^{lo} DCs had intermediate CD4$^+$ T cell activation potential (Fig. 4C). Additionally, 33D1$^+$ CD11b^{int} DCs induced greater CD4$^+$ T cell proliferation compared to their 33D1$^-$ CD11b^{lo} counterparts as measured by CFSE dilution (Fig. 4D). To investigate the ability of these subsets to drive Il-2 production from CD4$^+$ T cells, 33D1$^+$ CD11b^{int} and 33D1$^-$ CD11b^{lo} DCs were cultured with autologous polyclonal FoxP3$^-$ CD4$^+$ T cells for 72H and supernatants were used to stimulate STAT5 phosphorylation in T_r cells from freshly isolated CD4$^+$ T cells as outlined in Fig. 2A. Here, 33D1$^+$ CD11b^{int} DCs induced significantly more Il-2 from polyclonal CD4$^+$ T cells compared to 33D1$^-$ CD11b^{lo} DCs (Fig. 4E), while Il-2 was undetectable when either DC subset was cultured alone demonstrating DCs do not spontaneously secrete Il-2.

While our data indicated that 33D1$^-$ CD11b^{lo} (CD8α) DCs are relatively poor CD4$^+$ T cell activators and inducers of Il-2 production *in vitro*, these cells express high levels of MHCII

and therefore should be capable of presenting MHCII-restricted antigens to $CD4^+$ T cells *in vivo*. Therefore, to further evaluate their role in the homeostatic maintenance of T_r cells, we examined mice lacking BATf3, a basic leucine zipper transcription factor that is required for the development of CD8α DCs (37). Here we observed a profound reduction in the frequency and absolute number of $33D1^-$ $CD11b^{lo}$ DCs which mirrored the overall reduction in CD8α-expressing DCs reported for these mice. Furthermore, $33D1^+$ $CD11b^{int}$ DCs were expanded in $BATf3^{-/-}$ spleens, presumably the result of enhanced availability of shared DC growth- and survival factors such as Flt3L which may be efficiently consumed by $33D1^-$ $CD11b^{lo}$ DCs (Fig. 5A). Despite the substantial reduction in $33D1^-$ $CD11b^{lo}$ (CD8α) DCs, T_r cell Il-2 activation was unchanged in $BATf3^{-/-}$ mice demonstrating this subset is not necessary for the homeostatic maintenance of Il-2-dependent T_r cells. Furthermore, T_r cells from $BATf3^{-/-}$ spleens were more actively proliferative (based on Ki-67 staining) than those from WT mice suggesting enhanced antigen presentation to auto-reactive $CD4^+$ T cells by $33D1^+$ $CD11b^{int}$ DCs in the absence of $33D1^-$ $CD11b^{lo}$ DCs (Fig. 5B). In combination with our *in vitro* observations, these data collectively demonstrate that although multiple DC populations are capable of inducing Il-2 production from auto-reactive $CD4^+$ T cells, $33D1^+$ $CD11b^{int}$ DCs are the most potent $CD4^+$ T cell activators and likely play a key role in the maintenance of Il-2-dependent T_r cells.

$33D1^+$ $CD11b^{int}$ and $33D1^-$ $CD11b^{lo}$ (CD8α) DCs are both sufficient for the generation of T_r cell supportive Il-2 through antigen presentation on MHCII and CD80/86 co-stimulation

$33D1^-$ $CD11b^{lo}$ DCs are not necessary for the homeostatic maintenance of Il-2 dependent T_r cells (Fig. 5). However, given their ability to activate and drive Il-2 production from $CD4^+$ T cells *in vitro*, we next examined if either $33D1^+$ $CD11b^{int}$ or $33D1^-$ $CD11b^{lo}$ DCs were sufficient for the generation of T_r cell supportive Il-2 *in vivo*. For this, sorted DCs were adoptively transferred into DTx treated CD11c-DTR-Tg mice to evaluate their ability to rescue Il-2 activation in T_r cells from DC-depleted animals (Fig. 6A). At a high transfer dose, both $33D1^+$ $CD11b^{int}$ and $33D1^-$ $CD11b^{lo}$ DCs rescued Il-2 activation in T_r cells from DC-depleted mice, and this required MHCII-restricted antigen presentation. Titrating down the number of adoptively transferred cells, $33D1^+$ $CD11b^{int}$ DCs showed a clear superiority over $33D1^-$ $CD11b^{lo}$ DCs in their ability to rescue T_r cell pSTAT5 in DC-depleted mice (Fig. 6A). Furthermore, $33D1^+$ $CD11b^{int}$ DCs incapable of presenting MHCI-restricted antigens to $CD8^+$ T cells due to loss of β2-microglobulin ($\beta2M^{-/-}$) were equally capable of rescuing Il-2 activation in T_r cells from DC-depleted mice compared to their WT counterparts (Fig. 6B), supporting previous *in vitro* and *in vivo* observations that MHCII-mediated antigen presentation to $CD4^+$ T cells represents the principal source of homeostatic T_r cell-supportive Il-2. Reconciling these observations with those made in $BATf3^{-/-}$ mice, $33D1^-$ $CD11b^{lo}$ DCs are sufficient but not necessary for T_r cell Il-2 activation. Finally, we addressed the role of CD80/86 co-stimulation in the maintenance of Il-2-dependent T_r cells by treating mice individually or in tandem with blocking antibodies against CD80 and/or CD86. Here we observed an essential but redundant role for CD80 and CD86 in the support of Il-2-dependent T_r cells, likely through the ability of CD28-mediated co-stimulation to promote Il-2 production from conventional $CD4^+$ T cells (Fig. 6C).

DC migration to the splenic white pulp is critical for the homeostatic maintenance of Il-2 dependent T_r cells

33D1$^+$ CD11b^{int} DCs primarily reside within splenic marginal zone bridging channels (MZBCs); specialized regions at the nexus of the B cell follicle, T cell zone, and red pulp (38, 39). In this location 33D1$^+$ CD11b^{int} DCs are thought to capture particulate antigens in the blood and ferry them across the marginal sinus into T cell zones of the spleen. G-protein coupled receptors (GPCRs) including EBI2 (40, 41), $S1P_{1–5}$ (42), and CCR7 (43, 44) are critically important for DC positioning within MZBCs and essential for chemotaxis of migratory DCs into secondary lymphoid tissues. Pertussis toxin (PTx) is a $G_{\alpha i}$ inhibitor and can be used to antagonize $G_{\alpha i}$-linked GPCR function. Therefore, to address how inhibition of $G_{\alpha i}$-dependent GPCR signaling in DCs would influence DC positioning and subsequent Il-2-dependent T_r cell homeostasis in the spleen, CD11c$^+$ cells were treated with/without PTx *in vitro* and extensively washed prior to transfer into DC-depleted mice (Fig. 7A) where we assessed their ability to rescue STAT5 phosphorylation in splenic T_r cells. Compared to those receiving un-manipulated CD11c$^+$ cells, PTx pre-treatment dramatically compromised the ability of transferred DCs to rescue Il-2 activation in T_r cells from DC-depleted mice (Fig. 7B), without jeopardizing the survival of transferred cells (Fig. 7C). By injecting recipient mice intravenously with a PE-conjugated αCD45 antibody which differentially labels cells in the red/white pulp (45), we confirmed PTx pre-treatment blocked the ability of transferred DCs to access the white pulp as ~90% of recovered PTx pre-treated DCs stained positive for the injected label (Fig. 7D). Collectively, these experiments emphasize the critical importance of DC migration into the splenic white pulp for the efficient generation of homeostatic T_r cell-supportive Il-2.

DISCUSSION

It is now well established that phenotypic and functionally heterogeneous T_r cell subsets exist in lymphoid and non-lymphoid tissues, where they exhibit a differential dependence on Il-2 signaling or TCR/co-stimulatory receptor activation for their homeostatic maintenance and function, respectively (6). Because Foxp3 directly inhibits their Il-2 production, T_r cells within SLOs depend on paracrine Il-2 downstream of cellular and molecular interactions which are largely T_r cell-extrinsic. Yet to date a clear understanding of the cellular and molecular factors which comprise this circuit and how they operate to maintain Il-2-dependent T_r cells at homeostasis is critically lacking. For instance, although DCs and T_r cells are known to be linked in a ‘homeostatic loop’ (30), the mechanisms linking DCs and T_r cells have not been fully defined, and the relative abilities of different DC subsets to modulate T_r cell abundance is not known. In the present study we provide a comprehensive analysis of the precise cellular and molecular factors maintaining Il-2-dependent T_r cells in the spleen at homeostasis. In short, we found that the frequency and function of Il-2-dependent T_r cells in the spleen is contingent on the presentation of MHCII-restricted auto-antigens to self-reactive CD4$^+$ T cells, largely by CD80/86-bearing 33D1$^+$ CD11b^{int} DCs within the splenic white pulp. Deviations or malfunctions in this circuit likely impair the suppressive capacity of T_r cells through the inability to efficiently produce Il-2. Thus, we have provided empirical evidence supporting the notion that T_r cell frequencies are commensurate with the degree of T cell auto-reactivity. To this we add new insights that Il-2

released from 33D1^{+} CD11b^{int} DC-activated auto-reactive CD4^{+} T cells represents the major homeostatic signal calibrating the frequency and function of splenic T_r cells. Interestingly, 33D1^{+} DCs in the spleen were recently shown to promote the generation of CD4^{+} follicular helper T (T_{fh}) cells by upregulating CD25 and sequestering IL-2 (46). Thus, depending on the context, these cells can either promote Il-2 signaling to support T_r cell maintenance, or inhibit Il-2 signaling to generate T_{fh} cells, and through these reciprocal mechanisms influence both the regulation of autoimmunity and the generation of protective antibody responses.

SLO-resident T_r cells constitutively express high levels of CD25, inhibiting auto-reactive T cell priming in part by sequestering Il-2 from neighboring conventional T cells to establish an insurmountable threshold for complete T cell activation in the absence of additional inflammatory stimuli. Il-2 signaling has the supplementary effect of reinforcing T_r cell lineage stability through FoxP3 induction and co-opting bystander suppression by promoting CTLA-4 and CD39/73 expression (47, 48). Our lab has previously shown the acquisition of Il-2 by SLO resident T_r cells is largely dependent on the chemokine receptor CCR7 suggesting paracrine Il-2 is spatially constrained within SLOs (32). Indeed, T_r cells have recently been shown to perceive Il-2 within T cell/CD11c^{lo} DC clusters found in para-cortical regions of lymph nodes (48). This is consistent with our own observation that PTx pre-treatment of adoptively transferred DCs inhibited their ability to promote Il-2 activation in splenic T_r cells from DC-depleted mice; presumably a reflection of their inability to localize with and activate Il-2 producing CD4^{+} T cells inside T cell zones of the spleen. Interestingly, inducible genetic ablation of TCR expression on T_r cells does not alter lineage stability or the frequency of Il-2-dependent T_r cells within SLOs, but instead is required for the maintenance of non-lymphoid tissue-resident T_r cells (9). This is likely due to the fact that upon TCR engagement in the presence of inflammatory signals, T_r cells up-regulate tissue homing receptors and redistribute out of SLOs into peripheral tissues where they undergo successive rounds of TCR-dependent proliferation - establishing a repertoire of T_r cells specific for antigens typically encountered within those tissues (49–51). T_r cell which fail to engage their TCRs undergo programmed cell death, no longer supported by anti-apoptotic signals downstream of Il-2 (21). Therefore, SLO-resident T_r cells transition from naïve-like, Il-2 consuming “passive” suppressors to actively suppressive, TCR/co-stimulation dependent tissue-resident cells. Despite their unique homeostatic maintenance requirements, continual involvement from both Il-2-dependent and -independent T_r cells is necessary for the prevention of autoimmunity.

Given the observation that Il-2$^{+/-}$ mice have reduced T_r cell frequencies and enhanced effector T cell activation, our data strongly suggests T_r cells are under continual competition for a limiting supply of Il-2. Consequently, altering the Il-2 reservoir, either directly or through manipulation of the cells involved in its production, could dramatically enhance T cell-mediated immunity. These insights may help reconcile the somewhat paradoxical observation that mice constitutively lacking DCs (ΔDC) develop spontaneous fatal $Th_{1/17}$ mediated autoimmunity (52). While T_r cell frequencies from ΔDC mice were shown to be normal, it was suggested a critical sub-population of DC-dependent T_r cells may be absent in these mice allowing B cells or macrophages to compensatorily activate conventional CD4^{+} T cells, driving autoimmunity. We speculate this sub-population of T_r cells is in fact

SLO-resident Il-2-dependent T_r cells no longer supported in the absence of 33D1$^+$ CD11b^{int} DCs in the spleen and equivalent CD103$^-$ CD11b$^+$ DCs in lymph nodes.

The relevant antigens driving basal Il-2 production supporting Il-2-dependent T_r cells could in theory be derived from two major sources; commensal microorganisms and/or self. While critically important for intestinal T_r cell homeostasis (53), enhanced frequencies and intact Il-2 activation amongst circulating T_r cells from gnotobiotic mice demonstrate microbial-derived antigens are not required for the production of T_r cell-supportive Il-2 (54). Interrogating the role of "self" antigens in the generation of homeostatic Il-2 has relied heavily on TCR-transgenic mice in which model antigens are experimentally introduced or ectopically expressed (55). While this approach has been instrumental to our understanding of how cognate antigen recognition shapes conventional and regulatory T cell homeostasis and function, the supra-physiological levels of antigen and high precursor frequencies of antigen-specific T cells typically employed in these systems may misrepresent what occurs for self-antigen recognition at the steady-state. In contrast, by co-culturing cells in media devoid of any foreign protein, we have directly shown CD4$^+$ T cells generate Il-2 in response to *bona fide* self-antigens. Although activated CD8$^+$ T cells are known to produce Il-2, we show using CD4$^{-/-}$:Il-2$^{-/-}$ mixed bone marrow chimeras that CD8$^+$ T cell-derived Il-2 is insufficient for the maintenance of Il-2-dependent T_r cells. CD8$^+$ T cells may be inherently less auto-reactive than CD4$^+$ T cells and therefore fewer of the cells may contribute to base-line Il-2 production. Alternatively, production of Il-2 by CD8$^+$ T cells may be mechanistically or spatially segregated in a way that precludes its access to CD25^{hi} T_r cells. Although DCs have been reported to produce Il-2 upon activation (28), our data indicate that DCs are not a significant source of the Il-2 that acts on T_r cells. For instance, MHCII-deficient DCs failed to rescue Il-2 activation in T_r cells from DC-depleted mice despite Il-2-sufficiency. Furthermore, supernatants from DCs cultured alone or DCs cultured with Il-2-deficient CD4$^+$ T cells failed to stimulate pSTAT5 in freshly isolated T_r cells demonstrating CD4$^+$ T cells and not DCs are the critical source of T_r cell-supportive Il-2.

Given their propinquity to the blood-filtering red pulp, the presentation of serum proteins by 33D1$^+$ CD11b^{int} DCs to CD4$^+$ T cells likely represents the major antigenic source stimulating the production of homeostatic T_r cell-supportive Il-2. Indeed, a recent report has demonstrated 33D1$^+$ DCs within MZBCs can prime CD4$^+$ T cell responses to red blood cell antigens, and this response is abolished if recipient mice are given LPS prior to transfusion (39). We posit the redistribution of 33D1$^+$ CD11b^{int} DC into T cell zones of the spleen, which has been widely reported upon LPS injection (40, 56), is to blame for this tempered response. Therefore, 33D1$^+$ CD11b^{int} DCs must be optimally positioned within splenic MZBCs to acquire the particulate blood antigens they subsequently present to auto-reactive CD4$^+$ T cells to induce T_r cell supportive Il-2 release from CD4$^+$ T cells.

Recently, the manipulation of T_r cell abundance and/or function has become an attractive therapeutic strategy to either boost or inhibit immune responses in a variety of clinical settings. We propose that, in addition to directly targeting T_r cells, similar modulation of specific DC lineages could synergize existing T_r cell-based therapeutics. For example, while inferior at activating CD4$^+$ T cells, 33D1$^-$ CD11b^{lo} (CD8α) DCs mediate the cross-presentation of antigens to CD8$^+$ T cells (57). Accordingly, BATf3$^{-/-}$ mice show increased

susceptibility to viral infections (58), impaired syngeneic tumor rejection (37), and defective intracellular pathogen clearance (59) – all consequences of diminished $CD8^+$ T cell function in the absence of CD8α DCs. While we have demonstrated $CD8^+$ T cells do not generate T_r cell-supportive Il-2, they do produce robust IFNγ down-stream of Il-12 release from CD8α DCs (59, 60). As immunotherapies aimed at dampening T_r cell responses are currently used for the treatment of solid tumors, bolstering DCs responsible for cross-priming $CD8^+$ T cell responses could promote cytotoxic anti-tumor immunity at the expense of those encouraging Il-2 production (and subsequent T_r cell maintenance) from $CD4^+$ T cells. Likewise, given that Il-2 controls the frequency of Il-2-dependent T_r cells in the spleen and is of limiting supply, manipulating the Il-2 reservoir, either directly or through the cells or molecules which stimulate its release could be therapeutically beneficial in augmenting immune responses naturally constrained by T_r cells.

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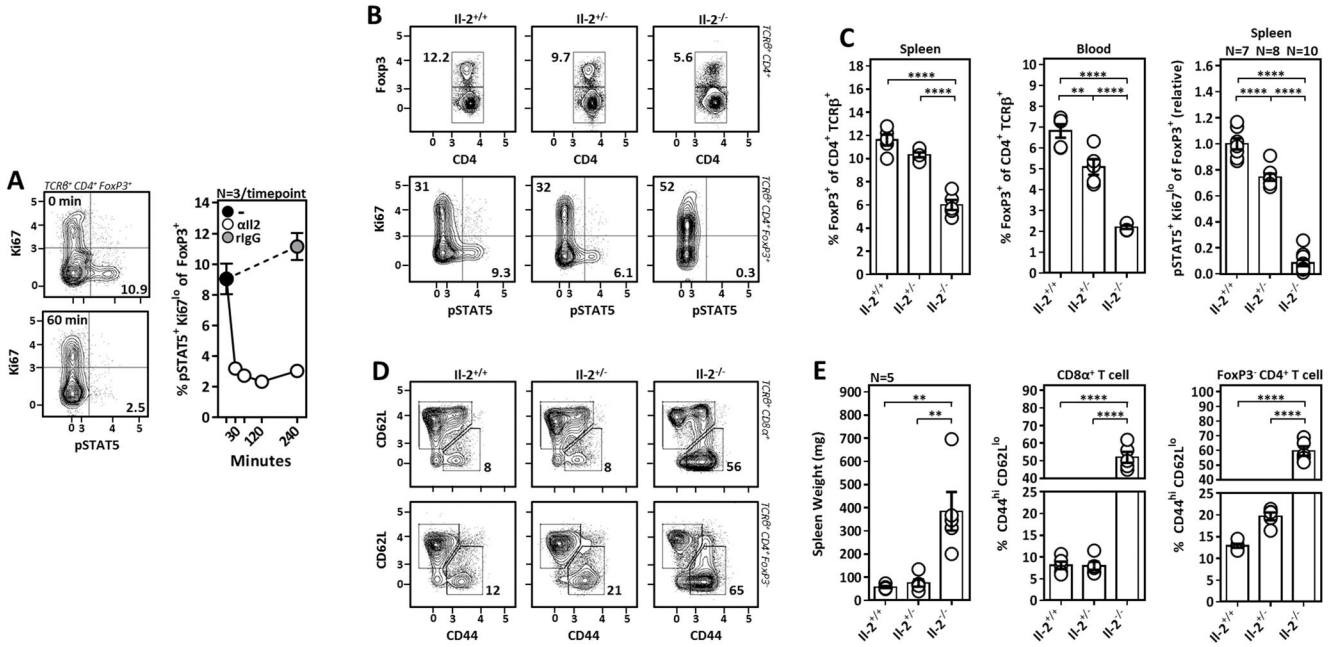

Figure 1. T_r cells compete for a limiting supply of Il-2

(A) Rat IgG or Il-2 blocking antibody (S4B6-1) was administered to mice I.V. at the indicated time-points prior to sacrifice to assess Il-2 activation kinetics in splenic T_r cells by phosphorylation of STAT5 (pSTAT5). (B and D) Representative flow cytometry plots assessing T_r cell frequencies and pSTAT5 (B), and activation of FoxP3$^-$ T cells (D), in WT (Il-2$^{+/+}$) Il-2 heterozygous (Il-2 $^{+/-}$) and Il-2 knockout (Il-2$^{-/-}$) mice. (C) T_r cell frequencies amongst TCRβ$^+$ CD4$^+$ T cells in the spleen (left) and blood (middle) of Il-2$^{+/+, +/-,}$ and $^{-/-}$ mice (N = 5). (Right) Compiled data for T_r cell pSTAT5 in mice of the indicated genotype (N = replicate mice per group). (E) Spleen weight (left) and frequency of activated (CD44^{hi} CD62L^{lo}) CD4$^+$ and CD8$^+$ T cells as gated in D (N=5). Data representative of at least 2 independent experiments. Error bars in all panels represent mean ±SEM. **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001, as calculated by one-way ANOVA with Tukey's post hoc test.

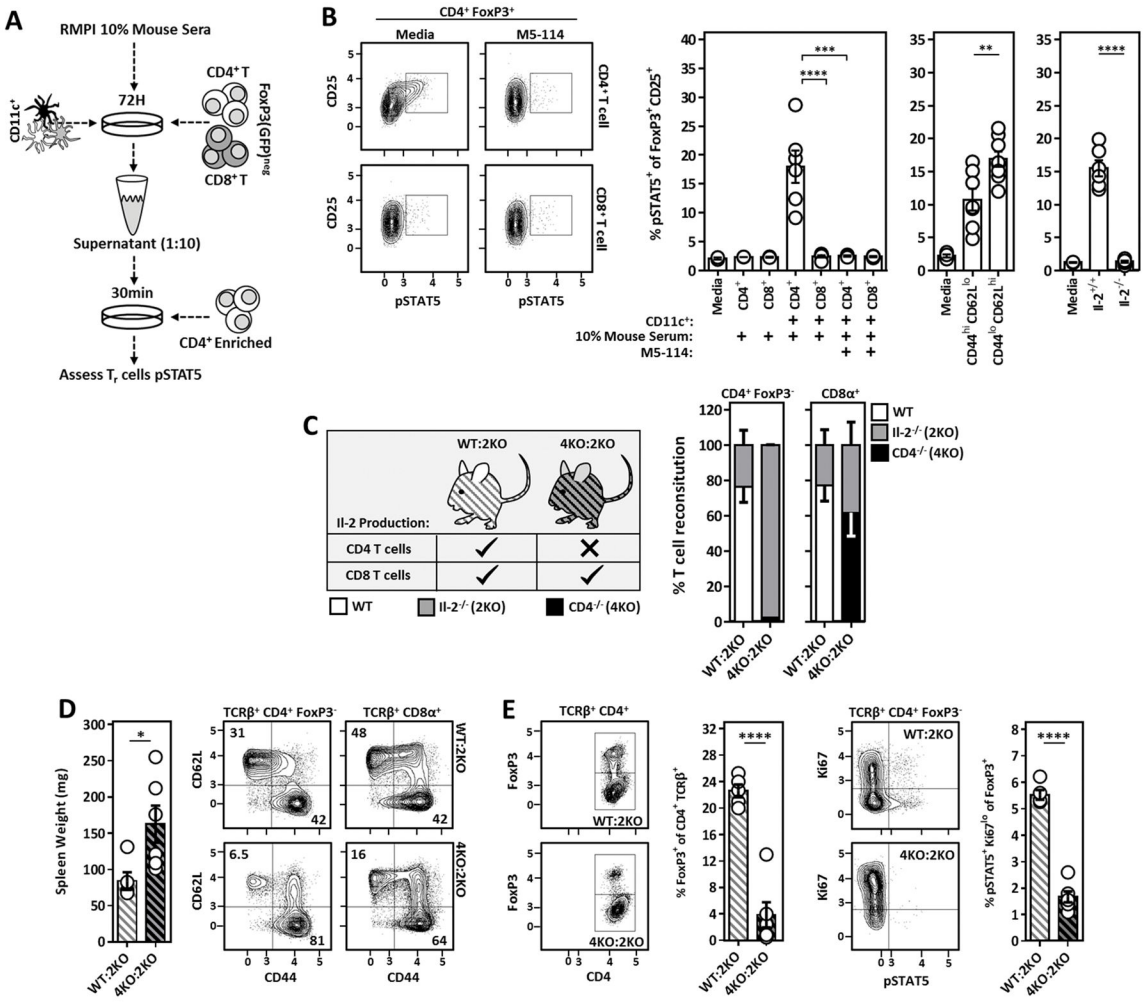

Figure 2. CD4$^+$ T cell-derived Il-2 sustains SLO resident T$_r$ cells and prevents the development of spontaneous autoimmunity

(A) Experimental design to assess qualitative Il-2 production in DC/T cell co-cultures via bio-assay. (B) Representative flow cytometry plots (left) and compiled data (right) assessing pSTAT5 amongst freshly isolated CD4$^+$ FoxP3$^+$ T cells stimulated with 1:10 diluted co-culture supernatants generated as outlined in A. Anti-MHCII (M5-114) antibodies were included in the original co-cultures where indicated. (Middle) Experiments were repeated using sorted CD44^{hi} CD62L^{lo} and CD44^{lo} CD62L^{hi} CD4$^+$ T cell subsets. (Right) Similar experiments were performed utilizing unfractionated Il-2$^{-/-}$ CD4$^+$ T cells as the co-culture T cell source. The frequency of pSTAT5$^+$ cells amongst CD25$^+$ FoxP3$^+$ T cells is quantified. (C) Design of WT:Il-2$^{-/-}$ and CD4$^{-/-}$:Il-2$^{-/-}$ chimeras used to assess the requirement of CD4$^+$ vs. CD8$^+$ T cell-derived Il-2 in the maintenance of Il-2 dependent T$_r$ cells *in vivo*. (Right) Reconstitution analysis of CD4$^+$ and CD8$^+$ T cells from WT:Il-2$^{-/-}$ and CD4$^{-/-}$:Il-2$^{-/-}$ chimeras 5–6 weeks post-reconstitution. ~98% of the CD4$^+$ T cells within CD4$^{-/-}$:Il-2$^{-/-}$ chimeras were Il-2-deficient. (D) Spleen weight and naïve vs. activated phenotype of splenic CD4$^+$ and CD8$^+$ T cells from WT:Il-2$^{-/-}$ and CD4$^{-/-}$:Il-2$^{-/-}$ chimeras at time of sacrifice. (E) Analysis of T$_r$ cell frequencies and Il-2 activation (pSTAT5) in

splenic T_r cells of the indicated chimeric mice at time of sacrifice. Chimera data is pooled from two independent experiments with at least 2 mice per group. Error bars in all panels represent mean ±SEM. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$, as calculated by unpaired students Student's *t*-test (D) or one-way ANOVA with Tukey's post hoc test (B).

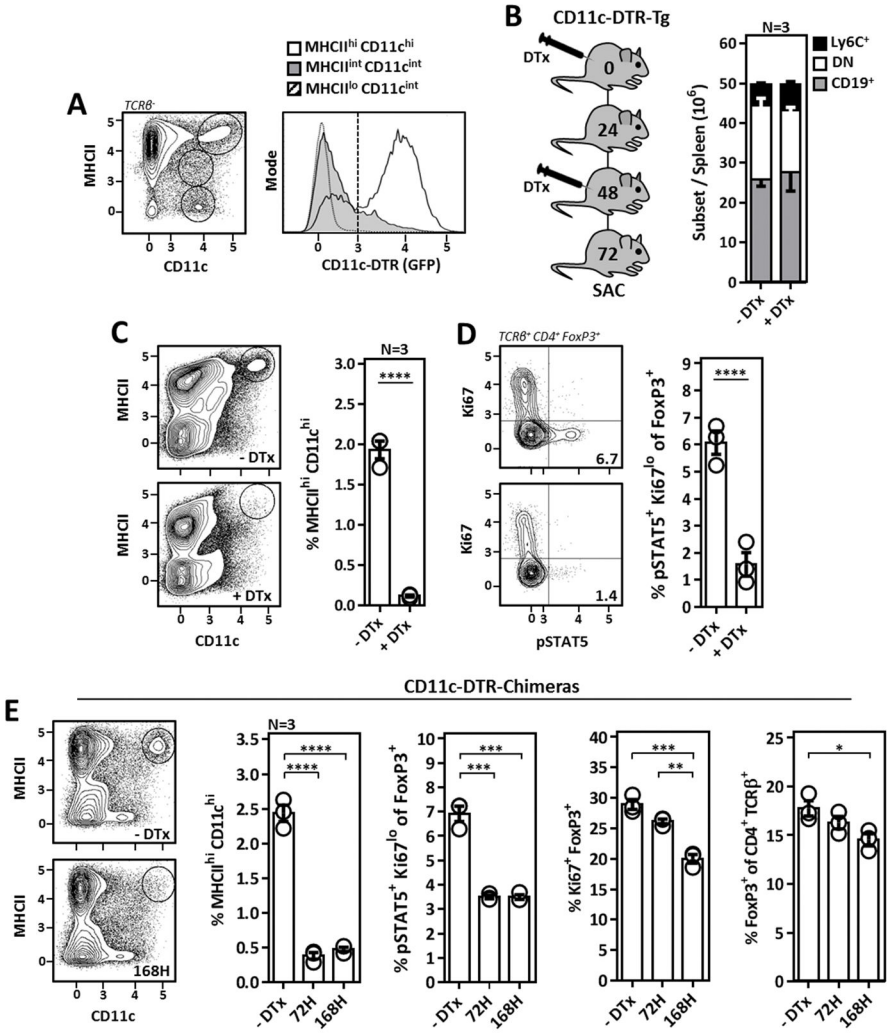

Figure 3. DCs are required for the homeostatic maintenance of Il-2-dependent T_r cells

(A) Flow cytometric analysis of GFP (DTR) expression in various splenic cell populations from CD11c-DTR-Tg mice. (B) Strategy for the elimination of DCs in CD11c-DTR-Tg mice (left) and broad cellular subset composition of splenocytes from CD11c-DTR-Tg mice ±DTx treatment (right). (C) Analysis of $MHCII^{hi}$ $CD11c^{hi}$ DCs in CD11c-DTR-Tg mice ±DTx treatment. (D) Il-2 activation (pSTAT5) in T_r cells from CD11c-DTR-Tg mice ±DTx treatment. (E) Analysis of the DC and T_r cell compartment in CD11c-DTR-Chimeric mice treated ±DTx for 3 or 7 days. T_r cell Il-2 activation (pSTAT5), proliferation (Ki67), and frequency amongst $TCR\beta^+$ $CD4^+$ T cells are plotted. Error bars in all panels represent mean ±SEM. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$, as calculated by unpaired students Student's *t*-test (C, D) or one-way ANOVA with Tukey's post hoc test (E).

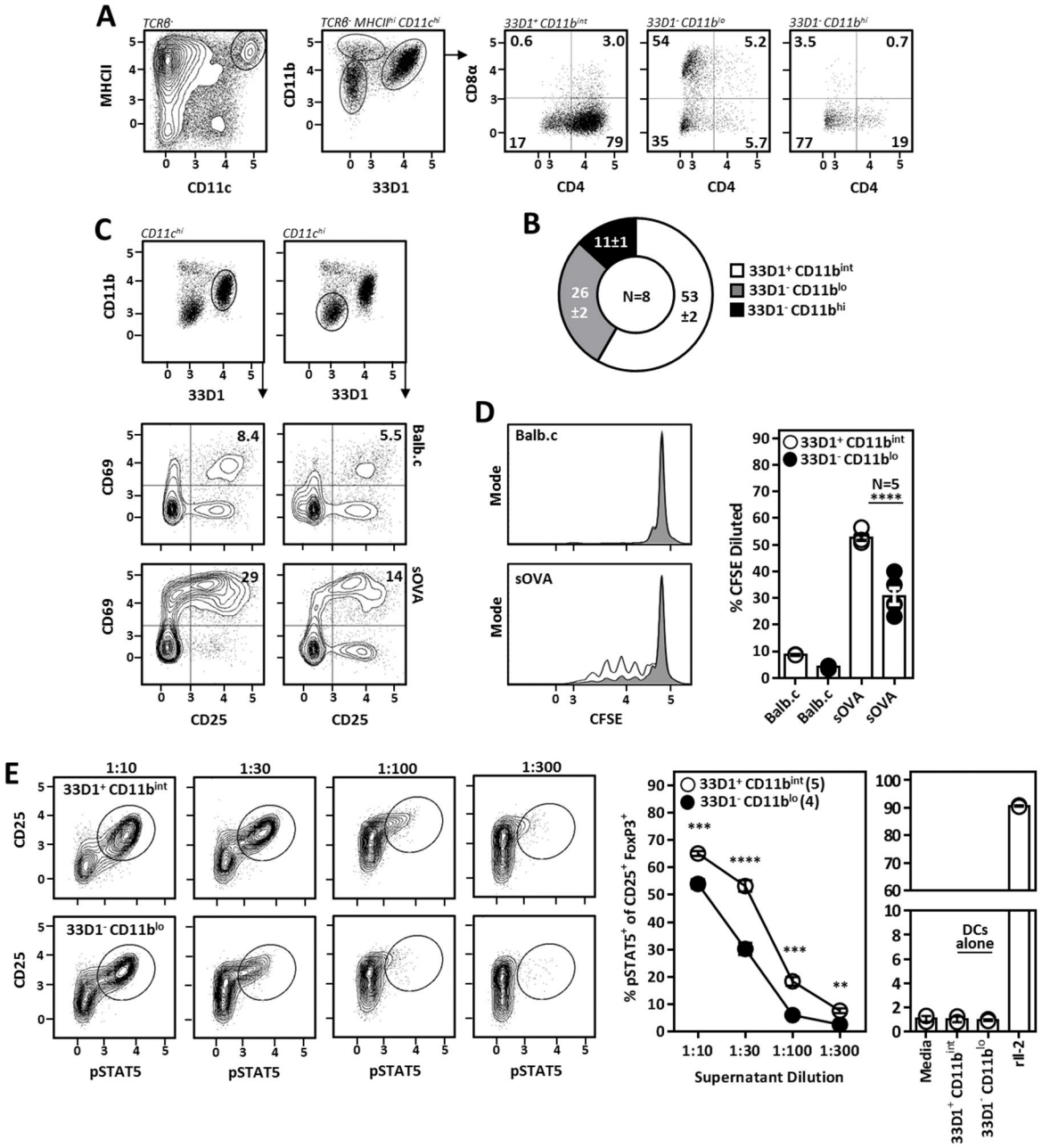

Figure 4. 33D1^{+} CD11b^{int} DCs are potent activators and inducers of Il-2 production from auto-reactive CD4^{+} T cells

(A) Gating strategy used to identify splenic DC subsets and how they relate to canonical "CD4" and "CD8α" DCs. (B) Relative abundance of individual splenic DC subsets identified as in A. Numbers indicate mean ±Standard Deviation. (C) Early T cell activation as assessed by CD25 and CD69 upregulation on OVA-specific CD4^{+} T cells following overnight culture with 33D1^{+} CD11b^{int} or 33D1^{-} CD11b^{lo} DCs from Balb.c or sOVA mice; 3:1 (T cell:DC ratio). (D) Proliferation of OVA-specific CD4^{+} T cells cultured for 72H as in E; 10:1 (T cell:DC ratio). (E) (Left) Il-2 activation (pSTAT5) amongst T_r cells from freshly isolated CD4^{+} T cells briefly cultured in supernatant diluted as indicated from CD4^{+} T cell/

33D1^{+} CD11b^{int} DC or CD4^{+} T cell/33D1^{-} CD11b^{lo} DC co-cultures as outlined in Fig. 2A. (Right) Supernatants from 33D1^{+} CD11b^{int} or 33D1^{-} CD11b^{lo} DCs cultured alone failed to stimulate T_r cell pSTAT5 demonstrating DCs do not serve as a direct source of Il-2 in this culture system. Recombinant mouse Il-2 (rIl-2) was used as a positive control. Error bars in all panels represent mean ±SEM. **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$, as calculated by unpaired students Student's *t*-test (D, G) or one-way ANOVA with Tukey's post hoc test (F).

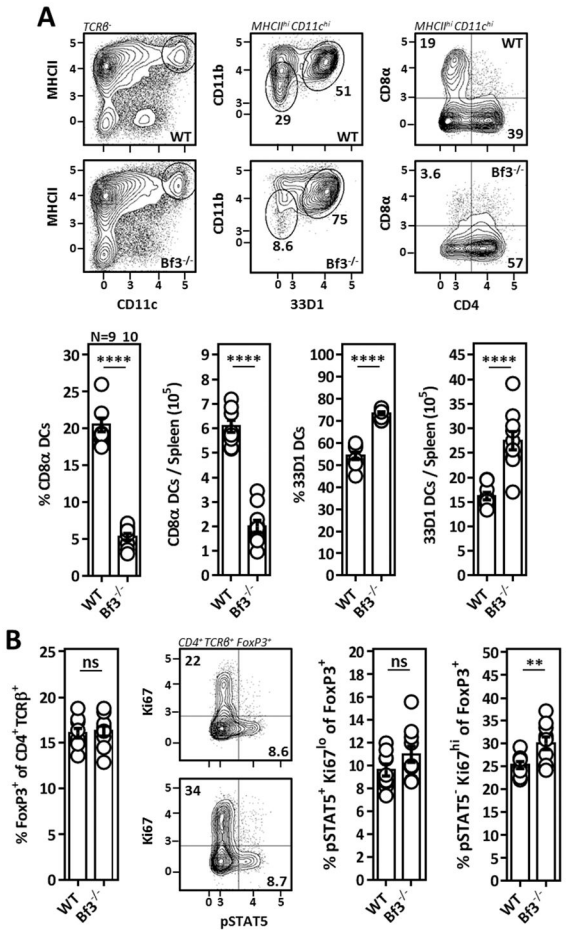

Figure 5. 33D1^{-} CD11b^{lo} (CD8α) DCs are dispensable for the maintenance of Il-2 dependent T$_r$ cells

(A) Representative flow cytometry (top) and compiled data (bottom) identifying splenic DC subsets from 7–8 week old WT or BATf3$^{-/-}$ (Bf3$^{-/-}$) mice. (B) T$_r$ cell frequencies, Il-2 activation (pSTAT5), and proliferation (Ki67) amongst T$_r$ cells from WT or BATf3$^{-/-}$ spleens in A. Data pooled from two independent experiments with at least 4 mice per group. Error bars in all panels represent mean ±SEM. **, P ≤ 0.01; ****, P ≤ 0.0001, as calculated by unpaired students Student's *t*-test. ns, not significant.

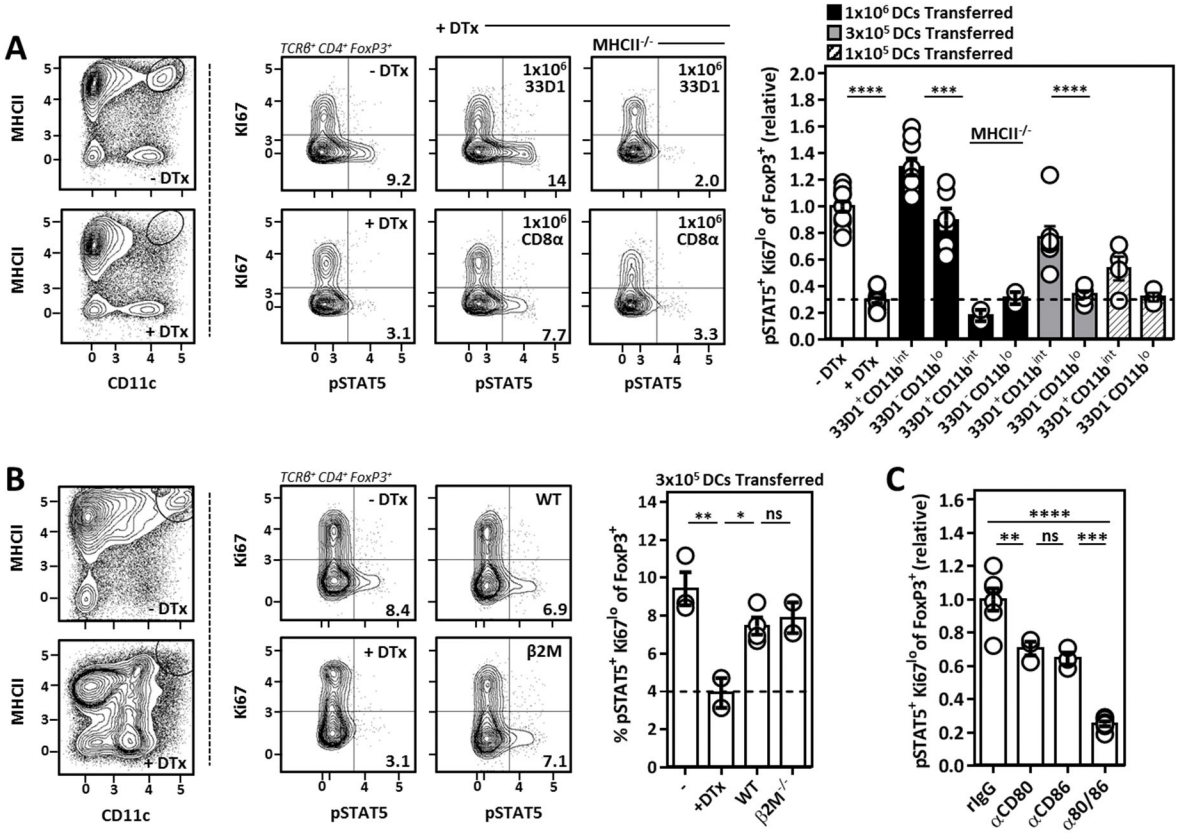

Figure 6. Sufficiency of DC subsets and their molecular requirements for the maintenance of Il-2-dependent T_r cells *in vivo*

(A) Representative flow cytometry and compiled data comparing T_r cell Il-2 activation (pSTAT5) from DC-deplete mice receiving varying amounts of adoptively transferred DCs of the indicated subset and genotype. Numbers listed above bar graphs indicate number of DCs used for the adoptive transfer. Data pooled from 4 independent experiments and pSTAT5 normalized to the untreated control T_r cells within each experiment. (B) Il-2 activation (pSTAT5) in T_r cells from DC-depleted mice receiving $3x10^5$ WT or $\beta2M^{-/-}$ $33D1^+$ $CD11b^{int}$ DCs. (C) Mice were treated with a single dose of 100μg of the indicated antibodies 48H prior to sacrifice. Histogram represents T_r cell Il-2 activation (pSTAT5) in mice receiving CD80/CD86 antibodies individually or in tandem. Error bars in all panels represent mean ±SEM. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$, as calculated one-way ANOVA with Tukey's post hoc test. ns, not significant.

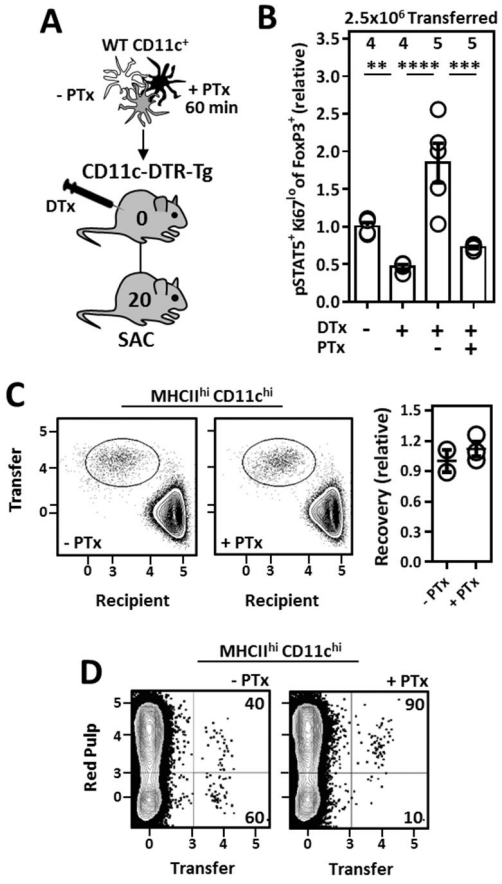

Figure 7. DC access to the splenic white pulp is required for the maintenance of Il-2-dependent T_r cells

(A) Strategy to assess the ability of GPCR-inhibited DCs to rescue Il-2 activation (pSTAT5) in T_r cells from DC-depleted mice. CD11c-enriched cells were incubated for 60 minutes in RP-10 ±1 μg/ml pertussis toxin (PTx) prior to adoptive transfer. (B) Il-2 activation (pSTAT5) in T_r cells from mice receiving $2.5x10^6$ $CD11c^+$ cells treated as in A. Data pooled from two independent experiments with at least 2 mice per group. (C) Recovery of adoptively transferred DCs ±PTx pretreatment 20H post-transfer into congenically marked recipients. (D) Localization of adoptively transferred DCs within recipient mice as assessed by in vivo antibody labeling. $2.5x10^6$ $CD11c^+$ cells ±PTx pretreatment were transferred i.v. Error bars in all panels represent mean ±SEM. **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$, as calculated by one-way ANOVA with Tukey's post hoc test