VARIATIONS IN ANTIGEN PRESENTATION DEFINE REGULATORY T CELL FORMATION AND ACTIVITY *IN VIVO*

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To Drew,

For your unfailing love

And infinite ability to make me laugh

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ABSTRACT

VARIATIONS IN ANTIGEN PRESENTATION DEFINE REGULATORY T CELL FORMATION AND ACTIVITY *IN VIVO*

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The studies presented in this dissertation examine how the formation, differentiation, and activity of Foxp3+ regulatory T (Treg) cells can be affected by variations in the amount of a cognate antigen (Ag) that is present, the cell types by which it is expressed and/or presented, and whether Ag recognition occurs in an inflammatory environment. Recognition of a weakly immunostimulatory self-peptide was able to promote both thymic $CD4^+Foxp3^+$ Treg cell development and the formation of $CD4^+Foxp3^+$ Treg cells from $CD4^+Foxp3^-$ T cell precursors in the periphery. Despite promoting Treg cell formation, this weakly immunostimulatory self-Ag was unable to induce Treg cell activity during influenza virus infection. Conversely, when the same Ag was derived from an influenza virus, it drove Treg cells to proliferate and acquire a $T\text{-}bet^+CXCR3^+$ phenotype, and to suppress the accumulation of $CD4^+Foxp3^-$ and $CD8^+$ effector T cells in the lungs of infected mice. Notably, when cognate Ag was expressed at high levels by antigen presenting cells, Treg cells also underwent substantial proliferation, but little or no differentiation into a $T\text{-}bet^+CXCR3^+$ phenotype occurred, and their ability to modulate an anti-viral immune response was impaired. This failure to promote the formation of $T\text{-}bet^+CXCR3^+Foxp3^+$ Treg cells could be attributed in part to

the presentation of the cognate Ag by B cells, and in part to their initial activation by a highly stimulatory Ag in a non-inflammatory environment. Interactions with cognate peptide:MHC complex in these contexts diminished the ability of Treg cells to differentiate upon subsequent exposure to cognate Ag when it was derived from virus and presented by dendritic cells in the lung-draining mediastinal lymph nodes. Collectively, these studies show that the mode of presentation of an Ag can critically affect the formation and activity of Foxp3+ Treg cells *in vivo*, and that the type of antigen presenting cell and inflammatory environment encountered during their initial activation can determine their ability to differentiate into novel effector phenotypes.

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LIST OF ABBREVIATIONS

Ag	antigen
APC	antigen presenting cell
CNS1	conserved noncoding sequence 1
d	day
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
eGFP	enhanced green fluorescent protein
GMFI	geometric mean fluorescence intensity
HA	hemagglutinin
HEL	hen egg lysozyme
HSV	herpes simplex virus
IFN-γ	interferon-γ
IL-2	interleukin-2
iMO	inflammatory monocyte
LN	lymph node
medLN	mediastinal lymph node
mesLN	mesenteric lymph node
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte protein
OVA	ovalbumin
PCC	pigeon cytochrome c
p.i.	post-infection
pLN	peripheral, non-gut draining lymph node

pTreg cell	peripherally-derived regulatory T cell
RSV	respiratory syncytial virus
TCR	T cell receptor
TGF-β	transforming growth factor-β
Treg cell	regulatory T cell
TS1	transgenic mouse expressing a TCR specific for the S1 determinant of influenza virus PR8 HA
TSDR	Treg cell specific demethylated region
tTreg cell	thymically-derived regulatory T cell
YFP	yellow fluorescent protein

CHAPTER 1: Introduction and Overview

1.1 Introduction

Regulatory T (Treg) cells are a subset of $CD4^+$ T cells that are crucial for the maintenance of self-tolerance and immune homeostasis in both mice and humans (Bennett et al. 2001; Brunkow et al. 2001). Treg cells are defined by expression of the transcription factor Foxp3, which is necessary for their development and persistence, and loss of Foxp3 function through either mutation or deletion leads to rapid onset of autoimmunity and lymphoproliferative disease in both mice and humans (Brunkow et al. 2001; Fallarino et al. 2003; Sakaguchi et al. 1995; Williams and Rudensky 2007). The majority of Treg cells also express the IL-2Rα chain CD25, and lymphoproliferative disease and autoimmune pathology develops in the absence of $CD4^+CD25^+$ T cells (Sakaguchi et al. 1995; Willerford et al. 1995). The majority of Treg cells are believed to form in the thymus following recognition of self-Ag (Hsieh et al. 2006), although conversion of naïve $CD4^+Foxp3^-$ cells into $Foxp3^+$ Treg cells in the periphery represents an alternative source of these cells (Shevach and Thornton 2014). In addition to their well-established role in the prevention of autoimmunity (Sakaguchi et al. 2006), there is increasing evidence that Treg cells also function in other settings in which immune activation occurs, such as during the immune response to a pathogen (Bedoya et al. 2013; Fulton, Meyerholz, Varga 2010; Lund et al. 2008). Moreover, it has recently become apparent that Treg cells must differentiate further and acquire novel characteristics in order to effectively regulate immune activation in different contexts (Gratz and Campbell 2014). A greater understanding of how signals transmitted through

the T cell receptor (TCR) can determine Treg cell differentiation, activity, and functional differentiation will be necessary if Treg cells are to be used in a therapeutic setting.

1.2 Regulatory T cell development in the thymus

T cells develop in the thymus, where somatic gene rearrangement in each developing thymocyte can lead to the creation of a unique TCR. This process results in the generation of a wide variety of TCR specificities with the potential to recognize a vast array of ever-changing pathogen-derived antigens, but also inevitably results in the generation of TCRs that recognize self-antigens. The number of circulating self-reactive T cells is limited by the fact that thymocytes must undergo a stringent selection process, which results in the positive selection of cells expressing a TCR that weakly recognizes self-peptide:MHC complexes, but the deletion of cells bearing a TCR that binds self-peptide:MHC complexes too strongly (Palmer 2003). However, not all thymocytes that recognize self-Ag undergo deletion, and an alternative fate for these self-reactive thymocytes is differentiation into Foxp3$^+$ Treg cells.

Multiple studies using TCR-transgenic mice in which the TCR is specific for a defined self-Ag have established that TCR-mediated recognition of self-Ag can drive developing thymocytes to differentiate into Foxp3$^+$ Treg cells. Our lab has demonstrated this concept using TS1 mice, which express a transgenic TCR that recognizes the Site 1 (S1) epitope of PR8 influenza virus hemagglutinin (HA) presented on I-E^d and that can be identified using the monoclonal antibody 6.5 (Kirberg et al. 1994). In single

transgenic TS1 mice, essentially none of the $6.5^{+}CD4^{SP}$ thymocytes or $6.5^{+}CD4^{+}$ T cells express Foxp3. However, when TS1 mice are mated with transgenic mice that express the PR8 HA as a neo-self-Ag (termed HA28 mice), the resultant TS1xHA28 mice contain significantly more $6.5^{+}CD4^{SP}$ thymocytes and $6.5^{+}CD4^{+}$ T cells that express Foxp3, and these $6.5^{+}CD4^{+}Foxp3^{+}$ cells are potently suppressive *in vitro* (Jordan et al. 2001; Larkin, Picca, Caton 2007; Lerman et al. 2004; Picca et al. 2009). Importantly, the development of 6.5^{+} Treg cells in TS1xHA28 mice does not require endogenous rearrangement of the TCR, as 6.5^{+} Treg cells still form in TS1xHA28 mice on a $TCR\alpha^{-/-}$ background and in HA28 mice reconstituted with $TS1.Rag^{-/-}$ bone marrow (Jordan et al. 2001; Larkin et al. 2008). Thus, TCR recognition of an agonist self-Ag can drive thymocytes to develop into $Foxp3^{+}$ Treg cells. Other groups using transgenic mice that expressed HA under control of an immunoglobulin promoter or an Aire promoter also found that recognition of self HA could induce HA-reactive thymocytes to differentiate into Treg cells (Apostolou et al. 2002; Aschenbrenner et al. 2007). Furthermore, studies involving TCR transgenic mice, in which the TCR is specific for OVA, crossed with mice expressing OVA as a neo-self-Ag provided evidence that recognition of a different self-Ag can also drive intrathymic Treg cell development (Kawahata et al. 2002; Walker et al. 2003). In the absence of a TCR transgene, Treg cells ($CD4^{SP}CD25^{+}Foxp3^{+}$) and Treg cell progenitors ($CD4^{SP}CD25^{+}CD122^{hi}Foxp3^{-}$) in the thymus express higher levels of Nur77, the expression of which reflects TCR signal strength, than do $CD4^{SP}CD25^{-}Foxp3^{-}$ thymocytes, indicating that thymocytes that upregulate Foxp3 have received a strong TCR signal (Moran et al. 2011). Furthermore, TCRs derived from $Foxp3^{+}$ T cells directed Treg cell differentiation more effectively than did TCRs derived from $Foxp3^{-}$ T cells, providing further evidence that Treg cell differentiation is a TCR instructive process (Bautista et al. 2009; Leung, Shen, Lafaille 2009). Finally, $CD4^{+}CD25^{-}$ T cells

transduced with TCRs taken from Treg cells proliferated to a greater extent when transferred into WT host mice than did T cells transduced with TCRs acquired from $CD4^+CD25^-$ cells, and T cells expressing a Treg cell-derived TCR caused more rapid wasting disease in $Rag^{-/-}$ hosts, further demonstrating their self-reactivity (Hsieh et al. 2004; Hsieh et al. 2006). Together, these studies provide evidence that thymically-derived Treg (tTreg) cells form based on TCR specificity for self-Ag.

Autoreactive thymocytes can differentiate into $Foxp3^+$ Treg cells, but they can also undergo deletion, and there is evidence that these processes are inter-related and that the abundance of cognate Ag may determine the extent to which each occurs. In many of the studies in which TCR transgenic thymocytes upregulated Foxp3 in the presence of cognate Ag, deletion of thymocytes expressing the clonotypic TCR was also observed, suggesting that Treg cell differentiation and deletion can occur simultaneously and that recognition of the same Ag can drive each process (Apostolou et al. 2002; Aschenbrenner et al. 2007; Kawahata et al. 2002; Larkin et al. 2008; Walker et al. 2003). Our lab has previously described studies in which TS1 mice were inter-mated with various lineages of HA transgenic mice that express HA at different levels, and the amount of 6.5^+CD4^{SP} thymocyte deletion that occurred directly correlated with the amount of HA mRNA present in the thymus, such that more 6.5^+CD4^{SP} deletion was observed in mice with higher levels of HA expression (Picca et al. 2009). Interestingly, the percentage of 6.5^+CD4^{SP} cells expressing Foxp3 remained similar in all HA transgenic mice examined, with the result that $6.5^+CD4^+Foxp3^+$ T cells accumulated more efficiently in mice with relatively lower levels of HA expression due to the less extensive deletion that occurred in these mice relative to mice with higher expression of

HA. Similarly, i.v. injection of HEL and subsequent HEL presentation in the thymus could induce both deletion and Foxp3 upregulation in TCR-transgenic thymocytes specific for HEL (Atibalentja, Byersdorfer, Unanue 2009). Injection of higher doses of HEL resulted in more extensive deletion of HEL-reactive thymocytes, while the percentage that upregulated Foxp3 remained consistent, such that more HEL-specific $CD4^{SP}Foxp3^{+}$ thymocytes accumulated in mice injected with low doses than higher doses of HEL (Atibalentja, Byersdorfer, Unanue 2009). Thus, thymocyte deletion biases Treg cell reactivity toward agonist self-peptides present at relatively low abundance.

1.3 Regulatory T cell differentiation from $CD4^{+}Foxp3^{-}$ T cells outside the thymus

tTreg cells appear to constitute the majority of the Treg cell population (Hsieh et al. 2006; Josefowicz et al. 2012), but in certain circumstances $CD4^{+}Foxp3^{-}$ cells that are present in the periphery can differentiate into $Foxp3^{+}$ Treg cells (termed peripherally-derived Treg (pTreg) cells) (Yadav, Stephan, Bluestone 2013). *In vitro*, exposure to TGF-β in conjunction with TCR stimulation induces $CD4^{+}CD25^{-}$ T cells to upregulate Foxp3 expression and differentiation into functionally suppressive Treg cells (Chen et al. 2003; Kretschmer et al. 2005). *In vivo,* several studies have suggested that pTreg cells may be important in establishing tolerance to foreign antigens that are present at mucosal barriers, such as the lungs and intestinal tract. For instance, a specialized subset of $CD103^{+}$ dendritic cells (DCs) exists in gut associated lymphoid tissues that can induce upregulation of Foxp3 in T cells that recognize orally-administered Ag in a TGF-β and retinoic acid-dependent manner (Coombes et al. 2007; Sun et al. 2007).

Additionally, one study found that multiple TCRs isolated from colonic Treg cells were specific Ag derived from commensal bacteria (Lathrop et al. 2011). Thymocytes that were retrovirally-transduced to express TCRs derived from colonic Treg cells did not upregulated Foxp3 in the thymus, but mature T cells expressing these TCRs could differentiate into pTreg cells and accumulated in the colon, although this only occurred when host mice had similar intestinal bacterial composition as the mice from which the TCR was originally isolated (Lathrop et al. 2011). These studies demonstrate that pTreg cell formation can occur in gut associated lymphoid tissues based on TCR specificity for Ag derived from food or commensal bacteria present in the gut. Furthermore, mice that lack the conserved non-coding sequence-1 (CNS1) element at the Foxp3 locus, which display normal tTreg cell formation but impaired Treg differentiation from $CD4^+Foxp3^-$ T cells both *in vitro* and *in vivo* following feeding with an Ag, develop gastritis and airway inflammation, indicating that the ability of conventional T cells to upregulate Foxp3 after encountering a harmless environmental Ag may be crucial to preventing damaging inflammation at barrier surfaces (Josefowicz et al. 2012; Zheng et al. 2010).

pTreg cells can also form in non-gut draining LNs in an Ag-dependent manner (Apostolou and von Boehmer 2004). For instance, injection of a fusion protein containing the S1 peptide of HA attached to the C terminus of an anti-DEC antibody to target HA for endocytosis and presentation by DCs resulted in the upregulation of Foxp3 and CD25 by adoptively transferred $6.5^+CD4^+Foxp3^-$ T cells (Kretschmer et al. 2005). In this study, 6.5^+ Treg cell differentiation occurred most efficiently when a relatively low dose of the anti-DEC-HA fusion protein was used (Kretschmer et al. 2005). Similarly, a higher percentage of moth cytochrome c (MCC)-specific $CD4^+$ T cells upregulated Foxp3

when a relatively lower dose of MCC was injected compared with relatively higher doses (Gottschalk, Corse, Allison 2010). Thus, cognate Ag that is injected, rather than present at mucosal barriers, can also drive pTreg cell formation in the periphery, and a relatively lower dose of Ag favors pTreg cell formation. However, exogenously administered peptides are subject to turn-over and clearance, and how specificity for naturally processed self-peptides can direct pTreg cell formation has not been well studied. Moreover, naturally processed self-peptides can be expressed at varying levels by diverse cell types and at distinct anatomical locations, and how these factors might influence pTreg cell formation has not been determined.

1.4 Treg cell stability

Foxp3 expression is absolutely required for Treg cell formation, however retroviral transduction of conventional $CD4^+Foxp3^-$ T cells with Foxp3 did not result in acquisition of the full Treg cell transcriptional signature, indicating that Foxp3 expression alone is not sufficient to induce a full, stable Treg cell phenotype (Hill et al. 2007). Subsequently, it was discovered that selective demethylation of the CNS2 element within the Foxp3 locus occurs in Treg cells but not conventional $CD4^+$ T cells, and is required for stable Foxp3 expression (Floess et al. 2007; Polansky et al. 2008). Treg cells isolated directly *ex vivo* display extensive demethylation at the Foxp3 locus and stable expression of Foxp3, but this demethylation was not observed in Treg cells formed *in vitro* in the presence of TGF-β, which can rapidly lose Foxp3 expression upon restimulation (Floess et al. 2007; Polansky et al. 2008). However, when $CD4^+Foxp3^-$ T cells were stimulated with anti-CD3 and TGF-β in the presence of the azacytidine, which

inhibits DNA methylation, the CNS2 element was demethylated and stable Foxp3 expression was induced (Polansky et al. 2008). Furthermore, Treg cells in which CNS2 is deleted fail to maintain Foxp3 expression during division (Zheng et al. 2009). Together, these studies demonstrate that Foxp3 expression and commitment to the Treg cell lineage are unstable in the absence of CNS2 demethylation. While Treg cells constitute a relatively stable lineage under homeostatic conditions (Komatsu et al. 2009; Miyao et al. 2012; Rubtsov et al. 2010), there is evidence that they can lose Foxp3 expression and adopt an effector phenotype under certain inflammatory and lymphopenic conditions (Duarte et al. 2009; Laurence et al. 2012; Zhou et al. 2009). For instance, a fraction of $CD4^+Foxp3^+$ cells transferred into $RAG2^{-/-}$ mice downregulate Foxp3 and acquire the ability to produce cytokines such as IL-2, IFN-γ, and IL-17, and this unstable subset was enriched among $CD4^+Foxp3^+$ cells that did not express CD25 (Duarte et al. 2009; Komatsu et al. 2009; Miyao et al. 2012). Furthermore, a subset of $CD4^+Foxp3^+$ cells cultured in the presence of IL-6 or IL-27 lost Foxp3 expression and gained the ability to produce IL-17, suggesting that cytokine stimulation may also drive loss of Foxp3 (Laurence et al. 2012). These studies indicate that Treg cells may downregulate Foxp3 under certain circumstances, but they all used $Foxp3^{GFP}$ reporter mice to sort their initial $CD4^+Foxp3^+$ T cell population, and concerns were raised that contaminating GFP^- cells in the sorted population may be the precursors for the $CD4^+Foxp3^-$ T cell population in each experiment (Sawant and Vignali 2014). Indeed, a subsequent study used a Foxp3 lineage tracing mouse strain in which tamoxifen treatment resulted in constitutive and heritable expression of YFP in a cohort of cells that expressed Foxp3 at the time of treatment, and found that YFP^+CD4^+ T cells retained Foxp3 expression at steady state for up to 5 months and during Th1-type inflammation induced by *L. monocytogenes* infection (Rubtsov et al. 2010). A separate group using a

different Foxp3 lineage tracing mouse model, in which Foxp3 expression resulted in constitutive and heritable expression of YFP constitutively rather than inducibly, found that transfer of $CD4^+Foxp3^+YFP^+$ T cells specific for a pancreatic islet autoantigen into NOD.TCR$\alpha^{-/-}$ mice resulted in a subset of these cells losing Foxp3 expression and initiating IFN-γ production (Zhou et al. 2009). The same group later showed that a population of myelin oligodendrocyte glycoprotein (MOG)-specific $YFP^+CD4^+Foxp3^-$ T cells developed in mice during experimental autoimmune encephalomyelitis (EAE), and, moreover, that MOG-specific $YFP^-Foxp3^-CD4^+$ T cells did not upregulated YFP during EAE. These results suggest that these so called "ex-Treg" cells derived from $CD4^+Foxp3^+$ cells that subsequently lost Foxp3 expression, rather than from transient Foxp3 upregulation and subsequent YFP expression in $CD4^+Foxp3^-$ T cells (Bailey-Bucktrout et al. 2013). Furthermore, it was determined that when a population of $CD4^+CD25^+Foxp3^+$ Treg cells displaying full demethylation of CNS2 was transferred into mice with developing EAE, a subset of these cells that were specific for MOG, as determined by tetramer staining, lost Foxp3 expression (Bailey-Bucktrout et al. 2013). Thus, questions remain regarding whether Foxp3 expression is stable in Treg cells and the circumstances in which Foxp3 expression may be lost.

1.5 Role of TCR specificity in Treg cell activity *in vivo*

Treg cells require TCR stimulation to expand and to suppress proliferation of effector cells (Cozzo, Larkin, Caton 2003; Takahashi et al. 1998; Thornton and Shevach 1998), but once activated through the TCR, Treg cells can suppress proliferation of $CD4^+$ T cells of other specificities both *in vitro* and *in vivo* (Takahashi et al. 1998; Yu et

al. 2005). Treg cells proliferate *in vivo* following recognition of cognate Ag, both when it is present as a self-Ag and when it is provided via immunization, but do not proliferate when cognate Ag is absent, and this observation remains true even in the context of lymphopenia, which can induce expansion of $CD4^{+}Foxp3^{-}$ T cells (Cozzo, Larkin, Caton 2003; Walker et al. 2003). Multiple studies have found that tissue Ag-specific Treg cells are enriched in the draining LNs of that tissue. For instance, OVA-specific DO11 Treg cells divide and accumulate preferentially in the pancreatic LN of mice expression OVA under control of the rat insulin-promoter (Walker et al. 2003). Furthermore, Treg cells isolated from the ovarian LNs are more effective than Treg cells isolated from non-draining LNs at suppressing the development of autoimmune ovarian disease in mice that were thymectomized at day 3 post-birth (Samy et al. 2005; Samy et al. 2008). Similarly, Treg cells capable of suppressing experimental autoimmune prostatitis were selectively enriched in the prostate LN, and Treg cells capable of suppressing dacryoadenitis were enriched in the lacrimal gland LN (Wheeler, Samy, Tung 2009). An examination of TCR repertoires revealed that Treg cell TCR repertoires vary by anatomical location (Lathrop et al. 2008). Together with the observation that tissue-specific Ag is selectively presented in draining LNs (Hoglund et al. 1999; Scheinecker et al. 2002), these studies provide evidence that tissue-draining LNs are enriched for Treg cells that can recognize antigens derived from that tissue, and these Treg cells more effectively suppress autoimmune disease in that tissue. Thus, a role for TCR specificity has been established in Treg cell activity as well as in Treg cell formation.

1.6 Treg cell activity during infection

It is becoming increasingly apparent that Treg cells constitute an active component of the immune response to a pathogen. In one of the first published experiments to demonstrate the involvement of Treg cells during an infection, $CD4^+CD25^+$ T cells were shown to accumulate within sites of chronic *Leishmania major* infection in the skin (Belkaid et al. 2002). Although Foxp3 had not been identified as the lineage-defining transcription factor for Treg cells yet, these $CD4^+CD25^+$ T cells displayed many of the hallmarks of Treg cells, including a high expression level of CTLA-4 and low expression of CD45RB, a hypoproliferative phenotype *in vitro*, and strong suppression of $CD4^+CD25^-$ cell proliferation following anti-CD3 stimulation (Belkaid et al. 2002). Expansion and/or recruitment of Treg cells also occurs in the lungs and mediastinal LN during acute respiratory syncytial virus (RSV) infection and in the vaginal tract following infection with herpes simplex virus (HSV) 2, suggesting that this may be a common occurrence during the response to any pathogen (Fulton, Meyerholz, Varga 2010; Lee et al. 2010; Lund et al. 2008). The Treg cells found at the site of infection (the lungs in RSV infection and the vaginal tract in HSV-2 infection) are not only present but appear to be dynamically responding, as they express activation markers and proliferate to a greater extent than Treg cells in naïve mice (Fulton, Meyerholz, Varga 2010; Lee et al. 2010; Lund et al. 2008; Scott-Browne et al. 2007). The source of the Treg cells that accumulate at infection sites has been a matter of considerable interest. One possibility is that $CD4^+Foxp3^-$ T cells that recognize pathogen-derived Ag may convert into $CD4^+Foxp3^+$ pTreg cells during an infection. However, when polyclonal populations of Treg cells and non-Treg cells were transferred into mice infected with either *L. major* or *M. tuberculosis*, the Treg cells isolated from infection sites had

expanded from the population of transferred Treg cell, and had not converted from the conventional T cells (Belkaid et al. 2002; Shafiani et al. 2010). Furthermore, when TCR-transgenic $CD4^+Foxp3^-$ T cells specific for either *M. tuberculosis* or influenza virus Ag were tracked during infection with each respective pathogen, no upregulation of Foxp3 occurred, but TCR-transgenic Treg cells expanded during infection when they were capable of recognizing the pathogen (Bedoya et al. 2013; Shafiani et al. 2010). Thus, the preponderance of evidence suggests that the Treg cells present at sites of infection expand from Treg cell that exist in the pre-immune repertoire and do not differentiate from $CD4^+Foxp3^-$ T cells.

There is evidence that the Treg cells that accumulate at infection sites can modulate the anti-pathogen immune response. Depletion of Treg cells using the anti-CD25 antibody PC-61 prior to infection resulted in increased virus-specific $CD8^+$ T cell proliferation and activation following foot pad injection of HSV-1 virus, and increased the number of effector immune cells in the lungs during influenza and RSV infection (Bedoya et al. 2013; Fulton, Meyerholz, Varga 2010; Lee et al. 2010; Suvas et al. 2003). Furthermore, depletion of Treg cells resulted in more efficient clearance of the pathogen in several different infection models (Belkaid et al. 2002; Kursar et al. 2007; Scott-Browne et al. 2007; Suvas et al. 2003), but in some cases also led to increased immunopathology (Cervantes-Barragan et al. 2012; Suvas et al. 2004). These studies suggest a role for Treg cells in curbing the effector immune response during an infection, thus potentially averting immunopathology but impairing clearance of the pathogen. Other groups have alternatively suggested that Treg cells may support the initiation of an immune response to a pathogen. For instance, in one study, depletion of Treg cells by

treatment with diphtheria toxin in Foxp3DTR mice, which express the human diphtheria toxin receptor under control of a Foxp3 promoter, prior to genital infection with HSV-2 led to increased viral titers in the vaginal tract and spinal cord and exacerbated disease (Lund et al. 2008). It was further shown that mice depleted of Treg cells had fewer adaptive and innate immune cells in the vaginal tract at day 2 post infection, although effector cell numbers had recovered by day 4 post infection, suggesting that Treg cells may be important in the initial recruitment of effector cell subsets into the infected tissue. Similarly, depletion of Tregs with anti-CD25 antibody prior to RSV infection resulted in decreased numbers of RSV-specific $CD8^+$ T cells in lungs at day 6 post-infection, but increased numbers by day 8 post-infection, again supporting the idea that Tregs may help direct the initial effector response (Fulton, Meyerholz, Varga 2010). However, a caveat to the use of a Treg cell depletion strategy to examine the function of Treg cells during infection is that it results in systemic immune activation and severe disruption of immune homeostasis that can eventually lead to death (Kim, Rasmussen, Rudensky 2007). Thus, it is possible that the differences in the anti-pathogen immune response that are observed in Treg cell-depleted mice are secondary to disruption of immune homeostasis, rather than revealing a direct effect of Treg cells on anti-viral effector cells. An alternative strategy for assessing the role of Treg cells in infection has been to adoptively transfer TCR-transgenic Treg cells that are specific for pathogen-derived antigens, and evaluate how their presence affects the immune response during infection. Our lab has shown that adoptive transfer of Treg cells specific for influenza virus results in decreased accumulation of $CD4^+Foxp3^-$ and $CD8^+$ T cells in the lungs of influenza virus-infected mice (Bedoya et al. 2013). Similarly, Treg cells expressing a TCR specific for an *M. tuberculosis* Ag accumulated in the lungs and draining LNs during infection, but only when the *M. tuberculosis* strain used contained their cognate Ag, and delayed the

arrival of effector cells in the lungs (Shafiani et al. 2010). These data show that TCR-mediated recognition of Ag is crucial for Treg cell activity during infection, and that Treg cells that recognize pathogen-derived Ag can modulate the effector response. However, the majority of Treg cells present in the pre-immune repertoire appear to be specific for self-Ag (Weissler and Caton 2014). Accordingly, an additional possibility is that Treg cells can be activated based on recognition of a selecting self-Ag that is being presented at the site of infection, and the extent to which Treg cell recognition of self might drive Treg cells to modulate the immune response to a pathogen has not been investigated.

1.7 Functional specialization of Treg cells

Recently, it has become apparent that Tregs cells can differentiate further in response to different stimuli, and acquire distinct transcriptional profiles that allow them to maintain immune homeostasis in different circumstances (Campbell and Koch 2011). The use of a Foxp3-driven Cre system to conditionally delete IRF4, a transcription factor required for differentiation of the Th2 subpopulation of $CD4^+$ T cells (Lohoff et al. 2002), in Foxp3-expressing cells resulted in autoimmune lymphoproliferative disease and an increase in the percentage of $CD4^+$ T cells producing Th2-associated cytokines but not those associated with a Th1 or Th17 $CD4^+$ T cell response (Zheng et al. 2009). Similarly, selective deletion of Stat3, which is associated with the Th17 subpopulation of $CD4^+$ effector T cells, in $Foxp3^+$ cells resulted in the development of inflammatory bowel disease, and the percentage of $CD4^+$ T cells producing IL-17 was selectively increased (Chaudhry et al. 2009). Finally, deletion of T-bet, a transcription factor required for Th1 effector $CD4^+$ T cell differentiation (Szabo et al. 2000), in Treg cells resulted in failure to

control Th1-mediated autoimmune lymphoproliferation (Koch et al. 2009). Studies from our lab have shown that Treg cells that recognize influenza virus Ag differentiate to acquire expression of T-bet, CXCR3, Blimp1, and IL-10 during influenza virus infection (Bedoya et al. 2013). Additional work has shown that signaling through the IFN-γR can induce T-bet expression in Treg cells, and T-bet can in turn drive CXCR3 expression, which suggests that costimulation with viral Ag and IFN-γ during influenza virus infection likely prompts Treg cell differentiation in this context (Koch et al. 2009). However, questions remain regarding how variations in Ag presentation and the cytokine environment might affect Treg cell differentiation towards a T-bet$^+$ subpopulation.

1.8 Manipulation of Treg cells for therapeutic use

Expansion or adoptive transfer of Treg cells has been suggested as a potential therapy in a variety of settings, including solid organ or hematopoietic stem cell transplantation, autoimmune diseases, and allergy (Allan et al. 2008). In patients with autoimmune diseases, such as type 1 diabetes, multiple sclerosis, and rheumatoid arthritis, Treg cells that are present are unable to suppress autoimmune disease development and show evidence of dysfunction (Ehrenstein et al. 2004; Lindley et al. 2005; Venken et al. 2008; Viglietta et al. 2004). It is possible that these Treg cells have failed to differentiate appropriately to acquire the necessary capabilities, or that they do not have the correct specificity. Furthermore, adoptive transfer of Treg cells limited disease development in mouse models of all three diseases, suggesting that they may be an effective treatment in human patients, as well (Morgan et al. 2005; Tang et al. 2004; Yu et al. 2005). In one study, umbilical cord blood-derived Treg cells were

expanded and transferred to patients receiving a hematopoietic stem cell graft, and the treatment was well accepted and showed promising results with respect to decreasing the incidence of acute graft-versus-host disease (Brunstein et al. 2011). Additional studies have further proven the safety and feasibility of Treg cell adoptive transfer in solid organ transplant, although efficacy has not been established (Ferrer et al. 2014). A protocol has recently been established for expanding human alloantigen-reactive Treg cells *in vitro* using good manufacturing practice-compliant reagents, supporting the feasibility of Treg cell expansion for adoptive immunotherapy (Putnam et al. 2013). As clinical trials move forward, a better understanding of how variations in Ag presentation can affect Treg cell activation and differentiation may increase our ability to induce Treg cells to be effective in different settings.

CHAPTER 2: Materials and Methods

2.1 Transgenic mice

TS1 mice express a transgenic TCR that is specific for the S1 determinant (SFERFEIFPKE) of influenza virus PR8 hemagglutinin presented in the context of MHCII I-E^d and can be identified by the monoclonal antibody 6.5 (Kirberg et al. 1994). HA28 and HA12 mice express a truncated form of PR8 HA (the NH_2 terminal 237 amino acids) under control of the SV40 early promoter-enhancer region (Cerasoli et al. 1995; Picca et al. 2009; Shih, Cerasoli, Caton 1997). HACII mice express a full length PR8 HA transgene driven by an MHCII promoter (Jordan et al. 2001; Reed et al. 2003). BALB/c.Foxp3^{eGFP} (or C.Cg-Foxp3$^{tm2(EGFP)}$Tch/J) mice (Haribhai et al. 2007) and congenic BALB/c.Ly5.1 mice were acquired from The Jackson Laboratory. BALB/c mice were purchased from Charles River Laboratories. Mice were inter-mated to produce TS1.Foxp3^{eGFP}.Ly5.1 mice and TS1xHA28 Foxp3^{eGFP}.Ly5.1 mice. All experimental mice were female, and in cases where transgenes were expressed, experimental mice were heterozygous for the TCR transgene, HA transgene, and Ly5.1 allele, and were homozygous for the Foxp3^{eGFP} reporter. All mice were bred and maintained on a BALB/c background, and were housed under specific pathogen-free conditions in the Wistar Institute Animal Facility. Experiments were performed according to protocols approved by the Wistar Institutional Animal Care and Use Committee.

2.2 Real time quantitative RT-PCR

RNA was isolated from harvested tissue using a Biospec Tissue Tearor and TRIzol reagent (Life Technologies), and purified and concentrated using an RNeasy kit (Qiagen). cDNA was synthesized with the High Capacity RNA to cDNA kit (Applied Biosystems), and RT-qPCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems) on a 7500 Fast Real-Time PCR system.

2.3 Cell sorting and adoptive transfers

For Treg cell induction experiments, $6.5^{+}CD4^{+}CD25^{-}eGFP^{-}$ cells were sorted from the LNs and spleens of TS1.Foxp3^{eGFP}.Ly5.1 mice. For Treg cell transfer experiments, $CD4^{+}eGFP^{+}$ cells were isolated from the LNs and spleens of TS1xHA28.Foxp3^{eGFP}.Ly5.1 mice. In both cases, 10^{6} cells in 150 µL PBS were adoptively transferred into recipient mice via tail vein injection. FACS was performed using a MoFlow (DakoCytomation) or FACSAria (BD Biosciences).

2.4 Cell staining and flow cytometry

Single cell suspensions of pLNs (pooled axillary, brachial, and inguinal), mesenteric LNs, spleens, thymii, mediastinal LNs and lungs were stained using the Live/Dead Fixable Aqua Dead Cell Stain Kit (Invitrogen), blocked with Fc block (BD Biosciences), then stained for surface markers at 4 ˚C for 25 minutes. For intracellular staining, cells were fixed and permeabilized after surface staining using the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer's instructions. Antibodies were purchased from eBioscience, BD Biosciences, or Invitrogen. Data was collected

using an LSR II flow cytometer (BD Biosciences) and analyzed on FlowJo software (Tree Star).

2.5 CellTrace Violet labeling

Cells were washed twice with PBS, then resuspended in pre-warmed PBS at $3x10^6$ cells/mL with 3 μM CellTrace Violet (Invitrogen), and incubated at 37 ˚C for 20 minutes, after which an equal volume of pre-warmed FBS was added, and cells were incubated for an additional 5 minutes. Cells were washed with PBS prior to adoptive transfer or addition to *in vitro* cultures.

2.6 *In vitro* suppression assay

In a 96-well U-bottom plate, 100,000 $CD4^-CD8^-$ BALB/c splenocytes, 50,000 FACS-isolated $CD4^+6.5^+eGFP^-$ responder cells from TS1.Foxp3 mice, and 1 μM S1 peptide were combined in supplemented IMDM plus 10% FBS. $CD8^-Ly5.1^+6.5^+eGFP^+$Treg cells were isolated from TS1xHA28. $Foxp3^{eGFP}$.Ly5.1 mice or from HA28 recipient mice at day 7 post-transfer of $6.5^+CD4^+CD25^-eGFP^-$ cells from TS1.$Foxp3^{eGFP}$.Ly5.1 mice; these Treg cells were added to the culture at a 1:2 or 1:4 ratio relative to the responder cells. Cells were stained for flow cytometric analysis after 3 days of culture.

2.7 Influenza viruses

The influenza viruses PR8 (A/Puerto Rico/8/1934 [H1N1]) and its derivative RV6, which contains a single amino acid substitution in the Site 1 peptide of PR8 HA (SFERF**E**IFPKE mutated to SFERF**K**IFPKE), were propagated in 10-d hen's embryonated eggs (Hurwitz et al. 1984).

2.8 Influenza virus infection

Mice were anesthetized by i.p. injection of ketamine/xylazine (70/7 mg/kg), and 0.003 HAU of PR8 virus or 0.005 HAU of RV6 virus or 0.017 HAU of J1 virus was administered intranasally in 50 µL PBS.

2.9 ELISA for IFN-γ

Blood was collected immediately post-mortem by cardiac puncture, allowed to clot, then spun down to separate out serum. To perform the ELISA, a 96-well plate was coated with anti-IFN-γ antibody (clone R4-6A2; BD Biosciences) overnight. Serum was plated undiluted and diluted 1:3 with PBS supplemented with 10%FBS. Biotinylated-anti-IFN-γ (clone XMG1.2; eBioscience) was used as the secondary antibody, and streptavidin-HRP and tetramethylbenzidine (eBioscience) were used to detect bound antibody, and the concentration was calculated based on a standard curve using an IFN-γ standard.

2.10 In vitro APC : T cell coculture

B cells ($B220^{+}CD11c^{-}CD11b^{-}$), DCs ($B220^{-}CD11c^{+}Ly6C^{-}Ly6G^{-}$), neutrophils ($B220^{-}CD11c^{-}CD11b^{+}Ly6G^{hi}Ly6C^{lo}$) and inflammatory monocytes ($B220^{-}CD11c^{-}CD11b^{+}Ly6G^{-}Ly6C^{hi}$) were isolated from the mediastinal LNs of mice at day 5 post-infection. These APCs were plated in a 96 well U-bottom plate either undiluted, or diluted into $CD4^{-}CD8^{-}$ feeder cells from the spleen of a BALB/c mouse, such that 100,000 cells were present in each well in supplemented IMDM plus 10% FBS. 60,000 cells isolated from the LNs of a TS1xHA28.$Foxp3^{eGFP}$ mouse and labeled with CellTrace Violet were then added to each well. Division and CXCR3 expression by the Treg cells from the TS1xHA28.$Foxp3^{eGFP}$ were determined after 4 days of culture at 37 ˚C.

2.11 Statistical analyses

Statistical tests were performed with GraphPad Prism software. A one-way ANOVA followed by Tukey's post-test was used for comparisons between groups within the same dataset. A two-tailed Student *t* test was used where indicated when comparing two independent groups. Mean +SEM represented in all bar graphs. *$p<0.05$; **$p<0.01$; ***$p<0.005$.

2.12 Solutions

Phosphate buffered saline (PBS): 2.2mM NAH_2PO_4 [Anhydrous], 12.6 mM Na_2HPO_4 [Anhydrous], 6.2 mM KCl, and 170 mM NaCl (solution pH 7.4)

FACS buffer: PBS, 2% heat-inactivated fetal bovine serum (FBS; Tissue Culture Biologicals, Tulare, CA)

Supplemented Iscove's Modified Dulbecco's Medium (IMDM; Mediatech, Inc., Herndon, VA), 10% FBS, $5x10^{-5}$ M 2-mercaptoethanol, and 100 µL/mL gentamycin

RBC lysis buffer: 0.15 M NH_4CL and 0.01 M Tris (pH 7.5)

CHAPTER 3: Distinct modes of Ag presentation promote Treg cell formation *in vivo*

3.1 Introduction

During T cell development within the thymus, a signal through the TCR is required for positive selection and survival of a thymocyte, and thymocytes that fail to interact with MHC molecules are deleted from the repertoire (Klein et al. 2014). However, thymocytes that receive too strong a TCR signal from recognition of self-peptide:MHC complexes are deleted, or alternatively, differentiate into Foxp3+ Treg cells (Klein et al. 2014). Although the precise mechanism that determines whether autoreactive thymocytes undergo deletion or become Treg cells remains undetermined, there is evidence that the abundance of the cognate self-Ag can play a role in fate determination, with Treg cell formation biased towards thymocytes that recognize a less prevalent cognate self-Ag (Jordan et al. 2001; Picca et al. 2009). The clonal frequency of thymocytes expressing a particular TCR can also affect the extent of Treg cell formation in the thymus, with less thymocytes upregulating Foxp3 when a higher percentage of thymocytes express a transgenic TCR (Bautista et al. 2009; Leung, Shen, Lafaille 2009). Perhaps due in part to the existence of this niche that limits the number of Treg cells of a given specificity that can develop, some autoreactive thymocytes are able to leave the thymus and enter the periphery, as evidenced by the sudden autoimmune lymphoproliferative disease that develops when Treg cells are eliminated (Kim, Rasmussen, Rudensky 2007). Whether these autoreactive T cells can later upregulate Foxp3, or if they are intrinsically incapable of doing so, perhaps due to changes in the epigenome, has not been explored.

Mature $CD4^+$ T cells that are signaled through the TCR complex upon recognition of cognate Ag:MHCII complex can proliferate and adopt distinct effector cell fates (i.e. Th1, Th2, Th17, pTreg, or follicular helper T cells), or alternatively they can undergo deletion or become anergic (Vahedi et al. 2013). Exposure of $CD4^+$ T cells to Mls superantigens *in vivo* resulted in expansion, then deletion, of Mls reactive T cells (McCormack et al. 1993; Webb, Morris, Sprent 1990). Furthermore, when $CD4^+$ T cells specific for HA were adoptively transferred into mice expressing HA as a self-Ag, cells were rapidly deleted and the remainder became anergic, in that they failed to proliferate or produce IFN-γ upon subsequent stimulation (Lanoue et al. 1997). Similarly, $CD4^+$ T cells isolated from a mouse that had been tolerized against human γ-globulin (HGG) by i.p. injection of HGG failed to proliferate or produce IL-2 or IL-4 upon subsequent stimulation with HGG (Chu et al. 1995). Anergy and deletion constitute two potential mechanisms of establishing peripheral tolerance, but tolerance can also be achieved by conversion of $CD4^+Foxp3^-$ T cells into $Foxp3^+$ Treg cells. While thymically-derived Treg (tTreg) cells appear to constitute the majority of the Treg cell population (Hsieh et al. 2006; Josefowicz et al. 2012), these tTreg cells are insufficient for maintaining immune homeostasis (Josefowicz et al. 2012), and the Treg cell repertoire is supplemented by the conversion of $CD4^+Foxp3^-$ T cells into $CD4^+Foxp3^+$ Treg cells in the periphery (Yadav, Stephan, Bluestone 2013). $CD4^+Foxp3^-$ conventional T cells that express a TCR specific for Ag derived from colonic microbiota or from food, neither of which would be present in the thymus during T cell development, can upregulate Foxp3 and become functional Treg cells (Lathrop et al. 2011; Sun et al. 2007). A specialized subset of $CD103^+$ DCs that exists within the gut draining LNs facilitates this Treg cell differentiation in a TGF-β and retinoic acid dependent manner (Coombes et al. 2007; Sun et al. 2007).

However, $CD4^+Foxp3^-$ T cells can also convert into $Foxp3^+$ Treg cells in the pLNs following injection of cognate Ag, and in some cases, low doses of the peptide were found to favor $Foxp3^+$ pTreg cell formation (Apostolou and von Boehmer 2004; Gottschalk, Corse, Allison 2010; Kretschmer et al. 2005). It has furthermore been shown that recognition of self-Ag can drive $CD4^+Foxp3^-$ cells to upregulate Foxp3 in lymphopenic mice (Knoechel et al. 2005). The extent to which recognition of self-Ag can induce pTreg cell differentiation in lymphoreplete mice, and how variations in presentation of a self-Ag might affect this process, have not been well studied.

The transcription factor Helios is expressed in nearly all $CD4^{SP}Foxp3^+$ cells in the thymus and marks thymocytes that have received a strong signal through the TCR, but Helios is not present in Treg cells formed *in vitro* by TCR stimulation of $CD4^+Foxp3^-$ T cells in the presence of TGF-β, or in pTreg cells that differentiate after mice are fed cognate Ag (Daley, Hu, Goodnow 2013; Gottschalk, Corse, Allison 2012; Hadis et al. 2011; Thornton et al. 2010). Due to these observations, Helios was originally identified as a marker of tTreg cells, however subsequent studies revealed that $CD4^+Foxp3^-$ T cells that upregulated Foxp3 upon recognition of an injected Ag additionally upregulated Helios (Gottschalk, Corse, Allison 2012). The discrepancy in these observations has not been satisfactorily resolved, and why Helios may be upregulated in pTreg cells in some circumstances but not in others remains unclear.

In this section, we describe studies investigating how varying the presentation of a self-Ag can affect the fate of autoreactive thymocytes and T cells. We made use of several different transgenic mouse lineages that express HA as a self-Ag at different

levels and in different cell types, and assessed the fate of TCR-transgenic thymocytes and T cells specific for HA in these various environments. A low dose of a self-Ag favored Treg cell formation in the thymus, while a higher dose resulted in increased deletion of reactive thymocytes. In the periphery, a relatively less immunostimulatory form of self-Ag induced a subset of reactive $CD4^+Foxp3^-$ T cells to upregulate Foxp3 and differentiated into $Helios^+$ Treg cells, but the same self-Ag expressed at higher levels on APCs and presented in a more immunostimulatory manner induced reactive $CD4^+Foxp3^-$ T cell proliferation but not Foxp3 expression. We have additionally assessed whether previous exposure to cognate Ag during development, or the presence of tTreg cells of the same specificity can affect the conversion of $CD4^+Foxp3^-$ cells into pTreg cells. Collectively, these studies reveal that the mode of presentation of a self-Ag plays a determining role in the fate of thymocytes and T cells that recognize that Ag.

3.2 Results

3.2.1 The HA transgene is expressed at lower levels and self-HA is presented with less stimulatory potency in HA28 mice relative to HACII mice

HA28 mice and HACII mice are previously described transgenic lineages that express the hemagglutinin molecule from the influenza virus A/PR/8/34 (PR8) as a neo-self Ag (Jordan et al. 2001; Reed et al. 2003; Shih, Cerasoli, Caton 1997). HA28 mice express a truncated form of PR8 HA (the NH_2-terminal 237 amino acids) driven by the SV40 early promoter-enhancer region (Cerasoli et al. 1995), while HACII mice express the full length PR8 HA under control of an MHC Class II I-Eα promoter (Reed et al.

2003). The different insertion sites and promoters for the HA transgene in the two lineages cause the HA to be expressed by different cell types and ultimately in drastically different amounts. Analysis by RT-PCR showed that the amount of HA RNA was increased in both lineages over background (BALB/c control) in the thymus, spleen, pLNs, and intestines (Fig. 3-1 A). However, over 1000-fold more HA RNA was found in HACII mice relative to HA28 mice in all tissues examined (Fig. 3-1 A). Adoptive transfer of CellTrace-labeled, HA-specific $CD4^+$ T cells from TS1 mice (transgenic mice expressing an HA-specific TCR recognized by the anti-clonotypic mAb 6.5 (Kirberg et al. 1994)) demonstrated that the endogenous HA could induce T cell division in both lineages (Fig. 3-1 B). In contrast, no division occurred in BALB/c mice, in which HA is not present. Furthermore, a higher percentage of T cells underwent more rounds of division in HACII mice compared with HA28 mice. Thus, higher levels of HA mRNA and targeted expression to MHC Class II^+ cells makes the neo-self HA a more potent stimulator of $CD4^+$ T cells in HACII mice than in HA28 mice.

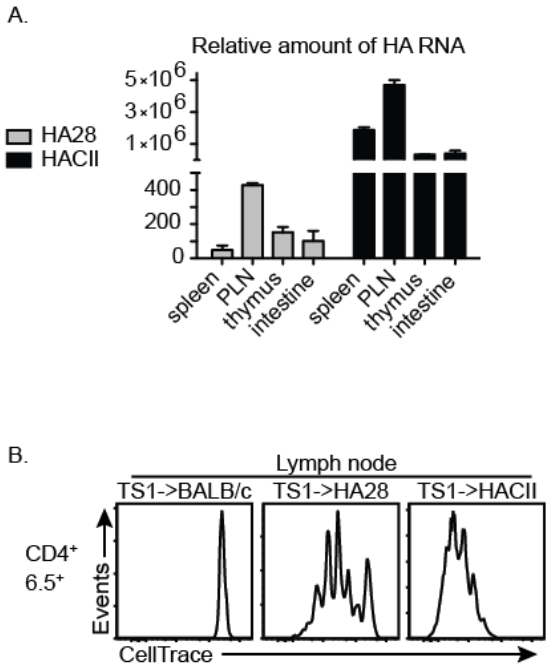

Figure 3-1 HA28 and HACII mice express the PR8 HA molecule systemically in different amounts and with unique stimulatory potencies. (**A**) RT-PCR was used to determine the amount of HA RNA in the spleens, pLNs, thymi, and intestines of HA28 and HACII mice relative to BALB/c controls. (**B**) $6.5^{+}CD4^{+}eGFP^{-}$ cells isolated from the LNs of TS1.Foxp3^{eGFP} mice were labeled with CellTrace Violet then adoptively transferred into either BALB/c, HA28, or HACII recipients. The extent of CellTrace Violet dilution was assessed at day 3 post-transfer.

3.2.2 Stimulatory potency of the HA self-Ag determines the extent of autoreactive thymocyte deletion and Treg cell formation

To evaluate how the differences in HA expression in HA28 versus HACII mice might influence autoreactive thymocyte development, we mated both lineages with TS1 mice to produce TS1xHA28 and TS1xHACII mice. In TS1xHA28 mice, there was a significant decrease in the number of $6.5^{+}CD4^{SP}$ thymocytes relative to TS1 mice, but the number of $6.5^{+}CD4^{SP}Foxp3^{+}$ thymocytes was significantly increased (Fig. 3-2 A). This result is consistent with previous studies showing that the self HA peptide can induce both $6.5^{+}CD4^{SP}$ thymocyte deletion and $6.5^{+}CD4^{SP}Foxp3^{+}$ thymocyte formation in TS1xHA28 mice through processes driven predominantly by HA expression on radioresistant cell types (Jordan et al. 2001; Lerman et al. 2004). In TS1xHACII mice, the number of $6.5^{+}CD4^{SP}$ cells was further reduced relative to either TS1 or TS1xHA28 mice (Fig. 3-2 A). Although the percentage of $6.5^{+}CD4^{SP}$ thymocytes that were $Foxp3^{+}$ was increased compared with TS1 mice, the severe deletion of $6.5^{+}CD4^{SP}$ thymocytes led to there being no significant change in the number of $6.5^{+}CD4^{SP}Foxp3^{+}$ thymocytes (Fig. 3-2 A).

The relative representations of $6.5^{+}CD4^{+}$ T cells in the peripheral LNs of TS1, TS1xHA28 and TS1xHACII mice mostly paralleled those of $6.5^{+}CD4^{SP}$ thymocytes (Fig. 3-2 B). However, in contrast to what we observed in the thymus, $6.5^{+}CD4^{+}$ T cells were no less abundant in the peripheral LNs of TS1xHA28 mice than TS1 mice, suggesting that homeostatic expansion of both $6.5^{+}CD4^{+}Foxp3^{-}$ and $6.5^{+}CD4^{+}Foxp3^{+}$ cells occurred in the periphery.

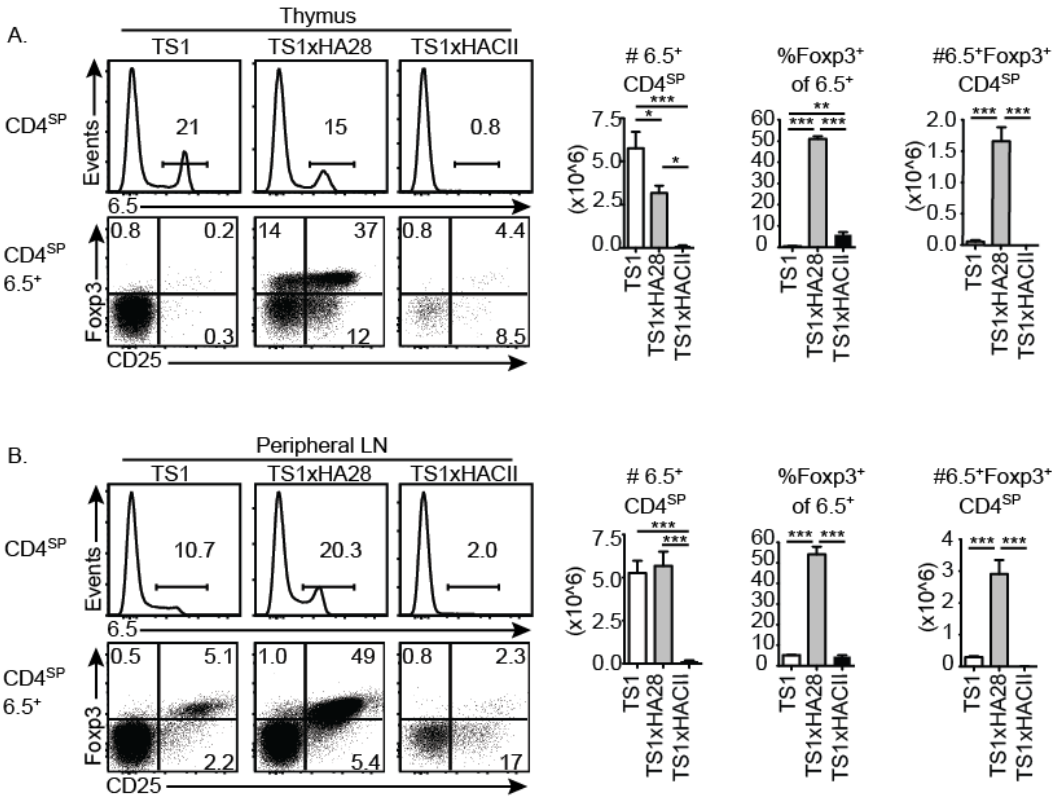

Figure 3-2 Recognition of self-antigen can drive intrathymic Treg cell formation The number and percentage of $CD4^{SP}$ cells expressing the 6.5^{+} clonotypic TCR and of $6.5^{+}CD4^{SP}$ cells expressing Foxp3 was assessed in the (**A**) thymus and (**B**) pLNs by flow cytometry.

Collectively, these studies show that differences in the amounts and/or cell types in which the HA self peptide is expressed affect the extent to which it induces both deletion of HA-specific thymocytes and their differentiation into Foxp3$^+$ Treg cells. Moreover, the HA self-Ag promotes formation of significantly higher numbers of 6.5$^+$CD4$^+$Foxp3$^+$ Treg cells in TS1xHA28 mice than in TS1xHACII mice, in part because less deletion occurs in the less immunostimulatory setting.

3.2.3 Autoreactive CD4^{SP} thymocytes upregulate CD25 and Helios

We additionally assessed the expression of CD25 and Helios on 6.5$^+$CD4^{SP} thymocytes in TS1xHA28 mice to determine how their expression correlated with exposure to cognate self-Ag and Foxp3 expression (Fig. 3-3). CD25 is part of the IL-2 receptor, and exposure to IL-2 represents a crucial second step (following TCR engagement) in thymic Treg cell development (Lio and Hsieh 2008). Helios upregulation in thymocytes can occur following a strong TCR signal, while outside the thymus, Helios is predominantly expressed in Foxp3$^+$ Treg cells (Daley, Hu, Goodnow 2013; Thornton et al. 2010). In the thymus of a TS1xHA28 mouse, CD25 was upregulated in approximately 60% of 6.5$^+$CD4^{SP}Foxp3$^+$ cells and 20% of 6.5$^+$CD4^{SP}Foxp3$^-$ cells (Fig. 3-3 A). Helios was present in nearly 100% of 6.5$^+$CD4^{SP}Foxp3$^+$ thymocytes and 70% of 6.5$^+$CD4^{SP}Foxp3$^-$ thymocytes. Neither molecule was expressed by 6.5$^+$CD4^{SP} thymocytes in TS1 mice, in which cognate Ag is not present.

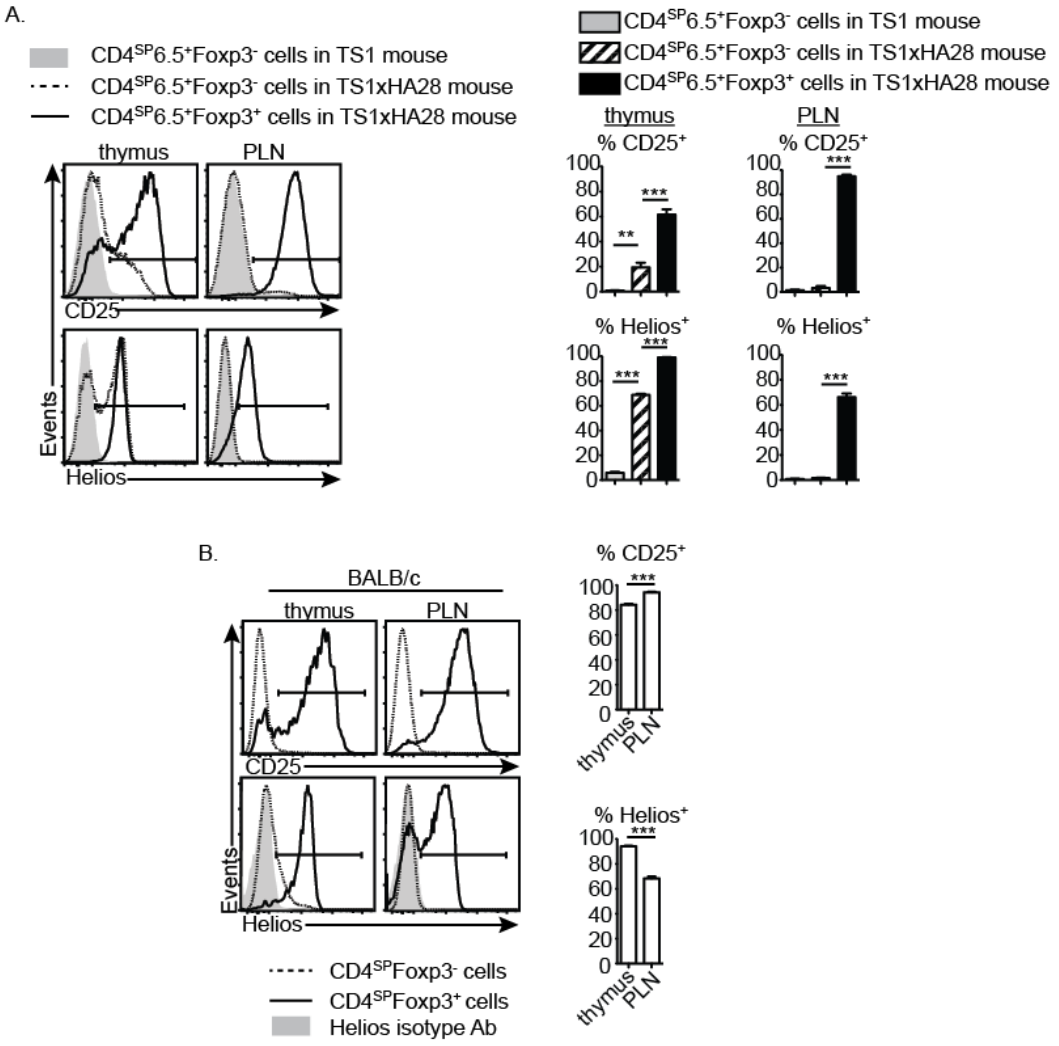

Figure 3-3 CD25 and Helios can be expressed by autoreactive Foxp3$^-$ and Foxp3$^+$ CD4^{SP} thymocytes (**A**) CD25 and Helios expression were assessed in the thymus and pLNs in 6.5$^+$CD4^{SP}Foxp3$^-$ cells from TS1 mice (shaded grey), 6.5$^+$CD4^{SP}Foxp3$^-$ cells from TS1xHA28 mice (dashed), and 6.5$^+$CD4^{SP}Foxp3$^+$ cells from TS1xHA28 mice (black). n=4-6 per group from 3 independent experiments. (**B**) Histograms show representative plots of CD25 and Helios expression on CD4^{SP}Foxp3$^-$ cells (dashed) and CD4^{SP}Foxp3$^+$ cells isolated from BALB/c mice (black line). Graphs show percent of CD4^{SP}Foxp3$^+$ cells that express CD25 and Helios in either the thymus or pLNs. n=3 mice.

Thus, expression of both molecules is driven by recognition of self-Ag, but is not dependent on Foxp3 expression. In the pLNs, $6.5^{+}CD4^{+}$ cells in a TS1xHA28 mouse expressed neither CD25 nor Helios, indicating that these molecules either were lost or that $6.5^{+}CD4^{SP}$ thymocytes on which they were expressed were either deleted or differentiated into Treg cells (Fig. 3-3 B). A higher percentage of $6.5^{+}CD4^{SP}Foxp3^{+}$ cells expressed CD25 in the pLNs compared with the thymus, while the percentage expressing Helios was lower in the pLNs. To determine how expression of CD25 and Helios in TS1xHA28 mice corresponded to that in mice with unmanipulated TCR repertoires, we assessed their expression in a BALB/c mouse. In the thymus, approximately 80% of $CD4^{SP}Foxp3^{+}$ cells expressed CD25, and again this percentage was increased in the pLNs (Fig. 3-3 B). Very little CD25 expression was seen in $CD4^{SP}Foxp3^{-}$ thymocytes or T cells. Similar to the $6.5^{+}CD4^{SP}Foxp3^{+}$ thymocytes in a TS1xHA28 mouse, almost all $CD4^{SP}Foxp3^{+}$ thymocytes in a BALB/c mouse expressed Helios, and the percentage of $Helios^{+}$ cells decreased in the pLN. A small subset of $CD4^{SP}Foxp3^{-}$ BALB/c thymocytes expressed Helios, but mature $Foxp3^{-}$ T cells in the periphery did not. These data show that upregulation of Helios marks autoreactive $CD4^{SP}$ thymocytes, as $6.5^{+}CD4^{SP}$ thymocytes express Helios in TS1xHA28 mice, but not in TS1 mice in which they are not exposed to cognate Ag. Moreover, Helios is present in both $6.5^{+}CD4^{SP}Foxp3^{-}$ and $6.5^{+}CD4^{SP}Foxp3^{+}$ cells, and similar populations of $Helios^{+}CD4^{SP}Foxp3^{+}$ and $Helios^{+}CD4^{SP}Foxp3^{-}$ thymocytes were found in BALB/c mice, suggesting that Ag-experienced $CD4^{SP}Foxp3^{+}$ and $CD4^{SP}Foxp3^{-}$ cells are present in the thymus of unmanipulated BALB/c mice.

3.2.4 Foxp3 upregulation by $CD4^+CD25^-Foxp3^-$ cells occurs following recognition of a self-Ag in cells that have undergone limited rounds of division

To investigate whether recognition of self-Ag could induce $CD4^+CD25^-Foxp3^-$ cells to differentiate into $Foxp3^+$ Treg cells after they had left the thymus, we purified congenically-marked $6.5^+CD4^+CD25^-eGFP^-Ly5.1^+$ cells from the LNs of TS1.$Foxp3^{eGFP}$.Ly5.1 mice (the $Foxp3^{eGFP}$ reporter allele was used to purify $Foxp3^-$ cells based on the absence of eGFP expression), labeled the cells with CellTrace Violet, and transferred them into BALB/c, HA28, or HACII hosts (Fig. 3-4 A). eGFP expression in the $Foxp3^{eGFP}$ reporter mouse was previously described to accurately reflect Foxp3 expression in $CD4^+$ T cells (Haribhai et al. 2007), and intracellular staining with anti-Foxp3 antibody revealed that Foxp3 and eGFP were consistently expressed in the same cells (Fig. 3-4 B). Three days after transfer we examined the extent of division and eGFP upregulation (as an indication of Foxp3 expression) of 6.5^+CD4^+ cells in pooled non-gut associated (brachial, axillary and inguinal) peripheral LNs (pLNs). The 6.5^+CD4^+ T cells in the pLNs of BALB/c mice remained undivided and 99% did not express Foxp3, providing an internal control demonstrating the purity of the sort (Fig. 3-3 C). In contrast, many of the 6.5^+CD4^+ cells isolated from the pLNs of HA28 mice had undergone several rounds of division, and a significantly higher percentage of these cells expressed Foxp3 compared to those isolated from BALB/c mice (Fig. 3-3 C). Moreover, the 6.5^+CD4^+ cells that had upregulated Foxp3 in HA28 mice were almost exclusively among those that remained undivided or had undergone one or two divisions, while many of those that remained $Foxp3^-$ had undergone 3-5 rounds of division (Fig. 3-3 D).

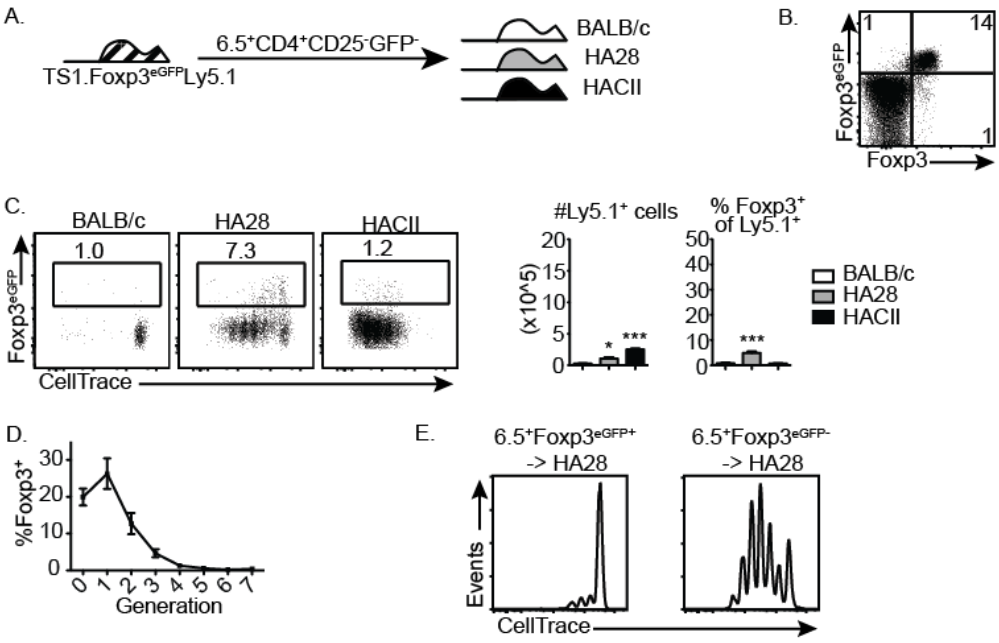

Figure 3-4 Foxp3 upregulation by autoreactive $CD4^+CD25^-Foxp3^-$ cells in the pLNs can occur in cells that have undergone limited rounds of division (**A**) Schematic showing adoptive transfer of CellTrace Violet-labeled $6.5^+CD4^+CD25^-GFP^-$ cells isolated from the pLNs of TS1.$Foxp3^{eGFP}$.Ly5.1 mice into BALB/c, HA28, and HACII recipients. (B) Foxp3 intracelluar stain versus $Foxp3^{eGFP}$ expression on $CD4^+CD8^-$ T cells in the pLN of a $Foxp3^{eGFP}$ reporter mouse. (C) Flow plots show CellTrace Violet dilution and $Foxp3^{eGFP}$ expression in $CD4^+Ly5.1^+$ cells isolated from the pLNs of recipient mice 3 days post-transfer. Graphs show the number of donor-derived cells and the percent expressing Foxp3. (D) Percent of $CD4^+Ly5.1^+$ cells expressing $Foxp3^{eGFP}$ in each generation in the pLNs of an HA28 mouse at day 3 post-transfer. n=3 mice from 2 independent experiments. (E) 6.5^+GFP^+ cells from TS1xHA28.$Foxp3^{eGFP}$ mice or 6.5^+GFP^- cells from TS1.$Foxp3^{eGFP}$ mice were transferred into HA28 recipients, and dilution of CellTrace Violet was assessed at day 3 post-transfer.

Interestingly, when naturally-occurring $6.5^{+}Foxp3^{+}$ Treg cells from TS1xHA28 mice were transferred into HA28 mice, they also underwent much less division in 3 days than did $6.5^{+}Foxp3^{-}$ cells isolated from TS1 mice, suggesting that a relatively hypoproliferative response may be an intrinsic part of the Treg cell identity (Fig. 3-3 E). In HACII recipients, all of the $6.5^{+}CD4^{+}CD25^{-}eGFP^{-}Ly5.1^{+}$ T cells had undergone multiple rounds of division, and the percentage that had upregulated Foxp3 expression was no higher than in BALB/c mice (Fig. 3-3 C). Thus, recognition of self can drive pTreg cell differentiation, and autoreactive T cells that upregulated Foxp3 had undergone limited rounds of division.

3.2.5 The extent of $6.5^{+}CD4^{+}Foxp3^{-}$ T cell conversion to $Foxp3^{+}$ Treg cells depends on the stimulatory potency of the self-Ag

Previous studies have variously shown that mature $CD4^{+}$ T cells are capable of undergoing expansion, deletion and/or conversion into $Foxp3^{+}$ Treg cells upon antigenic stimulation in the periphery. To assess how differences in the expression of self-HA in HA28 and HACII mice might influence these different fates, we again purified congenically-marked $6.5^{+}CD4^{+}CD25^{-}eGFP^{-}Ly5.1^{+}$ cells from the LNs of TS1.$Foxp3^{eGFP}$.Ly5.1 mice, and transferred them into BALB/c, HA28, or HACII hosts (Fig. 3-5 A). We then assessed the accumulation and Foxp3 expression of $Ly5.1^{+}CD4^{+}$cells at later time points. When we initially analyzed HA28 and HACII mice at 14 and 21 days post-transfer, we found that recoveries were variable and that some mice contained no $Ly5.1^{+}CD4^{+}$cells, while there were good recoveries from other individual mice (Fig. 3-5 B,C). One explanation for this irreproducibility could be that the

Ly5.1$^+$ cells were being rejected because expression of GFP by CD4$^+$ T cells was in some individuals promoting formation of GFP-specific CD8$^+$ T cells that were rejecting the transferred cells. Accordingly, we repeated the day 14 and day 21 transfers of Ly5.1$^+$6.5$^+$CD4$^+$Foxp3$^-$ cells into BALB/c.Foxp3^{eGFP}, HA28.Foxp3^{eGFP}, and HACII.Foxp3^{eGFP} mice, and in this setting we obtained consistent and equivalent recoveries of Ly5.1$^+$CD4$^+$ cells from all of the individual mice within a group (Fig. 3-6 A).

In HA28 mice, similar numbers of Ly5.1$^+$CD4$^+$Foxp3$^+$ T cells were recovered at day 7 after transfer as had been obtained 3 days after transfer (Fig. 3-6 B). By contrast, approximately 3 times as many Ly5.1$^+$CD4$^+$ T cells were recovered in HACII mice at 7 days post transfer than after 3 days, but there was again negligible accumulation of Ly5.1$^+$CD4$^+$Foxp3$^+$ T cells. In HA28.Foxp3^{eGFP} mice, the number of Ly5.1$^+$CD4$^+$ T cells that were recovered 21 days after transfer was no higher than in BALB/c mice, but nearly half of these cells expressed Foxp3 (which was undetectable in Ly5.1$^+$CD4$^+$ T cells recovered from BALB/c mice) (Fig. 3-6 B). By contrast, sizable populations of Ly5.1$^+$CD4$^+$ T cells were recovered 21 days after transfer into HACII.Foxp3^{eGFP} mice, but there again was little or no expression of Foxp3 by these cells. Thus, expression of HA as a self-Ag with a low stimulatory potency drives the differentiation and accumulation of CD4$^+$Foxp3$^+$ T cells from CD4$^+$Foxp3$^-$ precursors outside of the thymus more efficiently than expression of HA in a more immunostimulatory manner.

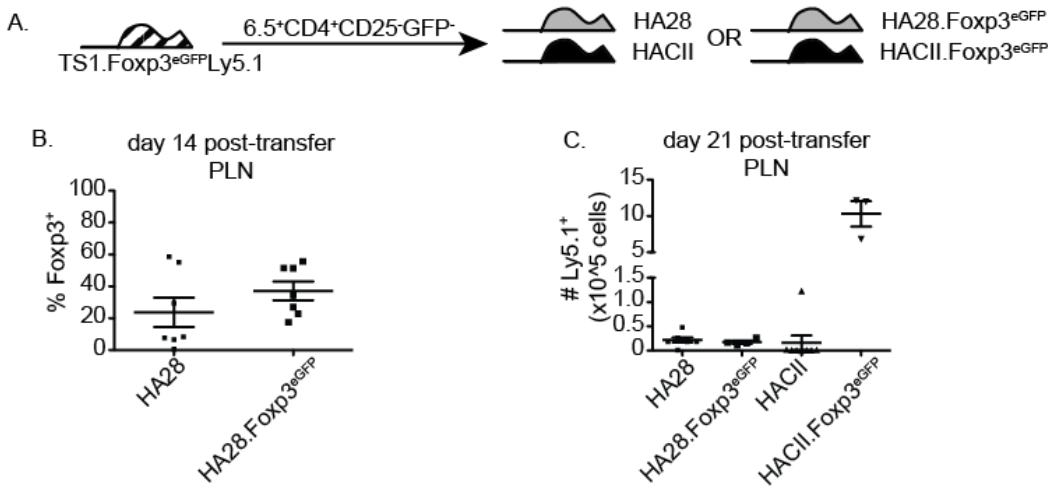

Figure 3-5. GFP expression may mediate rejection of transferred T cells in recipient mice that do not express the eGFP transgene. (**A**) Schematic showing adoptive transfer of $6.5^{+}CD4^{+}CD25^{-}GFP^{-}$ cells isolated from the pLNs of $TS1.Foxp3^{eGFP}.Ly5.1$ mice into HA28, $HA28.Foxp3^{eGFP}$, HACII or $HACII.Foxp3^{eGFP}$ recipients. (**B**) The percentage of $Ly5.1^{+}$ cells expressing Foxp3 at day 14, and (**C**) the number of $Ly5.1^{+}$ cells in the pLNs of recipient mice were assessed at day 21 post-transfer.

3.2.6 6.5^+ pTreg cells formed in HA28 mice suppress 6.5^+CD4^+ T cell proliferation *in vitro*

To determine the stability and suppressive capacity of the $CD4^+Foxp3^+$ T cells that were formed in HA28 mice, we isolated $6.5^+eGFP^+Ly5.1^+$ cells from the pLNs of HA28 mice at day 7 post-transfer of $CD4^+6.5^+CD25^-eGFP^-Ly5.1^+$ cells from TS1.Foxp3^{eGFP}Ly5.1 mice. These purified cells were then incubated for 3 days *in vitro* with S1 peptide and Ag presenting cells, and their expression of Foxp3 and ability to suppress proliferation of co-cultured $Ly5.1^-6.5^+Foxp3^{eGFP-}$ responder $CD4^+$ T cells from TS1.Foxp3^{eGFP} mice were assessed (Fig. 3-6 C). In parallel cultures, we included thymically-derived tTreg cells (i.e. $6.5^+CD4^+eGFP^+Ly5.1^+$ Treg cells from TS1xHA28.Foxp3^{eGFP} mice) in place of the pTreg cells in order to compare stability and suppressive ability between pTreg cells and tTreg cells of the same specificity. While nearly 100% of tTreg cells had maintained Foxp3 expression in culture, a higher percentage of $Ly5.1^+CD4^+$ pTreg cells formed in HA28 mice cells appeared to have lost Foxp3 expression (Fig. 3-6 C). Nevertheless, more than 70% of the $Ly5.1^+CD4^+$ pTreg cells maintained eGFP expression, and were as effective as tTreg cells in suppressing the proliferation of $Ly5.1^-CD4^+$ responder T cells. These data show that pTreg cells formed in HA28 mice had similar suppressive ability *in vitro* as tTreg cells of the same TCR specificity, but exhibited less stable expression of Foxp3.

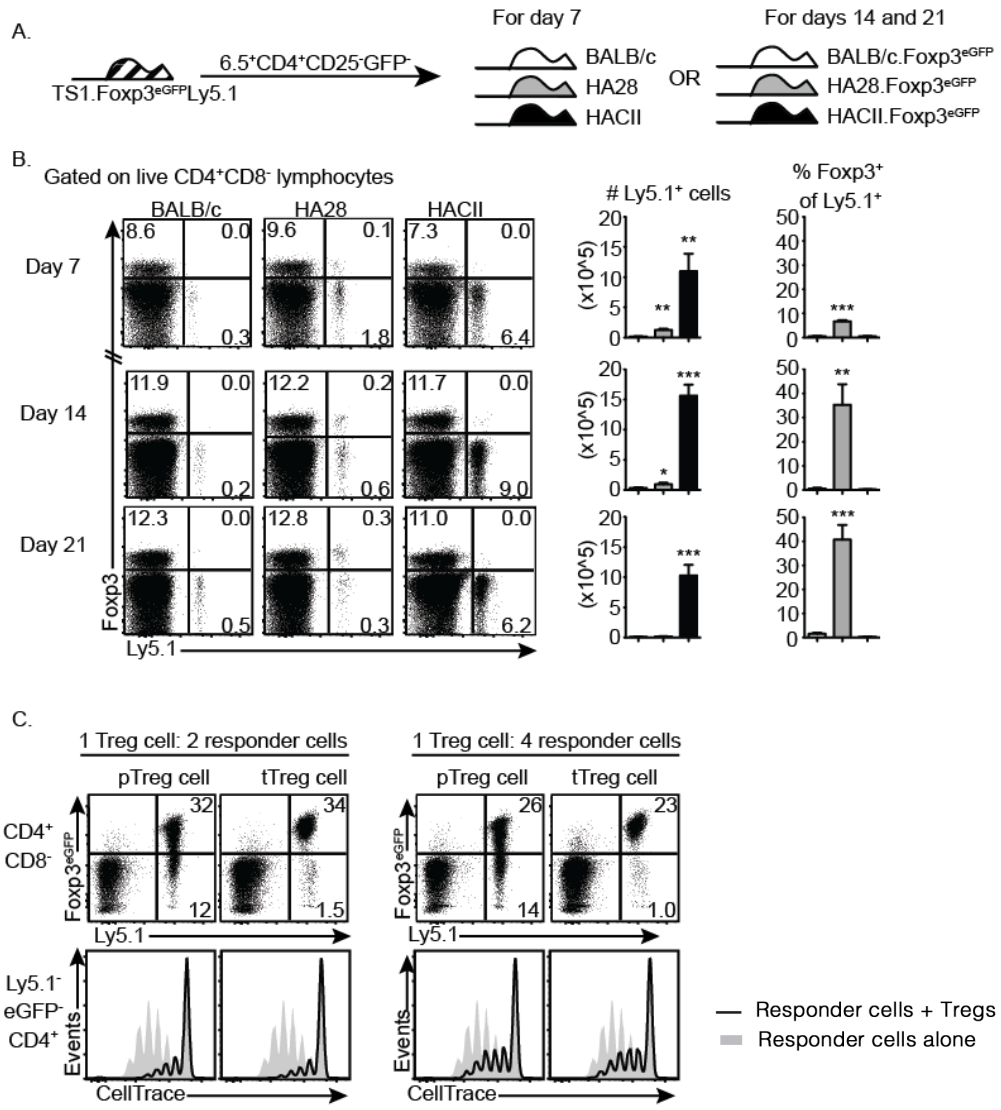

Figure 3-6 The stimulatory capacity of cognate self-antigen determines the extent of Foxp3+ pTreg cell formation (**A**) Schematic showing adoptive transfer of 6.5+CD4+CD25-GFP- cells isolated from the pLNs of TS1.Foxp3eGFP.Ly5.1 mice into BALB/c, HA28, and HACII recipients for analysis at day 7 post-transfer or BALB/c.Foxp3eGFP, HA28.Foxp3eGFP, and HACII.Foxp3eGFP recipients for analysis at days 14 and 21 post-transfer. (**B**) Flow plots show Ly5.1 and Foxp3 expression in CD4+CD8- cells isolated from the pLNs of recipient mice 7, 14, or 21 days post-transfer. Graphs show the number of donor-derived cells and the percentage expressing Foxp3. (**C**) CD4+6.5+CD25-eGFP- cells from congenically-marked TS1.Foxp3eGFP.Ly5.1 mice were transferred into Ly5.1-/- HA28 hosts. At day 7 post-transfer, Ly5.1+6.5+CD4+eGFP+ pTreg cells were re-isolated from host mice and cultured *in vitro* with CellTrace Violet-labeled 6.5+CD4+Ly5.1-Foxp3- responder T cells, 1 μM S1 peptide and feeder cells. In parallel cultures, Ly5.1+6.5+CD4+eGFP+ tTreg cells isolated from TS1xHA28.Foxp3eGFP.Ly5.1 were used in place of pTreg cells. After 3 days in culture, Foxp3eGFP expression in Ly5.1+ cells and CellTrace Violet dilution by responder T cells was assessed.

3.2.7 6.5^{+}CD4^{+} T cells that upregulate Foxp3 in HA28 mice lose the ability to produce IL-2 and IFN-γ

To determine the ability of autoreactive CD4^{+}Foxp3^{-} T cells to produce cytokines, we isolated cells from the pLNs of BALB/c.Foxp3^{eGFP}, HA28.Foxp3^{eGFP}, and HACII.Foxp3^{eGFP} mice that had received 6.5^{+}CD4^{+}CD25^{-}eGFP^{-}Ly5.1^{+} cells from the LNs of TS1.Foxp3^{eGFP}.Ly5.1 mice 14 days previously, stimulated them *in vitro* with PMA and ionomycin, and performed an intracellular cytokine stain. We found that Ly5.1^{+}CD4^{+} cells re-isolated from BALB/c.Foxp3^{eGFP} mice were capable of producing little or no IL-2 and IFN-γ (Fig. 3-7). When Ly5.1^{+}CD4^{+} cells were re-isolated from HA28.Foxp3^{eGFP} mice, the Foxp3^{-} subset were capable both IL-2 and IFN-γ. In contrast, Ly5.1^{+}CD4^{+}Foxp3^{+} pTreg cells isolated from HA28.Foxp3^{eGFP} mice showed little or no evidence of cytokine production, consistent with a reported role for Foxp3 in inhibiting IL-2 and IFN-γ production (Bettelli, Dastrange, Oukka 2005; Hori, Nomura, Sakaguchi 2003). Interestingly, despite proliferating more heavily in HACII mice, the Ly5.1^{+}CD4^{+}Foxp3^{-} cells isolated from this environment were less capable of IFN-γ and IL-2 production than when they were isolated from an HA28 mouse (Fig. 3-7). We additionally saw little evidence of IL-17 production by these cells (data not shown). 6.5^{+}CD4^{+}Foxp3^{-} cells adoptively transferred into HACII mice may adopt a different effector fate, or alternatively, decreased cytokine production has been observed during CD4^{+} T cell anergy and exhaustion, and the ultimate fate of these cells was not explored.

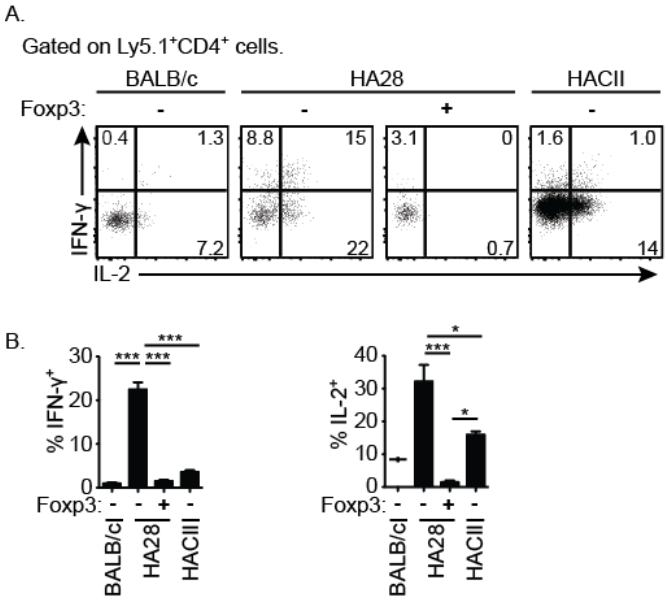

Figure 3-7 Adoptively-transferred 6.5^{+}CD4^{+}Foxp3^{-} cells produced more IFN-γ and IL-2 in HA28 hosts than in HACII hosts 6.5^{+}CD4^{+}CD25^{-}GFP^{-} cells isolated from the pLNs of TS1.Foxp3^{eGFP}.Ly5.1 mice were adoptively transferred into BALB/c.Foxp3^{eGFP} or HA28.Foxp3^{eGFP}, or HACII.Foxp3^{eGFP} recipients. (**A**) At day 14 post-transfer, cell suspensions from the pLNs of recipient mice were stimulated in vitro with PMA and ionomycin and an intracellular cytokine stain was performed. (**B**) The percentage of donor-derived Ly5.1^{+} cells expressing IFN-γ and IL-2 was determined. n=3-7 mice

3.2.8 pTreg cells formed in pLNs of HA28 mice gradually acquire a Helios^{high} and CD25^{high} phenotype

To further characterize the Foxp3^{+}CD4^{+} T cells that were being formed in response to the HA self-Ag in HA28 mice, we examined expression of the transcription factor Helios and the IL-2Rα chain CD25. Expression of both molecules increased progressively in the population of Ly5.1^{+}CD4^{+}Foxp3^{+} pTreg cells isolated from HA28 mice (Fig. 3-8). This observation could be explained by either the selective persistence of cells with high expression of Helios and CD25, or by their gradual upregulation within all cells that acquired Foxp3. Notably, the Ly5.1^{+}CD4^{+} T cells that had not acquired Foxp3 expression appeared to transiently upregulate Helios and CD25 relative to cells that had been transferred into BALB/c mice at early stages post transfer, but very little expression of these molecules could be detected by days 14 and 21 post-transfer (Fig. 3-8). These data show that Treg cells that develop in peripheral lymph nodes can acquire a Helios^{hi} and CD25^{hi} phenotype that is similar to what we have observed in tTreg cells.

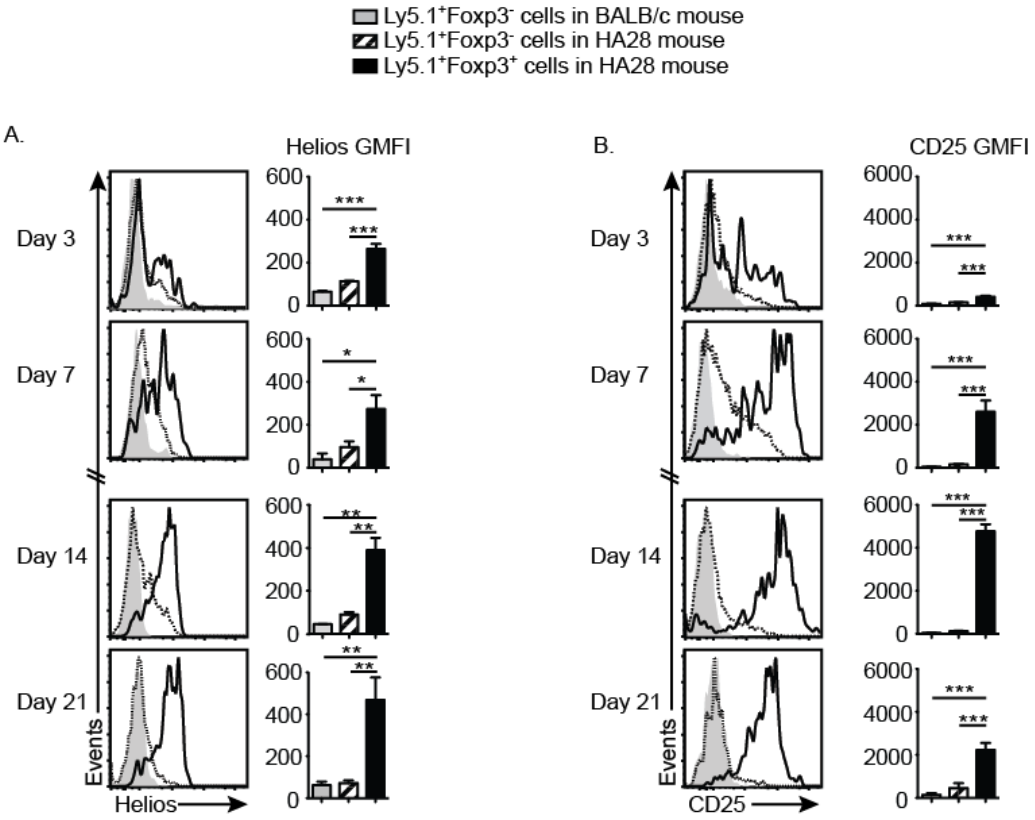

Figure 3-8 The 6.5$^+$ pTreg cell population that formed in the pLNs of HA28 mice gradually acquired expression of Helios and CD25 6.5$^+$CD4$^+$CD25$^-$GFP$^-$ cells isolated from the pLNs of TS1.Foxp3^{eGFP}.Ly5.1 mice were adoptively transferred into BALB/c or HA28 recipients (for analysis at days 3 and 7 post-transfer) or BALB/c.Foxp3^{eGFP} or HA28.Foxp3^{eGFP} recipients (for analysis at days 14 and 21 post-transfer). (A) Helios and (B) CD25 expression was determined by flow cytometry in Ly5.1$^+$Foxp3$^-$ cells isolated from pLNs of BALB/c recipients, and Ly5.1$^+$Foxp3$^-$ cells and Ly5.1$^+$Foxp3$^+$ cells from HA28 recipients at indicated days post-transfer.

3.2.9 6.5$^+$ pTreg cells isolated from the mesLNs of HA28 hosts expressed lower levels of Helios than 6.5$^+$ pTreg cells isolated from pLNs

Since previous studies have identified Ag-presenting cells in gut mucosal tissue that promote Foxp3$^+$ Treg cell formation (Coombes et al. 2007; Sun et al. 2007), we also assessed Ly5.1$^+$ cell accumulation and Foxp3 upregulation in the mesenteric LN following transfer of 6.5$^+$CD4$^+$CD25$^-$eGFP$^-$ cells from TS1.Foxp3^{eGFP}.Ly5.1 mice into BALB/c, HA28, or HACII recipients. For the most part, we found no significant differences between the extent of either Ly5.1$^+$CD4$^+$ T cell accumulation or Foxp3 upregulation in the mesLN relative to non-gut-draining pLNs (Fig. 3-9 A). The one exception was that a significantly higher percentage of donor-derived Ly5.1$^+$ cells had upregulated Foxp3 in the mesLNs of HACII mice than BALB/c mice at day 3 post-transfer (Fig. 3-9 A).

We also examined Helios levels on Ly5.1$^+$CD4$^+$Foxp3$^+$ T cells that had been isolated from the mesLNs of HA28 mice (Fig. 3-9 B). Interestingly, the mesLN contained significantly higher percentages of Ly5.1$^+$CD4$^+$Foxp3$^+$ T cells that did not express Helios than were found in the non-gut-draining PLN (Fig. 3-9 B). Since more than 90% of the Ly5.1$^+$CD4$^+$Foxp3$^+$ isolated from each site expressed the 6.5 clonotypic TCR, this indicates that factors other than TCR specificity must determine the extent of Helios upregulation by Foxp3$^+$CD4$^+$ T cells. These studies suggest that anatomical location may be a crucial factor in determining the extent to which Helios upregulation occurs in pTreg cells.

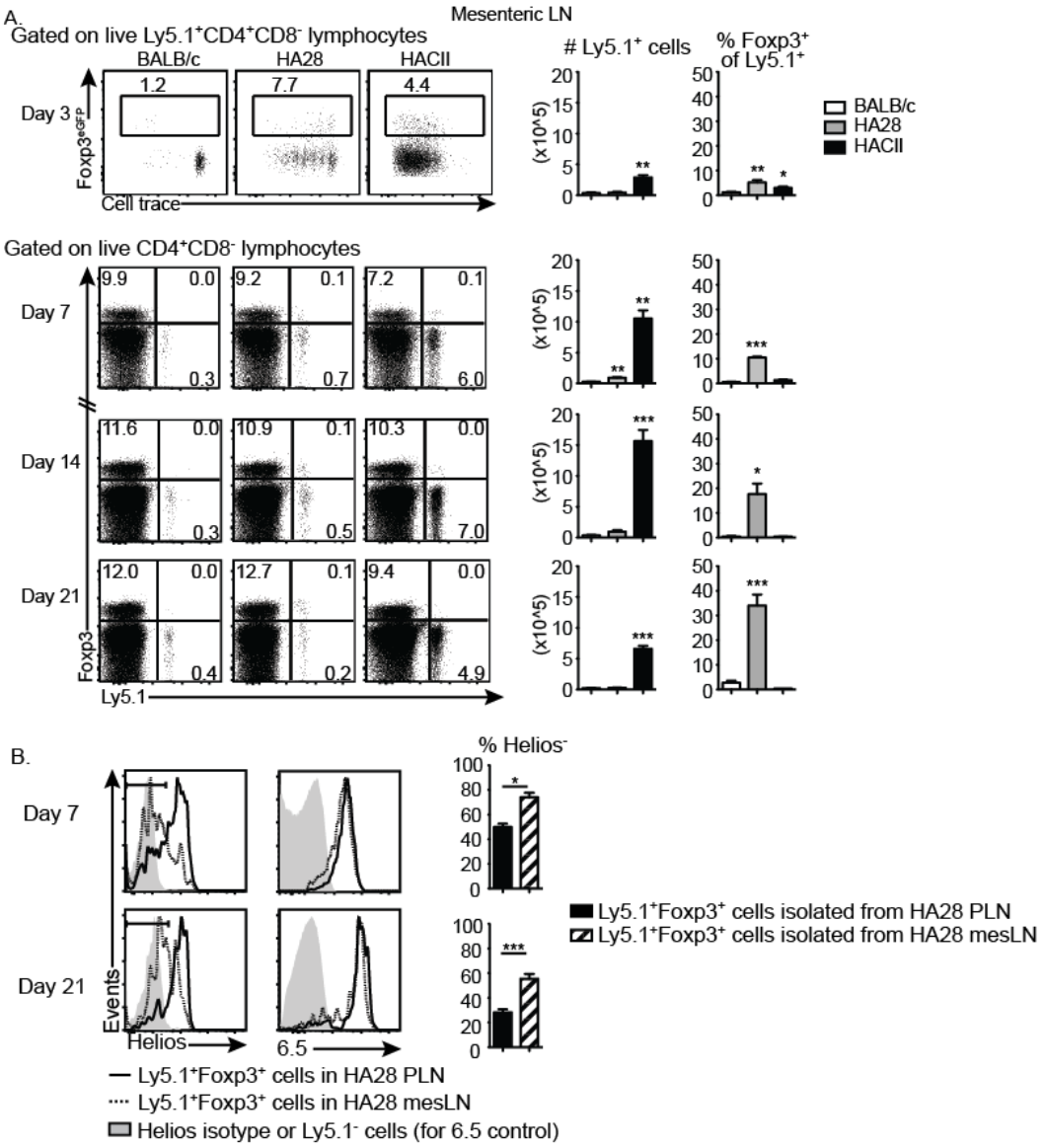

Figure 3-9 The extent of 6.5+ pTreg cell formation was similar in the mesLNs of HA28 and HACII mice as in the pLNs, but expression of Helios by pTreg cells differed between the two locations (**A**) 6.5+CD4+CD25-GFP- cells isolated from the pLNs of TS1.Foxp3^{eGFP}.Ly5.1 mice were transferred into BALB/c, HA28, and HACII recipients for analysis at days 3 or 7 post-transfer or BALB/c.Foxp3^{eGFP}, HA28.Foxp3^{eGFP}, and HACII.Foxp3^{eGFP} recipients for analysis at days 14 and 21 post-transfer. Flow plots show Ly5.1 and Foxp3 expression among CD4+CD8- cells isolated from the mesLNs of recipient mice 3, 7, 14, or 21 days post-transfer. Graphs show the number of donor-derived cells and the percentage expressing Foxp3. (**B**) Expression of Helios and the 6.5+ TCR on Ly5.1+Foxp3+ cells in the pLNs and mesLNs of HA28 recipients was assessed by flow cytometry.

3.2.10 In HA12 mice, a higher percentage of autoreactive 6.5^+CD4^+ T cells upregulate Foxp3 in the mesLN than in the non-gut-draining LNs

In addition to adoptively transferring $6.5^+CD4^+CD25^-eGFP^-$ cells from TS1.$Foxp3^{eGFP}$.Ly5.1 mice into BALB/c, HA28, and HACII recipients, we also transferred them into HA12 mice. The HA12 transgenic lineage has been described previously, and, like the HA28 mouse, express a truncated form of HA under control of an SV40 promoter. Studies involving the use of bone marrow chimeras have shown that the HA in HA12 mice is expressed on both radioresistant and bone marrow-derived cells, while in HA28 mice, HA is primarily expressed by non-hematopoietic cells (Picca et al. 2009). RT-PCR analysis showed an intermediate amount of HA being transcribed in the spleens, pLNs, mesLNs and intestines of HA12 mice relative to HA28 and HACII mice (Fig. 3-10 A). Accordingly, 6.5^+CD4^+ T cells transferred into HA12 mice had undergone, on average, more divisions than in an HA28 mouse but less than in an HACII mouse in both the pLNs and mesLNs at day 3 post-transfer (Fig. 3-10 B). The percentage of $Ly5.1^+$ cells that upregulated Foxp3 in the pLNs of an HA12 mouse was significantly lower than in an HA28 mouse at days 3 and 7 post-transfer, and was again confined to cells that have undergone little or no division (Fig. 3-10 C). In contrast, in the mesLNs, the percentage of $Ly5.1^+$ cells expressing Foxp3 was significantly higher in an HA12 mouse than an HA28 mouse, and the difference in the proliferation history of $Foxp3^+$ cells versus $Foxp3^-$ cells was less dramatic. A similar result was obtained when transferred cells were re-isolated at day 7 post-transfer (Fig. 3-10 D). Thus, the fate of autoreactive $CD4^+Foxp3^-$ T cells can vary by location even when cognate Ag is expressed systemically.

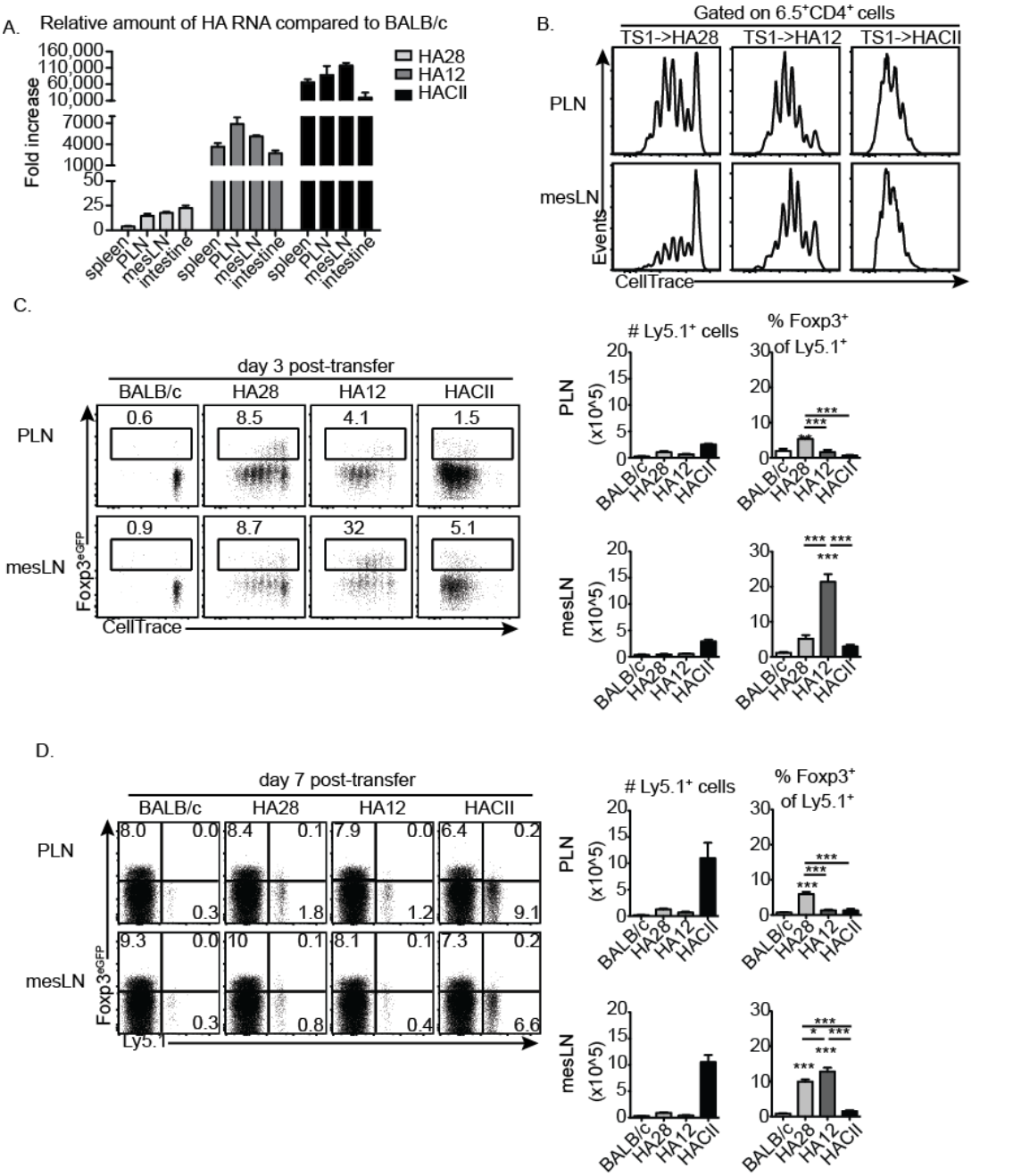

Figure 3-10 In HA12 transgenic mice, a higher percentage of 6.5$^+$CD4$^+$Foxp3$^-$ cells upregulated Foxp3 in the mesLN than in the pLNs (A) RT-PCR was used to determine the amount of HA RNA in the spleens, pLNs, thymi, and intestines of HA28 and HACII mice relative to BALB/c controls. (B) 6.5$^+$CD4$^+$eGFP$^-$ cells isolated from the LNs of TS1.Foxp3^{eGFP} mice were labeled with CellTrace Violet then adoptively transferred into either BALB/c, HA28, HA12, or HACII recipients. The extent of CellTrace Violet dilution and (C) Foxp3 expression were assessed at day 3 post-transfer. (D) Ly5.1$^+$CD4$^+$ cell accumulation and Foxp3 expression were determined at day 7 post-transfer of 6.5$^+$CD4$^+$CD25$^-$eGFP$^-$ cells isolated from TS1.Foxp3^{eGFP}.Ly5.1 mice into indicated recipients.

3.2.11 $6.5^+CD4^+CD25^-Foxp3^-$ cells isolated from the periphery of TS1xHA28 mice can upregulate Foxp3 in HA28 mice

We were curious as to whether $6.5^+CD4^+Foxp3^-$ T cells in the periphery of TS1xHA28 mice were intrinsically incapable of differentiating into $Foxp3^+$ Treg cells, given that they had so far failed to do so despite the presence of HA in both the thymus and periphery expressed in a manner that was capable of driving 6.5^+Foxp3^+ Treg cell formation. In order to address this question, we isolated $6.5^+CD4^+CD25^-eGFP^-.Ly5.1^+$ cells from the LNs of either TS1.$Foxp3^{eGFP}$.Ly5.1 mice or TS1xHA28.$Foxp3^{eGFP}$.Ly5.1, labeled them with CellTrace Violet, and transferred them into HA28 mice (Fig. 3-11 A). At day 3 post-transfer, cells from both types of donor mice had undergone similar numbers of divisions, but a slightly higher percentage of transferred 6.5^+CD4^+ cells from TS1xHA28.$Foxp3^{eGFP}$.Ly5.1 mice than from TS1.$Foxp3^{eGFP}$.Ly5.1 had upregulated Foxp3 (Fig. 3-11 B). By contrast, at day 14 post-transfer, similar numbers of $Ly5.1^+$ cells from each donor source had accumulated and the percentage of each expressing Foxp3 was not significantly different (Fig. 3-11 C). Thus, 6.5^+Foxp3^- cells that exist in TS1xHA28 mice retain the ability to upregulate Foxp3, and may actually be primed to do so compared with 6.5^+Foxp3^- cells that have developed in the absence of exposure to cognate Ag.

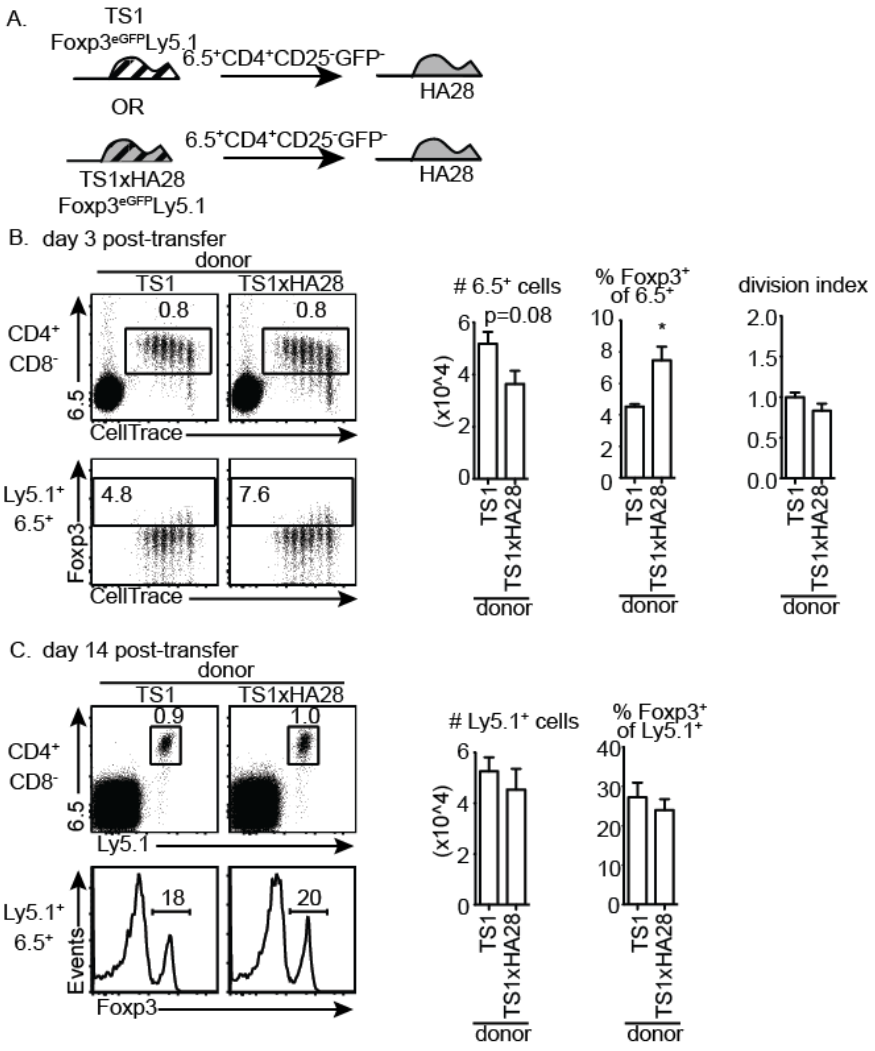

Figure 3-11 6.5$^+$CD4$^+$Foxp3$^-$ T cells in the periphery of TS1xHA28 mice retain the ability to upregulate Foxp3, and may be primed to do so (**A**) 6.5$^+$CD4$^+$CD25$^-$GFP$^-$ cells isolated from the pLNs of TS1.Foxp3^{eGFP}.Ly5.1 or TS1xHA28.Foxp3^{eGFP}.Ly5.1 mice were adoptively transferred into HA28 recipients. (**B**) CellTrace Violet dilution by 6.5$^+$Ly5.1$^+$CD4$^+$ cells was assessed at day 3 post-transfer, and accumulation and Foxp3 expression by these cells was assessed at days 3 and (**C**) 14 post-transfer. n=3 for day 3 post-transfer; n=6 from 3 independent experiments for day 14 post-transfer.

3.2.12 The presence of Treg cells that recognize self-Ag can affect the fate of $CD4^+Foxp3^-$ T cells with the same TCR specificity

To determine whether the presence of Treg cells with the same TCR specificity might affect the fate of autoreactive $CD4^+Foxp3^-$ T cells, we transferred $6.5^+CD4^+CD25^-eGFP^-$ cells isolated from TS1.$Foxp3^{eGFP}$.Ly5.1 mice into HA28 recipients either alone or in conjunction with either polyclonal $CD4^+eGFP^+$ Treg cells from BALB/c. $Foxp3^{eGFP}$ mice or $6.5^+CD4^+eGFP^+$ cells from TS1xHA28.$Foxp3^{eGFP}$ mice (Fig. 3-12 A). We found that the presence of 6.5^+ Treg cells, but not polyclonal Treg cells, reduced the number of transferred $Ly5.1^+6.5^+Foxp3^-$ cells that accumulated in the pLNs at day 7 post-transfer (Fig. 3-12 B). However, co-injection of 6.5^+ Treg cells did not significantly affect the number of $Ly5.1^+6.5^+Foxp3^+$ cells that accumulated in HA28 recipients, resulting in an increased percentage of $Ly5.1^+6.5^+$ cells that expressed Foxp3 (Fig. 3-12 B). In contrast to this result, when $6.5^+CD4^+CD25^-eGFP^-$ T cells were transferred into TS1xHA28 mice, no upregulation of Foxp3 was observed (Fig. 3-12 C). These results show that the presence of 6.5^+ Treg cells limits $6.5^+CD4^+Foxp3^-$ T cell accumulation but not $6.5^+CD4^+Foxp3^+$ pTreg cell accumulation in an HA28 mouse. In contrast, no upregulation of Foxp3 by 6.5^+CD4^+ T cells occurred following transfer into TS1xHA28 mice, suggesting that while a small number of Treg cells of identical specificity can result in preferential accumulation of $6.5^+CD4^+Foxp3^+$ cells relative to $6.5^+CD4^+Foxp3^-$ cells, a saturation point may exist beyond which no more pTreg cells of that specificity will form.

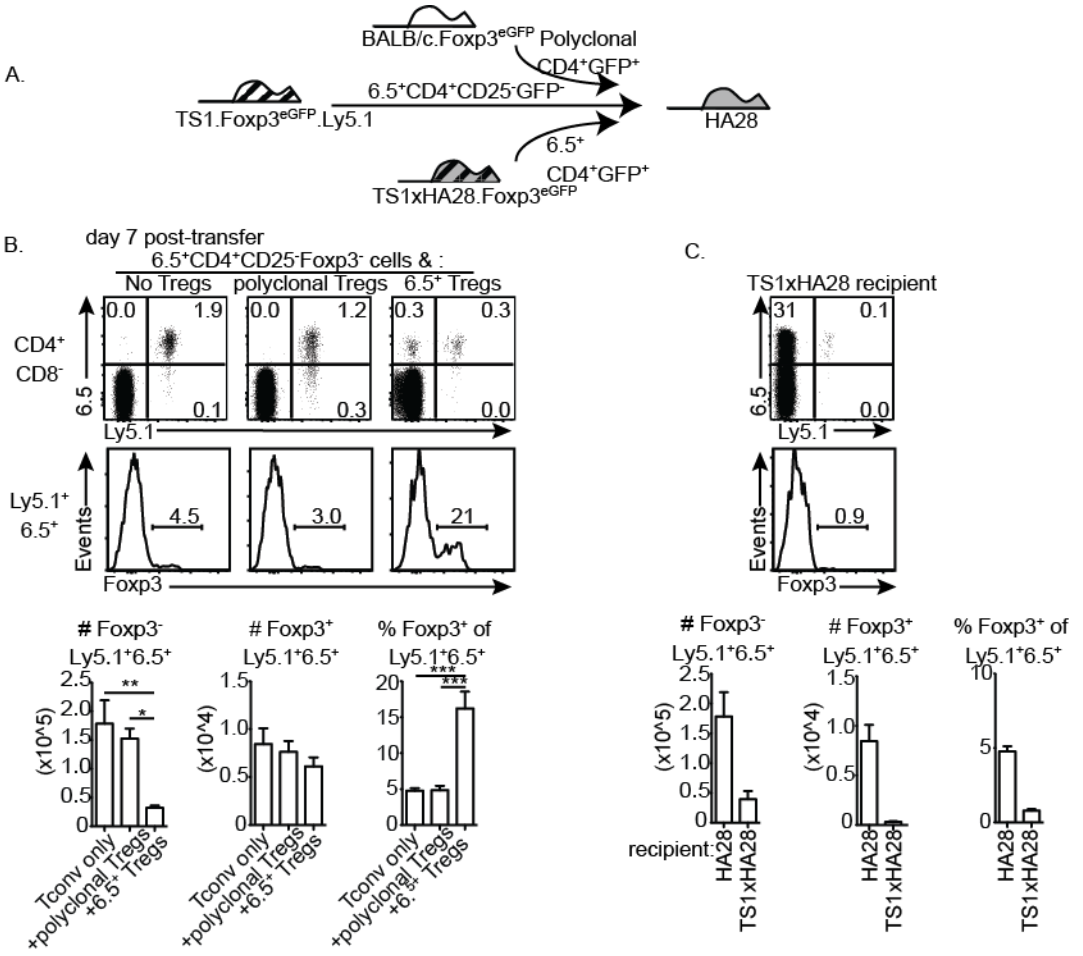

Figure 3-12 6.5$^+$CD4$^+$Foxp3$^+$ Treg cells suppressed the proliferation of 6.5$^+$CD4$^+$Foxp3$^-$ cells, but not the differentiation or accumulation of 6.5$^+$CD4$^+$Foxp3$^+$ pTreg cells in HA28 hosts. (**A**) 6.5$^+$CD4$^+$CD25$^-$eGFP$^-$ cells were isolated from TS1.Foxp3^{eGFP}.Ly5.1 mice and 8x10^5 cells were transferred into HA28 mice either alone or in conjunction with 4x10^5 6.5$^+$CD4$^+$eGFP$^+$ cells isolated from TS1xHA28.Foxp3^{eGFP}.Ly5.1 or with 4e5 CD4$^+$eGFP$^+$ cells from BALB/c.Foxp3^{eGFP} mice. (**B**) The number of 6.5$^+$Ly5.1$^+$CD4$^+$ cells in the pLNs at day 7 post-trasnfer and the percentage of these cells expressing Foxp3 was determined. n=5-6 mice combined from 3 independent experiments. (**C**) 8x10^5 6.5$^+$CD4$^+$CD25$^-$eGFP$^-$ cells isolated from TS1.Foxp3^{eGFP}.Ly5.1 mice were transferred into HA28 recipients or TS1xHA28 recipients, and accumulation and Foxp3 expression were determined at day 7 post-transfer. n=2 from 1 experiment for TS1xHA28 recipients.

3.3 Summary of Results and Discussion

3.3.1 The stimulatory potency of cognate self-Ag determines the fate of autoreactive thymocytes

Recognition of cognate self-Ag can drive thymocyte deletion or, alternatively, differentiation into a Foxp3$^+$ Treg cell (Jordan et al. 2001; Kawahata et al. 2002). Thymocytes expressing the transgenic, HA-specific 6.5$^+$ TCR undergo deletion in the thymus of a TS1xHACII mouse, in which the self-HA is expressed at high levels and under control of an MHCII promoter that directs its expression preferentially to APCs (Fig. 3-2). Foxp3 expression in 6.5$^+$CD4^{SP} thymocytes in TS1xHACII mice is slightly higher than in single transgenic TS1 mice, indicating that recognition of self-HA can induce Foxp3 upregulation. However, the extensive deletion that occurs in these mice ultimately results in no significant increase in the number of 6.5$^+$CD4^{SP}Foxp3$^+$ thymocytes or 6.5$^+$CD4$^+$Foxp3$^+$ cells in the pLNs. In contrast, some 6.5$^+$ thymocytes in TS1xHA28 mice, in which self-HA is expressed at much lower levels and predominantly by non-bone marrow-derived cells (Lerman et al. 2004), undergo deletion, but significantly less than in a TS1xHACII mouse. Furthermore, the percentage of 6.5$^+$CD4^{SP} thymocytes expressing Foxp3 was significantly higher than in TS1xHA28 mice than in TS1 or TS1xHACII mice, as was the number of 6.5$^+$CD4^{SP}Foxp3$^+$ thymocytes and of 6.5$^+$CD4$^+$Foxp3$^+$ cells in the periphery (Fig. 3-2). Thus, recognition of a self-Ag expressed at relatively low levels induced limited thymocyte deletion and differentiation into Foxp3$^+$ Treg cells, while recognition of the same self-Ag expressed at relatively higher levels resulted in extensive deletion and no significant increase in the

number of $CD4^{SP}Foxp3^{+}$ cells in the thymus or the periphery. This idea is consistent with previous publications from the lab using transgenic mice (Picca et al. 2009), as well as with one study demonstrating that injection of high doses of HEL (and subsequent processing and presentation within the thymus) resulted in more efficient deletion of thymocytes expressing an HEL-specific TCR, while injection of relatively lower doses of HEL resulted in increased accumulation of HEL-specific $CD4^{+}Foxp3^{+}$ cells (Atibalentja, Byersdorfer, Unanue 2009).

Further characterization of 6.5^{+} thymocyte development in TS1 and TS1xHA28 mice revealed that CD25 was upregulated on approximately 20% of $6.5^{+}CD4^{SP}Foxp3^{-}$ thymocytes and 65% of $6.5^{+}CD4^{SP}Foxp3^{+}$ thymocytes (Fig. 3-3 A). The transcription factor Helios, which has previously been found to be upregulated in thymocytes following strong TCR stimulation (Daley, Hu, Goodnow 2013), was upregulated in nearly 70% of $6.5^{+}CD4^{SP}Foxp3^{-}$ thymocytes and 100% of $6.5^{+}CD4^{SP}Foxp3^{+}$ thymocytes (Fig. 3-3 A). These data show that the majority of the $6.5^{+}CD4^{SP}$ thymocytes in TS1xHA28 mice have encountered cognate self-HA that induced CD25 and/or Helios upregulation, as $6.5^{+}CD4^{SP}$ thymocytes in TS1 mice express neither Helios nor CD25. The majority of $6.5^{+}CD4^{+}Foxp3^{+}$ T cells in the pLNs of TS1xHA28 mice expressed CD25 and Helios, and no expression of these molecules was observed in $6.5^{+}CD4^{+}Foxp3^{-}$ cells. We observed similar patterns of CD25 and Helios expression among $CD4^{SP}$ thymocytes in BALB/c mice with unmanipulated TCR repertoires (Fig. 3-3 B). Thus, recognition of self-Ag can induce upregulation of CD25 and Helios independently of Foxp3 expression in $CD4^{SP}$ thymocytes, and autoreactive $Helios^{+}CD4^{SP}Foxp3^{-}$ thymocytes can be observed both in TS1xHA28 mice and in BALB/c mice in the absence of a TCR transgene.

However, mature $CD4^+Foxp3^-$ cells either have lost expression of CD25 and Helios, or derive from a small population of 6.5^+CD4^{SP} thymocytes that do not express CD25 or Helios. The fate of the $Helios^+CD4^{SP}Foxp3^-$ thymocytes remains unclear, but one possibility is that they enter peripheral circulation as autoreactive $CD4^+$ T cells.

3.3.2 Stimulatory potency of a self-Ag determines the extent to which reactive $CD4^+Foxp3^-$ T cells differentiate into pTreg cells *in vivo*

Using the HA28 and HACII transgenic mice, we assessed the ability of the HA self-Ag to induce $6.5^+CD4^+Foxp3^-$ T cells to differentiate into $Foxp3^+$ pTreg cells. In HA28 mice, a subset of $6.5^+CD25^-Foxp3^-$ cells had upregulated Foxp3 expression at day 3 post-transfer, but this group was limited to cells that had undergone 0-2 rounds of division, while many of those that remained $Foxp3^-$ had divided 3-5 times (Fig. 3-4 C,D). In contrast, $6.5^+CD25^-Foxp3^-$ T cells transferred into HACII hosts had divided more extensively, and did not upregulate Foxp3. These data are consistent with a previous study in which pTreg cells differentiated following injection of cognate peptide that showed that a low dose of peptide induced more pTreg cell formation than a high dose, and that those cells that upregulated Foxp3 had undergone fewer rounds of division than those that did not (Gottschalk, Corse, Allison 2010). At day 21 post-transfer into HA28 recipients, nearly 50% of $6.5^+CD4^+Ly5.1^+$ donor-derived T cells expressed Foxp3, and the number of $CD4^+Ly5.1^+$ cell had begun to decrease, indicating that expression of Foxp3 was relatively stable, while those $Ly5.1^+$ cells that remained $Foxp3^-$ were gradually deleted (Fig. 3-6 B). Moreover, the 6.5^+ pTreg cells that formed in HA28 recipients were able to suppress proliferation of $6.5^+CD4^+Foxp3^-$ cells *in vitro* as well as

tTreg cells isolated from TS1xHA28 mice (Fig. 3-6 C). The use of eGFP-tolerant animals as recipients was crucial for examining the fate of Ly5.1$^+$ Treg cells at days 14 and 21 post-transfer, as evidence of deletion of donor cells that upregulated eGFP was found in both HA28 and HACII recipients, despite the fact that no upregulation of Foxp3 (and thus eGFP) was ever identified in HACII hosts (Fig. 3-5). These data suggest that Foxp3 may be transiently expressed by 6.5$^+$CD4$^+$Foxp3$^-$ transferred into HACII mice without leading to pTreg cell differentiation, as has been previously reported in both mouse and human T cells (Miyao et al. 2012; Wang et al. 2007).

The pTreg cells that formed in HA28 mice upregulated CD25 and Helios, such that by day 21 post-transfer, nearly 100% of Ly5.1$^+$Foxp3$^+$ cells, and none of the Ly5.1$^+$Foxp3$^-$ cells, isolated from the pLNs expressed these molecules. This Helios$^+$CD25$^+$ phenotype is very similar to that of 6.5$^+$CD4$^+$Foxp3$^+$ tTreg cells that formed in TS1xHA28 mice (Fig. 3-3 and Fig. 3-8). Additionally, Ly5.1$^+$ cells that upregulated Foxp3 in HA28 mice displayed a decreased ability to produce the inflammatory cytokines IL-2 and IFN-γ upon restimulation with PMA/ionomycin *ex vivo* relative to those cells that did not upregulate Foxp3, consistent with previous descriptions of Treg cells (Hori, Nomura, Sakaguchi 2003) (Fig. 3-7). Interestingly, a lower percentage of Ly5.1$^+$Foxp3$^-$ cells in HACII mice than in HA28 mice were capable of producing IL-2 and IFN-γ at day 14 post-transfer, and little IL-17 production by these cells was observed (Fig. 3-7). While 6.5$^+$CD4$^+$Foxp3$^-$ cells adoptively transferred into HACII recipients expanded to huge numbers and resulted in enlargement of the spleen and LNs, no signs of autoimmune disease were observed, which could be explained by the reduced cytokine production observed in these mice. 6.5$^+$ T cells adoptively

transferred into HACII mice may differentiate into a different helper T cell, thus explaining their decreased production of IL-2 and IFN-γ. Alternatively, in other systems, transfer of TCR-transgenic $CD4^+$ T cells into recipient mice that expressed cognate Ag as self resulted in T cell expansion prior to adoption of an anergic phenotype characterized by decreased cytokine production and proliferation upon subsequent stimulation (Lanoue et al. 1997; Tanchot et al. 2004). We did not directly assess the ability of 6.5^+ T cells to proliferate after 14 days in HACII hosts, although the number of $CD4^+Ly5.1^+$ cells in the pLNs of HACII recipients began to decrease following day 14, so it is possible that the rate of division had slowed by this time point. Moreover, $CD4^+$ T cells specific for viral Ag can become exhausted during a chronic viral infection, a condition that is again characterized by decreased proliferation and IL-2 and IFN-γ production in response to TCR stimulation following initial activation (Crawford et al. 2014). More studies would be needed to determine whether 6.5^+ T cells transferred into HACII mice may adopt one of these alternative fates.

3.3.3 pTreg cell differentiation in the mesenteric LN

Previous studies have demonstrated that specialized mechanisms dependent on retinoic acid and TGF-β exist in gut associated lymphoid tissues, such as the mesLN, to support the development of Treg cells (Coombes et al. 2007; Sun et al. 2007). Thus, we examined accumulation of $Ly5.1^+Foxp3^+$ Treg cells in the mesLNs of HA28 and HACII mice that received $6.5^+CD4^+CD25^-Foxp3^-Ly5.1^+$ cells isolated from TS1.Foxp3^{eGFP}.Ly5.1 donors at various time points post-transfer. $CD4^+Ly5.1^+$ T cell accumulation and the extent of Foxp3 upregulation was similar in the mesLNs to what we had observed in the

pLNs, although the percentage of $CD4^+Ly5.1^+$ expressing Foxp3 was significantly higher in the mesLN of an HACII recipient than a BALB/c recipient at day 3 post-transfer, which was not true in the pLNs (Fig. 3-9 A). This observation suggests that the requirement for a lower degree of stimulation in pTreg cell induction may be less stringent in the mesLNs than the pLNs. However, by days 7, 14, and 21 post-transfer, the percentage of $CD4^+Ly5.1^+$ cells from the mesLN expressing Foxp3 was no longer higher in an HACII mouse than a BALB/c mouse, which demonstrates that highly immunostimulatory self-Ag cannot support the continued differentiation or survival of Ag-specific pTreg cells. Interestingly, we found that a higher percentage of 6.5^+ pTreg cells in the mesLN than the pLNs of an HA28 mouse remained $Helios^-$ (Fig. 3-9 B). Helios was originally described as a marker of tTreg cells, and controversy remains over whether and under what circumstances it may be upregulated in pTreg cells (Gottschalk, Corse, Allison 2012; Shevach and Thornton 2014; Thornton et al. 2010). Our data suggests that the anatomical location in which a pTreg cell resides may determine whether or not it upregulates Helios; the self-HA and the 6.5^+ TCR on the transferred T cells did not differ between the pLNs and the mesLN of an HA28 mouse, but Helios was upregulated to a lesser extent by $6.5^+CD4^+Ly5.1^+$ cells in the mesLNs. Specialized $CD103^+$ DCs are present in the gut, and mediate pTreg cell differentiation through retinoic acid and TGF-β dependent mechanisms, leading us to speculate that these $CD103^+$ DCs may play a role in the formation of $Foxp3^+Helios^-$ pTreg cells.

In addition to assessing Foxp3 upregulation following adoptive transfer of $6.5^+CD4^+CD25^-Foxp3^-Ly5.1^+$ cells into HA28 and HACII hosts, we additionally transferred these cells into HA12 mice to determine the extent of their accumulation and

Foxp3 upregulation. HA12 mice express an intermediate amount of self-HA in the spleens, pLNs, mesLNs and intestines that is higher than what was found in an HA28 mouse but lower than in an HACII mouse. Additionally, HA expression on both radio-resistant and radio-sensitive cell types can drive 6.5^+ Treg cell formation in the thymus of TS1xHA12 mice, while in TS1xHA28 mice, HA expression on radio-resistant cell types is predominantly responsible for driving 6.5^+ Treg cell formation (Picca et al. 2009). We found that a higher percentage of donor-derived $CD4^+Ly5.1^+$ cells isolated from the pLNs had upregulated Foxp3 in HA28 mice versus HA12 mice, however when $CD4^+Ly5.1^+$ cells were isolated from the mesLNs instead, a higher percentage had upregulated Foxp3 in HA12 mice (Fig. 3-10). It may be that the optimal dose of a self-Ag for inducing Ag-dependent Treg cell formation is slightly higher in the mesLN than in the pLNs. Alternatively, it is possible that HA is expressed in $CD103^+$ DCs in the mesLNs of HA12 mice, thus increasing presentation of HA by these cells that preferentially induce pTreg cell formation. However, HA would also be expressed by $CD103^+$ DCs in HACII mice since it is under control of the MHCII promoter in this lineage, and yet little or no 6.5^+ pTreg cell formation occurred in the mesLNs of HACII mice, demonstrating that the immunostimulatory potency of the cognate self-Ag is still an important factor in determining the extent of pTreg cell formation.

3.3.4 Effects of prior exposure to cognate self-Ag and the presence of tTreg cells of the same specificity on pTreg cell formation

A substantial population of 6.5^+CD4^+ T cells that remain $Foxp3^-$ exists in the pLNs of TS1xHA28 mice despite having access to cognate self-Ag both in the thymus

and the pLNs. One possible explanation for their failure to upregulate Foxp3 was that they were intrinsically incapable of doing so, perhaps due to epigenetic differences that prevented access to the Foxp3 promoter. To determine whether these cells were capable of upregulating Foxp3, we transferred CellTrace Violet-labeled $6.5^{+}CD4^{+}CD25^{-}Foxp3^{-}$ cells, which were isolated from either TS1.$Foxp3^{eGFP}$mice or TS1xHA28.$Foxp3^{eGFP}$mice, into HA28 recipients, and assessed their accumulation and upregulation of Foxp3 at day 3 or 14 post-transfer. Division and accumulation of $6.5^{+}CD4^{+}$ cells from the two donor lineages was similar at day 3 post-transfer, but Foxp3 expression was significantly higher among $6.5^{+}CD4^{+}$ cells derived from from TS1xHA28 donors relative to TS1 donors (Fig. 3-11 B). These data suggest that $CD4^{+}Foxp3^{-}$ cells that develop in the presence of cognate self-Ag may be primed for Foxp3 upregulation relative to naïve $CD4^{+}Foxp3^{-}$ cells. Ultimately, however, $CD4^{+}Foxp3^{-}$ cells that developed in the presence of cognate self-Ag were neither more nor less capable of upregulating Foxp3 than their naïve counterparts, as accumulation and Foxp3 upregulation were identical between the two sources of $6.5^{+}CD4^{+}Foxp3^{-}$ cells by day 14 post-transfer (Fig. 3-11 B). Thus, it appears that no intrinsic reason exists to explain why some $6.5^{+}CD4^{+}$ T cells in TS1xHA28 do not upregulate Foxp3.

An alternative explanation for why $6.5^{+}CD4^{+}Foxp3^{-}$ T cells in the pLNs of TS1xHA28 mice failed to upregulate Foxp3 was that the presence of $6.5^{+}CD4^{+}Foxp3^{+}$ cells might limit their differentiation. When $6.5^{+}CD4^{+}CD25^{-}Foxp3^{-}Ly5.1^{+}$ cells were isolated from TS1.$Foxp3^{eGFP}$.Ly5.1 mice and transferred into HA28 recipients, the simultaneous transfer of 6.5^{+} Treg cells, but not of polyclonal Treg cells, limited their accumulation in the pLNs, demonstrating that 6.5^{+} Treg cells suppressed 6.5^{+} T cell

accumulation more effectively than Treg cells of other diverse specificities (Fig. 3-12 B). Furthermore, the percentage of $6.5^+CD4^+Ly5.1^+$ cells expressing Foxp3 was higher in HA28 mice that also received 6.5^+ Treg cells than in HA28 mice that received either polyclonal Treg cells or no Treg cells. However, the number of $6.5^+CD4^+Ly5.1^+Foxp3^+$ pTreg cells that formed in HA28 mice at day 7 post-infection was no higher when 6.5^+ Treg cells were co-transferred than when polyclonal Treg cells or no Treg cells were co-transferred, indicating that the presence of 6.5^+ Treg cells limited accumulation of $6.5^+CD4^+Foxp3^-$ cells, but did not impact $6.5^+CD4^+Foxp3^+$ pTreg cell formation (Fig. 3-12 B). In contrast, adoptively transferred $6.5^+CD4^+Ly5.1^+$ cells accumulated less in the pLNs of a TS1xHA28 recipient than an HA28 recipient, and no upregulation of Foxp3 occurred, suggesting that the 6.5^+ Treg cell niche may be saturated in TS1xHA28 mice, and that $6.5^+CD4^+Foxp3^-$ cells may not have sufficient access to cognate self-Ag or to cytokines, such as IL-2, to induce differentiation (Fig. 3-12 C). A limited niche for Treg cells of a given specificity has been found to exist in the thymus, and increasing the clonal precursor frequency of thymocytes expressing a transgenic TCR can result in a decreased number of clonotypic thymocytes upregulating Foxp3 (Bautista et al. 2009; Leung, Shen, Lafaille 2009). In this case, competition for cognate Ag may limit the size of the clonotypic tTreg cell niche, and the same phenomenon could occur in the periphery.

CHAPTER 4: Variations in Ag presentation determine Treg cell activity *in vivo*

4.1 Introduction

In addition to their well-established role in maintaining immune homeostasis at steady-state, Treg cells have more recently been revealed to participate in and modulate immune responses generated against pathogens (Maizels and Smith 2011). However, the signals required for Treg cell accumulation and activity during an infection remain poorly understood. TCR stimulation is required for Treg cell activity *in vitro* (Thornton and Shevach 1998), and TCR-transgenic Treg cells failed to expand or enter the lungs during *M. tuberculosis* or influenza virus infection in the absence of cognate Ag (Bedoya et al. 2013; Shafiani et al. 2010). Thus, it appears that a TCR signal is required for Treg cell accumulation during an infection, and that exposure to an inflammatory environment is not sufficient for their activation. It has been suggested that $CD4^{+}Foxp3^{-}$ T cells may differentiate into pTreg cells upon recognition of pathogen-derived Ag (Mills and McGuirk 2004), and there is good evidence that recognition of commensal microbiota can induce pTreg cell formation (Lathrop et al. 2011). Thus, peripheral differentiation of pathogen-specific pTreg cells represents one possible source of Treg cells that accumulate during infection. Alternatively, Treg cells that exist in the pre-immune repertoire may expand and modulate the effector immune response following recognition of either pathogen-derived Ag or self-Ag present at sites of infection. Indeed, in both *L. major* and *M. tuberculosis* infection models, the Treg cells isolated from infection sites derived from pre-existing Treg cells (Belkaid et al. 2002; Shafiani et al. 2010; Suffia et al. 2006). A subset of the Treg cells present at the site of *L. major* infection were found to be specific for microbial Ag, but this observation does not necessarily rule out a role for self-reactive

Treg cells during infection, and whether activation on self-antigen can drive Treg cell activity during infection has not been explored (Suffia et al. 2006).

Previous studies in our lab have demonstrated that 6.5^+ Treg cells isolated from TS1xHA28 mice expanded and accumulated in the lungs and lung-draining mediastinal LNs (medLNs) during infection with PR8 influenza virus, which contains the cognate HA (Bedoya et al. 2013). Thus, Treg cells selected on a self-Ag in the thymus can become activated during infection in response to cognate viral Ag. Adoptive transfer of 6.5^+ Treg cells prior to PR8 virus infection reduced the accumulation of $CD4^+Foxp3^-$ T cells and both total and virus-specific $CD8^+$ T cells in the lungs at day 8 post-infection (Bedoya et al. 2013). Notably, the presence of 6.5^+ Treg cells resulted in a decrease in $CD4^+Foxp3^-$ and $CD8^+$ T cell proliferation in the lungs but not in the medLNs at this time point, and expression of the chemokine receptor CXCR3 on effector cells in the medLN was not affected. These findings suggest that 6.5^+ Treg cells limit effector cell accumulation by suppressing proliferation within the lungs rather than by limiting migration of T cells into the lungs from the medLN. 6.5^+ Treg cells isolated from the lungs during PR8 virus infection were found to be capable of producing IL-10, suggesting one potential mechanism by which they may be exerting suppression (Bedoya et al. 2013); IL-10 limits immunopathology in the lungs during influenza virus infection, and treatment with IL-10R blocking antibody was found to result in increased mortality and more rapid death in infected mice (Sun et al. 2009). These findings are reminiscent of another study in which pathogen-specific Treg cells delayed accumulation of pathogen-specific $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes and lungs during *M. tuberculosis* infection (Shafiani et al. 2010).

It has recently become apparent that Treg cells can differentiate further and acquire novel characteristics that allow them to effectively suppress the activity of different T helper cell subsets in a process termed functional specialization (Campbell and Koch 2011). Selective ablation of IRF4, which is associated with Th2 cell differentiation, in Foxp3$^+$ cells results in uncontrolled Th2 cell activity, while expression of STAT3 by Treg cells was required to prevent Th17-mediated autoimmune lymphoproliferative disease (Chaudhry et al. 2009; Zheng et al. 2009). Alternatively, Treg cells can acquire expression of T-bet and CXCR3, and these T-bet$^+$ Treg cells are required to prevent Th1 cell-mediated immunopathology and limit IFN-γ production by CD4$^+$ T cells at steady state (Koch et al. 2009). We have found that 6.5$^+$ Treg cells differentiate towards this T-bet$^+$ phenotype during a PR8 influenza virus infection, and increase expression of T-bet, CXCR3, IL-10, and Blimp1 compared to donor 6.5$^+$ Treg cells (Bedoya et al. 2013). The transcription factor T-bet is necessary for CXCR3 expression, and IFN-γ is thought to be at least partially responsible for induction of T-bet expression, as IFN-γR$^{-/-}$ Treg cells express much lower levels of both T-bet and CXCR3 (Koch et al. 2009). Additional studies suggested that T-bet is dispensable for IL-10 production, and instead Blimp1 appears to be required (Cretney et al. 2011).

In this section, we describe studies examining the extent to which recognition of HA derived from self or from influenza virus can activate 6.5$^+$ Treg cells to proliferate, differentiate, and modulate the anti-viral immune response. Although a weakly stimulatory form of self-HA induced 6.5$^+$ Treg cell formation both in the thymus and the periphery, recognition of self-HA in this context did not induce 6.5$^+$ Treg cells to

accumulate or differentiate in the lungs and medLN during infection with influenza virus that lacked the cognate HA epitope. In contrast, when HA was present as a viral Ag, 6.5^+ Treg cells proliferated extensively, acquired a T-bet$^+$CXCR3$^+$ phenotype, and suppressed the accumulation of effector T cells in the lungs. Increasing the stimulatory potency of the self-HA by using HACII mice did not result in efficient Treg cell activation during influenza virus infection, and instead inhibited the ability of 6.5^+ Treg cells to differentiate and mediate suppression upon subsequent exposure to PR8 virus-derived HA. Further studies revealed that Ag presentation by B cells in HACII mice, and a non-inflammatory environment at the time of initial TCR stimulation, both likely contributed to the lack of 6.5^+ Treg cell differentiation and activity in HACII mice. Together, the studies presented in this section demonstrate that the type of APC presenting Ag to a Treg cell and whether presentation occurs in the presence of an inflammatory response can affect the extent of Treg cell differentiation, and further reveal that exposure to a highly stimulatory cognate self-Ag can result in changes to the Treg cell phenotype.

4.2 Results

4.2.1 Treg cells accumulate in the mediastinal LNs and lungs during PR8 influenza virus infection

To assess the accumulation of Treg cells during an immune response to influenza virus, we infected otherwise unmanipulated BALB/c mice with PR8 influenza virus and examined expression of CD25 and Foxp3 on $CD4^+$ T cells isolated from the medLNs and lungs (Fig. 4-1). The number of $CD4^+Foxp3^+$ Treg cells in each location

was 5-6 times higher at day 8 post-infection with PR8 influenza virus than in uninfected mice. Thus, Treg cells were recruited to and/or expanded at sites of influenza virus infection in BALB/c mice.

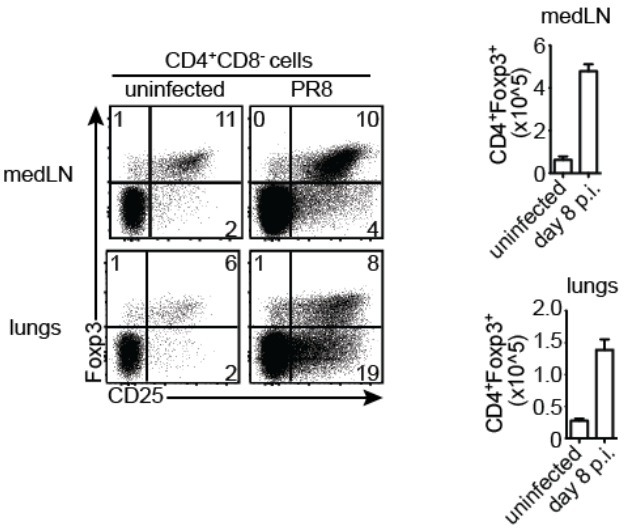

Figure 4-1 The number of Treg cells in the medLNs and lungs increases during influenza virus infection CD25 and Foxp3 expression on $CD4^+$ T cells in the medLNs and lungs of uninfected mice and at day post-PR8 influenza virus infection was assessed, and the number of $CD4^+Foxp3^+$ cells was determined.

4.2.2. $6.5^+CD4^+Foxp3^-$ T cells do not differentiate into $Foxp3^+$ Treg cells following recognition of viral Ag

One possible source of the Treg cells that accumulated during influenza virus infection was $CD4^+Foxp3^-$ cells that converted into $Foxp3^+$ pTreg cells upon recognition of viral Ag. To determine whether recognition of viral Ag might drive $CD4^+Foxp3^-$ T cells to differentiate into $Foxp3^+$ Treg cells, we transferred CellTrace Violet-labeled $6.5^+CD4^+CD25^-eGFP^-$ T cells isolated from TS1.$Foxp3^{eGFP}$ mice into BALB/c recipients that were infected intranasally the following day with PR8 influenza virus, which contains the HA epitope recognized by the 6.5^+ TCR (Fig. 4-2 A). At day 5 post-infection, 6.5^+CD4^+ T cells isolated from the medLNs, lungs, and spleens were analyzed for dilution of CellTrace Violet and expression of Foxp3. The 6.5^+CD4^+ T cells isolated from each location had all undergone multiple rounds of division, indicating that they had been exposed to cognate viral HA as part of a peptide:MHC complex, but no upregulation of Foxp3 occurred (Fig. 4-2 B). These data show that recognition of viral-HA induced $6.5^+CD4^+Foxp3^-$ T cell division but not Foxp3 upregulation, and suggests that the Treg cells that accumulate at infection sites likely derive from expansion of pre-existing Treg cells, and not from conversion of virus-reactive $CD4^+Foxp3^-$ T cells into pTreg cells.

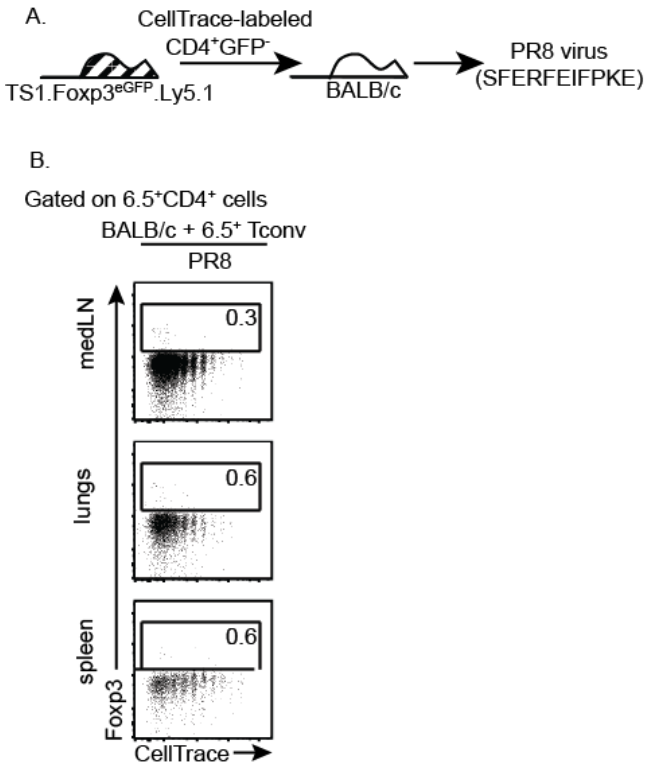

Figure 4-2 Recognition of viral antigen did not induce upregulation of Foxp3 in $6.5^{+}CD4^{+}$ T cells (**A**) $CD4^{+}eGFP^{-}$ cells were isolated from TS1.$Foxp3^{eGFP}$.Ly5.1 mice, labeled with CellTrace Violet, and transferred into BALB/c recipients, which were infected with PR8 influenza virus on the following day. (**B**) CellTrace Violet dilution and Foxp3 expression by $6.5^{+}CD4^{+}Ly5.1^{+}$ cells isolated from the medLNs, lungs, and spleen at day 5 post-infection were assessed. Plots are representative of 3 independent experiments.

4.2.3. The self-HA in HA28 mice is optimal for 6.5^+ Treg cell formation, but not for 6.5^+ Treg cell recruitment or expansion during an influenza virus infection

The studies in Chapter 3 showed that the HA self-peptide in HA28 mice can support the formation of $6.5^+CD4^+Foxp3^+$ Treg cells both during thymocyte development and in the periphery by promoting conversion of conventional $CD4^+$ T cells into $Foxp3^+$ Treg cells, and we were interested in whether the HA self-peptide in these mice could also activate Treg cells to exert regulatory function during an infection. To examine this question, we modified an adoptive transfer system that had previously been used in the lab to demonstrate that $CD4^+Foxp3^+$ Treg cells generated in TS1xHA28 mice could modulate immune responses to influenza virus following recognition of PR8 virus-derived HA. In the previous studies, BALB/c mice were seeded with $CD4^+Foxp3^+$ Treg cells from TS1xHA28 mice and then infected with PR8 virus on the following day, and the accumulation of donor-derived Treg cells, their differentiation, and their effects on the immune response was evaluated (Bedoya et al. 2013). To assess the ability of HA to activate the 6.5^+ Treg cells when it was present as a self-Ag but not as a viral-Ag, we repeated these studies in HA28 mice that had received $CD4^+Foxp3^+Ly5.1^+$ Treg cells from TS1xHA28.$Foxp3^{eGFP}$.Ly5.1 mice and were then infected with a PR8 mutant virus (RV6) bearing a single amino acid substitution in the S1 determinant of the HA that is recognized by the 6.5 TCR (Hurwitz et al. 1984). This mutation impairs recognition of the viral Ag by the transferred Treg cells, and thus the extent to which 6.5^+ Treg cells can become activated by the self-HA Ag could be assessed by comparison with Treg cell-recipient RV6-infected BALB/c mice (which do not express the HA self-Ag), and with Treg cell-recipient PR8-infected BALB/c mice (in which the transferred Treg cells can recognize the HA as a viral Ag, but not as a self-Ag). We first transferred CellTrace

Violet-labeled $CD4^+eGFP^+$ cells isolated from TS1xHA28.$Foxp3^{eGFP}$.Ly5.1 mice into BALB/c or HA28 recipients, which were infected the following day with PR8 virus, RV6 virus, or J1 virus (a reassortant virus that is genetically identical to PR8 except that it contains a serologically non-crossreactive H3 subtype HA (Palese 1977)) (Fig. 4-3 A,C). At day 5 post-infection, dilution of CellTrace Violet and Foxp3 expression were assessed in 6.5^+CD4^+ cells isolated from the medLNs, lungs, and spleens of the recipient mice. In BALB/c mice infected with PR8 virus, the majority of cells isolated from each anatomical location had divided and most had undergone between 4 to 8 rounds of division (Fig. 4-3 B). Within the lungs of these mice, no undivided 6.5^+CD4^+ cells were found, and these cells had undergone on average more divisions than those in the medLNs and spleens. RV6 virus induced some division of 6.5^+CD4^+ cells in the medLNs of infected BALB/c mice, but cells isolated from the lungs and spleens had undergone little or no division (Fig. 4-3 B). Importantly, the extent of 6.5^+CD4^+ Treg cell division that occurred in response to RV6 virus was much less than what occurred in response to PR8 virus infection, reflecting the reduced reactivity of the 6.5 TCR toward the RV6 mutant. Finally, in J1 virus infected BALB/c mice, 6.5^+CD4^+ Treg cells did not divide in any of the locations examined, despite a clear evidence of infection (data not shown), indicating that inflammatory signals alone in the absence of TCR stimulation were not sufficient to drive 6.5^+ Treg cell division at sites of infection (Fig. 4-3 B). In the same set of experiments, we also transferred CellTrace Violet-labeled $CD4^+eGFP^+$ cells isolated from TS1xHA28.$Foxp3^{eGFP}$.Ly5.1 mice into uninfected HA28 recipients; we found that 6.5^+CD4^+ Treg cells isolated from the medLN and spleens at day 6 post-transfer had undergone approximately 0 to 2 rounds of division, indicating that recognition of self-Ag could induce 6.5^+ Treg cell proliferation in HA28 mice, but that very few of these cells could be identified in the lungs (Fig. 4-3 D). Notably, even though HA28 mice expressed

the HA as a self-peptide, there was no greater accumulation of 6.5^+ Treg cells in the medLNs and lungs of RV6-infected HA28 mice than occurred in RV6-infected BALB/c mice.

We additionally assessed the accumulation of donor-derived $Ly5.1^+CD4^+$ cells in the medLN and lungs at day 8 post-infection, and found that the number of 6.5^+CD4^+ Treg cells in the lungs of PR8 virus-infected BALB/c mice increased nearly 8 fold between days 5 and 8 post-infection, while the increase in the medLNs was much less dramatic (Fig. 4-3 E,F). These data suggest that 6.5^+ Treg cells accumulate first in the medLNs, then in the lungs during infection with virus containing the cognate HA Ag. In contrast, 6.5^+CD4^+ Treg cells did not accumulate in the lungs of RV6 virus-infected BALB/c or HA28 mice at day 8 post-infection, and recognition of self-HA in HA28 mice did not cause 6.5^+ Treg cells to accumulate to a greater extent during RV6 virus-infection relative to BALB/c hosts (Fig. 4-3 E). At both days 5 and 8 post-infection, significantly more donor-derived Treg cells were present in the medLN and lungs of PR8-infected BALB/c mice compared with both RV6-infected BALB/c mice and RV6-infected HA28 mice, and in all conditions, transferred Treg cells maintained expression of Foxp3 (Fig. 4-3 F). Notably, both RV6 and PR8 virus induced similar amounts of weight loss and IFN-γ accumulation in the serum, suggesting that any differences observed in the 6.5^+ Treg cell response to the different viruses were not the result of disparities in the overall infection and immune response (Fig. 4-3 G,H).

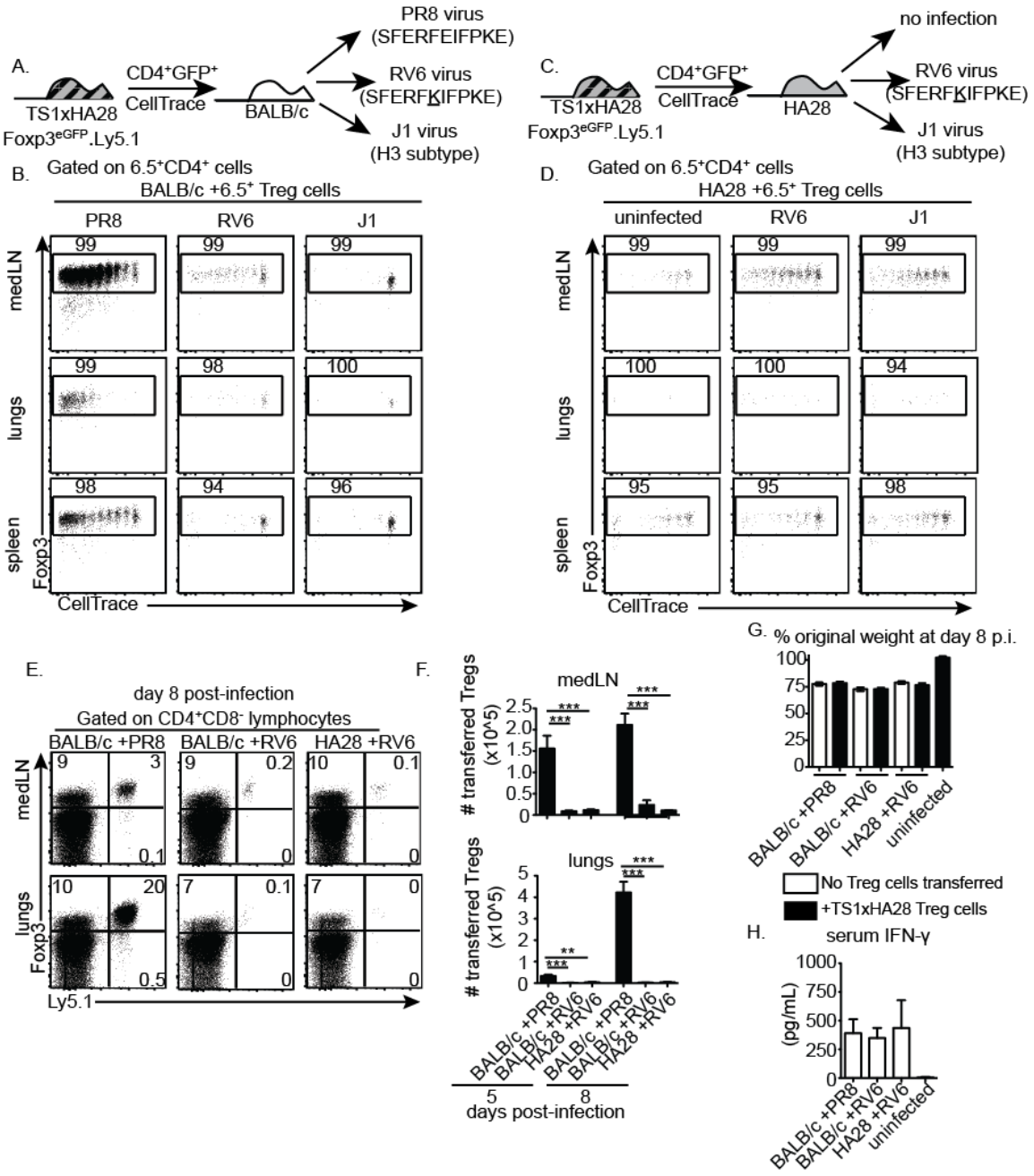

Figure 4-3 Recognition of neo-self HA in HA28 mice did not drive 6.5+ Treg cell accumulation in influenza virus-infected mice to the same extent as recognition of viral HA CD4+eGFP+ cells were isolated from the spleens and LNs of TS1xHA28.Foxp3eGFP.Ly5.1 mice, labeled with CellTrace Violet, and transferred into BALB/c (**A** and **C**) or HA28 (**B** and **D**) recipients. Recipient mice were infected with PR8, RV6, or J1 influenza virus the following day, and dilution of CellTrace Violet and Foxp3 expression were assessed at day 5 post-infection. (**E**) Experiment was set-up as above, but Ly5.1 and Foxp3 expression in total CD4+CD8- cells in the medLN and lungs at day 8 post-infection was assessed, then accumulation of Ly5.1+CD4+ cells was determined at days 5 and 8 post-infection (**F**) (n≥4 at day 5 p.i.; n≥6 at day 8 p.i. for each group). (**G**) Percent remaining of original body weight in otherwise unmanipulated mice (white bar) or mice that received 6.5+ Treg cells (black bar) (n=4-7) and (**H**) concentration of IFN-γ in the serum of indicated mice at day 8 post-infection (n=3-8).

These data show that recognition of self-HA can induce 6.5$^+$ Treg cell division in HA28 mice, but recognition of viral-HA during PR8 virus infection induced more extensive division. In PR8 virus-infected BALB/c mice, the majority of 6.5$^+$ Treg cell accumulation in the medLN occurred by day 5 post-infection, while accumulation in the lungs mostly took place between days 5 and 8 post-infection. In contrast, in RV6 virus-infected infected HA28 mice, 6.5$^+$ Treg cells isolated from the medLN had divided, but significantly less cells were present than in the case of PR8 virus infection, and 6.5$^+$ Treg cells did not accumulate in the lungs of RV6 virus-infected BALB/c or HA28 mice.

4.2.4 Recognition of virus HA, but not self-HA in HA28 mice, induced 6.5$^+$ Treg cell differentiation during influenza virus infection

Studies from our lab have previously shown that 6.5$^+$CD4$^+$Foxp3$^+$ Treg cells differentiat into T-bet$^+$CXCR3$^+$ Treg cells in PR8-infected BALB/c mice (Bedoya et al. 2013). Consistent with these previous studies, the 6.5$^+$CD4$^+$Foxp3$^+$ Treg cells that had undergone division in the medLNs of PR8-infected BALB/c mice at day 5 p.i. had uniformly upregulated CXCR3 and contained cells with increased T-bet expression, and CXCR3 and T-bet were also upregulated in the 6.5$^+$CD4$^+$Foxp3$^+$ Treg cells in the lungs of PR8-infected BALB/c mice at day 8 p.i. (Fig. 4-4). By contrast, significantly lower percentages of the 6.5$^+$CD4$^+$Foxp3$^+$ Treg cells in RV6-infected BALB/c or HA28 mice expressed CXCR3, and no significant upregulation of T-bet occurred. The ability to recognize self-HA did not result in CXCR3 upregulation in uninfected HA28 mice, and did not increase CXCR3 or T-bet upregulation by 6.5$^+$ Treg cells in RV6-infected HA28 mice relative to RV6-infected BALB/c mice.

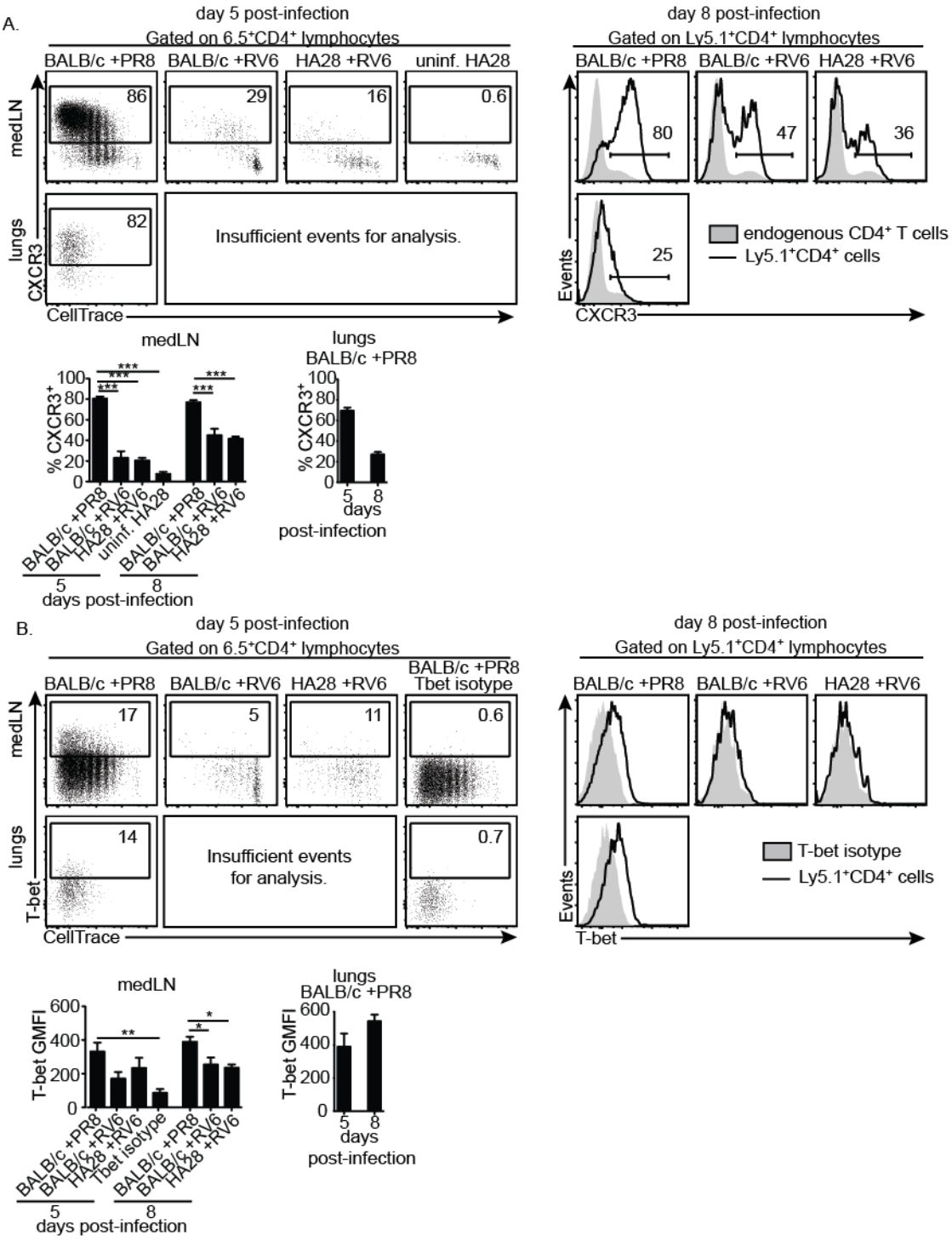

Figure 4-4 Recognition of viral HA, but not self-HA in HA28 mice, induced 6.5+ Treg cells to acquire expression of T-bet and CXCR3 during influenza virus infection CD4+eGFP+ cells were isolated from the spleens and LNs of TS1xHA28.Foxp3^{eGFP}.Ly5.1 mice and transferred into BALB/c or HA28 recipients, which were infected with PR8 or RV6 influenza virus the following day. CXCR3 (A) and T-bet (B) expression were assessed on donor-derived Treg cells isolated from the medLNs and lungs at days 5 and 8 post-infection. (n≥4 at day 5 p.i.; n≥6 at day 8 p.i. for each group)

Thus, recognition of the differentiation-inducing self-Ag did not result in 6.5^+ Treg cell differentiation towards a T-bet^+ phenotype, either in the presence or absence of infection. In contrast, recognition of the same peptide when it was present as a viral Ag induced upregulation of T-bet and CXCR3.

4.2.5 Recognition of PR8 virus-derived HA induced 6.5^+ Treg cells to suppress the effector T cell response during influenza virus infection more efficiently than recognition of self-HA

In the experiments described in the previous two sections, we also examined the frequencies of $CD4^+Foxp3^-$ and $CD8^+$ effector cells in the lungs of mice that had or had not received $6.5^+CD4^+Foxp3^+$ Treg cells. In the absence of transferred Treg cells, the magnitudes of the $CD4^+Foxp3^-$ and $CD8^+$ effector T cell responses were similar between all of the mice irrespective of whether they had been infected with PR8 or RV6 (Fig. 4-5). Adoptive transfer of $6.5^+CD4^+Foxp3^+$ Treg cells prior to infection caused a decrease in the effector T cell response only in BALB/c mice infected with PR8 virus. Thus, even though interactions with the self-HA can promote the formation and persistence of 6.5^+Foxp3^+ Treg cells in HA28 mice, such interactions did not direct $6.5^+CD4^+Foxp3^+$ Treg cells to accumulate, differentiate, or limit effector T cell accumulation at infection sites during an infection with a non-cognate influenza virus, such as occurred when the $6.5^+CD4^+Foxp3^+$ Treg cells could recognize HA derived from viral Ag.

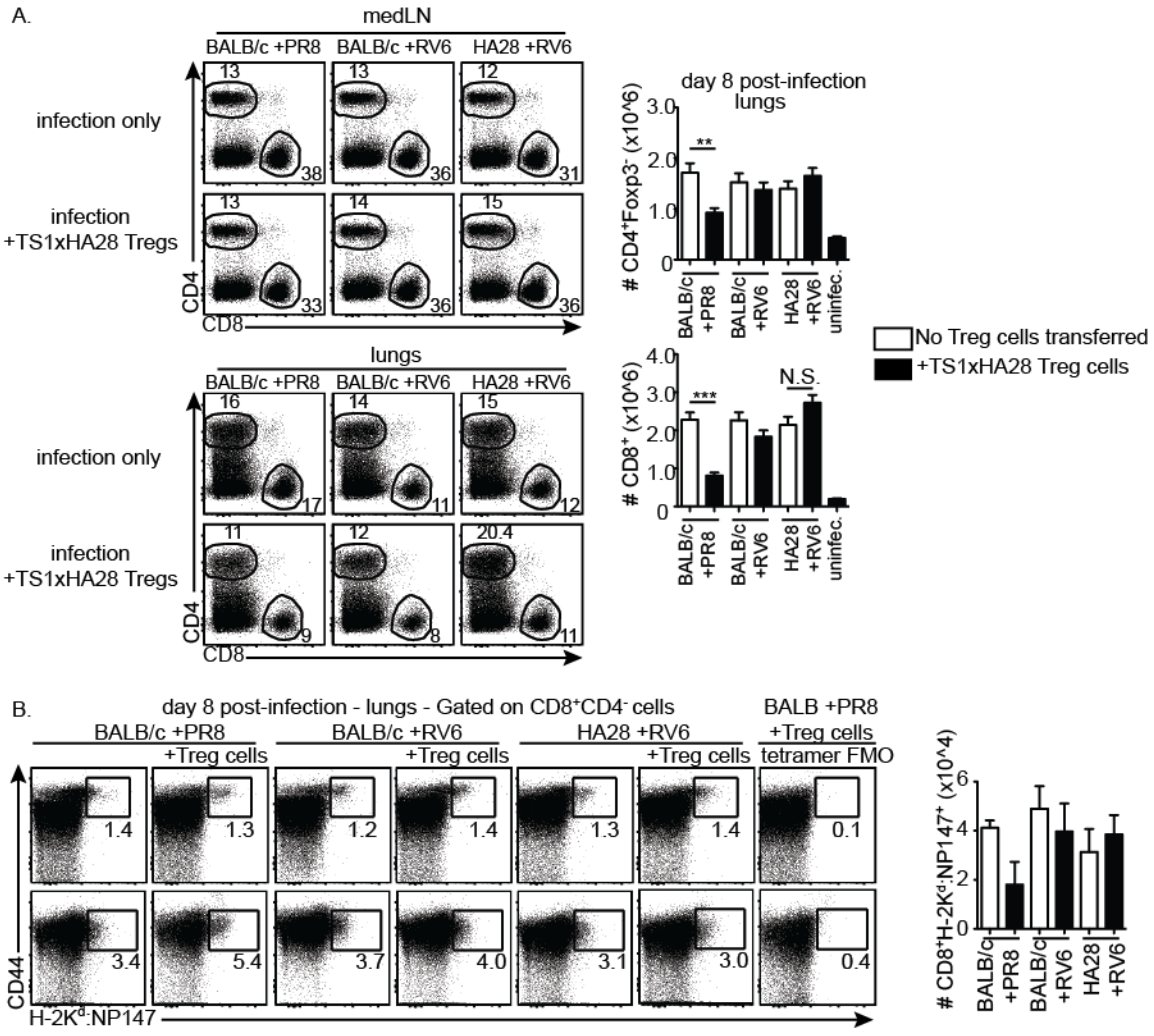

Figure 4-5 Adoptive transfer of Treg cells that could recognize viral antigen, but not of Treg cells that could recognize self-antigen, prior to influenza virus infection resulted in decreased accumulation of $CD4^+Foxp3^-$ and $CD8^+$ T cells in the lungs of infected mice $CD4^+eGFP^+$ cells were isolated from the spleens and LNs of TS1xHA28.$Foxp3^{eGFP}$.Ly5.1 mice and transferred into BALB/c or HA28 recipients, which were infected with PR8 or RV6 influenza virus the following day. (**A**) The number of endogenous $CD4^+Foxp3^-$ and $CD8^+$ T cells in the lungs of mice that had or had not received Treg cells at day 8 post-infection was determined (n≥6 for each group). (**B**) Expression of CD44 and binding of H-$2K^d$:NP147 tetramer by $CD8^+$ T cells isolated at day 8 post-infection (n=3-4).

4.2.6 Increasing the immunostimulatory strength of the self-HA inhibited 6.5$^+$ Treg cell accumulation and differentiation in response to viral Ag

It was possible that 6.5$^+$ Treg cells expanded and suppressed the immune response following recognition of viral-HA in a PR8 virus-infected BALB/c mouse, but not following recognition of self-HA during an RV6 virus infection in an HA28 mouse, because Treg cell proliferation and activation requires the high levels of the HA Ag that are generated during infection. To test whether increasing the level of expression of the HA as a self-Ag could promote 6.5$^+$ Treg cell activity in virus-infected mice, we made use of HACII mice, in which the HA transgene is expressed at high levels by APCs. To compare 6.5$^+$ Treg cell division in response to PR8 virus HA versus self-HA in HACII mice, we first infected BALB/c mice with PR8 virus and infected HACII mice with the weakly crossreactive RV6 virus. Then on the following day, we transferred CellTrace Violet-labeled, Ly5.1 congenically-marked 6.5$^+$CD4$^+$Foxp3$^+$ T cells from TS1xHA28.Foxp3^{eGFP}.Ly5.1 mice into the infected mice and into uninfected HACII mice. By performing the adoptive transfers the day after infection, we were able to assess division of 6.5$^+$ Treg cells after exposure to viral-HA or self-HA for the same amount of time. At day 5 post-infection (day 4 post-transfer) the Ly5.1$^+$CD4$^+$ Treg cells in the medLN and lungs of both uninfected and RV6-virus infected HACII mice had undergone similar levels of division as had occurred in PR8-infected BALB/c mice, suggesting that the self-HA in the medLN and lungs of HACII mice was equally capable of inducing division of 6.5$^+$ Treg cells as was the virus-derived HA (Fig. 4-6 B).

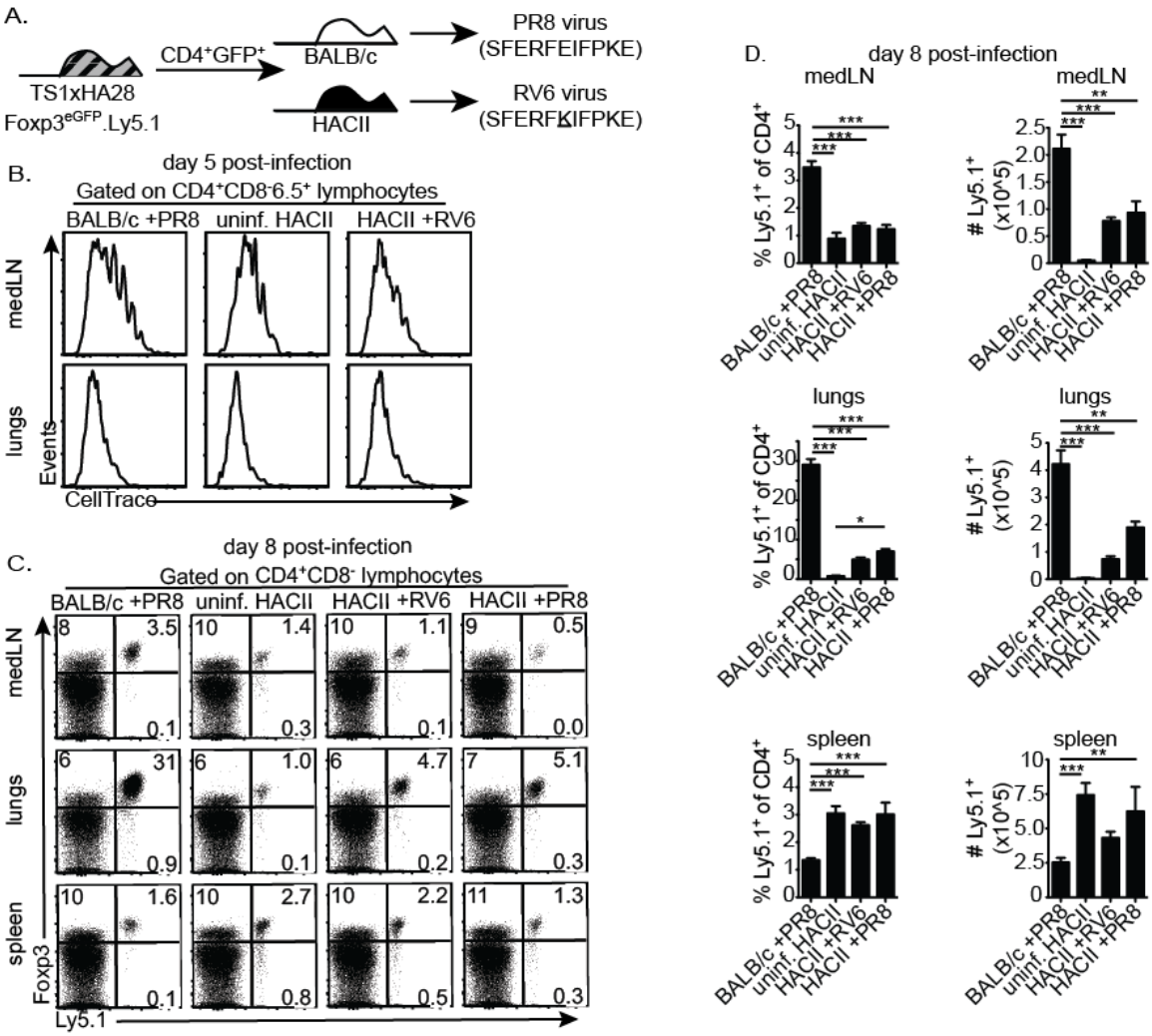

Figure 4-6 Recognition of self-HA in HACII mice drove 6.5$^+$ Treg cell proliferation but accumulation in the lungs and LNs was inefficient (**A**) CD4$^+$eGFP$^+$ cells isolated from the spleens and LNs of TS1xHA28.Foxp3^{eGFP}.Ly5.1 mice were transferred into BALB/c or HACII recipients, which were infected with PR8 or RV6 influenza virus. (**B**) CellTrace Violet dilution by adoptively-transferred CD4$^+$6.5$^+$eGFP$^+$ cells (from TS1xHA28.Foxp3^{eGFP}.Ly5.1 mice) isolated from indicated mice at day 5 post-infection (day 4 post-transfer). (**C**) Ly5.1 and Foxp3 expression on CD4$^+$CD8$^-$ cells isolated from indicated mice at day 8 post-infection (day 9 post-transfer) was assessed by flow cytometry, and (**D**) the percentage of CD4$^+$ cells expressing Ly5.1 and the total number of Ly5.1$^+$CD4$^+$ cells was determined. n≥5 for each group combined from at least 3 independent experiments.

The self-HA in HACII mice could also support the accumulation of 6.5^+ Treg cells, as $Ly5.1^+$ donor-derived cells made up a larger percentage of total $CD4^+$ cells and were present at higher numbers in the spleens of uninfected HACII than of PR8 virus-infected BALB/c mice at day 8 p.i. (Fig. 4-6 C,D). Notably, however, at day 8 p.i., significantly fewer $CD4^+Foxp3^+Ly5.1^+$ cells had accumulated in the medLNs of either uninfected or RV6-infected HACII mice than occurred in PR8-infected BALB/c mice, and the percentage of $CD4^+$ cells expressing Ly5.1 in the medLN did not increase in RV6 virus-infected HACII mice relative to uninfected HACII mice (Fig. 4-6 D). Furthermore, the 6.5^+ Treg cells that were found in the medLN of RV6 virus-infected HACII mice expressed significantly lower levels of CXCR3 and T-bet than were found in PR8-infected BALB/c mice (Fig. 4-7 A,B). Consistent with impaired differentiation, fewer $CD4^+Ly5.1^+Foxp3^+$ cells accumulated in the lungs of either uninfected or RV6 virus-infected HACII mice, and the cells that did accumulate again expressed lower levels of T-bet than occurred in PR8 virus-infected BALB/c mice.

To further explore how differences in the presentation of HA as a self- versus viral peptide might affect 6.5^+ Treg cell differentiation, we also examined the ability of 6.5^+ Treg cells to differentiate in HACII mice that had been infected with PR8 virus. Remarkably, the accumulation of $Ly5.1^+CD4^+Foxp3^+$ cells in the medLN and lungs was significantly lower in PR8 virus-infected HACII mice than in PR8 virus-infected BALB/c mice (Fig. 4-6 D), and the levels of CXCR3 and T-bet that were expressed were much more similar to those observed in RV6-infected HACII mice than was the case in PR8-infected BALB/c mice (Fig. 4-7 A,B). Moreover, the provision of Treg cells led to a significant decrease in the accumulation of $CD4^+$ and $CD8^+$ effector cells in the lungs of

PR8-infected BALB/c mice, but these were not significantly reduced in RV6- or PR8-infected HACII mice (Fig. 4-7 C). These differences in the suppressive effects of 6.5^+ Treg cell transfer in PR8 virus-infected BALB/c mice versus RV6 virus-infected HACII mice did not appear to be the result of differences in production of IL-10, which has been shown to limit inflammation in the lungs during influenza virus infection (Sun et al. 2009) (Fig 4-7 D). Thus, recognition of the self-HA in HACII mice both failed to support the efficient recruitment of 6.5^+ Treg cells in virus-infected mice, and actively suppressed the accumulation and differentiation of these cells in response to a viral Ag.

4.2.7 Recognition of self-HA in HACII mice caused 6.5^+ Treg cells to decrease expression of the 6.5^+ TCR, CD25, and Foxp3

6.5^+ Treg cells failed to efficiently upregulate T-bet and CXCR3 in HACII mice, and this impaired differentiation likely plays a role in their decreased ability to suppress the effector T cell response in the lungs during infection. However, adoptive transfer of 6.5^+ Treg cells into HACII hosts resulted in additional changes in the phenotype of the transferred cells that could potentially impair their suppressive capabilities. When $CD4^+Foxp3^+Ly5.1^+$ Treg cells from TS1xHA28.$Foxp3^{eGFP}$.Ly5.1 mice were transferred into BALB/c or HACII recipients, and host mice were infected with influenza virus the following day, we found that expression of the 6.5^+ TCR was significantly lower on $Ly5.1^+CD4^+$ cells isolated from the medLN, lungs, and spleens of HACII recipients than PR8 virus-infected BALB/c mice at day 8 p.i. (Fig. 4-8).

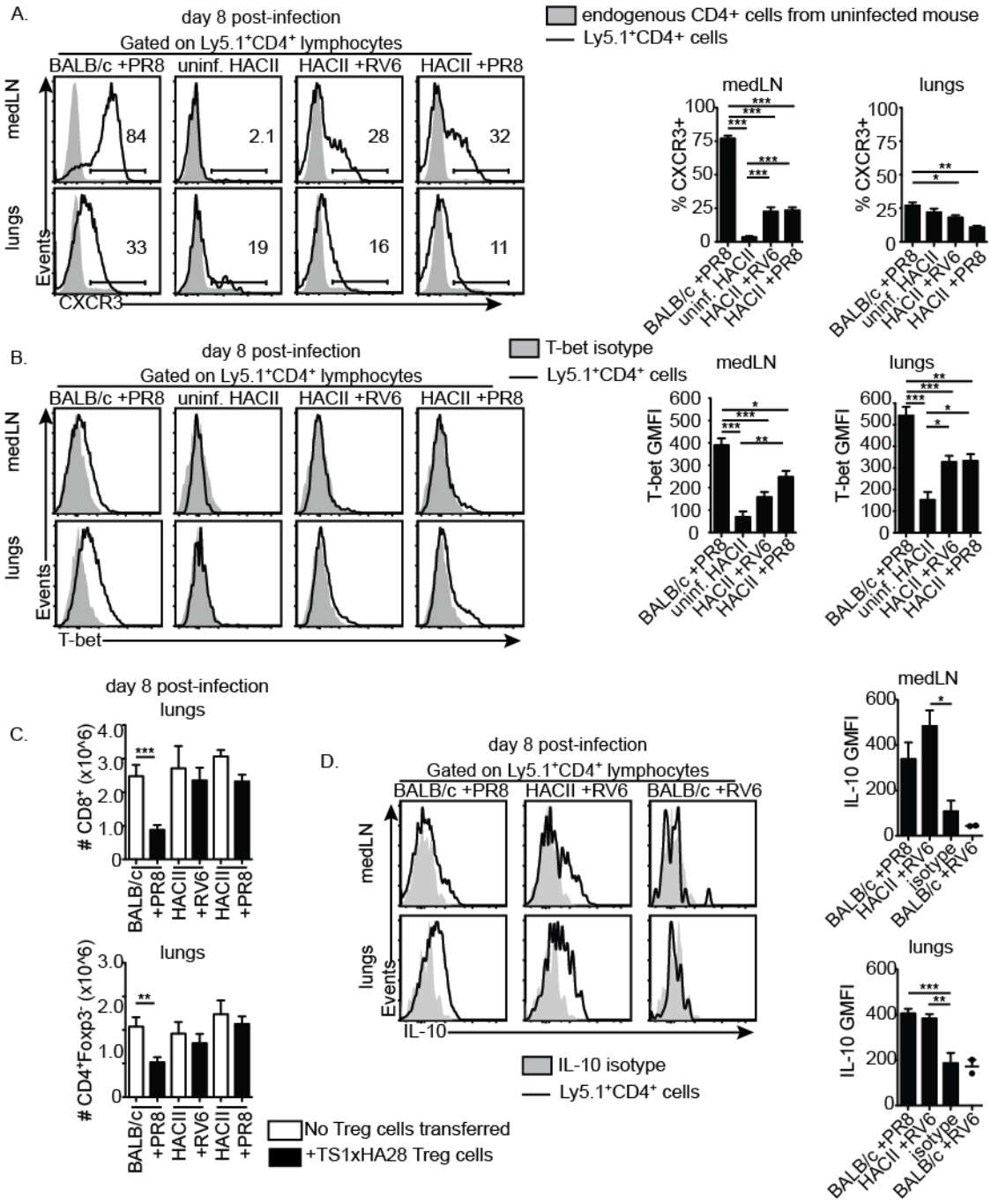

Figure 4-7 Increasing the immunostimulatory potency of self-HA inhibited 6.5+ Treg cell differentiation and activity during PR8 virus infection CD4+eGFP+ cells isolated from the spleens and LNs of TS1xHA28.Foxp3eGFP.Ly5.1 mice were transferred into BALB/c or HACII recipients, which were infected with PR8 or RV6 influenza virus on the following day. (**A**) CXCR3 and (**B**) Tbet expression on donor-derived Ly5.1+ cells was assessed at day 8 post-infection. (**C**) The number of endogenous CD4+Foxp3- and CD8+ T cells in the lungs of mice that had or had not received 6.5+ Treg cells was determined at day 8 post-infection. n≥5 for each group combined from at least 3 independent experiments. (**D**) Intracellular IL-10 was determined in indicated cell types isolated at day 8 p.i. from the medLN and lungs following PMA and ionomycin stimulation.

Furthermore, the percentage of Ly5.1^{+}CD4^{+} cells that had downregulated Foxp3 was significantly higher in the medLN and spleens of uninfected, RV6 virus-infected, or PR8 virus-infected HACII mice than in PR8 virus-infected BALB/c mice (Fig. 4-8). Interestingly, the presence of an RV6 or PR8 virus infection actually appeared to somewhat ameliorate the reduction in 6.5^{+} TCR and Foxp3 expression in the medLNs and lungs of HACII mice. PD-1 expression on Ly5.1^{+}CD4^{+} cells was higher in the spleens and medLNs of HACII mice, regardless of infection status, than of PR8 virus-infected BALB/c mice, while expression of PD-1 was elevated on Ly5.1^{+}CD4^{+} cells in the lungs of all mice (Fig. 4-8). PD-1 expression in this context likely reflects increased TCR stimulation in HACII mice and in the lungs of PR8 virus-infected BALB/c mice, but high levels of PD-1 expression have been shown to limit the accumulation of Foxp3^{+} follicular regulatory T cells and to reduce their suppressive ability, and PD-1 could play a similar role in this context (Sage et al. 2013).

4.2.8 The inflammatory environment at the time of initial TCR stimulation can determine the extent of Treg cell differentiation

We considered the possibility that initial recognition of self-HA in a non-inflammatory context could impede the ability of Treg cells to subsequently differentiate following TCR stimulation with viral Ag during infection. To test this theory, we infected HACII mice with RV6 virus, then waited 4 days until the immune response to the virus was already well underway prior to injecting CD4^{+}Foxp3^{+} T cells from TS1xHA28.Foxp3^{eGFP}.Ly5.1 mice (Fig. 4-9 A).

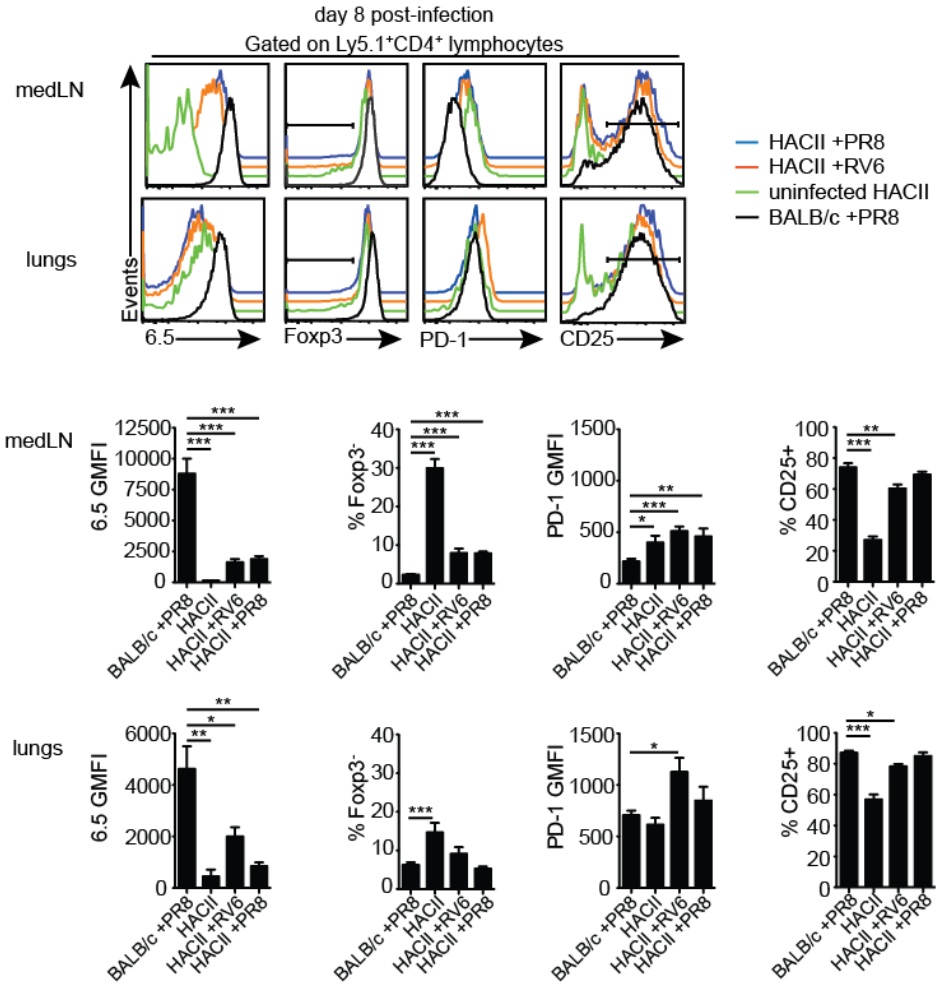

Figure 4-8 6.5^{+} Treg cells transferred into HACII hosts decreased expression of the TCR, CD25, and Foxp3, and upregulated PD-1 CD4^{+}eGFP^{+} cells isolated from the spleens and LNs of TS1xHA28.Foxp3^{eGFP}.Ly5.1 mice were transferred into BALB/c or HACII recipients, which were infected with PR8 or RV6 influenza virus on the following day. Expression of the 6.5 TCR, Foxp3, PD-1 and CD25 on donor-derived Ly5.1^{+} cells was assessed at day 8 post-infection. A one way ANOVA followed by a Dunnett multiple comparison test was performed to compare CD4^{+}Ly5.1^{+} cells isolated from a BALB/c mouse infected with PR8 virus with CD4^{+}Ly5.1^{+} cells isolated from various other mice.

We then compared CXCR3 expression on Ly5.1$^+$CD4$^+$ cells from these mice with CXCR3 expression on Ly5.1$^+$CD4$^+$ cells when they had been transferred into HACII hosts one day prior to infection. At day 8 p.i., a higher percentage of Ly5.1$^+$CD4$^+$ cells in the medLN of HACII mice expressed CXCR3 when they had been adoptively transferred at day 4 p.i. than at day 1 prior to infection, although the level of CXCR3 expression observed in PR8-infected BALB/c mice was not achieved (Fig. 4-9 B, C). Furthermore, the level of CXCR3 expression Ly5.1$^+$CD4$^+$ cells transferred at day 4 p.i. with RV6 virus was not significantly higher in an HACII mouse than in a BALB/c mouse. This observation suggests that activation on self-HA in the presence of an established inflammation-inducing viral infection results in more Treg cell differentiation than exposure to self-HA prior to viral infection, but still induces less Treg cell differentiation than does activation by viral-HA.

4.2.9 Recognition of self-HA can induce 6.5$^+$ Treg cells to upregulate CXCR3 and T-bet during J1 influenza virus infection

Since CXCR3 expression by 6.5$^+$ Treg cells during infection with the weakly cross-reactive RV6 virus was not increased in HACII hosts relative to BALB/c hosts, we wondered whether recognition of self-HA alone could ever induce Treg cell differentiation in the context of an infection. To this end, 6.5$^+$ Treg cells isolated from TS1xHA28.Foxp3^{eGFP} donors were transferred into HACII mice, which were then infected with J1 influenza virus. The J1 virus contains a serologically non-crossreactive H3 subtype HA that is not recognized by the 6.5$^+$ TCR (Palese 1977), and 6.5$^+$ Treg cells in J1 virus-infected BALB/c mice do not divide or upregulate Foxp3 (Fig. 4-10 A).

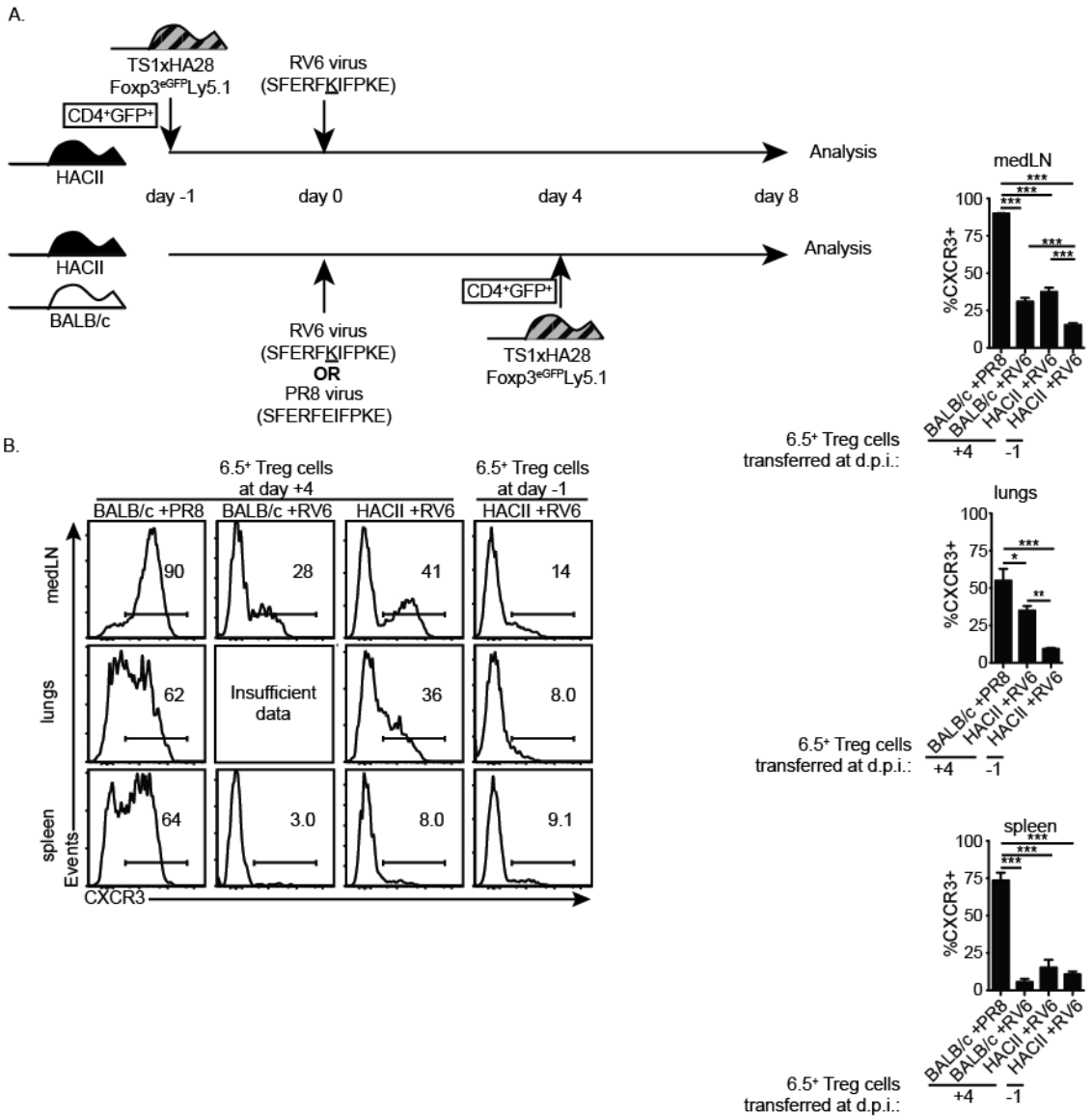

Figure 4-9 Transferring 6.5⁺ Treg cells into HACII mice with an ongoing immune response to RV6 virus increased CXCR3 expression on donor-derived cells relative to when adoptive transfer occurred prior to infection.
(**A**) CD4⁺eGFP⁺ cells isolated from TS1xHA28.Foxp3^{eGFP}.Ly5.1 mice were transferred into HACII or BALB/c recipients either one day prior to, or 4 days after, infection with influenza virus. Differentiation of Ly5.1⁺ cells was assessed at day 8 post-infection. (**B**) CXCR3 expression on Ly5.1⁺CD4⁺ cells isolated from the medLN, lungs, and spleen at day 8 post-infection

However, 6.5$^+$ Treg cells expressed higher levels of CXCR3 and T-bet in the medLNs and lungs of HACII mice infected with J1 virus than in uninfected HACII mice, and accumulated in the lungs to a greater extent (Fig. 4-10 B). These data provide evidence that recognition of self-HA during infection can result in limited differentiation of 6.5$^+$ Treg cells towards a T-bet$^+$ phenotype.

4.2.10 B cells and DCs are the predominant Ag presenting cells in the medLNs of influenza virus infected mice

The differing capacities of the HA to induce 6.5$^+$ Treg cell differentiation when expressed as a viral versus a self-Ag in infected mice might also have been a reflection of its presentation by different APC subsets. To examine this possibility, we first characterized the leukocyte subsets present in the medLNs and lungs of HACII mice infected with RV6 virus, and of BALB/c mice infected with PR8 virus, at day 6 post-infection. The numbers and distribution of a variety of APC subsets were quite similar in both sets of mice, suggesting that differences in APC populations present were not the cause of the observed differences in Treg cell activity Fig. (4-11 A). In each case, B cells were the most prevalent APC subset and substantially outnumbered dendritic cells (DCs), which were the second most numerous APC population in the medLNs and third most prevalent in the lungs behind inflammatory monocytes (iMOs). We next wanted to assess the ability of different APC subsets to present Ag and induce T cell proliferation during influenza virus infection.

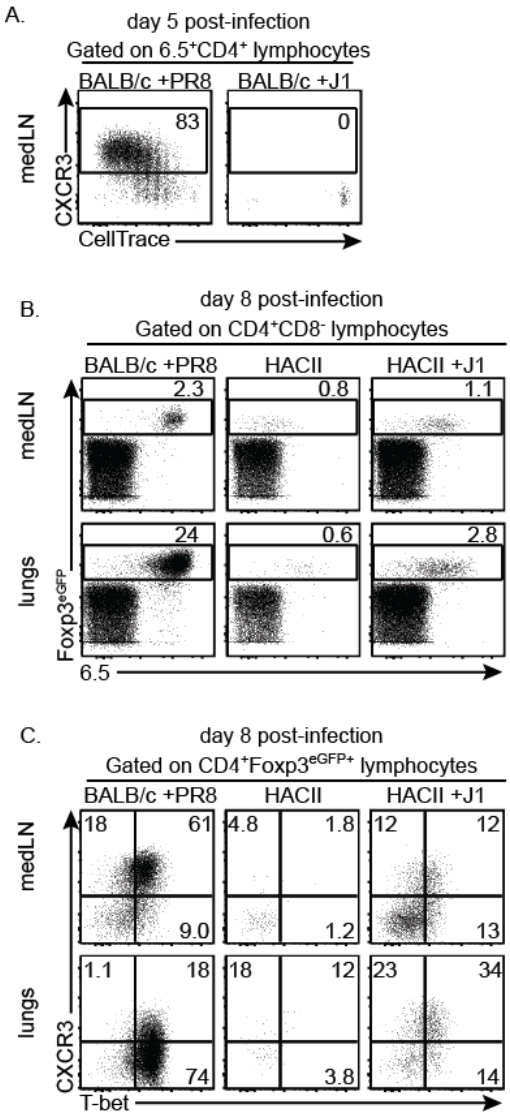

Figure 4-10 Recognition of self-HA can induce 6.5$^+$ Treg cells to upregulate CXCR3 and T-bet during J1 influenza virus infection (**A**) CD4$^+$eGFP$^+$ cells were isolated from the spleens and LNs of TS1xHA28.Foxp3^{eGFP} mice, labeled with CellTrace Violet, and transferred into BALB/c recipients. Recipient mice were infected with PR8 or J1 influenza virus the following day, and dilution of CellTrace Violet and CXCR3 expression were assessed at day 5 post-infection. (**B**) Experiment was set up as above, and expression of 6.5 and Foxp3^{eGFP} on total CD4$^+$CD8$^-$ cells, and (**C**) CXCR3 and T-bet expression on CD4$^+$eGFP$^+$ cells in the medLN and lungs at day 8 post-infection was assessed.

To this end, we isolated B cells, iMOs, neutrophils, and cDCs from the medLNs and lungs of PR8 virus-infected BALB/c mice and RV6 virus-infected HACII mice at day 5 post-infection, and cultured them *in vitro* for 5 days with CellTrace Violet-labeled unfractionated cells isolated from the LNs of TS1xHA28.Foxp3^{eGFP} mice (which contain 6.5^{+}CD4^{+}Foxp3^{+} cells and 6.5^{+}CD4^{+}Foxp3^{-} cells). Of the cells isolated from the medLNs of PR8 virus-infected HACII mice, DCs were the only subset to induce substantial division by the CD4^{+} T cells (both Foxp3^{eGFP+} and Foxp3^{eGFP-}) from TS1xHA28.Foxp3^{eGFP} mice (Fig. 4-11 B). In contrast, both B cells and DCs from the medLNs of RV6 virus-infected HACII mice drove CD4^{+} T cell proliferation (Fig. 4-11 B). When we assessed the ability of APCs isolated from the lungs of PR8 virus-infected BALB/c mice to induce CD4^{+} T cell proliferation, we found that DCs again drove the most division, but that B cells and, to a lesser extent, iMOs were also presenting PR8-derived HA Ag in a stimulatory manner (Fig. 4-11 C). Interestingly, neutrophils by themselves did not induce CD4^{+} T cell division, but dilution of neutrophils in CD4^{-}CD8^{-} BALB/c splenocytes did result in minimal proliferation of co-cultured CD4^{+} cells, suggesting that neutrophils act as viral reserves to be taken up and presented by other cells, but do not present Ag to CD4^{+} T cells themselves (Fig. 4-11 C). In the lungs of RV6 virus-infected HACII mice, B cells and DCs were also the primary Ag presenting cells. These data show that DCs are the predominant cell type presenting viral Ag, and thus driving CD4^{+} T cell proliferation, in the medLNs of PR8 virus-infected mice, while both B cells and DCs isolated from the medLNs of RV6 virus-infected HACII mice can present self-HA and drive CD4^{+} T cell division.

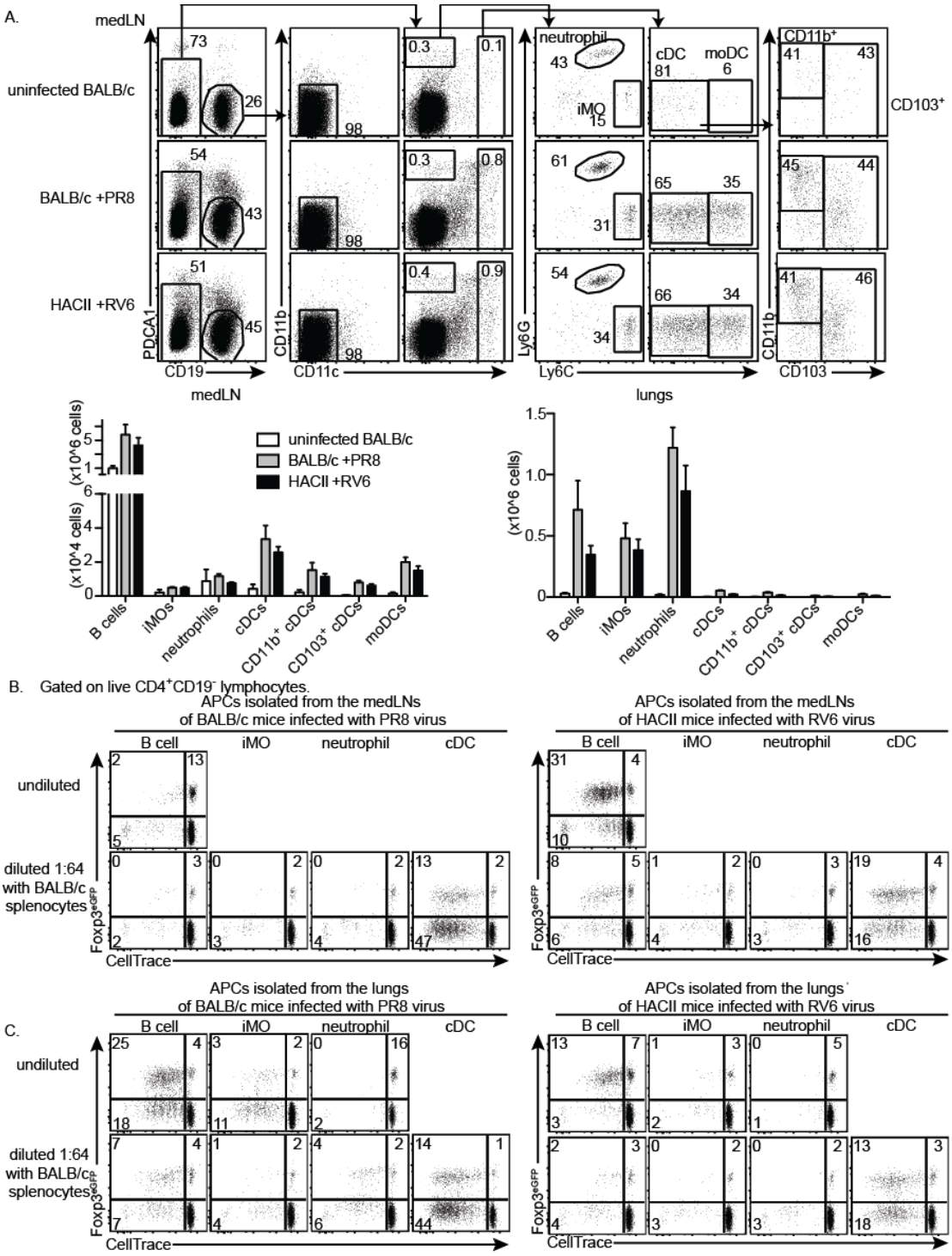
A.
medLN
uninfected BALB/c
BALB/c +PR8
HACII +RV6
PDCA1
CD19
CD11b
CD11c
Ly6G
Ly6C
CD11b+
CD103+
CD103
neutrophil
cDC
moDC
iMO
medLN
lungs
uninfected BALB/c
BALB/c +PR8
HACII +RV6
(x10^6 cells)
(x10^4 cells)
B cells
iMOs
neutrophils
cDCs
CD11b+ cDCs
CD103+ cDCs
moDCs
B. Gated on live CD4+CD19- lymphocytes.
APCs isolated from the medLNs of BALB/c mice infected with PR8 virus
APCs isolated from the medLNs of HACII mice infected with RV6 virus
B cell
iMO
neutrophil
cDC
undiluted
diluted 1:64 with BALB/c splenocytes
Foxp3eGFP
CellTrace
C.
APCs isolated from the lungs of BALB/c mice infected with PR8 virus
APCs isolated from the lungs of HACII mice infected with RV6 virus

Figure 4-11 DCs and B cells are the most prevalent APCs in the medLNs and the primary drivers of T cell proliferation in the medLNs and lungs of influenza virus infected mice (**A**) The number of B cells, inflammatory monocytes (iMOs), neutrophils, cDCs (conventional DCs; includes $CD11b^+$ and $CD103^+$), and monocyte-derived DCs (moDCs) was assessed in the medLNs of the indicated mice at day 6 post-infection (n=3 for each condition). CellTrace-labeled LN cells from TS1xHA28.$Foxp3^{eGFP}$ mice (containing both $6.5^+CD4^+Foxp3^-$ and $6.5^+CD4^+Foxp3^+$ cells) were cultured with indicated APC populations (either undiluted or diluted 1:64 with $CD4^-CD8^-$ feeder BALB/c splenocytes) isolated from the medLNs (**B**) or lungs (**C**) of PR8-infected BALB/c or RV6-infected HACII mice at day 5 post-infection. CellTrace Violet dilution and $Foxp3^{eGFP}$ expression by $CD4^+$ cells were determined by flow cytometry after 4 days of co-culture.

4.2.11 Recognition of cognate Ag presented by DCs induces Treg cells to upregulate CXCR3

Using the *in* vitro co-culture system described in the previous section, we assessed CXCR3 expression on $CD4^+Foxp3^+$ cells after they were cultured with cDCs or B cells from influenza virus-infected mice for 4 days (Fig. 4-12 A). For these experiments, the cDCs were diluted either 1:16 or 1:64 with $CD4^-CD8^-$ feeder cells isolated from splenocytes of uninfected BALB/c mice, while the B cells were used either undiluted or diluted 1:64 with feeder cells. The majority of the Treg cells cultured with cDCs from the medLNs of PR8 virus-infected BALB/c mice underwent division and many upregulated CXCR3, whereas B cells isolated from the medLNs or PR8-infected BALB/c mice induced very little division or CXCR3 upregulation (Fig. 4-12 A). These observations are consistent with previous studies that demonstrated that DCs collect viral Ag in the lungs during influenza virus infection, then migrate to the medLN where they constitute the predominant APC subset presenting viral Ag, while B cells in the medLN present very little viral Ag (Hamilton-Easton and Eichelberger 1995; Legge and Braciale 2003). By contrast, while both cDCs and B cells obtained from the medLNs of RV6-infected HACII mice (which present the HA self-Ag) could induce division of the Treg cells, only the cDCs induced CXCR3 upregulation. As noted above, B cells are the major population of APCs in the medLNs, and 6.5^+ Treg cells transferred into intact RV6-infected HACII mice showed a similar pattern of division with limited CXCR3 upregulation as was seen during co-culture of 6.5^+ Treg cells with B cells (Fig. 4-12 B). Furthermore, we observed that when TS1xHA28 LN cells were cultured with equal numbers of different APCs isolated from the spleen of an RV6-infected HACII mouse, a higher percentage of $CD4^+$ cells expressed Foxp3 after culture with B cells than with

other APC subsets, suggesting that B cells may be more capable of inducing expansion and/or survival of Treg cells than other APCs (Fig. 4-13). Thus, presentation of the self HA by B cells in HACII mice can induce selective expansion of Treg cells without prompting CXCR3 upregulation, thereby enlarging the $CXCR3^-$ Treg cell pool and potentially limiting the formation of Treg cells that could efficiently traffic to the lungs of infected mice.

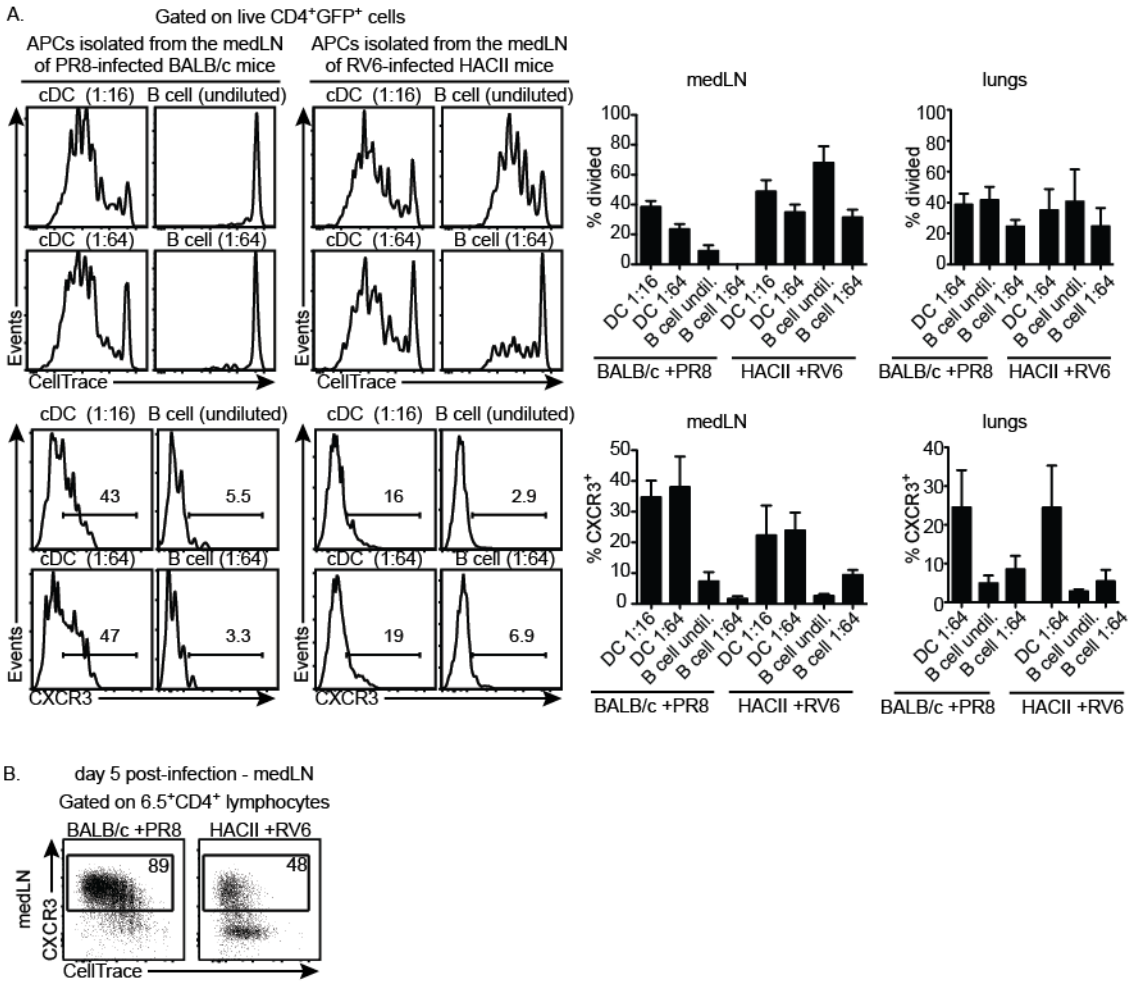

Figure 4-12 Ag presentation by both B cells and DCs can driveTreg cell division, but only DCs can induce upregulation of CXCR3 (**A**) CellTrace-labeled LN cells from TS1xHA28.Foxp3^{eGFP} mice (containing both 6.5^{+}CD4^{+}Foxp3^{-} and 6.5^{+}CD4^{+}Foxp3^{+} cells) were cultured with indicated APC populations (either undiluted or diluted with CD4^{-}CD8^{-} feeder BALB/c splenocytes) isolated from the medLNs or lungs of PR8-infected BALB/c or RV6-infected HACII mice at day 6 post-infection. CellTrace Violet dilution and CXCR3 expression by CD4^{+}Foxp3^{eGFP+} cells were determined by flow cytometry after 4 days of co-culture. Graphs show cumulative data from 3 independent experiments, in which LNs or lungs from 4 mice were pooled prior to isolation of DCs and B cells. (**B**) BALB/c mice were infected with PR8 virus and HACII mice were infected with RV6 virus, then CellTrace-labeled CD4^{+}eGFP^{+} cells isolated from TS1xHA28.Foxp3^{eGFP} mice were transferred into infected mice on the following day. CellTrace dilution and CXCR3 expression by 6.5^{+}CD4^{+} cells were assessed at day 5 post-infection.

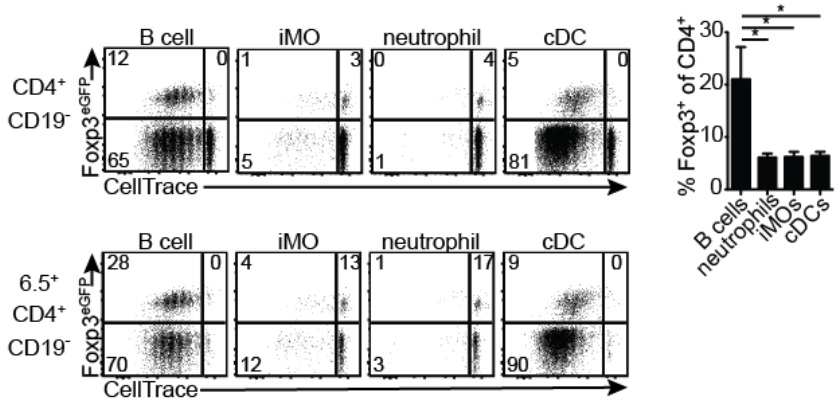

Figure 4-13 B cells support Treg cell expansion and/or survival CellTrace-labeled LN cells from TS1xHA28.Foxp3^{eGFP} mice (containing both 6.5^{+}CD4^{+}Foxp3^{-} and 6.5^{+}CD4^{+}Foxp3^{+} cells) were cultured with indicated APC populations isolated from the spleens of RV6-infected HACII mice at day 5 post-infection. CellTrace Violet dilution and Foxp3^{eGFP} expression by CD4^{+}CD19^{-} cells were asssessed by flow cytometry after 4 days of co-culture. Graph shows cumulative data from 3 independent experiments.

4.3 Summary of Results and Discussion

4.3.1 Treg cells present at infection sites expand from pre-existing Treg cells

One potential source of Treg cells that accumulate at infection sites is pTreg cells that differentiate from $CD4^+Foxp3^-$ cells upon recognition of pathogen-derived Ag. However, we found that $6.5^+CD4^+Foxp3^-$ cells divided extensively during infection with PR8 virus, but did not upregulate Foxp3 (Fig. 4-2. This result is reminiscent of a previous study in which $CD4^+Foxp3^-$ T cells expressing a transgenic TCR specific for *M. tuberculosis* also did not differentiate into $Foxp3^+$ Treg cells during *M. tuberculosis* infection (Shafiani et al. 2010). Moreover, Treg cells isolated from the sites of *Leishmania major* or *M. tuberculosis* infection were found to expand from a pre-existing pool of polyclonal Treg cells, while a congenically-marked population of polyclonal T cells that did not express either CD25 or Foxp3 did not upregulate these molecules during infection (Belkaid et al. 2002; Shafiani et al. 2010). Thus, these studies collectively provide evidence that Treg cells that accumulate during infection expand from pre-existing Treg cells, and do not convert from $CD4^+Foxp3^-$ cells.

4.3.2 Treg cells differentiated and suppressed the anti-viral immune response more efficiently when cognate Ag was derived from virus than from self

To assess whether recognition of self-HA could drive 6.5^+ Treg cell activity during influenza virus infection, 6.5^+ Treg cell accumulation and differentiation in RV6 virus-infected HA28 mice was compared with 6.5^+ Treg cell activity in BALB/c mice infected

with RV6 virus, where self-HA is not present. 6.5^+ Treg cells underwent very little division in the medLNs of RV6 virus-infected BALB/c mice and failed to accumulate within the lungs, while 6.5^+ Treg cells in PR8 virus-infected BALB/c mice proliferated extensively and accumulated in the lungs in large numbers; 6.5^+ Treg cells in HA28 mice underwent relatively low levels of division in response to the self-HA in these mice, and RV6 virus infection did not induce more extensive division or accumulation in the lungs in HA28 mice than in BALB/c mice (Fig. 4-3). Moreover, consistent with our previous report, activation of 6.5^+ Treg cells was associated with significant upregulation of both CXCR3 and T-bet in the medLNs, followed by an accumulation of $6.5^+CXCR3^+T\text{-}bet^+$ Treg cells in the lungs of PR8-infected BALB/c mice and suppression of the lung effector T cell response (Bedoya et al. 2013). By contrast, significantly lower levels of CXCR3 and T-bet were found in the $6.5^+CD4^+Foxp3^+$ Treg cells in the medLNs of RV6-infected HA28 or BALB/c mice, few of these cells accumulated in the lungs, and the effector T cell responses were not significantly affected (Fig. 4-4 and Fig. 4-5). Thus, even though interactions with the self-HA as it is presented in HA28 mice can promote the formation and persistence of $6.5^+CD4^+Foxp3^+$ Treg cell both intrathymically and in the periphery, reactivity with this self-peptide was unable to induce activation of the $Foxp3^+$ Treg cells sufficiently to allow them to modulate an acute influenza virus infection. In contrast, a similar number of Treg cells with identical specificity could modulate anti-viral immunity when cognate Ag was present as a viral Ag.

It was possible that the 6.5^+ Treg cells failed to differentiate efficiently and modulate anti-influenza virus immunity in response to recognition of the HA self-peptide in HA28 mice because the level of TCR stimulation they received was much lower than

when HA was present as a PR8 virus-derived Ag, as evidenced by the differences in 6.5^+ Treg cell proliferation in these two environments. To determine whether increasing the stimulatory potency of the self-Ag might induce 6.5^+ Treg cell activity during RV6 virus infection, we transferred $6.5^+Ly5.1^+$ Treg cells into HACII mice. Transferred 6.5^+ Treg cells proliferated more in an HACII host than in an HA28 host, and the extent of proliferation closely resembled that in a PR8-infected BALB/c mouse, but very few Treg cells accumulated in the medLNs of uninfected HACII mice, and no upregulation of CXCR3 or T-bet occurred (Fig. 4-6 and 4-7). When the HACII recipient mice were also infected with the weakly crossreactive RV6 virus, the $Ly5.1^+$ Treg cells accumulated in increased numbers in the medLNs, although the percentage of $CD4^+$ cells that were donor-derived did not increase, suggesting that $Ly5.1^+$ Treg cells were not being specifically recruited to the medLN as a result of infection. Additionally, a subset of $Ly5.1^+$ Treg cells upregulated CXCR3 and T-bet and trafficked to the lungs. Based on previous studies using IFN-$\gamma R^{-/-}$ Treg cells, it is likely that the high levels of IFN-γ induced by the RV6 virus infection promoted this CXCR3 upregulation, which coupled with TCR stimulation by the self-HA, was able to promote the formation of 6.5^+T-bet^+Foxp3^+ Treg cells in HACII mice (Koch et al. 2009). However, this process occurred with significantly lower efficiency in RV6 virus-infected HACII mice than in PR8-infected BALB/c mice, and 6.5^+ Treg cells did not significantly impair the anti-viral effector response in the lungs of RV6 virus-infected mice. Thus, in two different mouse lineages in which HA was expressed as a self-Ag, either in a weakly immunostimulatory manner capable of inducing 6.5^+ Treg cell differentiation or in a strongly immunostimulatory form in which expression was targeted to APCs, recognition of self-HA induced less efficient accumulation at infection sites, differentiation, and modulation of the effector immune response than did recognition of virus-derived HA.

4.3.3 Recognition of a strongly stimulatory self-Ag can impair the ability of Treg cells to respond upon subsequent recognition of a viral Ag

Unexpectedly, 6.5^+ Treg cell recognition of cognate self-HA expressed in a strongly immunostimulatory manner in HACII mice actually inhibited the ability of these Treg cells to respond to subsequent recognition of viral HA. A lower percentage of 6.5^+ Treg cells expressed CXCR3 and T-bet in the medLN of PR8-infected HACII mice than PR8-infected BALB/c mice, and many fewer of these cells accumulated in the medLNs and lungs of infected HACII mice (Fig. 4-6 and Fig. 4-7). Furthermore, the limited number of 6.5^+ Treg cells that did enter the lungs of PR8-infected HACII mice did not significantly affect effector T cell accumulation in these mice, despite their ability to produce IL-10.

Several phenotypic changes occurred in 6.5^+ Treg cells that were adoptively transferred into HACII hosts. Most notably, these cells exhibited decreased expression of Foxp3, CD25, and the 6.5 TCR and increased expression of PD-1 relative to 6.5^+ Treg cells isolated from a PR8 virus-infected BALB/c mouse (Fig. 4-8). Treg cells that lose Foxp3 expression also lose their ability to suppress, and Treg cells from $CD25^{-/-}$ mice fail to expand or to prevent autoimmune lymphoproliferative disease (Furtado et al. 2002; Williams and Rudensky 2007). Thus, it is possible that downmodulation of CD25 and/or Foxp3 renders 6.5^+ Treg cells adoptively transferred into HACII mice less suppressive. Downregulation of CD25 and Foxp3 by 6.5^+ Treg cells in HACII hosts occurred to a lesser extent during influenza virus infection than in an uninfected host, which is

somewhat surprising given reports that ongoing inflammation can cause loss of Foxp3 in Treg cells (Laurence et al. 2012; Zhou et al. 2009). However, we have previously shown that IL-2 is increased in the serum of PR8 virus-infected BALB/c mice (Bedoya et al. 2013), and IL-2 signaling can increase levels of CD25 and Foxp3 expression on Treg cells, providing a potential explanation for their increased stability in the presence of infection (Fontenot et al. 2005). Downmodulation of the TCR could render 6.5^+ Treg cells less responsive to antigenic stimulation, making them less able to mediate suppression or to continue proliferating once they have been activated and trafficked to the lungs (Schonrich et al. 1991). Finally, high levels of PD-1 expression could potentially reduce Treg cell the suppressive capabilities of Treg cells and/or limit their accumulation, as has been shown for $Foxp3^+$ follicular regulatory T cells (Sage et al. 2013).

The failure of 6.5^+ Treg cells to efficiently upregulate expression of T-bet and CXCR3 during influenza virus infection in HACII mice could also affect their ability to modulate the immune response, as CXCR3 has been shown to direct $CD4^+$ T cell trafficking into infected lungs (Kohlmeier et al. 2009). We found that 6.5^+ Treg cells that were transferred into HACII mice at day 4 post-infection upregulated CXCR3 more efficiently than did 6.5^+ Treg cells adoptively transferred into HACII mice one day prior to infection (Fig. 4-9). Furthermore, a higher percentage of 6.5^+ Treg cells in the medLNs than in the spleens of RV6 virus-infected HACII mice had upregulated CXCR3. These data suggest that the surrounding inflammatory milieu at the time of initial encounter with cognate self-Ag may play a crucial role in determining the differentiative fate of Treg cells.

4.3.4 6.5^+ Treg cells respond differently depending on which APC subset presents cognate Ag

Different APC subsets were isolated from the medLNs and lungs of PR8 virus-infected BALB/c mice and RV6 virus-infected HACII mice, and cultured with $6.5^+CD4^+Foxp3^-$ cells and $6.5^+CD4^+Foxp3^+$ cells isolated from the pLNs of TS1xHA28 mice to assess their ability to drive Treg cell division and differentiation. B cells isolated from the medLNs of RV6 virus-infected HACII mice were able to present self-HA to Treg cells in a manner that induced division but not CXCR3 upregulation (Fig. 4-12). In contrast, B cells isolated from the medLNs of PR8 virus-infected BALB/c mice failed to induce Treg cell proliferation, which is consistent with a previous study showing that B cells present little or no viral Ag in the medLN during influenza virus infection (Hamilton-Easton and Eichelberger 1995). Interestingly, DCs from both RV6 virus-infected HACII mice and from PR8 virus-infected BALB/c mice, presenting self-HA and virus-derived HA, respectively, induced both division and CXCR3 upregulation by Treg cells. Furthermore, B cells were the most prevalent APC subset within the medLNs, and could support Treg cell expansion/survival more efficiently than the other APCs that we examined (Fig. 4-11 and Fig. 4-13). Together, these data support a model in which 6.5^+ Treg cells in the medLN of influenza virus-infected HACII mice encounter B cells presenting cognate self-Ag with greater regularity than DCs, and interactions with B cells induce the expansion of 6.5^+ Treg cells that do not express CXCR3. As a result of this failure to differentiate, 6.5^+ Treg cells cannot efficiently traffic to the lungs, and therefore do not suppress effector cell accumulation in the lungs.

CHAPTER 5: Discussion and Speculation

5.1 Introduction

Treg cells are required to prevent widespread immune activation and autoimmune lymphoproliferative disease, and these cells can also participate in and modulate anti-pathogen immune responses (Fontenot, Gavin, Rudensky 2003; Kim, Rasmussen, Rudensky 2007; Maizels and Smith 2011; Sakaguchi et al. 1995). However, how variations in expression and presentation of cognate Ag can affect the ability of Treg cells to undergo activation and differentiation has not been well characterized. We have shown that cognate self-Ag presented in a relatively less immunostimulatory manner can induce both reactive thymocytes and $CD4^+$ T cells in the periphery to upregulate Foxp3 and differentiate into functional Treg cells, while the same self-Ag expressed at higher levels and in different cell types did not drive Treg cell formation. Recognition of the formation-inducing Ag when it was present as a self-Ag did not induce Treg cells to enter the lungs and modulate the anti-pathogen immune response during influenza virus infection. Contrarily, recognition of the same Ag when it derived from viral protein resulted in Treg cell differentiation and suppression of the anti-viral immune response. Moreover, initial recognition of a highly stimulatory form of self-Ag in the absence of inflammatory cytokines inhibited the ability of Treg cells to respond to subsequent recognition of viral Ag. In this chapter, we discuss how these results relate to previous studies describing the signals required for Treg cell formation and activity.

5.2 Treg cell formation *in vivo*

TCR-mediated recognition of self-peptide:MHC complexes can induce thymocytes to undergo deletion or differentiate into $Foxp3^+$ Treg cells, as has been shown using TCR-transgenic mice crossed with mice that express the cognate Ag as self (Apostolou et al. 2002; Jordan et al. 2001; Walker et al. 2003). Evidence suggests that the amount of cognate peptide that is present at this stage can be a crucial factor in determining the fate of autoreactive thymocytes: relatively higher doses of cognate peptide lead to more extensive deletion, whereas lower doses induce less deletion and greater accumulation of $CD4^+Foxp3^+$ Treg cells (Atibalentja, Byersdorfer, Unanue 2009; Picca et al. 2009). Our results are in agreement with these published observations, as we found that recognition of self-HA drove extensive deletion of 6.5^+CD4^{SP} thymocytes in TS1xHACII mice, and very little Foxp3 expression in 6.5^+CD4^{SP} thymocytes and 6.5^+CD4^+ T cells was observed (Fig. 3-2). In contrast, in TS1xHA28 mice, in which the self-HA was expressed at much lower levels in both the thymus and peripheral tissues, deletion of 6.5^+CD4^{SP} thymocytes occurred, but at much lower levels than in the TS1xHACII mouse. Moreover, more $6.5^+CD4^+Foxp3^+$ cells accumulated in the thymus and pLNs of TS1xHA28 mice than TS1 or TS1xHACII mice. Previous studies have shown that activation of the PI3K/Akt/mTOR pathway downstream of the TCR limits Foxp3 upregulation both *in vitro* and in the thymus, while blockade of this pathway increased Treg cell induction (Haxhinasto, Mathis, Benoist 2008; Sauer et al. 2008). Thus, increased activation of this pathway in 6.5^+ thymocytes in TS1xHACII mice where cognate HA is expressed at higher levels likely contributes to the paucity of $6.5^+CD4^{SP}Foxp3^+$ thymocytes in these mice.

The current 2-step model of Treg cell formation posits that $CD4^{SP}$ thymocytes first upregulate CD25 upon TCR stimulation, then upregulate Foxp3 after exposure to IL-2 (Lio and Hsieh 2008). However, we observed a substantial population of $6.5^{+}CD4^{SP}$ thymocytes in TS1xHA28 mice that had upregulated Foxp3, but not CD25 (Fig. 3-3 A). Recognition of self-HA drove Foxp3 upregulation in these cells, as no Foxp3 expression on $6.5^{+}CD4^{SP}$ thymocytes was observed in TS1 mice. Moreover, the percentage of $6.5^{+}CD4^{+}Foxp3^{+}$ cells expressing CD25 was higher in the pLNs than in the thymus, and a similar pattern was observed between $CD4^{SP}Foxp3^{+}$ thymocytes and $CD4^{+}Foxp3^{+}$ T cells in BALB/c mice with unmanipulated TCR repertoires. These observations raise questions regarding the fate of the $CD4^{SP}CD25^{-}Foxp3^{+}$ thymocytes because they must either upregulate CD25, downregulate Foxp3, or undergo apoptosis, since few $CD4^{+}CD25^{-}Foxp3^{+}$ T cells exist in the periphery. If they subsequently upregulate CD25, this would be in contrast to the established model of Treg cell development. Alternatively, Foxp3 expression may be transient and unstable in these $CD4^{SP}CD25^{-}Foxp3^{+}$ thymocytes. IL-2 signaling enhances expression of both CD25 and Foxp3 (Fontenot et al. 2005), and $CD4^{+}Foxp3^{+}$ cells with low expression of CD25 have been found to be less capable of maintaining Foxp3 expression than $CD25^{hi}CD4^{+}Foxp3^{+}$ cells; thus it is possible that in the absence of CD25 expression, $CD4^{SP}Foxp3^{+}$ thymocytes cannot compete for IL-2 and subsequently fail to maintain Foxp3 (Komatsu et al. 2009; Komatsu et al. 2014). We additionally found that the transcription factor Helios, which has recently been identified as a marker of strongly autoreactive thymocytes (Daley, Hu, Goodnow 2013), was upregulated in the majority of $6.5^{+}CD4^{SP}Foxp3^{-}$ thymocytes in TS1xHA28 mice, indicating that they had been exposed to self-HA (Fig. 3-3 A). Previous studies have shown that while some

$CD4^{SP}Helios^{+}Foxp3^{-}$ thymocytes undergo Bim-mediated deletion, a subset can survive through a Card11-dependent mechanism and remain $Foxp3^{-}$ (Daley, Hu, Goodnow 2013). Thus, it is likely that some of the $6.5^{+}CD4^{+}Foxp3^{-}$ cells in the periphery of TS1xHA28 mice encountered cognate Ag during development in the thymus and still failed to upregulate Foxp3. Furthermore, autoreactive $Helios^{+}CD4^{SP}Foxp3^{-}$ thymocytes were identified in BALB/c mice (Fig. 3-3 B), confirming that this population of autoreactive $CD4^{SP}$ thymocytes does not form solely as a consequence of the TCR transgene, and raising the question as to whether these cells are able to enter the periphery as Ag-experienced autoreactive $CD4^{+}Foxp3^{-}$ T cells.

Thymically-derived Treg (tTreg) cells appear to constitute the majority of the Treg cell population (Hsieh et al. 2006; Josefowicz et al. 2012), but in certain circumstances $CD4^{+}Foxp3^{-}$ cells in the periphery can differentiate into $Foxp3^{+}$ pTreg upon recognition of cognate Ag (Yadav, Stephan, Bluestone 2013). Most of the work establishing that Treg cells can differentiate in the periphery from $CD4^{+}Foxp3^{-}$ cells following recognition of Ag has been done using either exogenous administration of a cognate Ag (through injection or feeding) or in a lymphopenic environment (Coombes et al. 2007; Gottschalk, Corse, Allison 2010; Knoechel et al. 2005; Kretschmer et al. 2005; Lathrop et al. 2008; Sun et al. 2007). However, exogenously administered antigens are subject to clearance and turnover, and the extent to which specificity for a naturally processed, continuously present self-Ag under steady-state conditions might also drive pTreg cell formation, and the conditions under which this may occur, are still being determined. Given that self-Ags exist in abundance and can be expressed at varying levels and by many different cell types, we were interested in how varying the presentation of self-HA might

affect the fate of autoreactive 6.5$^+$CD4$^+$Foxp3$^-$ T cells. In HA28 mice, a subset of 6.5$^+$CD4$^+$CD25$^-$Foxp3$^-$ T cells had upregulated Foxp3 expression at day 3 post-transfer, but this group was limited to T cells that had undergone relatively few rounds of division (Fig. 3-4). In contrast, 6.5$^+$CD4$^+$CD25$^-$Foxp3$^-$ T cells transferred into HACII hosts had all divided extensively, and no expression of Foxp3 was observed. These data are consistent with a previous study in which pTreg cells were formed by injection of cognate peptide that showed that a low dose of peptide induced more pTreg cell formation than a high dose, and that those cells that upregulated Foxp3 had undergone fewer rounds of division than those that did not (Gottschalk, Corse, Allison 2010). One possible explanation for these observations is that relatively weaker activation of TCR signaling (such as occurs in HA28 mice) is more conducive to Treg cell formation than relatively strong TCR signaling. In support of this theory, there is evidence to suggest that a strong TCR signal is detrimental to Treg cell formation, as activation of the PI3K/Akt/mTOR pathway downstream of the TCR has been shown to limit Foxp3 upregulation both *in vitro* and in the thymus, while blockade of this pathway increased Treg cell induction (Haxhinasto, Mathis, Benoist 2008; Sauer et al. 2008). Additionally, a strong TCR signal abrogated Treg cell induction *in vitro* in response to TGF-β through NF-κB-dependent production of inflammatory cytokines (Molinero et al. 2011). However, if TCR signal strength is the sole determinant of pTreg cell formation, this would necessitate that some 6.5$^+$ T cells in HA28 mice receive a relatively weak signal from the HA (becoming Foxp3$^+$ and undergoing limited division), while other cells receive a stronger signal from the HA self-peptide. Competition for Ag could limit exposure to cognate peptide:MHC complexes such that some cells receive a stronger TCR signal than others, and indeed intraclonal competition for Ag has been shown to occur within the thymus, although in this case, competition for Ag limited the niche size for Treg cells

and reduced the percentage of clonotypic cells that upregulated Foxp3 (Bautista et al. 2009). Alternatively, intrinsic factors, such as epigenetic differences, may dictate which $CD4^+Foxp3^-$ cells upregulate Foxp3 in an HA28 mouse, and the fact that the $Foxp3^+$ cells have undergone relatively fewer rounds of division could be a consequence of the ability of Foxp3 to interact with downstream targets and induce a relatively non-proliferative (or "anergic") state in those cells. Evidence in support of this possibility comes from studies showing that retroviral transduction of Foxp3 into naïve $CD4^+$ T cells resulted in less robust proliferation upon TCR stimulation with anti-CD3 mAb compared with cells transduced with a control retrovirus (Hori, Nomura, Sakaguchi 2003), and from our own observations that $6.5^+CD4^+Foxp3^-$ cells underwent more rounds of division than naturally-occurring $6.5^+CD4^+Foxp3^+$ cells after transfer into HA28 recipients.

Ultimately, nearly half of the donor-derived cells in HA28 recipients expressed Foxp3 at days 14 and 21 post-transfer of $6.5^+CD4^+CD25^-Foxp3^-Ly5.1^+$ cells isolated from TS1.$Foxp3^{eGFP}$.Ly5.1 mice (Fig. 3-6). The numbers of $Ly5.1^+$ cells recovered were declining at these time points, suggesting that acquired Foxp3 expression was relatively stable in these pTreg cells and that 6.5^+CD4^+ T cells that had undergone division without upregulating Foxp3 were gradually deleted, as has been observed in some other adoptive transfer settings (Walker et al. 2003). This stable expression of Foxp3 contrasts some findings in human and mouse systems in which Foxp3 was found to be transiently expressed by $CD4^+$ T cells at early stages following activation, but was not indicative of Treg cell development (Miyao et al. 2012; Wang et al. 2007). 6.5^+ pTreg cells that formed in HA28 mice upregulated expression of CD25 and Helios, and lost the ability to produce IFN-γ and IL-2. When we re-isolated Treg cells generated in the

periphery of an HA28 mouse at day 7 post-transfer, we found that they were able to suppress $6.5^{+}Foxp3^{-}$ T cell division *in vitro* to a similar extent as thymically-generated Treg cells of the same specificity. However, while nearly all of the tTreg cells maintained Foxp3 expression after 4 days of *in vitro* culture, some of the pTreg cells began to lose Foxp3 expression. Foxp3 expression is stabilized by demethylation of the conserved non-coding DNA sequence 2 (CNS2) of the Foxp3 locus, a Treg cell-specific demethylated region (TSDR) that is normally methylated in $CD4^{+}Foxp3^{-}$ Treg cells (Ohkura, Kitagawa, Sakaguchi 2013), and CNS2 deficiency results in loss of heritable Foxp3 expression (Feng et al. 2014; Zheng et al. 2010). While *in vitro* generated Treg cells failed to completely demethylate CNS2, which is consistent with their unstable Foxp3 expression (Floess et al. 2007; Miyao et al. 2012), pTreg cells formed *in vivo* can achieve demethylation of the *Foxp3* locus under some conditions (Ohkura et al. 2012; Polansky et al. 2008). It is possible that complete CNS2 demethylation had not occurred in $6.5^{+}CD4^{+}Foxp3^{+}$ pTreg cells at day 7 post-transfer into HA28 hosts, potentially explaining their lack of stability of this time point. Further experiments would be needed to determine whether hypomethylation of TSDRs, including CNS2, occurred in the 6.5^{+} pTreg cells generated in HA28 mice, and the time period over which this demethylation might take place. In contrast to the relatively stable Foxp3 expression in a subset of $6.5^{+}CD4^{+}$ pTreg cells formed in HA28 mice, we suspect that transient upregulation of Foxp3 may occur in $6.5^{+}CD25^{-}Foxp3^{-}$ cells after transfer into HACII hosts. Although we were never able to observe Foxp3 expression in donor-derived cells this setting, we failed to obtain consistent engraftment unless the recipient HACII mice also expressed a $Foxp3^{eGFP}$ allele (Fig. 3-5). Additional studies would be required to confirm this conclusion, but this observation provides indirect evidence that these cells might

transiently express Foxp3, and concurrent expression of the GFP reporter transgene may mediate rejection in recipient mice that do not induce tolerance to GFP.

We looked for evidence of 6.5^+CD4^+ T cell deletion in HACII recipients, as previous studies have shown that exogenous administration of minor lymphocyte stimulating (*Mls*) superantigen or staphylococcal enterotoxin A superantigen resulted in deletion of superantigen-reactive T cells in the periphery, but acute deletion of 6.5^+CD4^+ T cells did not occur (McCormack et al. 1993; Webb, Morris, Sprent 1990). However, we did find that $6.5^+CD4^+Foxp3^-$ cells were less capable of producing IL-2 and IFN-γ at day 14 post-transfer in HACII hosts than HA28 hosts, despite undergoing more extensive proliferation in the HACII hosts (Fig. 3-7). Thus, recognition of a relatively weakly immunostimulatory self-Ag can induce pTreg cell differentiation, but reactive $CD4^+$ T cells that remained $Foxp3^-$ retained the ability to produce IL-2 and IFN-γ. In contrast, the same self-Ag presented in a more immunostimulatory manner failed to drive pTreg formation, but significantly fewer of the transferred $CD4^+Foxp3^-$ cells were capable of producing IL-2 and IFN-γ after two weeks *in vivo*. It is possible that $6.5^+CD4^+Foxp3^-$ T cells transferred into HACII mice differentiate towards a different $CD4^+$ helper T cell phenotype, but we did not see evidence of IL-17 production, such that it is unlikely that they are becoming Th17 cells. Alternatively, there have been descriptions of both $CD4^+$ T cell anergy and exhaustion following an initial TCR stimulation and strong proliferative response, both of which are characterized by a loss of ability to divide and produce cytokine upon subsequent TCR engagement (Crawford et al. 2014; Lanoue et al. 1997; Tanchot et al. 2001). Further studies would be necessary to determine whether $6.5^+CD4^+Foxp3^-$ cells transferred into HACII mice may be adopting one of these fates,

and to assess whether these cells develop an impaired proliferative response to subsequent TCR stimulation. The fact that the number of donor-derived T cells begins to decline after day 14 in HACII hosts suggests that they may no longer be dividing at the same high rate as when they were first transferred. It is perhaps somewhat surprising that $6.5^{+}CD4^{+}Foxp3^{+}$ T cells transferred into HACII were not rapidly deleted and did not completely lose the ability to produce IL-2; a previous report determined that the majority of $6.5^{+}CD4^{+}$ T cells transferred into a different HA-transgenic mouse lineage, in which the HA transgene is under control of the Igκ promoter and enhancer elements, were deleted within 14 days of transfer after an initial expansion period, and those that did not undergo deletion became anergic (Lanoue et al. 1997). However, in a separate study, pigeon cytochrome c (PCC)-reactive $CD4^{+}$ T cells transferred into T cell-deficient mice that expressed PCC as a self-Ag under control of an MHCI promoter expanded and adopted an anergic phenotype, but no deletion was observed (Tanchot et al. 2001). In this instance, anergy was characterized by reduced division and decreased production of IL-2 and IFN-γ upon subsequent stimulation with PCC peptide *in vitro* relative to PCC-reactive $CD4^{+}$ T cells transferred into mice that lacked the PCC transgene (Tanchot et al. 2001). However, PCC-reactive $CD4^{+}$ T cells re-isolated from PCC transgenic mice could produce some IL-2 and IFN-γ following stimulation with a high concentration of PCC peptide (Tanchot et al. 2001). It is possible that differences in the relative amount of cognate self-Ag or in the cell type on which it is expressed may determine the extent to which deletion occurs. For instance, in HACII mice, HA is expressed and presented by DCs, which may support $6.5^{+}CD4^{+}Foxp3^{-}$ T cell persistence, and the high level of expression may continue to induce some cytokine production.

It was noteworthy that the 6.5$^+$CD4$^+$Foxp3$^+$ pTreg cells that were isolated from the pLNs of HA28 mice also acquired expression of Helios, the levels of which increased progressively following transfer, either through selection of Helios$^+$ cells or gradual upregulation of the molecule itself, such that by days 14 and 21 post-transfer the large majority of these cells were Helios$^+$ (Fig. 3-8). This pattern of upregulation was different than what was seen using injected Ag, as in that case Helios was upregulated prior to Foxp3, and thus all Foxp3$^+$ pTreg cells initially expressed Helios (Gottschalk, Corse, Allison 2012). In both studies, Helios was upregulated in a subset of Foxp3$^-$ cells, but by day 21 post-initial antigenic exposure, only Foxp3$^+$ cells expressed Helios. Additionally, Gottschalk, *et al*. observed a subsequent decline in Helios levels among the Foxp3$^+$ pTreg cells that could be reversed by administering a second dose of peptide. We did not detect any similar decrease in Helios levels, likely because pTreg cells continue to receive continuous low-level TCR signal from recognition of a self-Ag.

Since Helios was not upregulated in Ag-specific Treg cells formed *in vitro* or *in vivo* by Ag feeding, it was initially suggested that Helios was a marker for Treg cells that had formed within the thymus (Thornton et al. 2010). This conclusion was challenged by subsequent studies showing that pTreg cells that developed following i.v. injection of cognate Ag did express Helios, and it was unclear whether differences in the route of Ag administration might be causing these differences in Helios expression (Gottschalk, Corse, Allison 2012). Consistent with this possibility, we found that a significantly higher percentage of the 6.5$^+$CD4$^+$Foxp3$^+$ pTreg cells isolated from the medLNs of HA28 mice were Helios$^-$ than was the case for those isolated from non-gut-draining pLNs (Fig. 3-9). It is tempting to speculate that the presence of distinct APC populations and/or

differences in the cytokine environment in non-gut draining versus gut-draining LNs may promote these differences in Helios expression. For example, specialized $CD103^+$ DCs that reside in the gut and mesenteric LN and that can induce Treg cell differentiation via a TGF-β- and retinoic acid-dependent mechanism may be responsible for the development of $Helios^-$ pTreg cells (Coombes et al. 2007; Sun et al. 2007), while a separate APC population in non-gut draining LNs may drive both Foxp3 and Helios upregulation. Alternatively, elevated levels of TGF-β in the gut-draining mesLNs may promote their formation, since "induced" $Foxp3^+$ Treg cells that are generated by activation of conventional $CD4^+$ T cells in the presence of TGF-β also lack Helios expression (Thornton et al. 2010). Whatever the mechanism, these findings demonstrate that pTreg cells with identical specificity for a self-Ag and formed in the same mouse can express different amounts of Helios according to the anatomical location from which they are isolated.

If recognition of self-Ag can drive Treg cell formation in both the thymus and the periphery, then one may wonder why all T cells with a TCR specificity that allows recognition of self-Ag, and that escape deletion, do not ultimately develop into $Foxp3^+$ Treg cells. The fact that this does not occur is evidenced by the coexistence of $Foxp3^+$ and $Foxp3^-$ 6.5^+ T cells in the periphery of TS1xHA28 mice (Picca et al. 2009) and of $CD25^-$ and $CD25^+$ T cells expressing a TCR specific for OVA in mice co-expressing OVA as a neo-autoantigen (Walker et al. 2003). Furthermore, some overlap has been found to occur in the TCR repertoire of naturally-occurring autoreactive $Foxp3^+$ and $Foxp3^-$ T cells (Hsieh et al. 2006), suggesting that $CD4^+Foxp3^-$ cells can recognize the same antigens as autoreactive $CD4^+Foxp3^+$ cells. Finally, depletion of Treg cells with

diphtheria toxin (DT) in Foxp3-DT receptor mice results in fatal autoaggressive lymphproliferatvie disease (Kim, Rasmussen, Rudensky 2007), which implies that autoreactive cells exist in steady-state, but are normally restrained by the presence of Treg cells. One possible explanation for why not all autoreactive T cells that escape thymic deletion ultimately develop into Treg cells is that there is an intrinsic difference in the cells themselves. However, $6.5^{+}CD25^{-}Foxp3^{-}$ T cells that developed in TS1xHA28 mice in the presence of cognate-autoantigen were no less capable of upregulating Foxp3 following transfer into HA28 hosts than were naïve $6.5^{+}CD25^{-}Foxp3^{-}$ T cells isolated from TS1 mice (Fig. 3-11). In fact, the Ag-experienced cells may have been primed to upregulate Foxp3, as the percentage expressing Foxp3 was slightly higher at day 3 post-transfer than among previously naïve T cells. Demethylation of TSDRs, including at the Foxp3 locus, can occur in a TCR stimulation-dependent manner in the absence of Foxp3 expression (Ohkura et al. 2012), and it would be interesting to know whether $6.5^{+}CD4^{+}Foxp3^{-}$ cells in TS1xHA28 mice exhibit a Treg cell-specific demethylation pattern. A second possibility is that a Treg cell niche exists that limits the accumulation of Treg cells of a defined TCR specificity. Within the thymus, it was found that the number of thymocytes expressing a transgenic TCR that could develop into $Foxp3^{+}$ Treg cells was limited, and that decreasing the clonal frequency of TCR-transgenic thymocytes resulted in an increased percentage of these cells expressing Foxp3 (Bautista et al. 2009; Leung, Shen, Lafaille 2009). The authors of these studies posit that competition for Ag may define the size of the Treg cell niche. Interestingly, in our studies, the percentage of 6.5^{+} T cells expressing Foxp3 in a TS1xHA28 mouse was similar to that in an HA28 mouse at days 14 and 21 post-transfer of $6.5^{+}CD4^{+}CD25^{-}Foxp3^{-}$ cells, despite the fact that the frequency of clonotypic cells was much higher in a TS1xHA28 mouse than in the adoptive transfer experiments. Although we do not know

for certain whether this percentage remains consistent beyond day 21 post-transfer, it is interesting to speculate that the Treg niche in this case may be defined as a percentage of T cells of a given specificity rather than as an absolute number. Furthermore, $6.5^+CD4^+CD25^-Foxp3^-$ T cells transferred into a TS1xHA28 mouse failed to upregulate Foxp3, perhaps indicating that the 6.5^+ Treg cell niche is already full in these mice (Fig. 3-12 C). Thus, Ag may not be limiting in a setting where it is expressed in a systemic and persistent manner, but another limiting factor, such as IL-2 may restrict the formation of Treg cells. IL-2 is required for Treg cell formation, expansion and homeostasis (Nelson 2004), and one group found that the accumulation of $CD4^+CD25^+$ Treg cells in a mouse correlated with the number of $CD4^+$ T cells capable of producing IL-2 that were present (Almeida, Zaragoza, Freitas 2006). Thus, it is possible that some equilibrium may develop between IL-2-producing autoreactive $CD4^+Foxp3^-$ T cells and IL-2-consuming autoreactive $CD4^+Foxp3^+$ Treg cells of the same specificity. Administration of IL-2:anti-IL-2 complexes can selectively expand Treg cells (Webster et al. 2009), and it would be interesting to know whether the percentage of 6.5^+ T cells expressing Foxp3 would change in a TS1xHA28 mouse or in an HA28 mouse in an adoptive transfer setting upon provision of exogenous IL-2.

Our studies have shown that recognition of self-Ag can drive $CD4^+Foxp3^-$ T cells in the periphery to upregulate Foxp3, CD25, and Helios, and to differentiate into functionally suppressive Treg cells. However, the extent to which this actually occurs *in vivo* and its relative importance in maintaining self-tolerance remains unclear. Thus far, no reliable marker has been found to differentiate tTreg cells and pTreg cells, making it difficult to determine the extent to which each subset contributes to the overall Treg cell

pool and to maintaining immune homeostasis in different circumstances. Helios and Neuropilin-1 have been suggested to be expressed exclusively by tTreg cells, but Helios can be upregulated on pTreg cells generated in pLNs, and Neuropilin-1 can be upregulated by Neuropilin-1^{-}CD4^{+}Foxp3^{+} cells in lymphopenic conditions (Gottschalk, Corse, Allison 2012; Yadav et al. 2012). pTreg cells can differentiate following recognition of food Ag or commensal bacteria, and likely play a role in preventing inflammation in response to innocuous antigens at mucosal barriers (Coombes et al. 2007; Fontenot et al. 2005; Lathrop et al. 2011; Sun et al. 2007). Mice that lack the intronic Foxp3 element CNS1 exhibit normal Treg cell development in the thymus, but impaired Foxp3 induction in CD4^{+} T cells following stimulation with anti-CD3 and TGF-β *in vitro* or feeding with cognate Ag *in vivo* (Josefowicz et al. 2012; Zheng et al. 2010). Furthermore, CNS1$^{-/-}$ mice have fewer Foxp3^{+} cells in the intestines and gut-associated lymphoid tissue, and develop inflammation in the gastrointestinal tract and lungs, suggesting that the ability of CD4^{+}Foxp3^{-} T cells to upregulate Foxp3 in response to TGF-β is critical to preventing inflammation at mucosal sites (Josefowicz et al. 2012; Zheng et al. 2010). However, while TGF-β is crucial for Foxp3 induction *in vitro* and in the gut, it may not be necessary for pTreg cell differentiation in non-gut draining LNs (Chen et al. 2003; Schlenner et al. 2012). Thus, these studies provide evidence that pTreg cells can be important in preventing pathological responses to commensals and innocuous antigens at mucosal surfaces, but do not necessarily address whether autoreactive pTreg cells may be important in maintaining self-tolerance. An additional study explored the relative importance of pTreg cells and tTreg cells in maintaining immune homeostasis through adoptive transfer of CD4^{+} T cells into newborn Foxp3-deficient mice. They found that less inflammation occurred when CD4^{+}Foxp3^{-} cells and CD4^{+}Foxp3^{+} were transferred concurrently than when CD4^{+}Foxp3^{+} T cells were

transferred alone, but $CD4^+Foxp3^-$ T cells were only beneficial when they were capable of upregulating Foxp3 and not when they were Foxp3-deficient (Haribhai et al. 2011). Additionally, when the transferred $CD4^+Foxp3^-$ cells contained a Foxp3-DTR reporter gene, depletion of pTreg cells that developed by administration of DT resulted in increased activation and IFN-γ production by host $CD4^+$ T cells, as well as inflammation in the lungs, colon, small intestine and liver. These data suggest that pTreg cells are necessary to reduce systemic inflammation and to limit immunopathology at barrier surfaces, although they come with the caveat that in adoptively transferring a limited number of tTreg cells into Foxp3-deficient mice, certain TCR specificities could be lacking from the tTreg cell pool that pTreg cells can, but perhaps normally would not, replace.

5.3 Signals driving Treg cell activity and differentiation during an infection

Treg cells have been found to suppress the anti-pathogen effector response in a multitude of infection models, but how TCR specificity for pathogen-derived and/or self-peptides can direct this activity is not well understood (Maizels and Smith 2011). There are two potential sources for the Treg cells that accumulate during an infection: (1) circulating $CD4^+Foxp3^-$ cells differentiate into $CD4^+Foxp3^+$ cells upon recognition of pathogen-derived Ag, or (2) Treg cells at infection sites expand from pre-existing Treg cells. We found that $6.5^+CD4^+Foxp3^-$ T cells that can recognize the HA molecule of PR8 influenza virus proliferated heavily during PR8 virus infection, but did not increase Foxp3 expression (Fig. 4-2). Similarly, in *L. major* and *M. tuberculosis* infection, no conversion of conventional $CD4^+$ T cells into $Foxp3^+$ Treg cells was found to occur, and Treg cells at

sites of infection expanded from pre-existing Treg cells (Shafiani et al. 2010; Suffia et al. 2006). It is possible that the amount of cognate Ag present in an infection may be too high and provide too strong of a TCR signal for pTreg cell differentiation to occur. As discussed in the previous section and shown by several different studies, a strong TCR signal can be detrimental to Treg cell differentiation (Haxhinasto, Mathis, Benoist 2008; Molinero et al. 2011). Certainly, the amount of proliferation undergone by $6.5^{+}CD4^{+}Foxp3^{-}$ cells during PR8 virus infection was more similar to what occurred in HACII mice, in which 6.5^{+} pTreg cell differentiation did not occur, than in HA28 mice. Furthermore, an ongoing inflammatory response can be detrimental to pTreg cell differentiation (Caretto et al. 2010; Oldenhove et al. 2009). For instance, the presence of a lethal *Toxplasma gondii* infection in the gut resulted in a decrease in the percentage of TCR transgenic T cells that upregulated Foxp3 when cognate Ag was fed to host mice, and the presence of IFN-γ inhibited Treg cell development *in vitro* (Caretto et al. 2010; Oldenhove et al. 2009). Thus, Treg cells that accumulate during infection likely do not derive from the conversion of $CD4^{+}Foxp3^{-}$ cells during infection, but rather expand from Treg cells already present in the pre-immune repertoire.

Studies from our lab had previously shown that 6.5^{+} Treg cells from TS1xHA28 mice, which are formed on self-Ag and are capable of recognizing an HA Ag derived from PR8 influenza virus, suppressed the anti-viral immune response during PR8 virus infection (Bedoya et al. 2013). Similarly, Treg cells expressing a transgenic TCR specific for *M. tuberculosis* were found to delay the accumulation of pathogen-specific $CD4^{+}$ and $CD8^{+}$ T cells in the lungs during *M. tuberculosis* infection (Shafiani et al. 2010), providing further evidence that pathogen-reactive Treg cells can modify the anti-

pathogen immune response. However, many studies have shown that Treg cells are predominantly formed based on specificity for self-Ag, leading us to question whether Treg cells might also be able to modulate an anti-pathogen immune response as a consequence of recognition of self-Ag within the inflammatory environment formed during infection (Apostolou et al. 2002; Hsieh et al. 2004; Jordan et al. 2001; Walker et al. 2003). Studies both *in vitro* and *in vivo* have shown that Treg cells require a TCR signal to be activated, but that they are able subsequently to suppress effector T cells with other specificities (Thornton and Shevach 1998; Yu et al. 2005), although this remains a matter of some debate (Huang, Zhu, Yang 2005; Tanchot et al. 2004). To determine whether activation on self-HA could induce 6.5^+ Treg cells to modulate the anti-influenza effector response, we assessed 6.5^+ Treg cell activity in HA transgenic mice during infection with RV6 virus, with which the 6.5 TCR has limited reactivity. In both HA28 mice, in which 6.5^+ Treg cells naturally form, and HACII mice, which express a potently stimulatory form of self-HA that induces similar division of 6.5^+ Treg cells as occurs during PR8 virus infection, 6.5^+ Treg cells failed to modulate the anti-viral immune response (Fig. 4-5 and Fig. 4-7). In contrast, recognition of HA derived from PR8 virus drove 6.5^+ Treg cells to suppress the effector T cell response in a PR8 virus-infected BALB/c mouse. Additionally, recognition of viral HA resulted in more accumulation in the medLNs and lungs and more upregulation of CXCR3 and T-bet than did recognition of self-HA. Curiously, recognition of self-HA in HACII mice impeded the ability of 6.5^+ Treg cells to respond to PR8 virus infection, as fewer cells accumulated in the medLNs and lungs of PR8 virus-infected HACII mice than PR8 virus-infected BALB/c mice (Fig. 4-6). Furthermore, 6.5^+ Treg cells in HACII mice displayed less upregulation of T-bet and CXCR3 during PR8 virus infection than in BALB/c hosts, and did not significantly affect effector T cell accumulation in the lungs (Fig. 4-7). Several

mechanisms likely contribute to the ability of the HA self peptide to inhibit 6.5^+ Treg cell differentiation and suppression of the anti-viral immune response in HACII mice. First, we found that 6.5^+ Treg cells transferred into HACII mice decreased expression of Foxp3, CD25, and the 6.5 TCR, and expressed higher levels of PD-1 than did 6.5^+ Treg cells isolated from PR8 virus-infected BALB/c mice (Fig. 4-8). Foxp3 and CD25 expression are both necessary for Treg cell persistence and function, and their loss could potentially explain the lack of 6.5^+ Treg cell activity in HACII mice (Furtado et al. 2002; Williams and Rudensky 2007). Perhaps surprisingly, given reports that ongoing inflammation can cause loss of Foxp3 in Treg cells (Laurence et al. 2012; Zhou et al. 2009), downmodulation of CD25 and Foxp3 by 6.5^+ Treg cells in HACII hosts was somewhat ameliorated during influenza virus infection. However, exposure to inflammation has previously been shown to induce changes in the transcriptional profile of Treg cells, including upregulation of CD25 and Foxp3 (Arvey et al. 2014). This observation could potentially result from the increase in IL-2 present during an inflammatory response, as IL-2 signaling can stabilize CD25 and Foxp3 expression (Fontenot et al. 2005). Additionally, downmodulation of the TCR complex has been shown to occur following TCR stimulation, and can render T cells insensitive to subsequent stimulation, which could limit the ability of 6.5^+ Treg cells to respond to virus-derived HA (San Jose et al. 2000; Schonrich et al. 1991). Finally, expression of PD-1 by $Foxp3^+CD4^+$ follicular regulatory T cells has been shown to limit their expansion and reduce their suppressive ability, and it is possible that the strong upregulation of PD-1 may have similar effects on 6.5^+ Treg cells in HACII mice (Sage et al. 2013). Studies from our lab have previously shown that adoptively transferred 6.5^+ Treg cells from TS1xHA28 mice are also unable to prevent the development of autoimmune arthritis in TS1xHACII mice, although transfer of polyclonal Treg cells can block disease

development (Oh et al. 2012). It is likely that a diverse TCR repertoire is required to suppress the effector cells that modulate disease development, but an alternative explanation could be that the phenotypic changes that occur in 6.5^+ Treg cells following recognition of self-HA in HACII mice render them incapable of suppression.

The idea that $CD4^+$ T cells must differentiate to acquire unique characteristics in response to specific stimuli in order to effectively control different pathogens is well appreciated (Zhu and Paul 2010). More recently, it has been found that Treg cells must also differentiate in order to efficiently suppress effector cells in different circumstances, and that Treg cells that are effective in one context may be ineffectual in other circumstances. For example, ablation of IL-10 specifically in Treg cells did not result in systemic immune activation, but did lead to the development of colitis and increased lung allergic inflammation, suggesting that production of IL-10 is required for Treg cell activity at mucosal surfaces, but not for prevention of systemic immune activation (Rubtsov et al. 2008). Furthermore, mice in which T helper cell subset (*i.e.*, Th1, Th2, Th17) lineage-defining transcription factors were selectively ablated in Treg cells developed immunopathology associated with the respective T helper subset (Campbell and Koch 2011). An important component of Treg cell differentiation is likely the acquisition of chemokine receptors that direct Treg cells to traffic to where they are needed. For instance, Treg cells genetically deficient in CCR6 accumulated less efficiently than their WT counterparts in the central nervous system during EAE (Yamazaki et al. 2008). Acquisition of CXCR3 expression has been shown to direct $CD4^+$ T cell trafficking into infected lungs (Kohlmeier et al. 2009), and the lack of CXCR3 expression by the majority of 6.5^+ Treg cells following activation in the medLN of infected

HA28 and HACII mice could explain the paucity of 6.5^+ Treg cells in the lungs of these mice. Interestingly, 6.5^+ Treg cells were able to limit the accumulation of $6.5^+CD4^+Foxp3^-$ cells in HA28 mice (Fig. 3-12), showing that activation on the self-HA in these mice can drive 6.5^+ Treg cell-mediated suppression in at least one context, and providing further evidence that the failure of the self-HA to induce 6.5^+ Treg cell differentiation may be responsible for their lack of activity in the context of an infection.

Previous studies demonstrated that STAT1 signaling, which occurs downstream of inflammatory cytokines such as IL-27 and IFN-γ, is necessary for T-bet and CXCR3 expression by Treg cells (Koch et al. 2012), and indeed, the inflammatory environment to which 6.5^+ Treg cells are exposed at the time of initial Ag encounter appears to contribute to their differentiation. CXCR3 upregulation on 6.5^+ Treg cells occurred more readily within the medLN, where the inflammatory response should be elevated, than the spleen of a PR8 virus-infected HACII mouse. Furthermore, waiting until the inflammatory response was established in an influenza virus-infected HACII mouse prior to adoptive transfer of 6.5^+ Treg cells resulted in an increase in the percentage of transferred cells expressing CXCR3 in the medLNs relative to when adoptive transfer occurred prior to infection (Fig. 4-9). These data demonstrate that TCR stimulation and exposure to inflammatory cytokine (such as IFN-γ) must occur concurrently, rather than sequentially, to induce Treg cell differentiation towards a Th1-like phenotype. However, despite being placed directly into an inflammatory setting, a sizable population of 6.5^+ Treg cells that underwent division still did not upregulate CXCR3, suggesting that the factors governing Treg differentiation may be slightly more complicated than simply TCR signal plus the general presence of cytokine.

We found that B cells that had been purified from RV6-infected HACII mice (presenting HA as a self-peptide) could induce extensive division of 6.5^+ Treg cells while inducing only limited upregulation of CXCR3, resembling the phenotype that is observed when 6.5^+ Treg cells are transferred into intact uninfected HACII mice (Fig. 4-12). This weak CXCR3 induction appears to be a property of the presentation of a target Ag by B cells, because B cells isolated from the lungs of PR8-infected BALB/c mice (i.e. B cells expressing the HA as a viral Ag) similarly induced division of 6.5^+ Treg cells with limited upregulation of CXCR3. In contrast, DCs isolated from the medLN of either PR8 virus-infected BALB/c mice or RV6 virus-infected HACII mice (and thus presenting either viral-HA or self-HA, respectively) induced both 6.5^+ Treg cell division and CXCR3 upregulation. Upregulation of CXCR3 appeared slightly more elevated when DCs presenting viral Ag were used, which could be explained by the fact that all of these DCs had taken up viral Ag, potentially triggering pattern recognition receptors that could induce further activation and differentiation of the DCs, while DCs presenting self-Ag in an HACII mouse had not necessarily come into direct contact with RV6 virus. B cells and DCs express different constellations of costimulatory molecules, which, in conjunction with TCR signaling, may drive Treg cells to unique fates, potentially including alternative differentiative fates not investigated here. Alternatively, DCs and B cells may produce different cytokines, or may induce production of different cytokines by the $6.5^+CD4^+Foxp3^-$ T cells also present in the cultures, that could affect Treg cell differentiation. Although serum levels of IFN-γ were similar in mice infected with PR8 or RV6 virus, it is possible that the localized concentration of IFN-γ around different APC subsets may differ. B cells are by far the most abundant APC in both uninfected HACII mice and in the medLNs of influenza virus-infected mice, and were found to

preferentially support the proliferation and accumulation of Treg cells, as has been observed previously (Chen and Jensen 2007; Sun et al. 2008). These findings provide evidence that efficient presentation of an Ag by B cells can exert a dominant effect on the differentiation of Treg cells even when DCs presenting the same Ag are also present. When we attempted to deplete B cells in HACII mice by treatment with anti-CD20 antibody prior to PR8 virus infection, there was no significant change in the accumulation of 6.5^+ Treg cells in the medLNs or lungs relative to isotype-treated mice, and the percentage expressing CXCR3 in the medLN did not significantly increase (data not shown). Unfortunately, we were unable to achieve complete depletion, and some B cells remained, particularly in the medLN. It is likely, given the ability of B cells to support Treg cell expansion and accumulation, that the remaining B cells were sufficient to drive expansion of 6.5^+ Treg cells that remain $CXCR3^-$, and further experiments in HACII.$Jh^{-/-}$ mice that constitutively lack B cells could explore the importance of B cells in 6.5^+ Treg cell differentiation. Together, these observations suggest that exposure of the 6.5^+ Treg cells to the HA expressed by B cells in HACII mice before an infection was established could induce substantial division and accumulation of 6.5^+ Treg cells without CXCR3 upregulation, and subsequent exposure to both the HA as a viral Ag and the inflammatory environment of the infection could not induce these cells to become $CXCR3^+$ in PR8-infected HACII mice.

These results may lead one to question how Treg cells ever come to participate in an anti-pathogen response, since most Treg cells are selected based on specificity for self-Ag. Presentation of self-Ag by DCs appears to drive some differentiation of Treg cells, and this may become more apparent when cognate self-Ag is presented primarily

by DCs, and is not expressed and presented by B cells. Certainly, recognition of self-HA in HACII mice was able to increase accumulation of 6.5^+ Treg cells in the lungs during RV6 infection, likely due to the increased presence of $MHCII^+$ cells (Fig. 4-6). However, a relatively strong TCR signal appears to be required to induce sufficient CXCR3 upregulation for trafficking into the lungs, since no 6.5^+ Treg cells were found in the lungs during RV6 virus infection in HA28 mice (Fig. 4-3), and most evidence suggests that highly immunostimulatory self-Ags do not induce Treg cell formation, which calls into question the extent to which self-reactive Treg cells may be able to contribute during an anti-pathogen immune response. Certainly, in our studies, TCR-mediated recognition of viral Ag was a much more efficient driver of Treg cell activity during infection than recognition of self. Others have shown that $CD4^+Foxp3^+$ that could recognize *M. tuberculosis*-derived Ag cells, as determined by tetramer staining, expanded from Treg cells present in a naïve mouse, confirming that pathogen-specific Treg cells exist in the steady state and proliferate after encountering cognate Ag (Shafiani et al. 2013). Further supporting evidence for the existence of Treg cells expressing a pathogen-reactive TCR comes from experiments in which Treg cells isolated from the site of *L. major* infection were found to expand from pre-existing Treg cells and demonstrated specificity for microbial Ag, as evidenced by their increased proliferation and production of IL-10 when cultured with infected *L. major*-DCs compared with uninfected DCs (Suffia et al. 2006). How pathogen-reactive Treg cells come to be part of the pre-immune repertoire remains undetermined, but it is conceivable that some degree of homology or cross-reactivity exists between self and viral antigens that allows Treg cells formed on self-Ag to respond to pathogen-derived Ag during an infection. In fact, both $CD8^+$ and $CD4^+$ T cells have been found to exhibit cross-reactivity that allows them to recognize unrelated pathogens (Su et al. 2013; Welsh et al. 2010). Using human $CD4^+$ T cells, it

was shown that clones of a $CD4^+$ T cell selected for HIV specificity by tetramer staining proliferated in response to peptides derived from *Micromonas* and *B. bifidum*, but not to an influenza virus HA epitope (Su et al. 2013). This phenomenon was not unique to this clone, as an HA-specific clone was also found to proliferate following exposure to peptides derived from *F. magna* and *T. vaginalis* (Su et al. 2013). These studies provide a potential explanation for how Treg cells from the pre-immune repertoire and formed based on recognition of self-Ag could potentially recognize pathogen-derived Ag, differentiate, and modulate the effector immune response during an infection.

5.4 Summary and conclusions

The work presented in this dissertation supports a model in which a less immunostimulatory form of self-Ag favors Treg cell formation both in the thymus and in the periphery. However, recognition of this weakly immunostimulatory self-Ag could not activate Treg cells to differentiate and suppress the effector immune response during influenza virus infection. In contrast, recognition of the same Ag when it was present as a viral Ag induced Treg cells to acquire expression of T-bet and CXCR3, to accumulate within the lungs, and to suppress accumulation of effector T cells. A highly stimulatory form of self-Ag expressed at high levels and predominantly by APCs could drive similar amounts of Treg cell division as the viral Ag, but was less efficient at inducing CXCR3 upregulation. Furthermore, Treg cells exposed to this highly stimulatory self-Ag displayed an impaired ability to differentiate, accumulate in the lungs, and modulate the anti-viral immune response upon subsequent exposure to viral Ag. Presentation of Ag

by B cells, the lack of an inflammatory environment at the time of initial TCR stimulation, and reduced expression of CD25, Foxp3, and the TCR may all contribute to this result.

Ultimately, the studies presented here show that the immunostimulatory capacity of a self-Ag can be an important parameter in determining Treg cell formation and activity in the periphery, and demonstrate that the activity of a Treg cell *in vivo* cannot necessarily be predicted based solely on Ag specificity. Rather, the cell type presentation Ag and the inflammatory environment at the time of initial antigenic encounter can also shape Treg cell activity. These are important ideas to consider as adoptive transfer of Treg cells is introduced as a clinical therapy. Transfer of Treg cells has been proposed as a therapy for autoimmune disease, solid organ transplant, and hematopoietic stem cell transplantation (Singer, King, D'Alessio 2014). In some settings, expansion and transfer of polyclonal Treg cell populations may be sufficient to achieve the desired result, but, in many cases Ag-specific Treg cells have been shown to more efficiently suppress development of autoimmune disease than polyclonal Treg cells (Jaeckel, von Boehmer, Manns 2005; Masteller et al. 2005; Tang et al. 2004). The recent successes with T cells expressing chimeric Ag receptors in treating acute lymphoblastic leukemia and chronic lymphocytic leukemia (Grupp et al. 2013; Kalos et al. 2011) suggest that similar protocols could be used to develop Ag-specific Treg cells for treatment of autoimmune disease. However, adoptively transferred Treg cells must be able to differentiate appropriately in order to traffic to where they are needed and suppress the necessary effector cell subsets. Clearly, a better understanding of the factors contributing to Treg cell formation, differentiation, and activity under different circumstances will be necessary to ensure the safety and efficacy of Treg cell adoptive therapy.

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