33D1^{+} Dendritic Cells in Tolerance and Immunity

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Abstract:

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CD4$^+$ Foxp3$^+$ regulatory T cells (T_r cells) are potent anti-inflammatory cells which naturally suppress autoimmune disease by tempering immune responses to self- and foreign-antigens. Given their integral role in the maintenance of self-tolerance, the manipulation of T_r cell abundance and/or function has tremendous implications for the treatment of a spectrum of diseases including autoimmunity, graft rejection, and cancer. Although once thought to represent a uniform group of immuoregulatory cells, work from our lab and others over the past several years has demonstrated considerable phenotypic and functional heterogeneity exist amongst T_r cells. Broadly, T_r cells can be subsetted based on their localization within secondary-lymphoid (SLO) and non-lymphoid tissues where they exhibit a unique dependence on IL-2 signaling and TCR/co-stimulatory receptor activation for their homeostatic maintenance and function, respectively. Loss of either of these distinct T_r cell subsets is sufficient to invoke lethal autoimmunity. However, T_r cells are incapable of IL-2 production due to transcriptional repression at the IL-2 locus by Foxp3 and are therefore dependent on paracrine sources of IL-2 downstream of cellular and molecular interactions which are largely T_r cell-extrinsic. The results presented in this dissertation comprehensively identify the components of the circuit sustaining IL-2-dependent T_r cells in the spleen.

Here we demonstrate the presentation of MHCII-restricted auto-antigens by CD80/86-bearing 33D1^{+} CD11b^{int} DCs to self-reactive CD4^{+} T cells within T cell zones of the spfleen synchronizes the frequency and function of IL-2-dependent T_r cells, preventing spontaneous autoimmunity. Furthermore, the amount of homeostatic IL-2 generated through this circuit is limiting for T_r cells and subtle perturbations in its availability can enhance immune activation directly through its impact on IL-2-dependent T_r cells. Therefore, altering the IL-2 reservoir, either directly or through the manipulation of 33D1^{+} CD11b^{int} DCs or additional molecules which stimulate its production, could be therapeutically beneficial in augmenting immune responses naturally constrained by T_r cells. Our findings also help reconcile the apparent paradox of lethal autoimmunity which develops in mice constitutively lacking DCs, which we posit results from the selective loss of IL-2-dependent T_r cells no longer supported by IL-2 release downstream of 33D1^{+} CD11b^{int} DCs. Moreover we report DC-intrinsic CD4 expression, historically used to delineate DCs in the spleen but functionally undefined, is dynamically regulated on 33D1^{+} DCs in a cell intrinsic manner and augments the function of 33D1^{+} DCs in part through its ability to influence IL-2 production from CD4^{+} T cells in response to complex antigens. Thus, 33D1^{+} DCs are integral for sustaining self-tolerance through their indirect maintenance of T_r cells, and modulate CD4 expression to potentially regulate the magnitude and/or duration of an immune response.

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For my dad

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List of Abbreviations

(in order of appearacne)

PAMP	Pathogen Accociated Molecular Pattern
TLR	Toll-Like Receptor
MHC	Major Histocompatability Complex
DC	Dendritic Cell
APC	Antigen Presenting Cell
TCR	T Cell Receptor
RP	Red Pulp
WP	White Pulp
LN	Lymph Node
MZ	Marginal Zone
MZBC	Marginal Zone Bridging Channel
NK	Natural Killer Cell
cDC	Conventional Dendritic Cell
Mo-DC	Monocyte-derived Dendritic Cell
pDC	Plasmacytoid Dendritic Cell
TAP	Transactivator Associated with Antigen Processing
RAG	Recombination-Activating Gene
RSS	Recombination Seignal Sequences
SLO	Secondary Lymphoid Organs
NLT	Non-Lymphoid Tissue
T_r	Regulatory T cell
cT_r	Centeral Regulatory T Cell
eT_r	Effector Regulatory T cell
STAT	Singal Transducer and Activator of Transcription
DTx	Diphtheria Toxin
PTx	Pertussus Toxin

Chapter 1:

Introduction

The immune system

The advent of multicellularity ~800 million years ago provided a niche for unicellular organisms to exploit as a source of sustenance and safe-haven. Therefore, the ability to identify and expel intruding pathogens represented a critical step in the evolution of complex organisms and today represents a universal feature of multicellular life (1). Principally, this is accomplished through the ability of an organism to distinguish its own cells and cellular components from those which don't naturally occur within that organism; a concept referred to as "self / non-self-recognition". The molecules, cells, and organs which participate in this critical distinction collectively encompass the immune system. Within vertebrates, two functionally distinct but overlapping branches – termed innate and adaptive immunity – work in unison to provide protection and immunologic memory against pathogens and malignancies.

Innate immunity

The innate immune system, so named because it is inherited and functional at birth, denotes a general strategy utilized by all metazoans to recognize conserved properties of the microbes that infect them. Innate immune recognition is embodied by the prototypic example of pathogen associated molecular pattern (PAMP) recognition by Toll-like receptors (TLRs). PAMPS encompass a broad range of carbohydrates, lipids, and proteins expressed on all microorganisms that are indispensable to their function but absent within the organisms they infect (2). Instead, the host organism expresses a series of invariable pattern-recognition receptors (amongst other receptors) termed TLRs, each recognizing a distinct "flavor" of PAMP. Thus, PAMP engagement by TLRs signifies a microbial presence and activates innate immune mechanisms to destroy PAMP-containing entities. The cellular mediators of the innate immune system are predominantly "myeloid" cells and can be broadly sub-divided into two categories;

granulocytes – which stockpile granules of anti-microbial molecules and effector proteins they secrete into the extracellular environment once activated for the destruction of pathogens, and phagocytes – which ingest and degrade large proteins and whole microbes. Given their abundance in barrier tissues, low activation threshold, and abrupt effector functions, innate immunity represents the first-line of defense within the vertebrate immune system (the exclusive defense strategy in invertebrates) and is typically sufficient to thwart host colonization by pathogenic microbes.

Adaptive immunity

Adaptive immunity is thought to have arisen around the time of the Cambrian explosion (~500 million years ago) in cartilaginous fish (3, 4) and provides a second-line defense against pathogenic organisms which escape detection or overwhelm the innate immune system. Transcending a reliance on invariant pattern-recognition receptors to discriminate self / non-self, adaptive immunity utilizes novel antigen receptors generated *in situ* to distinguish a virtual universe of foreign molecules (5). Antigen receptor diversity is accomplished through the random shuffling, like cards in a deck, of inherited receptor gene segments; generating a unique and exclusively expressed cell-surface antigen receptor. Adaptive immune cells which encounter antigens matching their receptor undergo extensive clonal expansion, generating an army of antigen-specific effector cells to control an infection. However, in contrast to the rapidity of the innate immune response, recognition and clonal expansion of adaptive immune cells develops over several days accounting for the lag between symptoms of infection and resolution. Advantageously, following pathogen clearance, a small number of antigen-specific cells survive within secondary lymphoid and non-lymphoid tissues indefinitely – poised to rapidly (hours) respond to secondary re-challenge. This latent reservoir of adaptive immune cells capable of rapidly eliminating previously-encountered pathogens is the defining feature of

immunologic “memory” and the basis of vaccination. Adaptive immune cells, or “lymphocytes” as they’re commonly referred, encompass B and T cells of varying subsets. Generally, B cells secrete vast quantities of antibodies (soluble B cell receptors) which aid in the immobilization, neutralization, and opsonization of pathogens for destruction by the innate immune system. T cells come in two main sub-classes; $CD8^+$ T cells which are directly cytolytic, and $CD4^+$ T cells which produce immune-instructive cytokines to synergize innate and adaptive immune responses once activated. However, unlike B cells which can recognize native antigen, antigen recognition by T cells requires cognate-antigen be “presented” on specialized platform molecules called major histocompatibility complex (MHC). In the case of $CD4^+$ T cells, antigen presentation is accomplished almost-exclusively by a specialized group of innate immune cells called dendritic cells.

A brief history of dendritic cells

In 1868, a 21 year old German undergraduate named Paul Langerhans identified a novel population “of branched skin cells resembling neurons” while analyzing human skin for an open competition organized by Berlin University (6). The origin and function of these eponymous “Langerhans cell” eluded dermatologists for the next century until the 1973 discovery by Ralph Steinmann and Zanvil Cohn of a similar population of rare cells in mouse spleen possessing a unique stellate morphology and tissue distribution (7, 8). Given their observed network of extensively branching pseudopods, Steinmann and Cohn supposed the term “dendritic” cell – after the Greek word for tree (Dendron) – was a fitting name. For the seminal discovery of dendritic cells (DCs) and the subsequent elucidation of their unique ability to capture antigen and initiate T cell-mediated immunity, Ralph Steinman was awarded the Nobel Prize in Physiology or Medicine in 2011. In the decades following their discovery, Steinman’s DCs were observed in nearly all non-lymphoid tissues including the skin (where

Langerhans had mistakenly identified them as nerve cells) where they were shown to represent a functionally unified collection of antigen presenting cells (APCs) capable of transporting antigen from peripheral tissues into T cell zones of draining lymph nodes for the initiation of adaptive immune responses (9–12). DCs were 30-50% more efficient than unfractionated splenocytes (~60-70% $MHCII^+$ B cells) at activating T cells in a mixed lymphocyte reaction (11, 13) and could efficiently prime T cells independently of exogenous stimulus – establishing their reputation as "nature's adjuvants" (14). These early studies helped define the paradigm of DCs as sentinel cells which survey tissues for the presence of pathogen or malignancy and orchestrate T cell-mediated immune responses once activated (14–16).

Dendritic cells: At the intersection of innate and adaptive immunity

The T cell receptor (TCR) recognizes peptide-antigen solely in the context of MHC molecules presented in *trans*; an additional level of complexity in receptor activation reflecting the need to avoid aberrant T cell activation (17). Peptide-fragments derived from actively translated proteins are presented on MHC-class I (MHCI) by all nucleated cells for presentation to cytotoxic $CD8^+$ T cells. Therefore, MHCI-mediated antigen presentation serves to alert $CD8^+$ T cells to the presence of viral infection (which utilize host transcription machinery for propagation) or cellular transformation (expressing mutated proteins). Recognition of cognate-antigen by $CD8^+$ T cells results in direct cytolysis of the antigen-bearing cell; allowing $CD8^+$ T cells to rapidly and autonomously eliminate malignant cells or those harboring intracellular infection. However, not all pathogenic microbes reside intracellularly where their growth and dissemination can be controlled by cytolysis of infected host cells. Rather, helper $CD4^+$ T cells are required to synchronize innate and adaptive immune responses necessary for the clearance of diverse intra- and extracellular pathogens including bacteria, protozoa, helminths, and fungi.

So named because they “help” orchestrate immune responses through the secretion of instructional effector cytokines, $CD4^+$ T cells are activated by peptide-fragments presented in the context of MHC-class II (MHCII) expressed by professional APCs. Ostensibly encompassing B cells, macrophages, and DCs, professional APCs acquire exogenous antigen through phagocytosis and macropinocytosis (18) and are thus capable of presenting activating peptides to $CD4^+$ T cells without harboring intracellular infection. However, in contrast to DCs, macrophages and B cells are insufficient to prime naïve $CD4^+$ T cells at steady-state and will not be discussed in further detail here (19–21). Instead, their sentinel position and capacity for antigen transport endows DCs with the extraordinary ability to activate and differentiate exceedingly rare antigen-specific $CD4^+$ T cells *in vivo*. Such differentiation requires three synergizing signals be delivered successively to a naïve $CD4^+$ T cells by an activating DC (22, 23). TCR recognition of cognate-antigen on MHCII provides “signal 1” while co-stimulatory receptor engagement by $CD4^+$ T cells with co-stimulatory ligands constitutively expressed on the DCs (and enhanced upon PAMP recognition) provides “signal 2”. While integral for $CD4^+$ T cell differentiation, signals 1 and 2 convey no qualitative information about the type of $CD4^+$ T cell help needed for clearance of a given infection. Instead, it is the secretion of a defined set of T cell-polarizing cytokines by the DC – termed “signal 3” – that instructs naïve $CD4^+$ T cells to adopt one of several well-defined helper T cell identities (T_h1, T_h2, T_h17, Tf_h, T_r) each optimized for a specific type of immune response (i.e. fungal) (24, 25). Importantly, the selection of cytokines delivered as signal 3 are determined downstream of innate-immune sensing mechanisms predominantly within the activating DC. Thus, the integration of environmental and inflammatory cues by DCs directs the outcome of naïve $CD4^+$ T cell differentiation and the ensuing adaptive immune response, placing DCs at the fulcrum of innate and adaptive immunity. Phenotypic, spatial, and functionally heterogeneity amongst DCs can also influence

their ability to differentially modulate T cell responses through their propensity to release varying "signal 3" cytokines.

Immune recognition in the spleen

Unlike most secondary lymphoid structures which receive antigen from peripheral tissues by way of afferent lymphatics (26), systemic blood-borne antigens are presented to T cells within the spleen (27). Partitioned into two functionally distinct anatomical regions, the splenic red pulp (RP) consists primarily of phagocytic cells which degrade ageing RBCs, while the white pulp (WP) encompasses numerous organized follicles of T and B lymphocytes architecturally resembling lymph nodes (LN). Segregating the splenic RP and WP is the marginal zone (MZ) –a dense network of reticular cells and extracellular matrix impermeable to the passive diffusion of large molecular weight proteins (28). However, specialized conduits called marginal zone bridging channels (MZBC) intersect the MZ, spanning the distance between the splenic RP and WP (29–31). At steady-state, these channels are occupied by aggregates of DCs which are thought to capture particulate blood antigens for transport into the WP while simultaneously occluding the stochastic ingress of native antigens (27, 30). Conceptually, MZBCs are therefore functionally analogous to afferent lymphatics; facilitating the transport of "peripheral antigens" (taken up in the RP/MZ) into organized lymphoid follicles (splenic WP) for the initiation of adaptive immune responses. Splenic DCs which facilitate antigen transport can similarly be considered "migratory". Thus, despite obvious differences in the tissues and organs they survey, the initiation of adaptive immunity is remarkably similar between the LNs and spleen.

DC subsets in the spleen

Originally identified by uniform expression of MHCII and the absence of CD3 (T cells), CD19/20 (B cells), CD14 (monocytes), and CD56/57 (NK) cells (14), the development of the N418 antibody against CD11c provided a fundamental tool for identifying novel DC subsets in myriad tissues (32). However given their relative paucity in non-lymphoid tissues at steady-state, splenic DCs remain the best-characterized in terms of ontogeny and function. It is now appreciated three ontologically and functionally distinct DC lineages exist in the spleen; termed classical DCs (cDCs), monocyte-derived DCs (Mo-DCs) and plasmacytoid DCs (pDCs) (16, 33). cDC are the most abundant DC lineage within the spleen at steady-state and encompass two distinct subsets possessing the differential ability to prime $CD4^+$ and $CD8^+$ T cell responses through classical- and cross-presentation pathways, respectively. In contrast to cDCs, Mo-DCs ($MHCII^{hi}$ $CD11c^{hi}$ $CD4^{lo/neg}$ $CD8\alpha^{lo/neg}$ $CD11b^{hi}$ $Ly6C^{+/-}$) DCs are exceedingly rare at steady-state but expand under inflammatory conditions where they coordinate innate antimicrobial responses through the release of TNFα and iNOS (34). pDCs ($MHCII^{int}$ $CD11c^{int}$ $B220^+$ $PDCA\text{-}1^+$) morphologically resemble plasma cells and secrete vast quantities of type-1 interferons upon TLR7/9 activation (35). However, in comparison to their cDC counterparts, Mo-DCs and pDCs lack efficient APC function and will not be considered here further.

Phenotypic and functional diversity of splenic cDCs

cDC heterogeneity was first reported in the mid-1990s when it was shown DCs could be categorized into two functionally distinct subsets based on expression of the T cell co-receptors CD4 and CD8α (36, 37). CD4 expression coincided with those DCs which bound the DC-specific monoclonal antibody 33D1 (developed in Ralph Steinmann's lab in the early 1980s) (38) and were poised to initiate $CD4^+$ T cell responses through classical antigen-presentation pathways, while $CD8\alpha^+$ DCs were subsequently shown to possess the additional ability cross-

present antigens (a mechanism discussed in more detail later) to $CD8^+$ T cells. Despite their 20+ year history as DC lineage markers, the function of CD4 and CD8α on DCs remains an open question and will be the focus of chapter 4 of this dissertation. Complicating matters, we have independently observed lability of CD4 and CD8α expression on activated DCs, demonstrating these markers are unreliable for delineating DC subsets under inflammatory conditions. We have obviated this issue by instead employing 33D1 and CD11b as our principal DC subset-defining markers.

33D1$^-$ CD11b^{lo} (CD8α) DCs

DCs expressing CD8α (but lacking CD8β) comprise 20-30% of total MHCII^{hi} CD11c^{hi} DCs in the spleen at steady-state (39) where they share a unique dependence on the transcription factors Id2, IRF8, and BATf3, as well as Flt3L signaling for their development and survival (40, 41). Classically referred to as "CD8α DCs", these cells are uniformly low for CD11b, lack expression of 33D1 (DCIR2), and have recently shown to express the chemokine receptor XCR1 (27). Moreover, 33D1$^-$ CD11b^{lo} DCs are predominantly expanded after provision of exogenous Flt3L-expressing tumors and selectively ablated in BATf3$^{-/-}$ mice (42). We therefore use negative expression of 33D1 and CD11b to define "CD8α" DCs, despite heterogeneity of CD8α expression on these cells. 33D1$^-$ CD11b^{lo} DCs have been shown to prime T_h1 responses through their propensity to secrete the signal 3 cytokine Il-12, and are thus critical for the control of intracellular parasites (42–45). However, the defining feature of 33D1$^-$ CD11b^{lo} DCs is their ability to cross-present exogenous antigens to $CD8^+$ T cells on MHCI (46).

Conventional antigen presentation involves the processing and presentation by all nucleated cells of endogenous (internal) antigens on MHCI to $CD8^+$ T cells, or exogenous (acquired) antigens to $CD4^+$ T cells on MHCII by professional APCs. However a third form of antigen presentation exists, termed cross-presentation, in which exogenous antigens are

presented on MHCI to $CD8^+$ T cells. Cross-presentation is thought to subvert immune evasion strategies of intracellular pathogens or transformed cells which block conventional MHCI-mediated antigen presentation in order to protect their host cells from $CD8^+$ T cell-mediated cytolysis (47, 48). Therefore, cross-presentation allows APCs to activate $CD8^+$ T cells in the absence of intracellular infection. $33D1^-$ $CD11b^{lo}$ DCs are poised to cross-present antigens due to constitutively high expression of proteasome components, transporter associated with antigen processing (TAP), and endoplasmic reticulum chaperone proteins which favor the loading of exogenous peptide-fragments onto MHCI (49). Indeed, $Batf3^{-/-}$ mice specifically lacking $33D1^-$ $CD11b^{lo}$ DCs are susceptible to intracellular pathogens including viral infections, and fail to clear highly immunogenic syngeneic tumors, reflecting impaired cytotoxic T cell immunity (42, 43, 50). Additionally $33D1^-$ $CD11b^{lo}$ DCs express high levels of MHCII (a hallmark of cDCs) and can activate $CD4^+$ T cells *in vitro*, albeit with less efficiency than $33D1^+$ DCs (independent observation). The c-type lectin receptors DEC205 and Clec9A are distinctly expressed on $33D1^-$ $CD11b^{int}$ DCs (51) and the former has been used to experimentally deliver antigens to this subset by way of α-DEC205 antibodies (49). Such experiments have revealed $DEC205^+$ DCs ($33D1^-$ $CD11b^{lo}$) are 10-times less efficient at driving antigen-specific $CD4^+$ T cell proliferation compared to their $33D1^+$ counterparts *in vivo*. More recent studies have revealed this disparity in the ability of $33D1^-$ $CD11b^{lo}$ DCs to activated $CD4^+$ T cells *in vivo* vs *in vitro* reflects differential steady-state co-localization of $CD4^+$ and $CD8^+$ T cells with cDC subsets in the spleen (27). $33D1^-$ $CD11b^{lo}$ DCs preferentially occupy regions of the spenic WP enriched in $CD8^+$ T cells at steady-state, and $XCR1^+$ ($33D1^-$ $CD11b^{lo}$) DCs residing outside the WP migrate into these regions once activated. Thus, $33D1^-$ $CD11b^{lo}$ DCs are functionally specialized to defend against viral and intracellular pathogens via their ability to co-localize with, and cross-present antigens to, $CD8^+$ T cells.

33D1^{+} CD11b^{int} (CD4) DCs

33D1^{+} CD11b^{int} DCs account for the majority (55-70%) of cDCs in the spleen at steady-state and are superior activators of CD4^{+} T cells through their ability to classically-present antigen on MHCII. Development of these DCs requires the transcription factor IRF4 and signaling downstream of NOTCH2 and GM-CSF (41, 52–54). Unlike their 33D1^{-} CD11b^{lo} DC counterparts which are specialized for cross-presentation on MHCI, 33D1^{+} CD11b^{int} DCs are highly enriched in cathepsins; proteases activated by the low pH environment of lysosomes where they serve to degrade phagocytosed material – specializing 33D1^{+} CD11b^{int} DCs for exogenous antigen presentation on MHCII (49). Furthermore, their steady-state localization within MZBCs juxtaposes them with blood-borne antigens they sample and subsequent present to CD4^{+} T cells (30, 31, 55). *In vivo* activation precipitates extensive re-localization of 33D1^{+} CD11b^{int} DCs from MZBCs into peripheral regions of the splenic WP enriched in CD4^{+} T cells. Therefore, 33D1^{+} CD11b^{int} DCs also co-localize with the T cell lineage they preferentially activate (27). However, in opposition to 33D1^{-} CD11b^{int} DCs which orchestrate T_h1 immunity, 33D1^{+} CD11b^{int} DCs prime T_h2 responses through their secretion of the “signal 3” cytokine Il-4, making them indispensable for the clearance of helminth infections. Additionally, through their ability to differentially secrete TGFβ, IL-6, and IL-23, 33D1^{+} CD11b^{int} DCs are poised to commit naïve CD4^{+} T cells towards a T_h17, T_{fh}, or T_r lineage (56–58). 33D1^{+} CD11b^{int} DCs therefore serve a dichotomous role – orchestrating the multifaceted immune responses necessary to eradicate complex extracellular pathogens while simultaneously limiting excessive immunopathology and autoimmunity through their ability to promote T_r cell differentiation and survival. T_r cell maintenance is further reinforced by the ability of 33D1^{+} CD11b^{int} DCs to stimulate paracrine T_r cell-supportive IL-2 production from conventional CD4^{+} T cells (49).

Regulatory T cells in the maintenance of self-tolerance

Mcfarlane Burnet's clonal selection theory proposed in 1959 provided an elegant Darwinian model for the immune system's extraordinary ability to defend against foreign pathogens of astronomical variety and complexity (59). Conceptually, rare pathogen-specific immune cells expressing unique antigen receptors and present before infection would expand – under selection pressures provided by the pathogen – to generate immunodominant "clones" sufficient for protective immunity. Yet, Burnet recognized such pre-immune receptor diversity required "….at some stage in early embryonic development a genetic process for which there is no available precedent". A theoretical basis for such diversity came in 1965 when Dreyer and Bennett proposed germ-line encoded DNA might be randomly reordered to generate novel gene sequences encoding diverse antigen receptors (60). However, it took advances in molecular cloning over the ensuing decade before the first demonstrative evidence for DNA rearrangement was reported by Hozumi and Tonegawa through antibody light chain DNA sequence disparities between mature B cells and their embryonic precursors (61, 62). These observations suggested series of linearly interspersed genes might be randomly combined to generate novel and functional antigen receptors. This notion was a further supported by the subsequent identification of recombination signal sequences (RSS) flanking antigen receptor variable, diversity, and joining gene segments, marking them for rearrangement (63, 64). The 1989 discovery of recombination activating gene (RAG) by David Baltimore's lab provided an enzymatic basis for DNA recombination (65, 66)– recognition and ligation of discrete RSS-flanked variable (V), diversity (D) and joining (J) gene segments – and thus a precedent for the pre-immune receptor diversity Burnet's clonal selection theory demanded.

While the mechanisms behind VDJ recombination were largely identified in B cells, T cells identically utilize RAG-mediated recombination to generate novel TCRs. However, given that T cells recognize short peptide fragments, their potential range of antigen recognition is

considerably larger than B cells at 10^{15}-10^{20} distinct epitopes (67). The actual number of T cell clonotypes (T cells bearing a unique TCR) in an adult human is closer to 10^{13} reflecting the stringency of T cell development on TCR specificity. This development process occurs in the thymus where T cells are scrutinized on their ability to recognize peptide-MHC complexes (positive selection) without cross-reactivity to self-antigens (negative selection) (68). Only ~5% of thymocytes successfully navigate thmyic selection to become peripheral mature-naïve T cells (69). However, a few auto-reactive clones inevitably escape negative selection and enter the periphery where they can clonally expand to coordinate destructive immune responses against host tissues and organs if left unregulated. To safe-guard against these rare self-reactive T cells, a parallel mechanism has evolved by which moderately self-reactive T cell clones seed the periphery and suppress aberrant immune responses to self.

<u>T_r cells: Function and heterogeneity</u>

The existence of a dedicated immune-suppressive cell was confirmed in 1995 when Sakaguchi et al. demonstrated day-three thymectomized mice developed multi-organ autoimmunity which could be reversed by transferring $CD4^+$ T cells expressing the high-affinity IL-2 receptor CD25 (IL2RA) (70). The subsequent identification that *scurfy* mice and IPEX patients (which developed congenital autoimmunity) uniformly lacked $CD4^+$ $CD25^+$ T cells, precipitated by mutations in the *Foxp3* gene, solidified $CD4^+$ $Foxp3^+$ T cells as this dedicated suppressive cell now collectively referred to as regulatory T cells (T_r cells) (71–73). While originally thought to represent a homogeneous group of immunosuppressive cells, work from our lab and others over the past decade has revealed considerable phenotypic and functional heterogeneity exists amongst T_r cells (74–76). Broadly, T_r cells can be subdivided based on localization within non-lymphoid tissues (NLT) or secondary lymphoid organs (SLO) where they display a unique and respective dependence on TCR/co-stimulatory engagement and IL-2

signaling for their survival and function. Within T cell zones of SLOs, paracrine IL-2 signaling maintains T_r cells independently of TCR through its ability to activate T_r cell pro-survival genes (77). In contrast, T_r cells occupying NLT or extra-follicular regions of SLO require constitutive TCR signaling for their survival and function (76). Here, apoptosis resulting from lack of IL-2 signaling is counterbalanced by constitutively high rates of TCR/co-stimulation-dependent proliferation. Therefore, T_r cells accumulate in tissues containing self-antigens for which they are specific for. This variable dependence on IL-2 signaling and TCR/co-stimulation can be exploited to phenotypically distinguish T_r cell subsets in various tissues. Actively proliferating ($Ki67^{hi}$) T_r cells encompass TCR-dependent T_r cells whereas non-proliferative ($Ki67^{lo}$) T_r cells represent IL-2-dependent T_r cells which can be further divided into those receiving active IL-2 signaling by phosphorylation of STAT5 (78). Importantly, T_r cells are incapable of IL-2 production due to transcriptional repression at the IL-2 locus by Foxp3 and are therefore dependent on paracrine sources of IL-2 for their survival. Thus, unlike NLT and extra-follicular resident T_r cells which directly express the molecules involved in their survival, IL-2-dependent T_r cells are supported by paracrine IL-2 signaling downstream of cellular and molecular interactions which are largely T_r cell-extrinsic. Conventional T cells do not spontaneously secrete IL-2, but rather are prompted to do so by APCs bearing cognate antigen. Therefore, it has been predicted that a circuit must exist by which APCs stimulate paracrine IL-2 release from conventional T cells in order to maintain neighboring IL-2-dependent T_r cells.

DC and T_r cell abundance are intimately linked

DC and T_r cell abundance were recently shown to be linked in a homeostatic loop: Reduced DC abundance in $Flt3L^{-/-}$ or α-Flt3L antibody-treated mice correlates with a loss of T_r cell frequencies, whereas DC expansion through provision of rmFlt3L or Flt3L-expressing tumors augments T_r cell abundance in a MHCII-dependent manner (79). Prolonged DC

depletion (in CD11c-DTR→WT chimeras) results in expansion and enhanced effector functions of T_h1 and T_h17 cells attributed to the reduction in T_r cell abundance. In contrast, expansion of endogenous DCs was shown to protect against $T_h1/17$-mediated auto-immunity in a T_r cell-dependent manner. It was simultaneously reported constitutive DC ablation caused lethal $T_h1/17$-mediated autoimmunity suggesting DCs were required for self-tolerance (80). While T_r cell abundance was not overtly affected in mice constitutively lacking DCs, it remained possible a specific DC-dependent T_r cells subset (i.e IL-2-dependent T_r cells) were critically absent in these mice, allowing B cells or macrophages to compensatorily activate $CD4^+$ T cells driving autoimmunity. The role of individual DC subset in the maintenance of T_r cells was not evaluated in these studies.

Summary and unanswered questions

Phenotypically and functionally diverse regulatory 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 NLTs depend on continual TCR signaling for their survival and function, phenotypically naïve T_r cells occupying 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. The link between DC and T_r cell abundance and the observations of autoimmunity in mice lacking DCs suggests DCs play an active role in the maintenance of self-tolerance through their ability to support T_r cells. However, given the phenotypic and functional heterogeneity that exists amongst T_r cells and DCs alike, self-tolerance is likely governed by unique DC/T_r cell interactions. **Chapter 3** will focus on determining the contribution of various splenic DC subsets in the maintenance of IL-2-

dependent T_r cells, the molecules they utilize to perform this function, and the antigens precipitating homeostatic IL-2 release. **Chapter 4** will summarize ongoing work elucidating a functional role for DC-intrinsic CD4 expression, a unique but undefined feature of $33D1^+$ DCs dynamically regulated upon DC activation in a cell-intrinsic manner.

Chapter 2:

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, MyD88$^{-/-}$ 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), S. Zeigler, 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 β2M$^{-/-}$ 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, or positively-selected $CD11c^+$ cells, were adoptively transferred into CD11c-DTR-Tg mice retro-orbitally (R.O.) directly following the initital 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 R.O. 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.

TLR agonist and ovalbumin injections: For in vivo DC activation experiments, mice were injected R.O. with either 10 ug/mouse LPS (vendor) or 25 ug/mouse R848 (vendor) in 200 ul PBS. Mice were immunized with OVAp (sequence) at a dose of 2 ug/mouse in 200 ul PBS via R.O. injection one day prior to the adoptive transfer of OVAp-specific $CD4^{+}$ T cells (OT-II).

Cell enumeration: Lymphocyte numbers were determined using Polybead polystyrene nonfluorescent microspheres (15 um, Polysciences, Inc.). Polybeads were resuspended in PBS (typically one drop Polybeads in 1 ml PBS) and the bead concentration was determined on a hemocytometer. A volume of stained cell suspension was added to an equal volume of bead suspension and samples were run on a flow cytometer. Beads could be easily distinguished from lymphocytes by their prominent side-scatter (log scale). The ratio of lymphocyte gate events (n_L) to bead gate events (n_B) was determined and used to calculate the concentration (C) of the original cell suspension as follows: $C = (n_L / n_B) * C_B$.

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.

Chapter 3:

A 33D1$^+$ DC / Auto-reactive CD4$^+$ T cell Circuit Maintains IL-2-dependent Regulatory T cells in the Spleen

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 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 (81, 82). 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 (71, 83).

Like phenotypically and functionally diverse effector T cells, T_r cell subsets exist in different tissues with unique homeostatic maintenance requirements (74, 75). 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) (84, 85), maintenance of T_r cells resident in non-lymphoid tissues is largely IL-2-independent, and distinct signals including TCR signaling (76), ICOS-mediated co-stimulation (86, 87), and IL-7 (88, 89), 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 (90, 91), 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.

RESULTS

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

IL-$2^{-/-}$, IL-2R$\alpha^{-/-}$ and IL-2R$\beta^{-/-}$ 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 (70, 92–95). 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 (77), 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 (96, 97), multiple sclerosis (98, 99), juvenile idiopathic arthritis (100), and systemic lupus erythematosus (101).

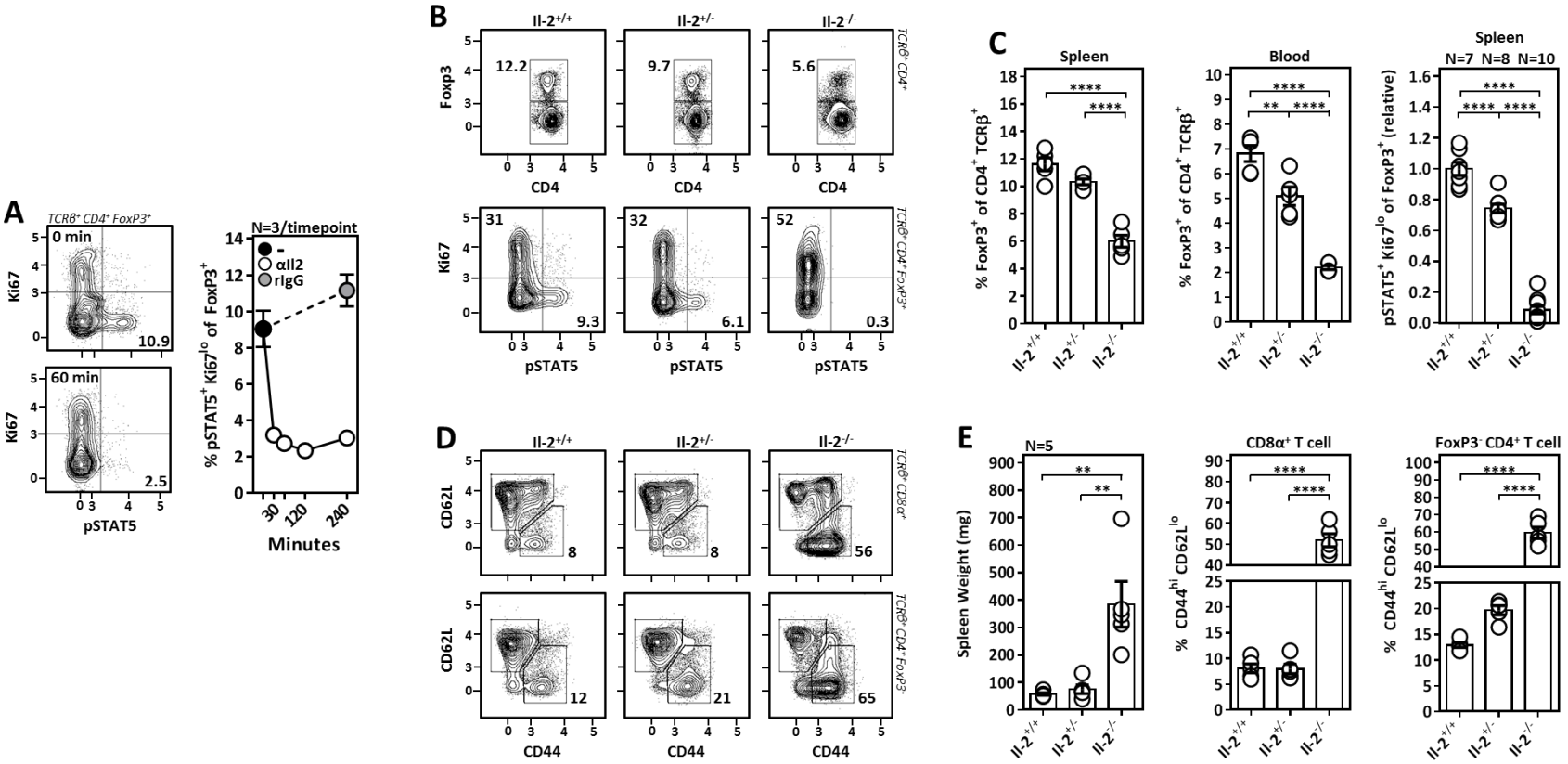


Figure 1. T_r cells compete for a limiting supply of Il-2: (A) Rat IgG or Il-2 blocking antibody (S4B6-1) was administered to mice I.V. at the indicated time-points prior to sacrifice to assess Il-2 activation kinetics in splenic T_r cells by phosphorylation of STAT5 (pSTAT5). (B and D) Representative 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.

$CD4^+$ 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 (102), 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-$2^{-/-}$ donor cells whereas other T cell populations derived from the $CD4^{-/-}$ donor cells remained IL-2-sufficient. By contrast, WT:IL-$2^{-/-}$ 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-$2^{-/-}$ 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-$2^{-/-}$ mixed chimeras compared to WT:IL-$2^{-/-}$ controls (Fig. 2E). Furthermore, those T_r cells recovered were absent of pSTAT5 to a degree reminiscent of IL-$2^{-/-}$ 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.

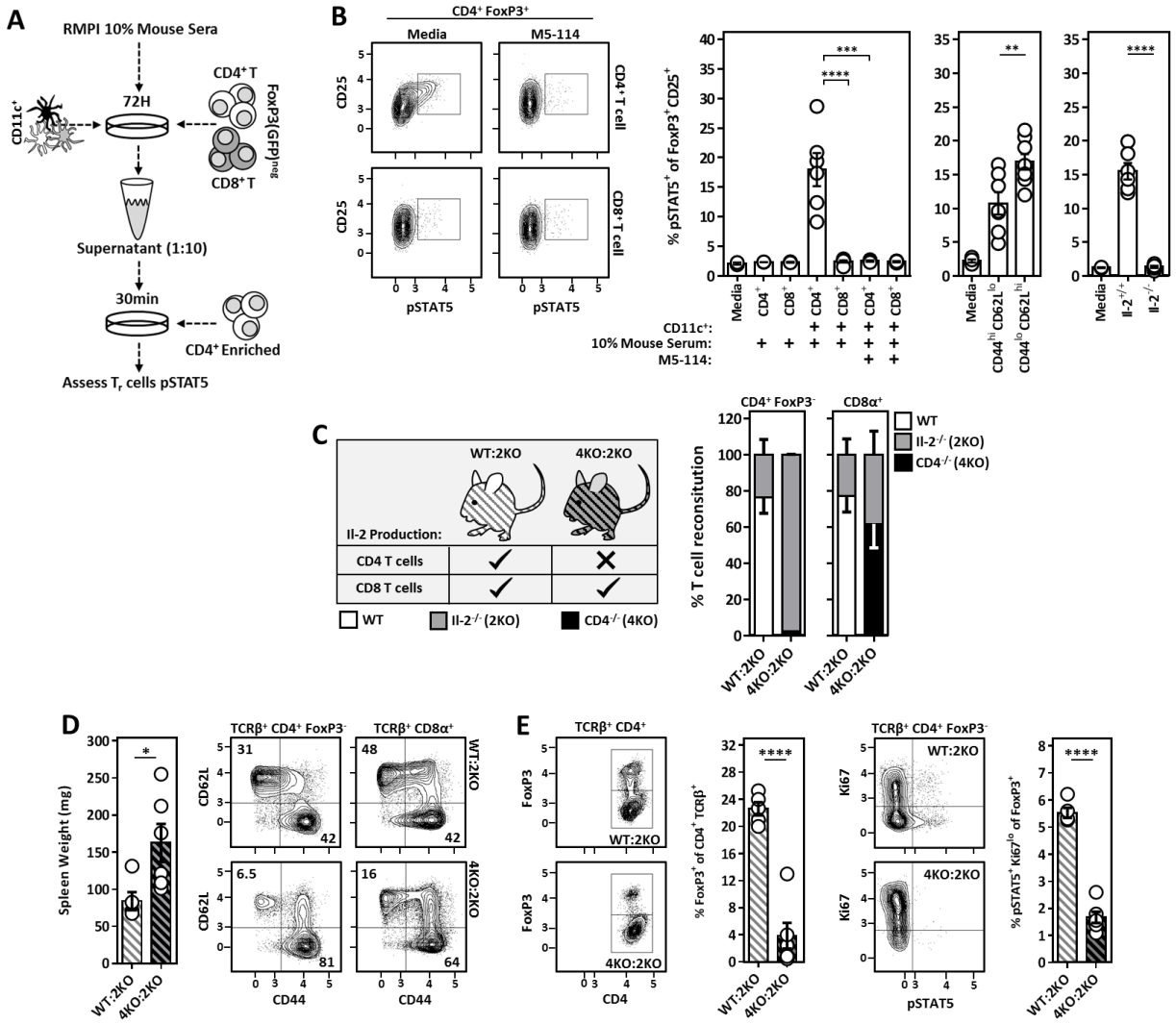


Figure 2. CD4⁺ T cell-derived IL-2 sustains SLO resident T_r cells and prevents the development of spontaneous autoimmunity: (A) Experimental design to assess qualitative IL-2 production in DC/T cell co-cultures via bio-assay. (B) Representative flow cytometry plots (left) and compiled data (right) assessing 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 ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001, as calculated by unpaired students Student's *t*-test (D) or one-way ANOVA with Tukey's post hoc test (B).

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 (79, 103). 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) (104), 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 (78), 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.

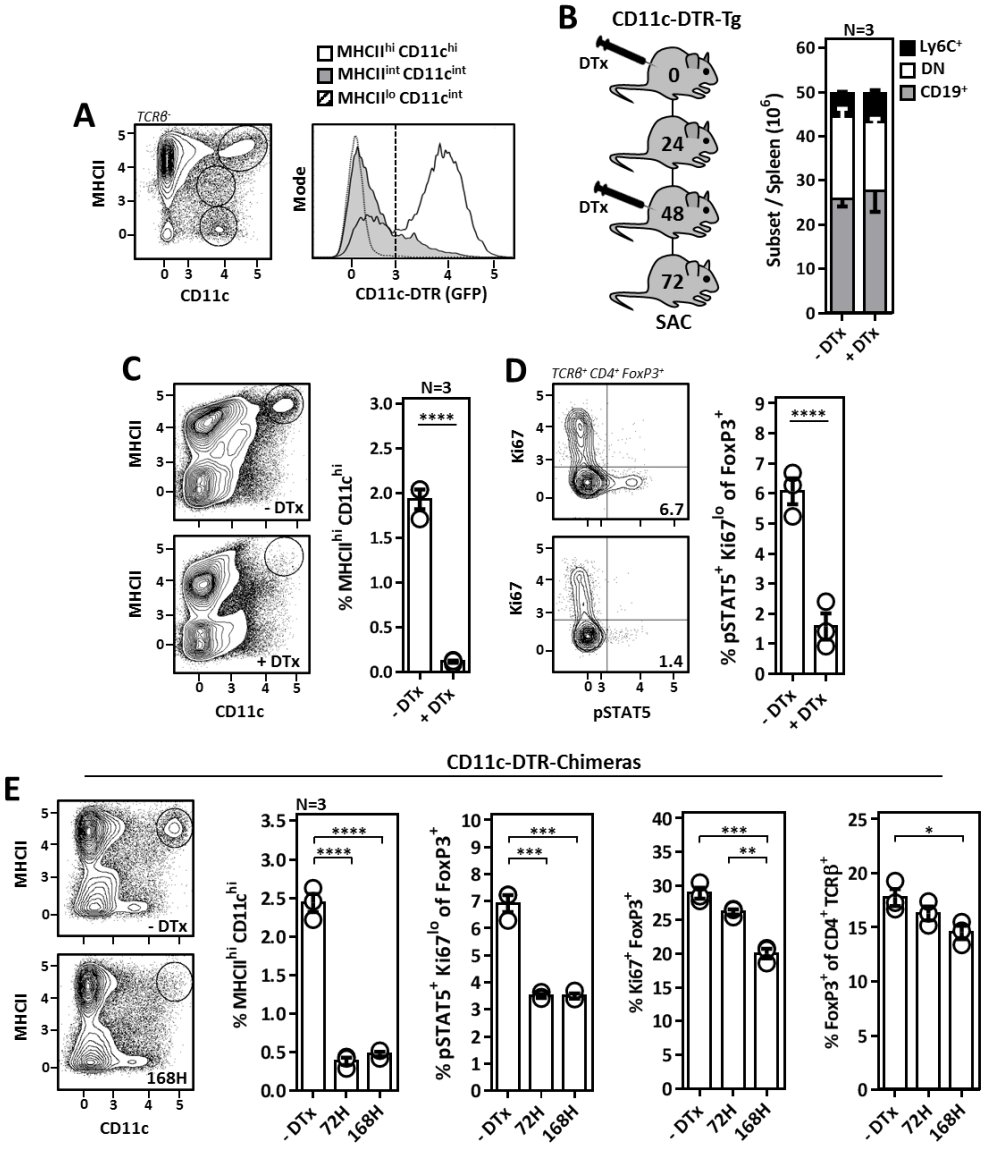


Figure 3. DCs are required for the homeostatic maintenance of IL-2-dependent T_r cells: (A) Flow cytometric analysis of GFP (DTR) expression in various splenic cell populations from CD11c-DTR-Tg mice. (B) Strategy for the elimination of DCs in CD11c-DTR-Tg mice (left) and broad cellular subset composition 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).

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 (37, 105), 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) (38). 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 (49), 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.

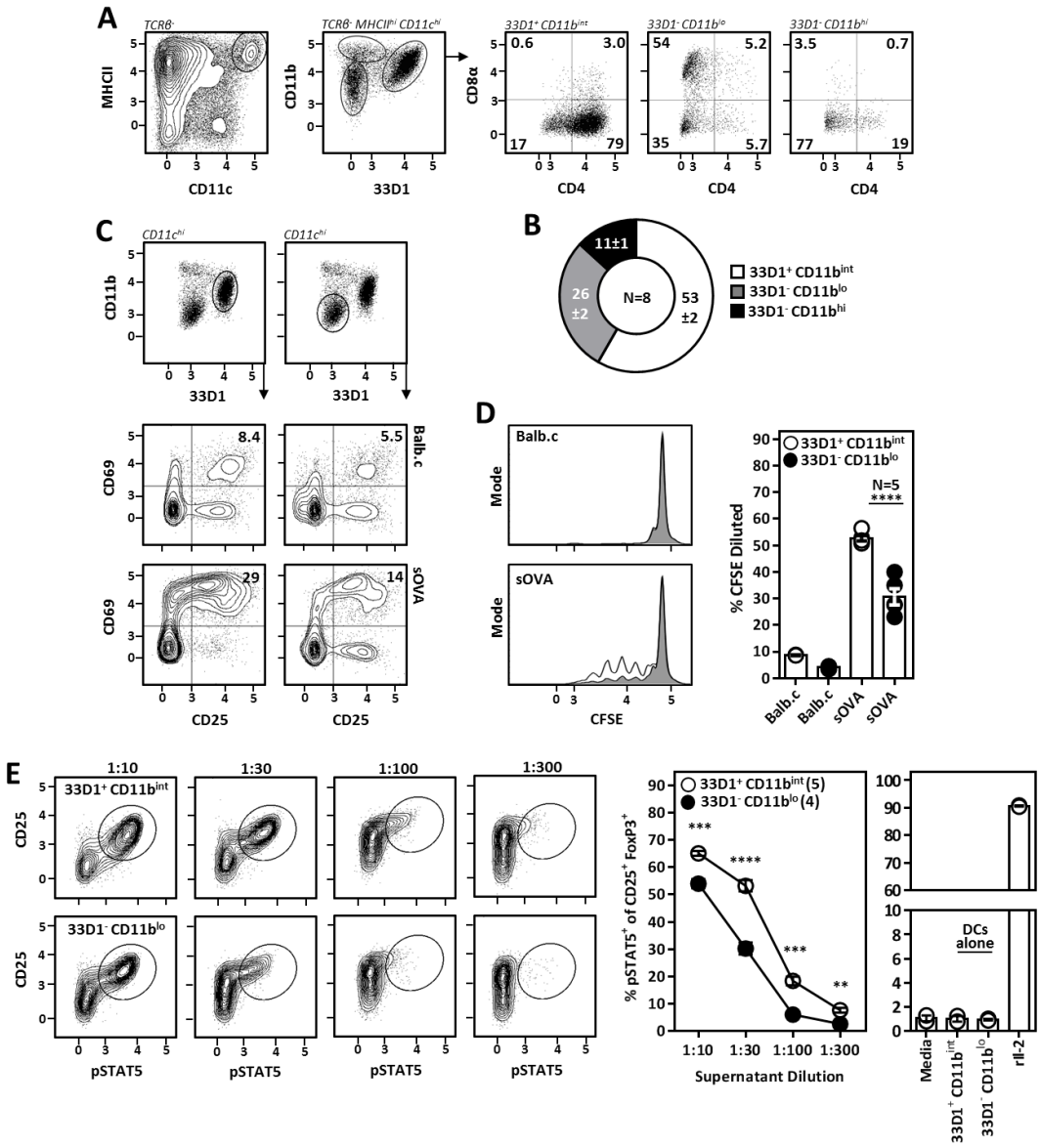


Figure 4. 33D1^{+} CD11b^{int} DCs are potent activators and inducers of IL-2 production from auto-reactive CD4^{+} T cells: (A) Gating strategy used to identify splenic DC subsets and how they relate to canonical "CD4" and "CD8α" DCs. (B) Relative abundance of individual splenic DC subsets identified 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 ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001, as calculated by unpaired students Student's *t*-test

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 (42). 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.

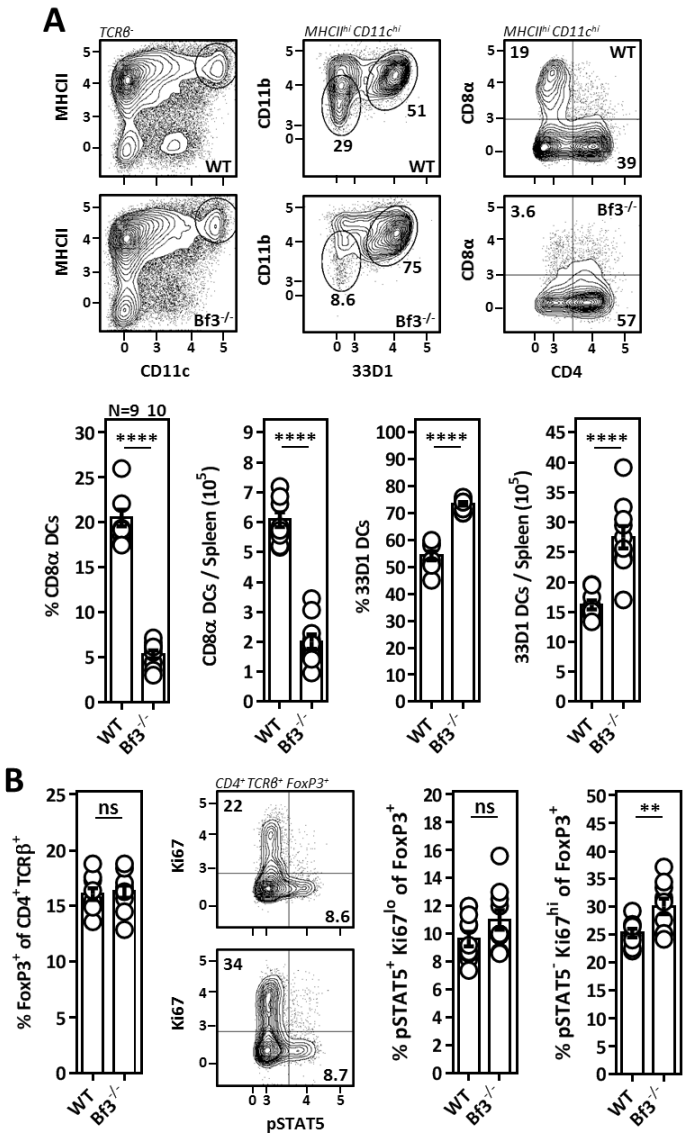


Figure 5. 33D1^{-} CD11b^{lo} (CD8α) DCs are dispensable for the maintenance of Il-2 dependent T_r cells: (A) Representative flow cytometry (top) and compiled data (bottom) identifying splenic DC subsets from 7-8 week old WT or BATf3$^{-/-}$ (Bf3$^{-/-}$) mice. (B) T_r cell frequencies, Il-2 activation (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.

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 (β2M$^{-/-}$) 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).

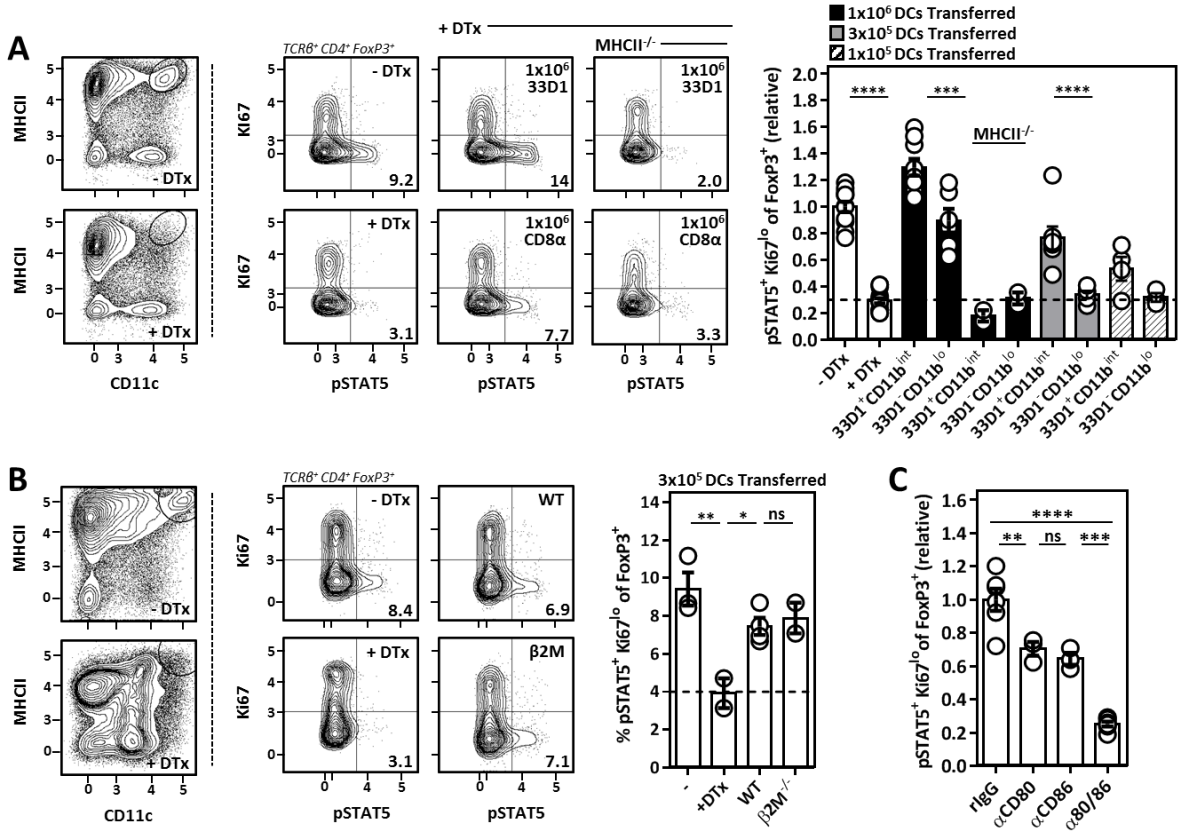


Figure 6. Sufficiency of DC subsets and their molecular requirements for the maintenance of IL-2-dependent T_r cells *in vivo*: (A) Representative flow cytometry and compiled data comparing T_r cell IL-2 activation (pSTAT5) from DC-deplete mice receiving varying amounts of adoptively transferred DCs 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.

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 (29, 55). 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 (30, 31), S1P$_{1\text{-}5}$ (106), and CCR7 (107, 108) 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 (28), 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.

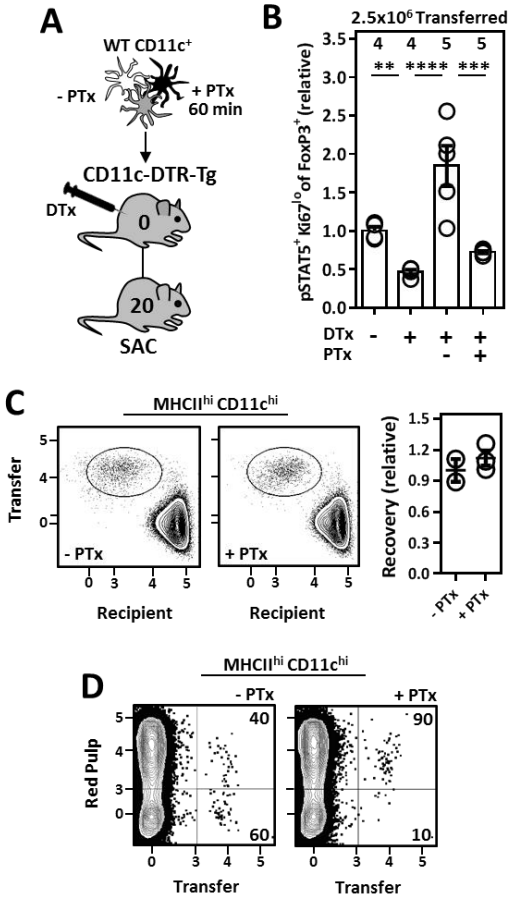


Figure 7. DC access to the splenic white pulp is required for the maintenance of Il-2-dependent T_r cells: (A) Strategy to assess the ability of GPCR-inhibited DCs to rescue Il-2 activation (pSTAT5) in T_r cells from DC-depleted mice. CD11c-enriched cells were incubated for 60 minutes in RP-10 ±1 µg/ml 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

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 (75). 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 (79), 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 (109). 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 (110, 111). 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 (78). 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 (111). 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 (76). 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 (112–114). T_r cell which fail to engage their TCRs undergo programmed cell death, no longer supported by anti-apoptotic signals downstream of IL-2 (77). 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 (80). 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 (115), 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 (116). 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 (117). 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 (102), 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 (55). We posit the redistribution of $33D1^+$ $CD11b^{int}$ DC into T cell zones of the spleen, which has been widely reported upon LPS injection (30, 118), 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 (119). Accordingly, $BATf3^{-/-}$ mice show increased susceptibility to viral infections (43), impaired syngeneic tumor rejection (42), and defective intracellular pathogen clearance (50) – 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 (44, 50). 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.

Chapter 4

DC-intrinsic CD4 Expression Enhances the Function of 33D1$^+$ DCs

"…studies using blocking antibodies or using gene knockout mice have failed to reveal any role for either CD4 or CD8 in DC development or function; I can only conclude that the good Lord put them there to help us delineate these subsets". Ken Shortman - Burnet Oration, 1999 (120)

Introduction

The immunological dogma of MHC-class restriction states helper T cells expressing the co-receptor CD4 perceive antigen in the context of MHC-class II while cytotoxic CD8$^+$ T cells recognize antigen presented on MHC-class I. Paradoxically, murine dendritic cells (DCs) which express high levels of MHC molecules (yet lack recombined antigen receptors) differentially express CD4 and CD8α, and these are in fact used to subset distinct populations of DCs within the spleen (37, 105). Why DCs concurrently express MHC molecules and T cell co-receptors has puzzled immunologists since their discovery on DCs in the mid 1990's, yet to date a functional role for DC-intrinsic CD4 expression has yet to be ascribed. Furthermore, human myeloid cell populations in addition to DCs have more recently been shown to express CD4 (but not CD8) (immgen.org) and therefore a functional understanding of DC-intrinsic CD4 expression in mice may inform its role on human non-T cell subsets. However, several issues have confounded the study of CD4 on DCs *in vivo;* namely the absence of CD4$^+$ T cells (the cells which DCs most directly influence) in CD4-deficient mice. Here, employing a series of transgenic- and chimeric-mouse approaches to standardize the environment in which CD4-sufficient and -deficient DCs operate, we have demonstrated DC-intrinsic CD4 expression is necessary for optimal CD4$^+$ T cell activation in response to exogenous and self-antigens*;* the first functional observation for CD4 on DCs in 20+ years.

Results

33D1^{+} CD11b^{int} DCs in the spleen express CD4 independently of T cells where it is dispensable for DC development and survival:

Mouse DC subsets differentially express CD4 and CD8α where they have historically been used to define DC populations in the spleen (16). Using the myeloid marker CD11b and the monoclonal antibody 33D1 (DCIR2) (38), we identified three distinct cDC subsets in the spleen defined as 33D1^{+} CD11b^{int}, 33D1^{-} CD11b^{lo}, and 33D1^{-} CD11b^{hi}. While largely absent on both 33D1^{-} DC subsets, 33D1^{+} DCs expressed abundant CD4 demonstrating these cells represent canonical "CD4" DCs (Fig. 1. A). To determine if CD4 was essential for the development or homeostatic maintenance of 33D1^{+} DCs, we first assessed their frequencies in WT and CD4$^{-/-}$ mice where we observed identical abundance of all DC subsets. Thus, CD4 is dispensable for the development and survival of DCs (Fig. 1 B). Additionally, RAG$^{-/-}$ mice were shown to express normal levels of CD4 suggesting its expression on 33D1^{+} DCs is cell-intrinsic and not the result of CD4 acquisition from neighboring CD4^{+} T cells (Fig. B). Finally, WT:CD4$^{-/-}$ mixed chimeras were generated to address the role of CD4 in DC development/survival in a competitive setting. Here, where we observed identical reconstitution of WT and CD4$^{-/-}$ 33D1^{+} DCs confirming CD4 is expendable for the development and homeostatic maintenance of 33D1^{+} DCs (Fig. 1 C).

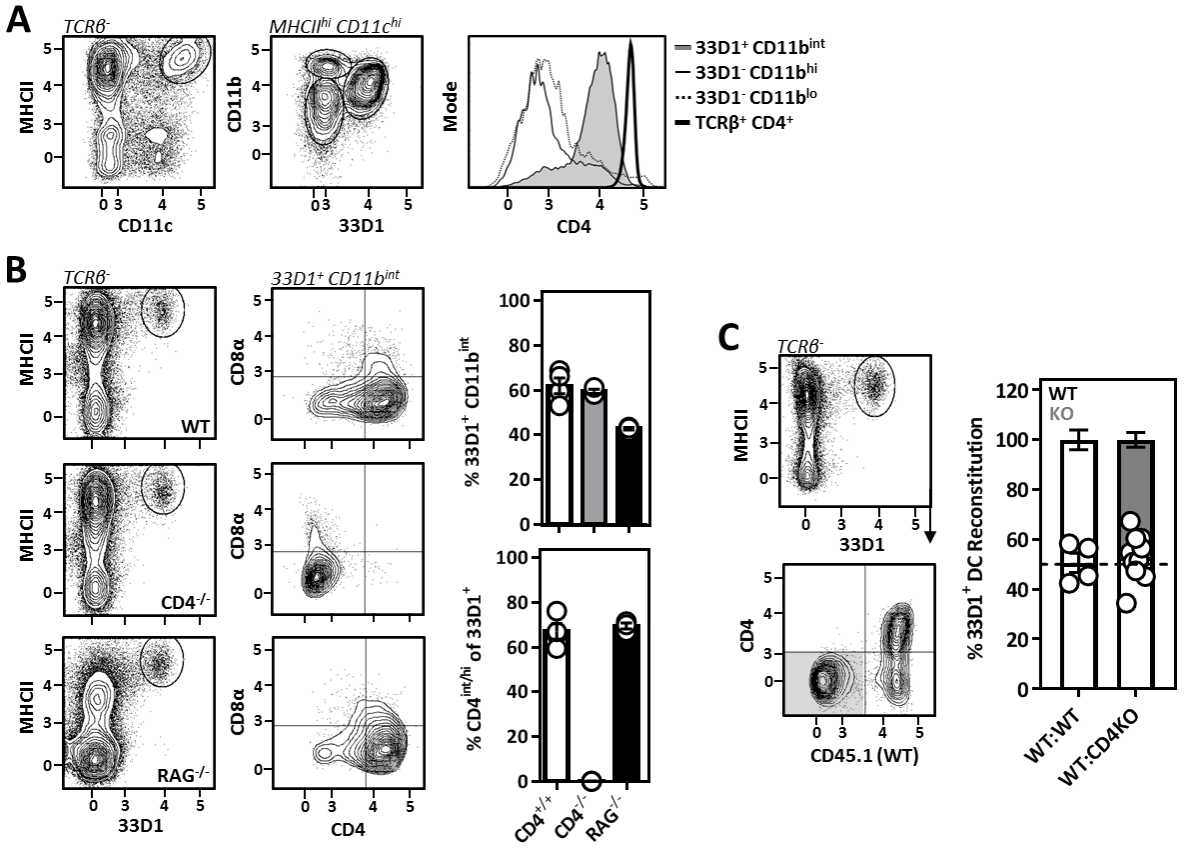


Figure 1. CD4 expression on DCs is cell-intrinsic and dispensable for DC development and survival. (A) CD4 expression as assessed by FACS staining on $CD4^+$ T cells (bold), $33D1^+$ $CD11b^{int}$ (Grey), $33D1^-CD11b^{lo}$ (black), and $33D1^-$ $CD11b^{hi}$ (dotted) DCs. (B) Analysis of $33D1^+$ DC frequencies and CD4 expression on $33D1^+$ DCs from WT, $CD4^{-/-}$, and $RAG^{-/-}$ mice. (C) WT:WT and WT:$CD4^{-/-}$ mixed chimeras were generated to evaluate the requirement for DC-intrinsic CD4 expression in DC development and survival in a competitive setting. Error bars in all panels represent mean ±SEM.

DC-intrinsic regulation of CD4 expression upon in vivo activation:

CD4 expression has been shown to increase on activated $CD4^+$ T cells, while mouse DCs lose CD4 expression following *ex vivo* culture (Fig. 2 A). We next wanted to determine if CD4 expression was similarly altered upon activation of $33D1^+$ DCs *in vivo*. Therefore WT mice were treated with the TLR4 agonist LPS 24H prior to assessing CD4 expression on $33D1^+$ DCs and $TCR\beta^+$ $CD4^+$ T cells by flow cytometry. In LPS treated animals, we observed a striking deterioration of CD4 expression specifically on $33D1^+$ DCs whereas CD4 expression on T cells remained intact (Fig. 2 B). Consistent with published reports on the behavior of *in vivo* activated DCs (30, 118), $33D1^+$ DCs migrated into T cell zones of the spleen early following LPS exposure where they remained protected from an injected PE-conjugated anti-CD45 antibody (Fig. 2 C) (28). To address whether this phenotype was DC-intrinsic or the result of bystander DC activation precipitated by the inflammatory milieu, WT:$MyD88^{-/-}$ mixed chimeras were generated in order to observe how CD4 expression and DC localization were altered on DCs capable/incapable of direct TLR sensing within the same inflammatory environment. Following treatment with the MyD88-dependent TLR agonist R848, we observed the selective loss of CD4 on WT, but not $MyD88^{-/-}$, $33D1^+$ DC, suggesting cell-intrinsic TLR sensing is required for CD4 loss on activated $33D1^+$ DCs (Fig. 2 D). Furthermore, in contrast to WT $33D1^+$ DCs which rapidly migrated into the splenic T cell zones upon activation, $MyD88^{-/-}$ $33D1^+$ DCs exhibited delayed migration kinetics in response to R848 exposure and appeared to prematurely egress from the white pulp (WP) (Fig. 2 E). Therefore, loss of CD4 expression and re-localization into the splenic WP following activation are $33D1^+$ DC-intrinsic processes, and maintenance of CD4 expression on $33D1^+$ DCs (as in the case of $MyD88^{-/-}$ $33D1^+$ DCs) may curtail their residence time within the splenic WP once activated.

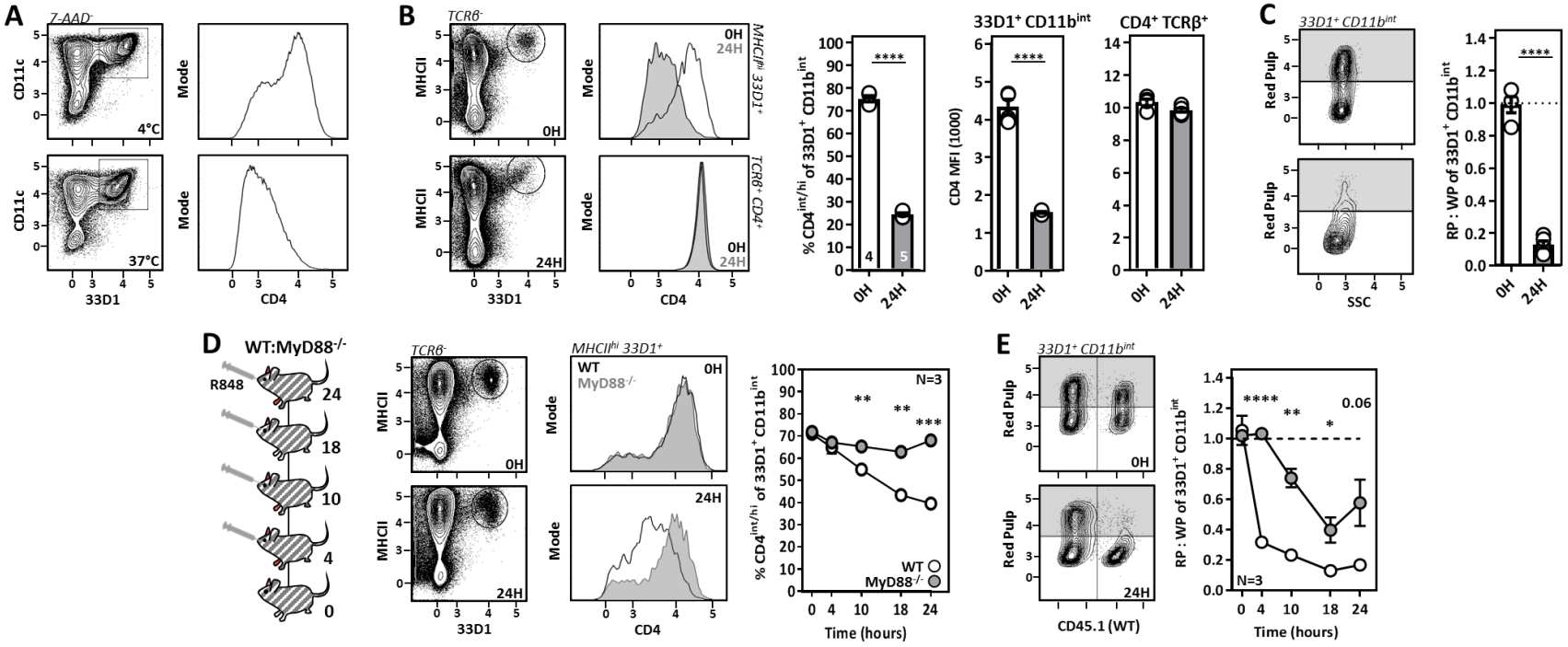


Figure 2. Dynamic regulation of DC-intrinsic CD4 expression upon *in vivo* activation. (A) CD11c^{+} cells were cultured for 24H in 96 well flat bottom plates at 4°C or 37°C before assessing CD4 expression on 33D1^{+} DCs by flow cytometry. (B) Mice were left untreated or treated 24H prior with 10ug/mouse LPS i.v. before assessing CD4 expression on 33D1^{+} DCs and CD4^{+} T cells. (C) LPS activated 33D1^{+} DCs migrated into the splenic white pulp where they were protected from staining with *an in vivo* injected PE-conjugated anti-CD45 antibody. (D) Loss of CD4 expression on WT and MyD88$^{-/-}$ 33D1^{+} DCs within WT:MyD88$^{-/-}$ mixed chimeras treated at various time-points with the MyD88-dependent TLR agonist R848. (E) Similar as in (C), *in vivo* localization of WT and MyD88$^{-/-}$ 33D1^{+} DCs following *in vivo* exposure to the MyD88-dependent TLR agonist R848 by *in vivo* PE-labeling. Error bars in all panels represent mean ±SEM. **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001, as calculated by unpaired Student's *t*-test.

Impaired proliferation of antigen-specific and endogenous CD4$^+$ T cells in mice harboring CD4-deficient DCs:

The absence of CD4$^+$ T cells in CD4$^{-/-}$ mice precludes the study of the function of CD4 on DCs from these mice. Therefore, to investigate the functional role of DC-intrinsic CD4 expression in a CD4$^+$ T cell-replete environment, we utilized a mixed chimera strategy in which CD4-deficiency could be restricted within the DC compartment. Taking advantage of CD11c-DTR-Tg mice in which diphtheria toxin (DTx) treatment induces the rapid and highly specific elimination of 33D1$^+$ DCs (Fig. 3 A) (104), we generated WT:CD11c-DTR (control) and CD4$^{-/-}$:CD11c-DTR (experimental) chimeras. Therefore, once reconstituted, a portion of 33D1$^+$ DCs would be sensitive to DTx-mediated ablation whereas the remaining DCs would be either WT (control) or CD4-deficient (experimental). DTx treatment would thus eliminate 33D1$^+$ DCs of CD11c-DTR-Tg origin whereas CD4$^+$ T cells derived from CD11c-DTR-Tg marrow would be spared due to absence of CD11c-expression (Fig. 3 B). This system would afford the ability to study the consequence of loss of DC-intrinsic CD4 expression in mice with an intact CD4$^+$ T cell compartment.

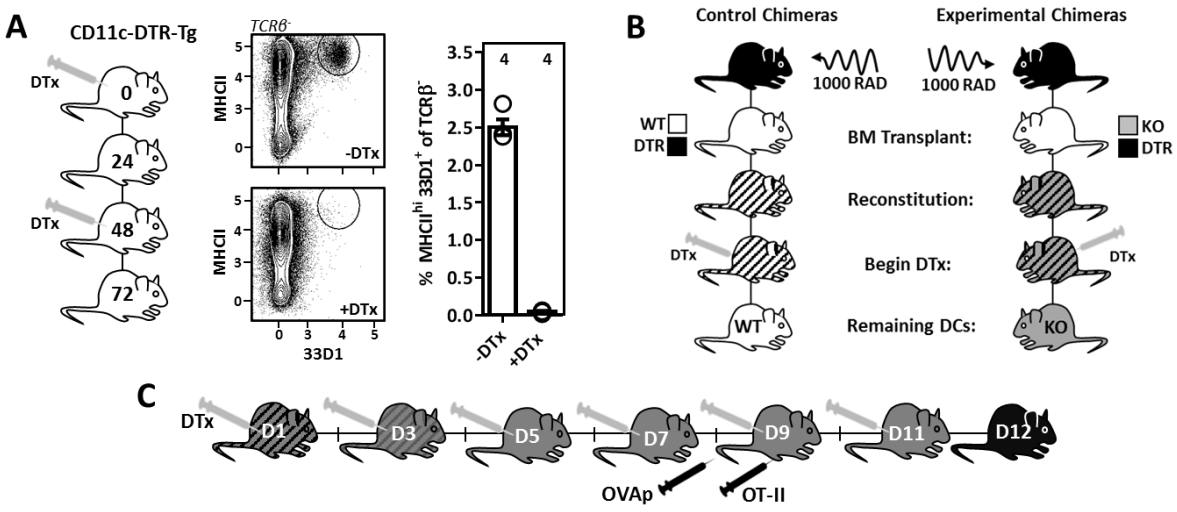


Figure 3. Strategy for the elimination of DC-intrinsic CD4 expression in CD4$^+$ T cell-replete mice. (A) Acute DTx treatment in CD11c-DTR-Tg mice can be used to rapidly and specifically eliminate 33D1$^+$ DCs (B) Generation of WT:DTR (control) and CD4$^{-/-}$:DTR (experimental) mixed chimeras to restrict CD4-deficiency within the CD4 compartment. (C) Strategy for evaluating Ag-specific CD4$^+$ T cell proliferation in control and experimental chimeras. Error bars in all panels represent mean ±SEM.

Assessing DC abundance within control and experimental chimeras 10 weeks post-reconstitution, identical numbers and frequencies of all DC subsets were observed (Fig. 4 A). Therefore, mice were treated with DTx every-other day for 12 days to repopulate the DC niche with DCs derived from the DTR^{neg} donor. Commencing DTx treatment, chimeras were immunized with OVA-peptide (OVAp) on D8 and CFSE-labeled OVA-specific $CD4^+$ T cells (OT-IIs) were adoptively transferred into DTx-treated chimeras on D9 post-DTx initiation (Fig. 3 C). At sacrifice, we observed a similar abundance of DCs within control and experimental chimeras with ~85% of $33D1^+$ DCs being CD4-deficient in experimental chimeras (Fig. 4 B). Assessing proliferation of transferred antigen-specific cells, OT-IIs underwent significantly fewer divisions when transferred into chimeric mice harboring CD4-deficient DCs compared to control chimeras (Fig. 4 C). Furthermore, homeostatic proliferation of endogenous CD4 (but not CD8) T cells was significantly reduced in experimental chimeras post-DTx treatment compared to controls, mirroring what was observed for antigen-specific $CD4^+$ T cells within these mice (Fig. 4 D). Collectively, these experiments strongly suggest DC-intrinsic CD4-expression is required for optimal activation of $CD4^+$ T cells *in vivo* in response to both exogenous and endogenous (self) antigens.

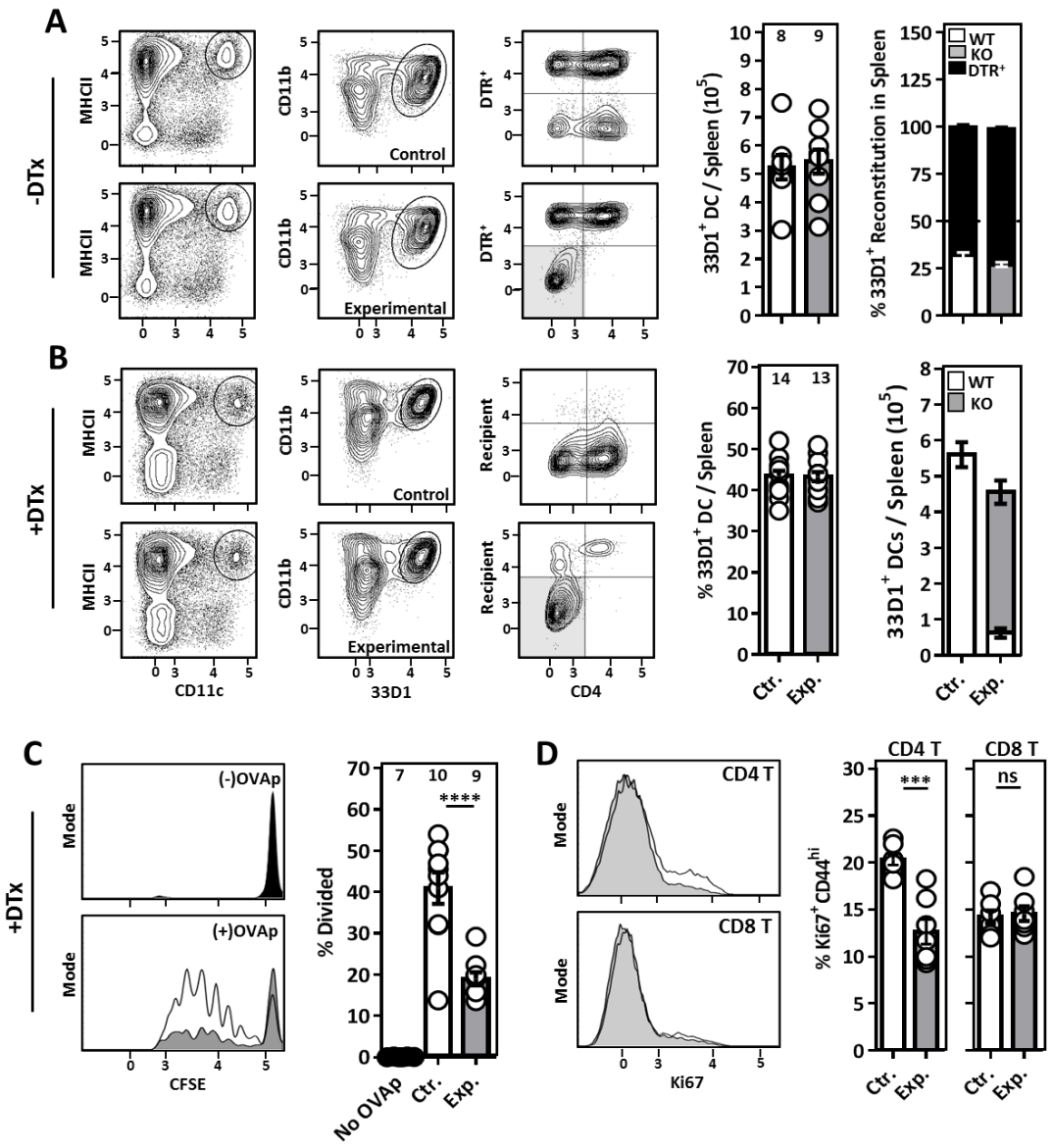
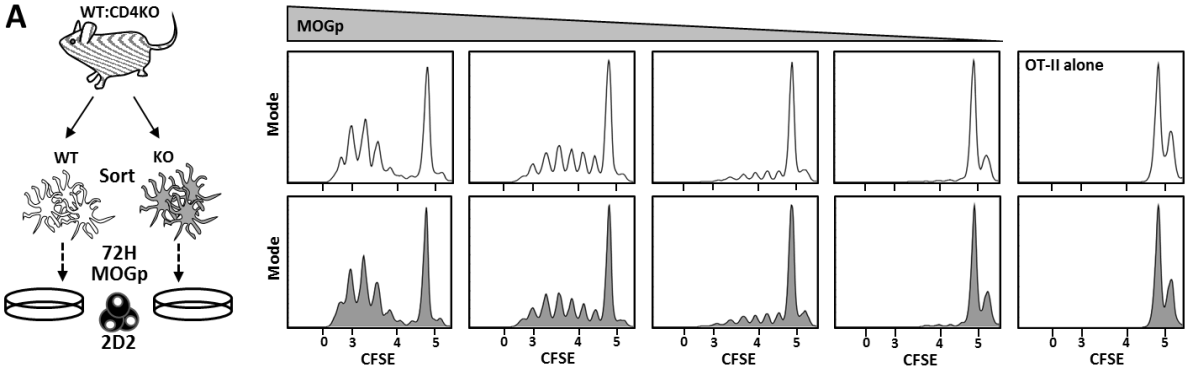


Figure 4. DC-intrinsic CD4 expression is required for the optimal activation of antigen-specific and endogenous CD4⁺ T cells *in vivo*. (A) Analysis of the DC compartment in WT:DTR (control) and CD4$^{-/-}$:DTR (experimental) chimeras 10 wks post-reconstitution prior to DTx treatment. (B) Similar analysis as in (A) in control and experimental chimeras treated every-other day for 12 days with DTx. (C) Proliferation of antigen-specific CD4^{+} T cells (OT-IIs) as assessed by dilution of CFSE in DTx-treated OVAp-immunized control and experimental chimeras 72H post-transfer (as outlined in Fig. 2 C). (D) Homeostatic proliferation of endogenous CD4 (left) and CD8 (right) T cells in DTx-treated control and experimental chimeras as assessed by Ki67 staining. Numbers above histograms represent replicates per group. Error bars in all panels represent mean ±SEM. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001, as calculated by unpaired students Student's *t*-test.

Intact peptide-antigen presentation by CD4-Deficient 33D1$^+$ DCs in vitro:

DC-intrinsic CD4 expression is dispensable for DC development and survival (Fig. 1), yet appeared to be necessary for the optimal activation of antigen-specific and endogenous CD4$^+$ T cells *in vivo* (Fig. 3*)*. We next investigated whether CD4-deficiencey impaired the ability of 33D1$^+$ DCs to present peptide-antigen to CD4$^+$ T cells an *in vitro* setting. To address this, WT and CD4$^{-/-}$ 33D1$^+$ DCs were sorted from WT:CD4$^{-/-}$ mixed chimeras so as to obtain WT and CD4$^{-/-}$ DCs from a T_r cell-replete environment. These DCs were co-cultured with CFSE-labeled MOG-peptide-specific CD4$^+$ T cells (2D2) at limiting concentrations of cognate peptide where we observed no difference in the ability of WT or CD4$^{-/-}$ DCs to induce antigen-specific CD4$^+$ T cell proliferation (Fig. 4 A). Next, WT:CD4$^{-/-}$ chimeras were immunized with OVAp 20H prior to sorting WT and CD4$^{-/-}$ 33D1$^+$ DCs, in addition to CD8α DCs which are known to be poor CD4$^+$ T cell activators. DCs were co-cultured with OVAp-specific CD4$^+$ T cells (OT-II) and activation of TCR-transgenic cells was assessed by upregulation of the early T cell activation markers CD25 and CD69. Under these conditions, antigen presentation by CD4-deficient 33D1$^+$ DCs was completely intact demonstrating DC-intrinsic CD4 expression is dispensable for the acquisition and retention of peptide-antigen, as well as its presentation to CD4$^+$ T cells *ex vivo* (Fig. 4 B).



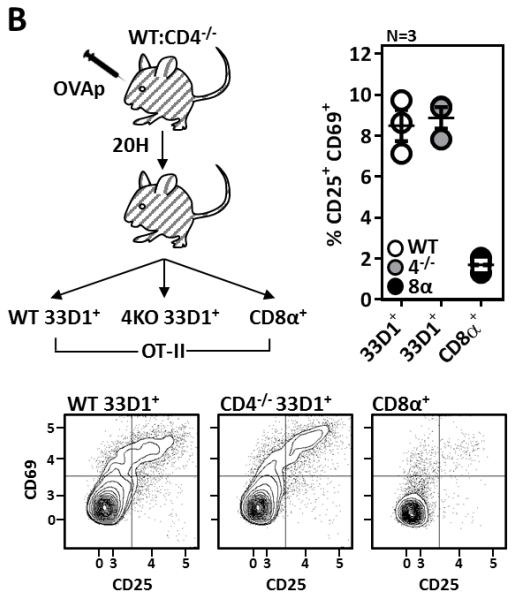


Figure 5. Intact peptide-antigen presentation by CD4-deficient 33D1$^+$ DCs *in vitro.* (A) WT and CD4$^{-/-}$ 33D1$^+$ DCs were sorted from WT:CD4$^{-/-}$ mixed chimeras and used to stimulate MOGp-specific CD4$^+$ T cells (2D2) at limiting concentrations of MOGp *in vitro*. (B) WT, CD4$^{-/-}$, and 33D1$^-$ CD11b^{lo} DCs were sorted from OVAp-imunized WT:CD4$^{-/-}$ chimeras and used to stimulate early activation of OVAp-specific CD4$^+$ T cells (OT-II) following overnight cultures. Error bars in all panels represent mean ±SEM.

CD4-deficient 33D1$^+$ DCs are localized normally within splenic MZBCs at homeostasis:

Given our inability to recapitulate *in vitro* the observation of impaired CD4$^+$ T cell proliferation *in vivo*, we next decided to focus on a potential role for DC-intrinsic CD4 expression in the coordination of splenic DC localization. At steady-state, 33D1$^+$ DCs primarily occupy splenic marginal zone bridging channels (MZBCs), specialized regions at the nexus of the B cell follicle, T cell zone, and red pulp (29, 30, 109). Here, 33D1$^+$ DC form dense aggregates partitioning the myeloid-rich red pulp (RP) from the lymphocyte-dense WP and are thought to transport particulate antigens from the blood into T cell zones of the spleen for the initiation of CD4$^+$ T cell responses (27, 55). Therefore MZBCs can be thought of as functionally analogous to afferent lymphatics; facilitating the transport of antigen by way of 33D1$^+$ DCs into T cell zones of SLOs for the generation of adaptive immune responses. Given their steady-state localization in proximity to MHCII^{hi} B cells and adjacent MHCII^{hi} 33D1$^+$ DCs, we hypothesized DC-intrinsic CD4 expression may facilitate 33D1$^+$ DC localization within MZBCs through interactions *in trans* with MHCII expressed on neighboring DCs or B cells. To text this, we generated WT (CD45.2):WT(CD45.1/2) → CD45.1 (control) and CD4$^{-/-}$ (CD45.2):WT(CD45.1/2) → CD45.1 (experimental) chimeras which we subsequently injected i.v with a PE-conjugated anti-CD45.2 antibody for 5 minutes prior to sacrifice (28). Therefore, localization of WT and CD4$^{-/-}$ 33D1$^+$ DCs could be approximated with respect to the MZBC by acquisition of the injected PE-conjugated antibody within the same mouse. While CD45.2-single positive DCs within both sets of chimeras displayed enhanced PE staining (likely due to 2x expression of CD45.2 over CD45.1/2 DCs), this difference was identical between WT and CD4$^{-/-}$ 33D1$^+$ DCs suggesting CD4-deficiency does not result in the gross mis-localization of 33D1$^+$ DCs (Fig. 5). Therefore, DC-intrinsic CD4 expression is negligible for the positioning of 33D1$^+$ DCs within MZBCs at steady-state.

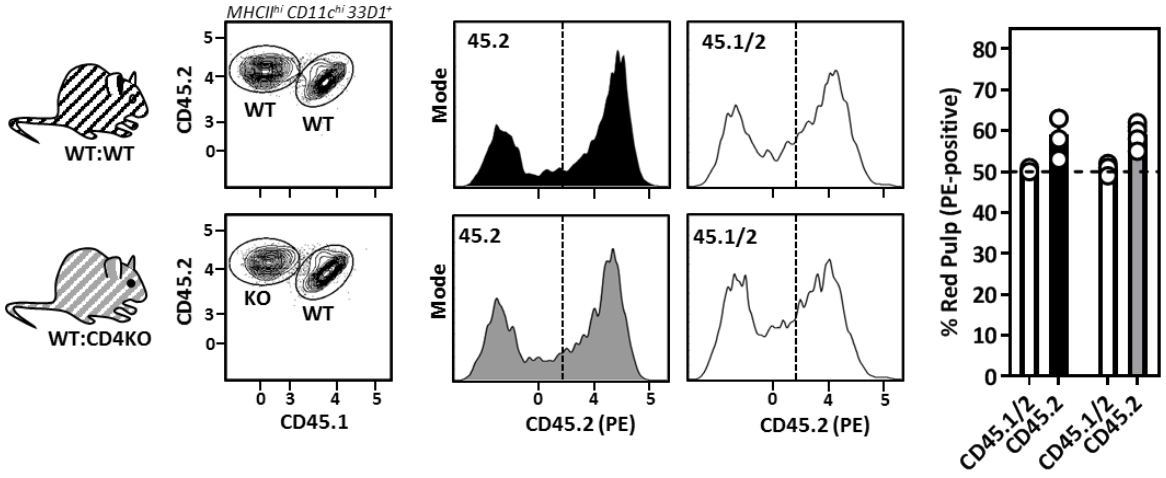


Figure 5. CD4-deficient 33D1^{+} DCs are properly localized within splenic MZBCs. WT:WT (control) and WT:CD4$^{-/-}$ (experimental) chimeras were injected with an PE-conjugated anti CD45.2 antibody to approximate the localization of WT and CD4$^{-/-}$ 33D1^{+} DCs within the same mouse. Error bars in all panels represent mean ±SEM.

CD4-deficient DCs fail to rescue IL-2 activation in T_r cells from DC depleted mice:

In a prior report, we had demonstrated 33D1$^+$ DCs indirectly promote regulatory T cell (T_r cell) - supportive IL-2 release from auto-reactive CD4$^+$ T cells, whereas CD8 T cells are non-contributory (121). IL-2 signaling in T_r cells lead to phosphorylation of STAT5 (pSTAT5) and was shown to be virtually absent in splenic T_r cells from IL-2$^{-/-}$ mice demonstrating a lack of compensatory signaling downstream of IL-7 and/or IL-15 in splenic T_r cells (111, 121). Thus, STAT5 phosphorylation in splenic T_r cells serves as a sensitive proxy for homeostatic IL-2 produced by 33D1$^+$ DC-activated CD4$^+$ T cells *in vivo.* We had also previously described an *in vitro* co-culture system to probe the molecules on DCs and CD4$^+$ T cells required for the efficient generation of T_r cell supportive IL-2. To accomplish this, bulk CD11c-enriched cells (70% MHCII^{hi} CD11c^{hi}) were cultured with polyclonal Foxp3$^-$ CD4$^+$ T cells in the presence of 10% autologous mouse serum. 72H later, co-culture supernatants were used to stimulate STAT5 phosphorylation in T_r cells amongst freshly isolated CD4$^+$ T cells (containing ~13-15% T_r cells). Thus, STAT5 phosphorylation in fresh Foxp3$^+$ CD25$^+$ T_r cells could serve as a qualitative measurement of soluble IL-2 produced within each co-culture (121). Importantly, given that all cell isolations/enrichments were performed in buffer devoid of foreign protein (e.g. fetal calf serum), IL-2 produced under these conditions reflected DC activation of self-reactive T cells. We confirmed the requirement for self-reactive TCRs in the generation of IL-2 in this system by culturing DCs with either polyclonal Foxp3$^-$ CD4$^+$ T cells or CD4$^+$ T cells of a fixed TCR specificity (OT-II). Here, only polyclonal CD4$^+$ T cells generated IL-2 demonstrating DC-mediated auto-antigen activation of self-reactive TCRs is required for the IL-2 produced in this experimental system (Fig. 6 A). Therefore, we next wanted to determine if CD4$^{+/-}$ or CD4$^{-/-}$ DCs were inferior at stimulating IL-2 production from auto-reactive CD4$^+$ T cells compared to their WT counterparts. Utilizing this same co-culture system, we observed CD4$^{-/-}$ DCs induced significantly less IL-2 production from polyclonal CD4$^+$ T cells compared to WT DCs (Fig. 6 B).

Considering the IL-2 generated in this system necessitates the presentation of complex self-antigens succeeding internalization and processing by the DC, these experiments suggested CD4-deficient DCs may be impaired in their ability to acquire and/or process complex antigens.

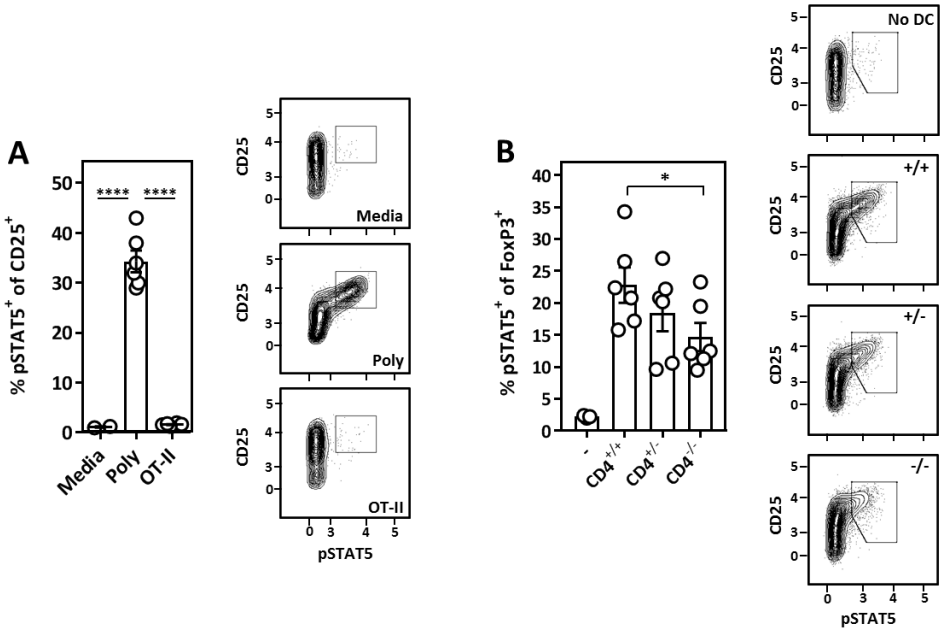


Figure 6. Impaired IL-2 production from CD4-deficient DC-stimulated auto-reactive CD4$^+$ T cells. (A) Polyclonal and OT-II$^+$ CD4$^+$ Foxp3$^-$ T cells were co-cultured with DCs for 72H in the presence of 10% mouse serum and supernatants were used to stimulate STAT5 phosphorylation in T_r cells amongst freshly isolated CD4$^+$ T cells. (B) Similar as in (A), CD4$^{-/-}$ DCs induce less robust IL-2 production from polyclonal Foxp3$^-$ CD4$^+$ T cells as compared to their WT counterparts. Error bars in all panels represent mean ±SEM. *, P ≤ 0.05; ****, P ≤ 0.0001, as calculated by unpaired students Student's *t*-test.

The observation that CD4-deficient DCs stimulated less IL-2 production from polyclonal $CD4^+$ T cells *in vitro* led us to next interrogate a role of DC-intrinsic CD4 expression in the generation of T_r cell-supportive IL-2 *in vivo*. DC ablation in CD11c-DTR-Tg mice results in a dramatic reduction in T_r cell IL-2 activation as assessed by loss of T_r cell pSTAT5; however this can be restored by transferring WT DCs into CD11c-DTR-Tg mice following the initial DTx treatment (121). Thus, CD11c-enriched cells from WT or $CD4^{-/-}$ mice were adoptively transferred into DTx-treated CD11c-DTR-Tg mice 72H prior to assessing pSTAT5 levels in recipient T_r cells at sacrifice. Due to a genotyping error, ~15% of adoptively transferred $CD4^{-/-}$ DCs were in fact heterozygous for CD4 yet despite this, in stark contrast to WT DCs which fully restored T_r cell IL-2 activation in these animals, (majority) CD4-deficiency dramatically compromised the ability of transferred DCs to rescue IL-2 activation in T_r cells from DC-depleted mice. While these experiments strongly supported a model by which CD4-deficient DCs were impaired in their ability to drive IL-2 production downstream of self-antigen activation of $CD4^+$ T cells *in vitro* and *in vivo*, it remained plausible that our *in vivo* observations alternatively reflected an inability of $CD4^{-/-}$ DCs to access and/or survive within recipient spleens following adoptive transfer. To address this, congenically unique WT and $CD4^{-/-}$ DCs were transferred into recipient mice i.v. at a 1:1 ratio (WT:WT and WT:$CD4^{-/-}$) where we assessed their recovery 18H post-transfer. Here identical frequencies of WT and $CD4^{-/-}$ DCs were recuperated from the spleens of recipient mice suggesting CD4-deficiency does not impair the ability of transferred DCs to access or survive within the spleens of recipient mice post-transfer. Instead, these data collectively argue for diminished self-reactive $CD4^+$ T cell activation and IL-2 production in the absence of DC-intrinsic CD4 expression.

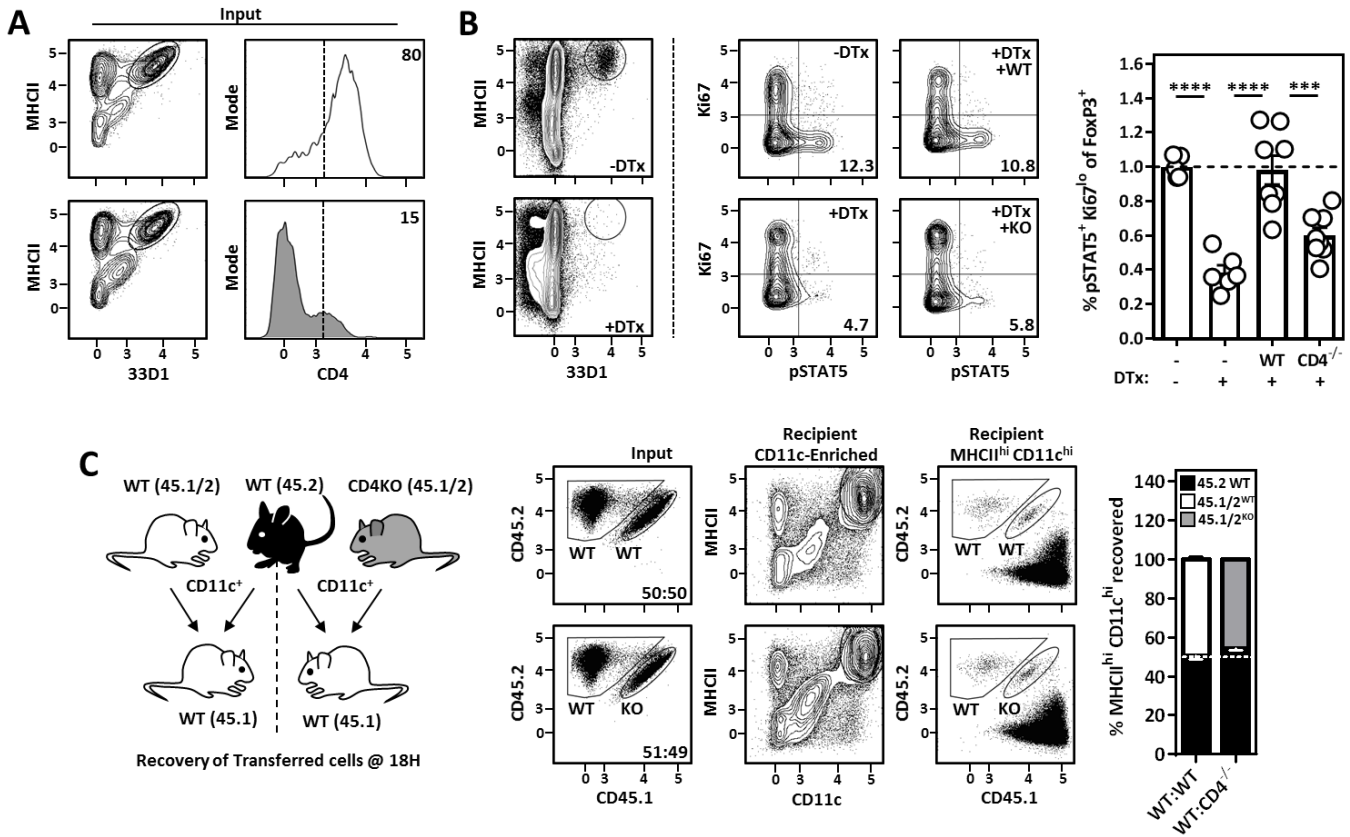


Figure 7. CD4-deficient DCs fail to rescue IL-2 activation in T_r cells from DC-depleted mice. (A) Analysis of CD11c-enriched cells from WT and $CD4^{-/-}$ mice prior to adoptive transfer into DTx-treated CD11c-DTR-Tg mice. Due to a genotyping error, a fraction of DCs were in fact $CD4^{+/-}$, although ~85% were CD4-deficient. (B) T_r cell IL-2 activation amongst recipient T_r cells from CD11c-DTR-Tg mice receiving no DCs, WT DCs, or $CD4^{-/-}$ DCs. (C) Congenically unique WT and $CD4^{-/-}$ DCs were co-transferred into recipient mice 18H prior to CD11c-enriching recipient spleens. Identical frequencies of WT and $CD4^{-/-}$ DCs were recovered from recipient mice demonstrating CD4-deficient DCs access and survive normally within recipient spleens. 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.

Discussion

After 20+ years of conjecture, we report the function of CD4 on DCs can now be extended beyond that of a DC lineage marker. Employing transgenic and chimeric mouse strategies to circumvent the limitations of studying CD4 on DCs in global CD4-deficient mice, we have demonstrated DC-intrinsic CD4 expression augments the function of 33D1$^+$ DCs *in vivo*. Mice harboring CD4-deficient DCs are impaired in their ability to optimally prime CD4$^+$ T cell responses to exogenous and endogenous antigens, in part through their inability to efficiently stimulate IL-2 production from CD4$^+$ T cells. Moreover, CD4 expression on 33D1$^+$ DCs is labile under inflammatory conditions where its loss occurs upon DC activation in a cell-intrinsic manner. These observations beget the intriguing possibility that DC-modulation of CD4 expression may represent a critical step in DC maturation; consequently influencing the magnitude and/or duration of an immune response.

We found DC-intrinsic CD4 expression was dispensable for DC development and survival, as well as for the acquisition, retention, and presentation of peptide-antigens to CD4$^+$ T cells *in vitro* and *ex vivo*. However, CD4-deficient 33D1$^+$ DCs were significantly impaired in their ability to drive antigen-specific and endogenous CD4$^+$ T cell proliferation in response to peptide-antigen *in vivo*. Given this disparity, we hypothesized DC-intrinsic CD4 expression may coordinate the proper micro-anatomical localization of 33D1$^+$ DCs within MZBCs. However, CD4-deficient 33D1$^+$ DCs were found to be properly localized along-side their WT counterparts within MZBCs. Therefore improper localization of CD4-deficient DCs could not explain the discordance in antigen-specific CD4$^+$ T cell proliferation observed for control and experimental chimeras *in vivo*. We therefore hypothesized the presence of polyclonal CD4$^+$ T cells (extant *in vivo* but absent *in vitro*), and their potential differential ability to generate IL-2 in response to self-antigen-activation by WT vs. CD4-deficient DCs *in vivo*, may explain this incongruity. Consistent with this hypothesis, endogenous CD4$^+$ T cells were shown to be hypo-proliferative

in mice harboring CD4-deficient DCs, reflecting defective basal self-antigen activation in these mice. Indeed, using our established autologous DC-T cell co-culture assay, we demonstrated CD4-deficient DCs induced less robust IL-2 production from polyclonal $CD4^+$ $Foxp3^-$ T cells *in vitro* in response to complex self-antigens compared to their WT counterparts. Furthermore, $CD4^{-/-}$ DCs were similarly impaired in their ability to rescue IL-2 activation in T_r cells from DC-depleted mice compared to those receiving WT DCs, suggesting impaired self-antigen activation of $CD4^+$ T cells *in vivo*. Collectively these experiments argue that differences in basal IL-2 production from bystander auto-reactive $CD4^+$ T cells in mice harboring WT or CD4-deficient DCs could account for the differential ability of antigen-specific $CD4^+$ T cells to effectively proliferate in these mice.

The activation of endogenous polyclonal T cells requires the coordinated capture, processing, and presentation of complex antigens by DCs. In contrast, peptide-antigen including OVAp can stochastically displace processed antigen within MHCII binding grooves on the DC surface, and therefore the stimulation of $CD4^+$ T cells specific for peptide-antigen requires only functional MHCII on DCs. Given that CD4-deficient DCs are normal in their ability to present peptide-antigen to antigen-specific $CD4^+$ T cells *in vitro* but impaired in their ability to drive IL-2 production in response to complex self-antigens from polyclonal $CD4^+$ T cells *in vitro* and *in vivo*, we hypothesize DC-intrinsic CD4 expression facilitates proper antigen capture and/or processing. DC activation and maturation has long been known to coincide with a reduction in antigen capture and processing – allowing DCs to focus on presenting only those antigens acquired before or during their initial activation (18, 122, 123). Concomitantly, limiting antigen capture likely prevents aberrant activation of self-reactive T cells by discouraging the processing and presentation of self-antigens subsequently liberated during the ensuing inflammatory response. Given the observation that CD4 expression wanes on activated $33D1^+$ DCs but appears to be required for the presentation of complex self-antigens to $CD4^+$ T cells at

steady-state, loss of DC-intrinsic CD4 expression may represent a critical step in DC maturation; discouraging further antigen capture and processing to limit the range of antigens DCs can present within those which stimulated their initial activation.

Chapter 5
Concluding remarks

Here, we have experimentally demonstrated the precise cellular and molecular factors comprising the circuit supporting IL-2-dependent T_r cells in the spleen at homeostasis. In summary, 33D1$^+$ CD11b^{int} DCs – the most abundant DC subset at homeostasis – supports IL-2-dependent T_r cells through their ability to efficiently present MHCII-restricted auto-antigens to self-reactive CD4$^+$ T cells within regions of the spleen in close proximity to T_r cells. The absence of CD4$^+$ T cell-derived Il-2 precipitates autoimmunity resulting from a selective loss of IL-2-dependent T_r cells. Therefore T_r cells require smoldering auto-immunity amongst conventional CD4$^+$ T cells for their homeostatic maintenance and survival.

Given the default setting of the immune system is self-tolerance and that constitutive DC ablation results in autoimmunity, we speculate a primary function of 33D1$^+$ CD11b^{int} DCs at homeostasis is the maintenance of self-tolerance through their ability to indirectly support IL-2-dependent T_r cells. 33D1$^+$ CD11b^{int} DCs localize within MZBCs where they survey the blood for disseminated infection. We propose a model by which the presentation of blood-borne auto-antigens to auto-reactive CD4$^+$ T cells at homeostasis precipitates IL-2 release immediately consumed by CD25^{hi} T_r cells. However, IL-2 is limiting and thus calibrates T_r cell with the degree of CD4$^+$ T cell auto-reactivity; ensuring sufficient numbers of T_r cells are present at homeostasis to subvert aberrant T cell priming without overwhelming beneficial T cell-mediated immune responses when desired.

Activation of 33D1$^+$ CD11b^{int} DCs *in vivo* results in their extensive re-localization out of MZBCs into regions of the splenic WP enriched in CD4$^+$ T cells. Coinciding with DC migration is a concomitant loss of DC-intrinsic CD4 expression. This observation led us to investigate a functional role for CD4 on DCs, where we observed its role in model- and self-antigen activation of TCR-transgenic and endogenous polyclonal CD4$^+$ T cells, respectively. Sub-optimal priming

of $CD4^+$ T cells in the presence of CD4-deficient DCs appears to be related (at least in part) to their inability to efficiently stimulate IL-2 production from polyclonal $CD4^+$ T cells. We believe this reduction in $33D1^+$ DC-stimulated IL-2 production reflects the inability of CD4-deficient DCs to acquire and present complex self-antigens to $CD4^+$ T cells. This notion complements previous reports of diminished antigen-acquisition by DCs upon DC maturation. Therefore, we propose a model by which loss of DC-intrinsic CD4 expression impairs their ability to present newly-acquired antigens post-maturation – focusing DCs to present only those antigens which stimulated their activation.

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