THE EFFECT OF T CELL RECEPTOR SPECIFICITY ON $CD4^+CD25^+$ REGULATORY T CELL FUNCTION IN AN AUTOIMMUNE SETTING

Soyoung Oh

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Andrew J. Caton, Ph.D., Supervisor of Dissertation

_____

Steven Reiner, M.D., Graduate Group Chairperson

Dissertation Committee:

Jan Erikson, Ph.D.

Christopher Hunter, Ph.D.

Terri Laufer, M.D.

Laurence A. Turka, M.D.

E. John Wherry, Ph.D.

To Mom and Dad,

For your unfailing love and support.

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ABSTRACT

THE EFFECT OF T CELL RECEPTOR SPECIFICITY ON $CD4^+CD25^+$ REGULATORY T CELL FUNCTION IN AN AUTOIMMUNE SETTING

Soyoung Oh

Andrew J. Caton, Ph.D.

The studies presented in this dissertation examine how the T cell receptor (TCR) specificity of $CD4^+CD25^+$ regulatory T (Treg) cells affects their function in a mouse model of autoimmune arthritis (TS1xHACII mice). TS1xHACII mice co-express $CD4^+$ T cells that express the transgenic 6.5 TCR, which is specific for the S1 determinant of influenza virus PR8 hemagglutinin (HA), and HA as a neo-self antigen under the MHC Class II I-Eα promoter. The majority of adult TS1xHACII mice develop inflammatory arthritis that is driven by recognition of S1 peptide by 6.5^+CD4^+ T cells. Notably, arthritis develops despite the presence of $CD4^+CD25^+Foxp3^+$ Treg cells, including a population of 6.5^+ cells that are specific for the disease target antigen S1 peptide. However, the administration of exogenous $CD4^+CD25^+$ Treg cells to pre-arthritic TS1xHACII mice can prevent arthritis in TS1xHACII mice, demonstrating that the disease is susceptible to $CD4^+CD25^+$ Treg cell activity.

Interestingly, we have found that the ability of $CD4^+CD25^+$ Treg cells to suppress arthritis in TS1xHACII mice is highly dependent on the TCR specificity(s) of the Treg cell population. Polyclonal $CD4^+CD25^+$ Treg cells (including cells that have not developed in the presence of the disease target antigen S1 peptide), but not $CD4^+CD25^+$ Treg cells that are enriched for S1-specific 6.5^+ cells are able to prevent arthritis development in TS1xHACII mice. Although polyclonal $CD4^+CD25^+$ Treg cells can suppress arthritis in TS1xHACII mice, our data suggest that successful $CD4^+CD25^+$ Treg cell mediated suppression of arthritis is remarkably sensitive to the balance of Treg cells and pathogenic 6.5^+CD4^+ T cells. We also present data indicating that $CD4^+CD25^+$ Treg cells that are strongly reactive for a highly expressed disease target antigen can be detrimental in the context of certain autoimmune diseases. Collectively, these studies indicate that the antigen-specificity of $CD4^+CD25^+$ Treg cells is a critical determinant of their ability to suppress arthritis in TS1xHACII mice. Moreover the data presented here also suggest that the balance of certain TCR specificities between $CD4^+CD25^+$ Treg and effector $CD4^+$ T cells plays an important role in determining the maintenance of tolerance versus the development autoimmunity.

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List of Abbreviations

AOD	autoimmune ovarian disease
APC	antigen presenting cell
BDC2.5	transgenic mouse expressing a TCR that is specific for a pancreatic β cell islet antigen
BDC-6.9	transgenic mouse expressing a TCR that is specific for a pancreatic antigen present in NOD but not BALB/c mice
CFSE	5-(and-6)-carboxyfluroscein diacetate, succinimidyl ester
CIA	collagen-induced arthritis
CNS	central nervous system
CNS2	conserved noncoding sequence 2
GFP	green fluorescent protein
GPI	glucose-6-phosphoisomerase
GVHD	graft-versus-host disease
EAE	experimental autoimmune encephalitis
EAP	experimental autoimmune prostatitis
HA	hemagglutinin
HLA	human leukocyte antigen
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
JIA	juvenile idiopathic arthritis
KRN	transgenic mouse expressing a TCR specific for GPI

K/BxN	transgenic mouse strain that is a cross between KRN and BxN mice; develops spontaneous arthritis driven by a $CD4^+$ T cell recognition of GPI and the production of GPI-specific antibodies by B cells
LN	lymph node
NOD	non-obese diabetic mouse strain
NOD.C6	non-obese diabetic mouse strain that does not express the peptide for the BDC-6.9 transgenic TCR
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte protein
MS	multiple sclerosis
PLP	myelin proteolipid protein
PLP1	pre-activated Ig-proteolipid protein 1
RA	rheumatoid arthritis
SCID	severe combined immunodeficiency
SKG	mouse strain described by Sakaguchi and colleagues; develops spontaneous arthritis due to altered negative selection of autoreactive thymocytes
TCR	T cell receptor
TS1	transgenic mouse expressing a TCR specific for the S1 determinant of influenza virus PR8 HA
TS1(SW)	transgenic mouse expressing a TCR specific for S1(SW) (an analog of S1 peptide) from the influenza virus SW HA
Treg	regulatory T cell

Chapter 1: Introduction and Overview

1.1 Introduction

The adaptive immune system is designed to be highly diverse, allowing for the recognition of a wide range of pathogens. However, this diversity must be balanced with the requirement of self-tolerance, and within tightly controlled numbers of peripheral lymphocytes. During T cell development, gene rearrangement mechanisms produce an enormous array of T cell receptors (TCRs). This diverse pool of TCRs is subsequently screened for autoreactivity, and highly self-reactive T cells are eliminated or functionally suppressed via central and peripheral tolerance mechanisms (Palmer 2003; Hogquist et al. 2005; Mueller et al. 2010), which include the activity of $CD4^+CD25^+$ regulatory T (Treg) cells (Sakaguchi 2004). The combination of these processes allows the adaptive immune system to achieve a diverse T cell repertoire while maintaining self-tolerance. However, these tolerance mechanisms are not foolproof, and approximately 3 to 5 percent of the population develops autoimmunity (Jacobson et al. 1997).

How and why $CD4^+CD25^+$ Treg cells fail to prevent the development of autoimmunity is poorly understood. The studies presented in this dissertation examine the role of $CD4^+CD25^+$ Treg cells in modifying a mouse model of

inflammatory arthritis that is dependent on the autoreactive $CD4^+$ T cell response. We also address how the antigen-specificity of the $CD4^+CD25^+$ Treg cells affects their *in vivo* suppressor function. This introductory chapter begins with an overview of rheumatoid arthritis and mouse models of arthritis with a particular focus on how $CD4^+$ T cells can contribute to disease. The following section presents a summary of relevant topics in $CD4^+CD25^+$ Treg cell biology. The chapter concludes with an overview of the current understanding of $CD4^+CD25^+$ Treg cell activity in human arthritis and mouse models of disease.

1.2 Rheumatoid Arthritis

Approximately 0.8 percent of the population develops rheumatoid arthritis (RA) (Lipsky). Although the etiology of the disease remains unknown, a major genetic risk factor for RA is the MHC Class II allele HLA-DR4 (DRβ1*0401). Interestingly, other MHC Class II alleles (HLA-DR5, HLA-DR2, HLA-DR3, and HLA-DR7) may be protective, as they are found at lower frequencies in RA patients than in individuals who have not developed RA (Lipsky). However, the mechanisms (e.g. deletion of autoreactive T cells, selection of Treg cells) by which these particular MHC Class II alleles could confer protection are unknown. In addition to the MHC Class II allele HLA-DR4, the gene *PTPN22* (protein tyrosine phosphatase non-receptor type 22), which encodes the lymphoid

tyrosine phosphatase (Lyp) that participates in TCR signal transduction, has been identified as another genetic risk factor for RA (Lipsky; (Lundy et al. 2007). Collectively, the association of genes that encode MHC Class II and TCR signaling molecules with RA development strongly supports a role for $CD4^+$ T cells in disease. Indeed, studies in multiple mouse models of arthritis have identified a critical role for $CD4^+$ T cells in arthritis development. A summary of these findings is presented in the following section.

1.3 The role of $CD4^+$ T cells in mouse models of arthritis

Mouse models of inflammatory arthritis possess several characteristics that resonate with human RA, and have been an instrumental tool in gaining better understanding of this autoimmune disorder. Work in several different arthritis models has clearly demonstrated a requirement for autoreactive $CD4^+$ T cells in arthritis pathogenesis. One of the most commonly used systems to study disease is the inductive model of collagen-induced arthritis (CIA). In CIA, disease development is initiated by immunizing mice with bovine type II collagen in complete Freund's adjuvant. The ensuing antigen-specific $CD4^+$ T cell response is required for B cell generation of collagen-specific antibodies that drive disease (Luross and Williams 2001). Although $CD4^+$ T cells are necessary for the initial immune response that leads to arthritis (Ranges et al. 1985), CIA is

ultimately dependent on the production of collagen-specific antibodies and arthritis does not develop in B cell deficient mice (Svensson et al. 1998).

K/BxN mice develop spontaneous arthritis that also requires T and B cell responses for disease development. Arthritis in K/BxN mice is initiated by KRN transgenic TCR $CD4^+$ T cell recognition of a glucose-6-phosphate isomerase (GPI) peptide presented by the MHC Class II molecule A^{g7}, which activates GPI-specific B cells, leading to the production of pathogenic autoantibodies that bind to articular surfaces and initiate an inflammatory cascade that leads to joint damage (Kouskoff et al. 1996; Matsumoto et al. 1999; Matsumoto et al. 2002). Chimeric mice, generated by administration of KRN.$RAG^{-/-}$ bone marrow to irradiated BxN.TCR$\alpha^{-/-}$ mice, develop arthritis, demonstrating that $CD4^+$ T cells of other specificities are not required for the initiation of arthritis development in K/BxN mice (Mangialaio et al. 1999). The transfer of serum from arthritic K/BxN mice into BxN.$RAG^{-/-}$ mice induces arthritis, indicating that the presence of autoantibodies alone is sufficient to mediate arthritis development, and that T cells are not required for the effector phase of disease (Korganow et al. 1999). However, although $CD4^+$ T cells are not necessary for the development of serum transfer induced arthritis, they can contribute to disease as co-administration of $CD4^+$ T cells with the serum transfers exacerbates arthritis by a mechanism that involves IL-17 (Jacobs et al. 2009).

While disease development in CIA and K/BxN arthritis models requires both T and B cell responses, it has been also shown that autoreactive $CD4^+$ T cells alone can initiate arthritis. Sakaguchi and colleagues have described the SKG mouse, which develops spontaneous arthritis that is attributed to a failure in negative selection of autoreactive thymocytes due to a mutation in the ZAP-70 (ζ-associated protein of 70 kDa) gene (Sakaguchi et al. 2003). The adoptive transfer of SKG $CD4^+$ T cells into $RAG^{-/-}$ mice can induce arthritis, demonstrating that B cell antibody production is not required for arthritis development, and that $CD4^+$ T cells alone can initiate disease (Hirota et al. 2007). However, while it is evident that autoreactive $CD4^+$ T cells drive arthritis development in SKG mice, the nature of the peptide(s) that the cells recognize is unknown. Moreover, arthritis development in SKG mice is also dependent on additional environmental cues, as SKG mice housed in specific pathogen free conditions do not develop disease (Yoshitomi et al. 2005). Our lab has recently described another mouse model of arthritis that is dependent on autoreactive $CD4^+$ T cells. TS1xHACII mice develop spontaneous inflammatory arthritis that is driven by $CD4^+$ T cell recognition of a single systemically presented peptide and does not require B cells (Rankin et al. 2008). Specific aspects of arthritis development in TS1xHACII mice will be discussed in greater detail in Chapter 3 of this dissertation.

While $CD4^+$ T cells clearly play a critical role in the initiation of the immune response that leads to arthritis development, other aspects of the immune response can also contribute arthritis pathogenesis. Dysregulation of inflammatory pathways can be a key contributing factor to disease, as the over-expression of cytokines such as TNF-α and IL-1α, or expression of a mutated IL-6R gp130 that enhances gp130-mediated signal transducer and activator of transcription (STAT3) activation also leads to arthritis development (Keffer et al. 1991; Niki et al. 2001; Sawa et al. 2006). Interestingly, the arthritis that develops in the IL-6R gp130 mutant mice is attributed to $CD4^+$ T cell activation resulting from enhanced homeostatic proliferation, and disease can be ameliorated by inhibiting the homeostatic proliferation of $CD4^+$ T cells (Sawa et al. 2006). In SKG mice, genetic deficiencies in IL-6, IL-1, and TNF-α resulted in either complete protection from arthritis or delayed disease onset accompanied by reduced incidence and disease severity (Hata et al. 2004), demonstrating that the autoreactive $CD4^+$ T cells are acting in concert with certain inflammatory pathways. In addition to IL-6, IL-1, and TNF-α, there are reports that IL-17, recently identified as defining a unique T helper cell lineage (Harrington et al. 2005; Park et al. 2005), is also involved in arthritis. In the next section we will summarize the relevant findings on IL-17 in human arthritis and mouse models of arthritis.

1.4 The role of IL-17 in arthritis

The cytokine IL-17 is thought to play a critical role in several autoimmune diseases, including experimental autoimmune encephalitis (EAE), psoriasis, and arthritis (Afzali et al. 2007; Korn et al. 2009). In human RA patients, IL-17 has been detected in the peripheral blood, serum, synovial fluid, and/or synovium (Chabaud et al. 1999; Kotake et al. 1999; Hirota et al. 2007; Sarkar et al. 2010). Interestingly in the synovium, IL-17 is primarily found in T cell enriched areas, and is mostly secreted by memory $CD4^+$ T cells isolated from the peripheral blood of RA patients (Sarkar et al. 2010), suggesting that $CD4^+$ T cells are a major source of IL-17 in RA. IL-17 has also been detected in the synovial fluid and joints of arthritic mice (Hirota et al. 2007). IL-17 may contribute to arthritis pathogenesis by a variety of mechanisms. IL-17 can induce the upregulation of cytokines such as IL-1β, TNF-α, and IL-6 (Chabaud et al. 1998; Jovanovic et al. 1998; Sarkar et al. 2010), which not only contribute to arthritis pathogenesis but can also promote additional differentiation of Th-17 cells (Weaver et al. 2007; Korn et al. 2009). IL-17 can also recruit innate immune cells such as monocytes or neutrophils, which are potential sources for the cytokines described above, to sites of inflammation (Lundy et al. 2007; Shahrara et al. 2009; Pelletier et al. 2010). Moreover, the IL-17R is found on chondrocytes, synoviocytes, and osteoclasts; and the activation of these cell types has direct implications for

arthritis pathogenesis (Sarkar et al. 2010). Indeed, *in vitro* studies have demonstrated that IL-17 can act upon osteoblasts to induce osteoclastogenesis (Sato et al. 2006), and potentially contribute to bone resorption. Thus, IL-17 can act upon cells in the joint and also activate and/or recruit other cell types to the primary sites of inflammation and autoimmune pathology.

Studies in several mouse models of arthritis have demonstrated that IL-17 plays an important role in disease pathogenesis. Mice lacking the IL-1 receptor antagonist (IL1-Ra$^{-/-}$) develop inflammatory arthritis, and interestingly the CD4^{+} T cells from these mice produce elevated amounts of IL-17 (Nakae et al. 2003). When IL1-Ra$^{-/-}$ mice are bred onto an IL17$^{-/-}$ background, the mice are protected from arthritis, demonstrating the critical role for IL-17 in mediating the disease that develops in IL1-Ra$^{-/-}$ mice (Nakae et al. 2003). Moreover, treating arthritic IL1-Ra$^{-/-}$ mice with anti-IL17 antibody can reduce disease severity (Koenders et al. 2008). Work in the CIA model has shown that increasing systemic IL-17 production in mice by gene transfer with an adenoviral vector expressing IL-17 accelerates diseases onset and severity (Lubberts et al. 2001). Complementary studies examining the effects of IL-17 or IL-17R blockade have demonstrated that inhibiting the biological activity of IL-17 reduces the severity of CIA (Lubberts et al. 2001; Lubberts et al. 2004; Egan et al. 2008). Furthermore, IL17$^{-/-}$ mice exhibit much lower incidence of CIA and reduced disease severity than wildtype

mice (Nakae et al. 2003). Similarly, in an LPS induced model of arthritis, disease incidence and severity are reduced in IL17$^{-/-}$ mice (Sato et al. 2006). Interestingly, studies in K/BxN and SKG mice indicate that IL-17 production by CD4^{+} T cells is important in arthritis development. In the K/BxN arthritis model, the augmentation of serum transfer induced disease by KRN CD4^{+} T cells is inhibited by treatment with anti-IL17 antibody, indicating that CD4^{+} T cell production of IL-17 was responsible for the exacerbation autoantibody induced arthritis (Jacobs et al. 2009). Additionally, Sakaguchi and colleagues have shown that IL-17$^{+/+}$CD4^{+}, but not IL-17$^{-/-}$CD4^{+} T cells from SKG mice, can transfer arthritis upon transfer into RAG$^{-/-}$ mice, demonstrating that CD4^{+} T cell generated IL-17 is critical for disease induction (Hirota et al. 2007).

1.5 CD4^{+}CD25^{+} regulatory T cells

The concept of suppressor T cells was first suggested by experiments in which thymectomized mice and adult rats developed autoimmune diseases that could be prevented by the transfer of spleen cells from healthy donors (Nishizuka and Sakakura 1969; Penhale et al. 1976). In the extensive work following those initial observations, Treg cell populations have been more clearly defined, and can be very broadly separated into thymically generated or peripherally generated (induced) Treg cells. Though several subpopulations of Treg cells

have been characterized, the best defined is the thymically generated $CD4^+CD25^+$ Treg cell population that comprises approximately 5 to 10 percent of the human and murine peripheral $CD4^+$ T cell repertoires. This population was first characterized by constitutive expression of the IL-2 receptor α chain (CD25) (Sakaguchi et al. 1995), which is also upregulated by activated $CD4^+$ T cells. Subsequently the forkhead transcription factor Foxp3 was identified as a more reliable marker for $CD4^+CD25^+$ Treg cells (Fontenot et al. 2003; Hori et al. 2003), and Foxp3 was considered to be the "master regulator" of the $CD4^+CD25^+$ Treg cell lineage.

However a recent set of studies has challenged the notion of Foxp3 as the key lineage commitment factor of $CD4^+CD25^+$ Treg cells. Multiple groups have reported that a subset of cells that have developed in the absence of Foxp3 express a genetic signature that is consistent with that of $CD4^+CD25^+Foxp3^+$ cells, suggesting that $CD4^+CD25^+$ Treg cell lineage commitment occurs upstream of Foxp3 expression (Gavin et al. 2007; Hill et al. 2007; Lin et al. 2007). Moreover, Foxp3 expression can be transiently upregulated in activated human T cells, which do not acquire regulatory function (Gavin et al. 2006; Mantel et al. 2006; Wang et al. 2007). Nonetheless, it is clear that Foxp3 expression is critical for $CD4^+CD25^+$ Treg cell function, as mice lacking Foxp3 develop severe autoimmunity (Fontenot and Rudensky 2005; Kim et al. 2007). Furthermore, in

humans, development of the autoimmune disorder IPEX has been attributed to mutations in the FoxP3 gene (Bennett et al. 2001; Gambineri et al. 2003).

1.5.1 The role of TCR specificity in the thymic selection of $CD4^+CD25^+$ Treg cells

The initial day three thymectomy experiments indicated that the thymus plays an important role in the generation of $CD4^+CD25^+$ Treg cells, however, the exact nature of the signals (e.g. TCR engagement, cytokines, co-stimulation) that guide the thymic development of $CD4^+CD25^+$ Treg cells remains unclear. While the observation that there is overlap between the TCR repertoires of $CD4^+CD25^+$ Treg cells and conventional $CD4^+$ T cells (Hsieh et al. 2004; Hsieh et al. 2006) suggests that other signals (e.g. cytokines, co-stimulation) likely also play a role in the thymic development of $CD4^+CD25^+$ Treg cells, a strong body of work supports a significant role for TCR interactions with self-peptides in the thymic selection of $CD4^+CD25^+$ Treg cells. The key findings in support of this concept are presented below.

Experiments using transgenic TCRs provided the first evidence that $CD4^+CD25^+$ Treg cells were selected via TCR interactions with self-peptide. It was observed that mice containing $CD4^+$ T cells expressing a transgenic TCR specific for the myelin basic protein (MBP) only developed EAE when on a $RAG^{-/-}$

background but not in intact mice (Olivares-Villagomez et al. 1998; Van de Keere and Tonegawa 1998; Hori et al. 2002). Subsequent studies showed that mice on an intact background did not develop disease because expression of a secondary TCR, resulting from endogenous α chain rearrangements, allowed for the selection of MBP-specific $CD4^+CD25^+$ Treg cells (Hori et al. 2002), suggesting a role for TCR and self-peptide interactions in $CD4^+CD25^+$ Treg cell selection. Indeed, studies in other transgenic systems have shown that introducing the cognate antigen for the transgenic TCR onto a $RAG^{-/-}$ background results in the development of $CD4^+CD25^+$ Treg cells expressing the transgenic TCR (Apostolou et al. 2002; Kawahata et al. 2002; Walker et al. 2003).

Work from our laboratory has further demonstrated the importance of TCR interactions with self peptides for the development of $CD4^+CD25^+$ Treg cells. Studies in TS1xHA-transgenic mice have demonstrated that $CD4^+$ T cells expressing the 6.5 TCR (Kirberg et al. 1994), which confers specificity for the S1 determinant of PR8 HA, undergo efficient $CD4^+CD25^+$ Treg cell selection in HA28 mice, which express PR8 HA driven by an SV40 promoter, demonstrating that interactions with the agonist peptide can guide $CD4^+CD25^+$ Treg cell development (Jordan et al. 2001). The requirement for peptide specificity for $CD4^+CD25^+$ Treg cell selection appears to be quite stringent, as $CD4^+$ T cells bearing a TCR with low reactivity to the S1 peptide are not selected to be

$CD4^+CD25^+$ Treg cells (Jordan et al. 2001). Interestingly, while $6.5^+CD4^+CD25^+$ Treg cells can also be selected in other TS1xHA lineages, the numbers are lower than what is generated in TS1xHA28 mice, and this appears to result from enhanced deletion of 6.5^+ thymocytes due to higher levels of HA expression in the thymus (Picca et al. 2006; Simons et al. 2010), indicating that lower levels of the cognate antigen favor more abundant $CD4^+CD25^+$ Treg cell selection.

1.5.2 The role of TCR specificity in $CD4^+CD25^+$ Treg cell function

TCR stimulation is required to activate $CD4^+CD25^+$ Treg cell suppressor function (Takahashi et al. 1998; Thornton and Shevach 1998). However, *in vitro* studies show that although $CD4^+CD25^+$ Treg cell activation is TCR specific, $CD4^+CD25^+$ Treg cells are able to inhibit responder $CD4^+$ T cells of different TCR specificities (Takahashi et al. 1998; Thornton and Shevach 2000), indicating that the suppression itself is not antigen-specific. Correspondingly, work in multiple murine autoimmune diseases (e.g. CIA, colitis, EAE, diabetes, gastritis) have shown that that the adoptive transfer of polyclonal $CD4^+CD25^+$ cells can prevent or ameliorate disease (Olivares-Villagomez et al. 1998; Van de Keere and Tonegawa 1998; Singh et al. 2001; Sarween et al. 2004; Morgan et al. 2005; Zwar et al. 2006; Kelchtermans et al. 2009), indicating that $CD4^+CD25^+$ Treg cells that are not enriched for disease-associated antigen specific cells can

mediate suppression of an organ specific disease. However, there is also a significant body of work suggesting that antigen-specific $CD4^+CD25^+$ Treg cells are more effective than polyclonal $CD4^+CD25^+$ Treg cells at suppressing organ-specific autoimmune diseases.

In the nonobese diabetes (NOD) model, the adoptive transfer of *in vitro* expanded $CD4^+CD25^+$ cells expressing the BDC2.5 TCR, which recognizes a pancreatic β cell islet antigen (Katz et al. 1993), into NOD.BDC2.5 mice (which also express the transgenic TCR) can suppress diabetes (Tang et al. 2004; Tarbell et al. 2004). Using a disease transfer model in which diabetes development is induced by the administration of splenocytes from diabetic NOD mice into NOD.scid mice, Steinman and colleagues also showed that lower numbers of *in vitro* expanded antigen-specific $CD4^+CD25^+$ cells could suppress disease, whereas greater numbers of *in vitro* expanded polyclonal $CD4^+CD25^+$ T cells could not (Tarbell et al. 2004). Work with induced $CD4^+CD25^+$ Treg cells also suggests that antigen-specific interactions are important for regulatory function. TGF-β induced regulatory cells expressing the BDC-6.9 TCR can suppress diabetes in NOD mice, which contain their cognate antigen, but not NOD.C6 mice, which do not contain their cognate antigen (Tonkin et al. 2008). It is possible that the inability to suppress diabetes in NOD.C6 mice may be linked

to poor trafficking, expansion, and/or survival at the primary site of autoimmune pathology.

Studies in the EAE model also emphasize that $CD4^+CD25^+$ Treg cell specificity for the target autoantigen is important for the prevention of autoimmunity. As described previously, mice expressing a transgenic TCR specific for the encephalotegenic MBP protein spontaneously develop EAE when on a RAG-deficient but not wildtype background, and the difference in disease development can be attributed to the absence of $CD4^+CD25^+$ Treg cells specific for EAE associated peptides in the $RAG^{-/-}$ mice (Hori et al. 2002). In another model of EAE where disease is driven by the immune response to the myelin proteolipid protein (PLP), the difference between two strains of mice with differing susceptibilities to disease appears to be the enhanced representation of PLP-peptide specific $CD4^+CD25^+$ Treg cells in the more resistant strain (Reddy et al. 2004). Interestingly, $CD4^+CD25^+$ cells isolated from healthy individuals can mediate better *in vitro* suppression of $CD4^+$ T cell proliferation when stimulated by MBP proteins than $CD4^+CD25^+$ cells isolated from multiple sclerosis (MS) patients (Kumar et al. 2006), suggesting that in humans protection may also correlate with the presence of $CD4^+CD25^+$ Treg cells that are specific for MS-associated peptides.

Studies in day 3 thymectomized mice that develop autoimmune ovarian disease (AOD), show that $CD4^+CD25^+$ Treg cells isolated from the draining lymph nodes are superior to $CD4^+CD25^+$ Treg cells isolated from non-draining lymph nodes at suppressing AOD (Samy et al. 2005; Samy et al. 2008). This apparent enrichment for disease-specific $CD4^+CD25^+$ Treg cells in the draining lymph nodes was evident for other organs as well, as $CD4^+CD25^+$ Treg cells isolated from the lacrimal or prostate draining lymph nodes were the most effective at suppressing dacryoadenitis and experimental autoimmune prostatitis (EAP) respectively (Samy et al. 2008; Wheeler et al. 2009). Collectively, these observations suggest that draining lymph nodes, which have been shown to uniquely present organ-specific peptides (Hoglund et al. 1999; Gagnerault et al. 2002; Scheinecker et al. 2002), are enriched for $CD4^+CD25^+$ Treg cells that are specific for antigens derived from the organ. Recent work examining the $CD4^+CD25^+$ Treg cell repertoires from various lymph nodes further corroborates the concept that regional lymph nodes may be biased towards $CD4^+CD25^+$ Treg cells that recognize organ-specific antigens, as Treg cells showed distinct patterns of TCR usage in different LNs, similar to the skewing of TCR usage among antigen-experienced conventional $CD4^+$ T cells in different anatomical locations (Lathrop et al. 2008).

1.5.3 Mechanisms of $CD4^+CD25^+$ Treg cell function

In spite of the clear observations that $CD4^+CD25^+$ Treg cells can suppress immune responses and prevent autoimmune disease, how they exert their regulatory function remains controversial. Indeed, a myriad of mechanisms by which $CD4^+CD25^+$ Treg cells can mediate suppression have been reported. *In vitro* studies indicate that direct cell contact between the regulatory and effector populations is necessary, as suppression is prevented when the two cell populations are separated in transwell experiments (Takahashi et al. 1998; Thornton and Shevach 1998). Additional work indicates that $CD4^+CD25^+$ Treg cell production of cytokines such as IL-10 or TGF-β is important for their suppressor function (Huang et al. 2005). Alternatively $CD4^+CD25^+$ Treg cells have been shown to act as a cytokine sink for cytokines such as IL-2, which can promote apoptosis of effector $CD4^+$ T cells (Pandiyan et al. 2007). It has also been suggested that $CD4^+CD25^+$ Treg cells may suppress immune responses by developing cytotoxic capabilities or modulating antigen presenting cells (APCs) (Kryczek et al. 2006; Shevach et al. 2006; Vignali et al. 2008). Indeed, Sakaguchi and colleagues have shown that a key contribution of CTLA-4 to $CD4^+CD25^+$ Treg cell function may be through regulation of APCs (Wing et al. 2008). Interestingly, recent work has also demonstrated that $CD4^+CD25^+$ Treg cells acquire the ability to suppress particular T-helper (Th) lineages by co-opting the transcription factor pathways (IRF-4, RORγt, T-bet) that are key for the

differentiation of the respective Th lineage cells (Chaudhry et al. 2009; Koch et al. 2009; Zheng et al. 2009). It is possible that expression of transcription factors specific for a certain Th lineage allows $CD4^+CD25^+$ Treg cells to acquire properties (e.g expression of chemokine or cytokine receptors) that allow them to traffic to the site of inflammation and/or compete for other soluble factors that may potentiate the $CD4^+$ Th cell response.

1.5.4 Plasticity of $CD4^+CD25^+$ Treg cells

$CD4^+CD25^+Foxp3^+$ Treg cells can produce Th-1 and Th-17 associated cytokines

Recent studies have challenged the notion of $CD4^+CD25^+$ Treg cells as a stable population of cells by demonstrating that under certain conditions $CD4^+CD25^+$ Treg cells can differentiate to acquire properties that are normally associated with effector $CD4^+$ T cells. While multiple groups have documented the loss of Foxp3 expression by $CD4^+CD25^+$ Treg cells (Hoffmann et al. 2006; Duarte et al. 2009; Komatsu et al. 2009; Murai et al. 2009; Tsuji et al. 2009; Zhou et al. 2009), there are also reports that $CD4^+CD25^+$ Treg cells that maintain Foxp3 expression can develop phenotypic properties (e.g. cytokine production) that are normally associated with effector $CD4^+$ T cells. During *Toxoplasma gondii* (*T. gondii*) infection, which induces a highly polarized Th-1 immune

response, a subset of $CD4^+CD25^+Foxp3^+$ Treg cells differentiates to express the transcription factor T-bet and to produce IFN-γ (Oldenhove et al. 2009). The acquisition of Th-1 cell properties by $CD4^+CD25^+$ Treg cells is at least partially dependent on a lack of IL-2 in the infectious setting, since treatment of *T. gondii* infected mice with an IL-2 α-IL-2 antibody complex that has been shown to enhance the biological activity of IL-2 (Boyman et al. 2006) reduces the presence of IFN-$\gamma^+CD4^+CD25^+Foxp3^+$ cells (Oldenhove et al. 2009).

In light of the complicated and interconnected relationship of $CD4^+CD25^+$ Treg cells and Th-17 cells (Bettelli et al. 2006; Veldhoen et al. 2006; Mucida et al. 2007; Zhang et al. 2008; Zhou et al. 2008), there has been much interest in determining whether $CD4^+CD25^+Foxp3^+$ Treg cells can differentiate to produce IL-17. Several groups have shown that IL-$17^+CD4^+CD25^+Foxp3^+$ cells can be isolated from human peripheral blood (Ayyoub et al. 2009; Beriou et al. 2009; Voo et al. 2009). The IL-17 producing $CD4^+CD25^+$ Treg cells also express CCR6 (Ayyoub et al. 2009; Beriou et al. 2009; Voo et al. 2009), a chemokine receptor that is associated with Th-17 cells (Acosta-Rodriguez et al. 2007; Annunziato et al. 2007). It appears that while IL-1β and IL-6 promote IL-17 production by human $CD4^+CD25^+Foxp3^+$ Treg cells (Beriou et al. 2009; Voo et al. 2009), the presence of TGF-β in *in vitro* cultures is inhibitory to this differentiation process (Voo et al. 2009). Interestingly, there is also evidence that stronger TCR

stimulation can favor IL-17 production, as a high but not low dose of α-CD3 induced IL-17 production by $CD4^+CD25^+Foxp3^+$ cells (Voo et al. 2009). However, despite producing IL-17, $CD4^+CD25^+$ Treg cells can inhibit responder $CD4^+$ T cells in *in vitro* suppression assays (Beriou et al. 2009; Voo et al. 2009). Murine $CD4^+CD25^+$ Treg cells can also be differentiated *in vitro* to generate a population of $IL\text{-}17^+CD4^+CD25^+Foxp3^+$ cells (Xu et al. 2007; Osorio et al. 2008; Yang et al. 2008). $CD4^+CD25^+Foxp3^+$ cells expressing RORγt, the transcription factor that governs Th-17 differentiation (Ivanov et al. 2006; Yang et al. 2008), can be found in mice *in vivo*, and while also present in the spleen are primarily enriched in the lamina propria and mesenteric lymph nodes (Lochner et al. 2008).

$CD4^+CD25^+$ Treg cells can lose Foxp3 expression

Recent studies have demonstrated that the expression of Foxp3, which is critical for maintenance of the $CD4^+CD25^+$ Treg cell phenotype (Wan and Flavell 2007; Williams and Rudensky 2007), can be unstable in $CD4^+CD25^+$ Treg cells. Using $Foxp3^{EGFP}$ reporter mice, Hori and colleagues showed that a subset of $CD4^+EGFP^+$ cells transferred into $RAG^{-/-}$ mice or lympho-replete mice lost Foxp3 expression, and this loss is exacerbated in the $RAG^{-/-}$ hosts (Komatsu et al. 2009). CD25 expression appeared to play a critical role in maintenance of Foxp3

expression, as $CD25^+$ cells were able to better maintain Foxp3 expression than $CD25^-$ cells isolated from the same mice. This observation is consistent with previous reports demonstrating that signaling through the IL-2R can potentiate Foxp3 expression (Fontenot et al. 2005; Zorn et al. 2006). Additionally, Foxp3 loss was most prevalent in the $CD4^+CD25^+$ Treg cells that had undergone extensive proliferation (Komatsu et al. 2009). This instability of Foxp3 can also be seen in human $CD4^+CD25^+$ Treg cells, as prolonged *in vitro* stimulation and expansion of $CD4^+CD25^+$ Treg cells isolated from peripheral blood mononuclear cells results in the loss of Foxp3 expression in a subset of cells (Hoffmann et al. 2006; Hoffmann et al. 2009). While exposure to lymphopenia can induce loss of Foxp3 expression in $CD4^+CD25^+$ Treg cells, exposure to inflammation during autoimmune disease also contributes to the instability of Foxp3 expression (Murai et al. 2009; Zhou et al. 2009). Interestingly, TCR engagement appears to enhance this process, as a greater percentage of $CD4^+CD25^+$ Treg cells expressing the BDC2.5 TCR, which is specific for a pancreatic islet antigen, lost Foxp3 than polyclonal $CD4^+CD25^+$ Treg cells in NOD mice (Zhou et al. 2009).

The loss of Foxp3 in $CD4^+CD25^+$ Treg cells can be prevented or reduced by the activity of cytokines such as TGF-β (Komatsu et al. 2009) or IL-10 (Murai et al. 2009), although the mechanisms by which these cytokines are acting remain unclear. Interestingly, a role for IL-10 in maintaining $CD4^+CD25^+$ Treg

cell Foxp3 expression only becomes apparent in conditions of inflammation. Kronenberg and colleagues showed that during colitis, wildtype $CD4^+CD25^+$ Treg cells transferred into an $IL10^{-/-}RAG^{-/-}$ host, or $IL10R\beta^{-/-}$ $CD4^+CD25^+$ Treg cells transferred into a $RAG^{-/-}$ host fail to suppress disease, which is associated with loss of Foxp3 expression in the $CD4^+CD25^+$ Treg cells (Murai et al. 2009). However, when $IL10R\beta^{-/-}$ $CD4^+CD25^+$ Treg cells were co-transferred with wildtype $CD4^+CD25^+$ Treg cells so that that $RAG^{-/-}$ mice did not develop colitis, the $IL10R\beta^{-/-}$ $CD4^+CD25^+$ Treg cells were able to maintain Foxp3 expression, indicating that IL-10 is important for the maintenance of Foxp3 expression only during conditions of inflammation (Murai et al. 2009).

Recent studies in both human and murine $CD4^+CD25^+$ Treg cells indicate that the methylation status of the Foxp3 promoter and the conserved noncoding sequence 2 (CNS2) is an important determinant of Foxp3 expression (Kim and Leonard 2007; Janson et al. 2008). Whereas natural $CD4^+CD25^+$ Treg cells could be characterized by demethylation at the Foxp3 promoter and CNS2, the DNA sequences remained methylated in induced $CD4^+CD25^+$ Treg cells or conventional $CD4^+$ T cells that had transiently upregulated Foxp3 (Baron et al. 2007; Floess et al. 2007; Polansky et al. 2008). Moreover, $CD4^+CD25^+$ Treg cells in which the CNS2 region of the Foxp3 locus has been knocked out show deficiencies in maintenance of Foxp3 expression when compared to wildtype

$CD4^+CD25^+$ Treg cells (Zheng et al. 2010). Whereas wildtype $CD4^+CD25^+$ Treg cells could maintain Foxp3 expression after multiple cell divisions, CNS2-deficient $CD4^+CD25^+$ Treg cells exhibited dramatic loss of Foxp3 expression after three to four cell divisions (Zheng et al. 2010). Interestingly, work with human $CD4^+CD25^+$ Treg cells shows that the cells that lose Foxp3 upon repeated *in vitro* stimulation have methylated the critical regions in the Foxp3 promoter (Hoffmann et al. 2009).

$CD4^+CD25^+$ Treg cells lose Foxp3 expression can acquire effector properties

$CD4^+CD25^+$ Treg cells that lose Foxp3 can differentiate to acquire effector functions. Strober and colleagues first showed that $CD4^+GFP^+$ cells purified from $Foxp3^{EGFP}$ reporter mice could differentiate to generate both $Foxp3^+$ and $Foxp3^-$ IL-17 producing cells when cultured *in vitro* in the presence of IL-6 (Xu et al. 2007). Subsequently other groups have also demonstrated that $CD4^+CD25^+$ Treg cells can lose Foxp3 and acquire the ability to produce IL-17, and suggest that the cytokines IL-6, IL-1, and IL-23 play important roles in this process (Osorio et al. 2008; Yang et al. 2008). The dichotomy between $CD4^+CD25^+$ Treg and Th-17 cells appears to be explained by antagonistic interactions between the lineage specific transcription factors Foxp3 and RORγt (Yang et al. 2008; Zhang et al. 2008; Zhou et al. 2008). Although a great deal of focus has been placed on

the conversion of $CD4^+CD25^+$ Treg cells to Th-17 cells, it has also been shown that $CD4^+CD25^+$ Treg cells that lose Foxp3 expression can acquire the ability to produce other cytokines such as IL-2 and IFN-γ (Duarte et al. 2009; Hoffman et al. 2009; Komatsu et al 2009; Murai et al 2009; Zhou et al 2009).

Upon transfer into T cell deficient mice, $CD4^+CD25^+$ Treg cells can lose Foxp3 and differentiate into T follicular helper (T_{FH}) cells, which promote germinal center formation and IgA secretion by B cells, in the Peyer's patches (Tsuji et al. 2009). Differentiation into T_{FH} cells, but not Foxp3 downregulation, required B cell interactions (Tsuji et al. 2009), indicating that environmental signals that do not contribute to Foxp3 downregulation can influence the differentiation of $CD4^+CD25^+$ Treg cells that have lost Foxp3 expression. The de-differentiation of $CD4^+CD25^+$ Treg cells can also have overtly negative physiological consequences. In the transfer colitis model, loss of Foxp3 by $CD4^+CD25^+$ Treg cells results in a failure to prevent disease (Murai et al. 2009). Work in the NOD mouse model of diabetes has demonstrated that the loss of Foxp3 expression in $CD4^+CD25^+$ Treg cells can directly contribute to disease pathogenesis, as the adoptive transfer of ex-Treg cells ($CD4^+CD25^+Foxp3^+$ cells that no longer express Foxp3) into NOD.$RAG^{-/-}$ mice can induce diabetes development (Zhou et al. 2009).

1.6 $CD4^+CD25^+$ Treg cells in human arthritis

In light of the compelling evidence that $CD4^+CD25^+Foxp3^+$ Treg cells play an active role in preventing the spontaneous development of systemic autoimmunity, many recent studies have aimed at determining whether some deficits in $CD4^+CD25^+$ Treg cell activity might contribute to the development of autoimmune diseases such as RA. Interestingly, many of these studies have reached the seemingly paradoxical conclusion that autoimmune arthritis can develop despite the presence of $CD4^+CD25^+$ Treg cells. For example, it appears that $CD4^+CD25^+$ Treg cells can be enriched in arthritic patients, since increased frequencies of $CD4^+CD25^+$ T cells have been found in synovial fluid (i.e. the primary disease site) (de Kleer et al. 2004; Mottonen et al. 2005; Ruprecht et al. 2005; Cao et al. 2006; Lawson et al. 2006) and in some cases also systemically in the peripheral blood of arthritic patients (Han et al. 2008). Indeed, an enhanced representation of $CD4^+CD25^+$ Treg cells in the joints and synovial fluid of affected individuals has been observed in patients with RA, with juvenile idiopathic arthritis (JIA), and with other rheumatic diseases in which arthritis is a secondary manifestation of disease (de Kleer et al. 2004; Mottonen et al. 2005; Ruprecht et al. 2005; Cao et al. 2006; Lawson et al. 2006; Han et al. 2008). Identifying $CD4^+CD25^+$ Treg cells based only on CD25 expression is limiting, however, in that it may also detect activated $CD4^+$ T cells that have upregulated the IL2Rα chain. However, analysis of Foxp3 mRNA and protein expression

supported the conclusion that the $CD4^+CD25^{bright}$ population isolated from RA patients was indeed enriched for Treg cells (de Kleer et al 2004; Ruprecht et al. 2005; Cao et al. 2006). A potential explanation for the enrichment of $CD4^+CD25^+$ Treg cells in arthritic joints is that the expression of specific patterns of chemokine receptors leads to preferential trafficking of $CD4^+CD25^+$ Treg cells to the disease site(s). Studies of human peripheral blood $CD4^+CD25^+$ Treg cells have shown that they express certain chemokine receptors, such as CCR4, and studies of mouse $CD4^+CD25^+$ Treg cells indicated that there are many different subsets of chemokine receptor expression on Treg cells, that could promote trafficking to specific locations (Iellem et al. 2001; Wei et al. 2006). A comparison of $CD4^+CD25^+$ T cells from the synovial fluid and peripheral blood of patients with active RA showed a significant enrichment in the synovial fluid of $CD4^+CD25^+$ Treg cells expressing the chemokine receptors CCR4, CCR5, and CXCR4 which are associated with migration to sites of inflammation (Jiao et al. 2007). Additionally, comparison of the chemokine profiles of dendritic cells and synovial tissue from RA patients and healthy individuals indicated that certain chemokines are enriched during RA, potentially resulting in the preferential recruitment of a variety of immune system cells, including $CD4^+CD25^+$ Treg cells (Radstake et al. 2005; Wei et al. 2006). Thus in RA patients, disease develops not only despite the presence of $CD4^+CD25^+$ Treg cells, but in spite of an enrichment of the $CD4^+CD25^+$ Treg cells at a primary site of autoimmune pathology.

These observations raise the question of whether the $CD4^+CD25^+$ Treg cells that are present in arthritic patients are perhaps dysfunctional, or are functional and are either unable to prevent disease, or are modifying it in some manner. There is evidence for both effective and dysfunctional $CD4^+CD25^+$ Treg cell activity in disease settings. Support for the beneficial effects of $CD4^+CD25^+$ Treg cells that localize in arthritic joints arose in studies of patients with JIA, where greater numbers of $CD4^+CD25^+$ T cells were found in patients with persistent-oligoarticular JIA (which is a relatively mild form of the disease) than in patients with the more severe extended-oligoarticular JIA (de Kleer et al. 2004). Additionally, $CD4^+CD25^+$ Treg cells isolated from patients with the milder form of JIA expressed higher levels of Foxp3 mRNA, which have been correlated with better suppressor function, than did $CD4^+CD25^+$ Treg cells from patients with more severe disease (de Kleer et al. 2004). Even within individual JIA patients, there appeared to be a divergence of $CD4^+CD25^+$ Treg cell function based on the location from which the $CD4^+CD25^+$ Treg cells were isolated. Results of *in vitro* suppression assays indicated that $CD4^+CD25^+$ T cells from the synovial fluid of JIA patients were more effective suppressors than those isolated from the peripheral blood, suggesting that the $CD4^+CD25^+$ Treg cells at the primary disease site possessed more potent regulatory function (de Kleer et al. 2004; Ruprecht et al. 2005). From a clinical standpoint, it has also been reported that

the duration of remission following corticosteroid treatment in JIA patients showed a positive correlation with the number of $CD4^+CD25^+$ Treg cells present in the synovial fluid (de Kleer et al. 2004). Thus, in JIA patients there seemed to be a correlation between an increased frequency of $CD4^+CD25^+$ Treg cells and a reduction in disease severity, with the possibility that more effective Treg cells localize to the joints and synovial fluid.

The alternative concept of dysfunctional $CD4^+CD25^+$ Treg cells in RA has been supported by findings that Treg cells isolated from RA patients exhibit reduced suppressor function (Ehrenstein et al. 2004; Valencia et al. 2006). Much of this work has examined the possible effects of the inflammatory environment in RA on $CD4^+CD25^+$ Treg cell function. Several groups have shown that $CD4^+CD25^+$ Treg cells isolated from RA patients post-Infliximab (anti-TNF-α) treatment show improved regulatory activity in *in vitro* suppression assays (Ehrenstein et al. 2004; Valencia et al. 2006; Nadkarni et al. 2007). $CD4^+CD25^+$ T cells isolated from patients with active RA, pre-Infliximab treatment, were able to suppress the *in vitro* proliferation, but not cytokine production, of responder $CD4^+$ T cells. However, after Infliximab treatment, $CD4^+CD25^+$ Treg cells originating from RA patients acquired the ability to suppress responder cytokine production (Ehrenstein et al. 2004). The improved suppressive activity of the $CD4^+CD25^+$ Treg cells also correlated with increased levels of Foxp3 mRNA, and

correspondingly, it has been shown that treatment of healthy donor Treg cells with TNF-α leads to a reduction in Foxp3 expression and loss of suppressor function (Valencia et al. 2006). Other *in vitro* work has shown that addition of cytokines such as IL-2, IL-7, and IL-15 to suppression assays can abrogate $CD4^+CD25^+$ Treg cell function, suggesting that multiple cytokines that may be elevated in RA patients can negatively affect Treg cell function (Ruprecht et al. 2005; Valencia et al. 2006; van Amelsfort et al. 2007).

There is also work suggesting that anti-TNF-α treatment may lead to the induction of peripheral $CD4^+CD25^+$ Treg cells rather than an improvement in the function of pre-existing Treg cells (Nadkarni et al. 2007). After Infliximab treatment, an increased percentage of $CD4^+Foxp3^+$ cells was observed in the peripheral blood of active RA patients. Corresponding *in vitro* studies showed that upon culture with Infliximab, a subset of $CD4^+CD25^-$ T cells from RA patients expressed Foxp3, which could be prevented by TGF-β blockade. Interestingly, this increase in Foxp3 expressing cells was not observed when $CD4^+CD25^-$ T cells from healthy donors were cultured with Infliximab (Nadkarni et al. 2007). The lack of Foxp3 induction in conventional $CD4^+$ T cells from healthy individuals upon Infliximab treatment suggests that not only $CD4^+CD25^+$ Treg cells, but also effector $CD4^+$ T cells from RA patients exhibit phenotypic changes in response to the inflammatory environment. Indeed, there is work suggesting that

conventional $CD4^+$ T cells isolated from the synovial fluid of RA patients are refractory to suppression by $CD4^+CD25^+$ Treg cells (de Kleer et al. 2004; van Amelsfort et al. 2007). While these studies of $CD4^+CD25^+$ T cells in RA have predominantly focused on the possibility of detrimental effects of the inflammatory environment on $CD4^+CD25^+$ Treg cell function, more recent work has shown that Treg cells from RA patients can exhibit deficiencies in CTLA-4 regulation that may also affect their suppressor capabilities (Flores-Borja et al. 2008). It has also been shown that higher percentages of $CD4^+CD25^+Foxp3^+$ T cells and monocytes from RA patients express GITR and GITR-L respectively than in healthy donors (Han et al. 2008). Ligation of GITR has been linked to abrogration of $CD4^+CD25^+$ Treg cell function (Shimizu et al. 2002; Ji et al. 2004), suggesting another possible mechanism by which Treg cells might be rendered dysfunctional in RA patients.

1.7 $CD4^+CD25^+$ Treg cells in mouse models of arthritis

Studies in multiple mouse models of inflammatory arthritis have indicated that $CD4^+CD25^+$ Treg cells are capable of modifying disease, and the role of Treg cells has been most extensively studied in the collagen-induced and K/BxN arthritis models. As seen in human arthritis, $CD4^+CD25^+$ Treg cells can be found in the synovial fluid, joints, and draining lymph nodes of arthritic mice (Morgan et

al. 2005; Gonzalez-Rey et al. 2007; Nguyen et al. 2007). $CD4^+CD25^+$ T cells isolated from arthritic mice are capable of exerting suppressor function in *in vitro* assays (Kang et al. 2008; Monte et al. 2008), although in some situations they have been found to be less functional than their counterparts in healthy mice (Manoury-Schwartz et al. 1997). $CD4^+CD25^+$ T cells from IFN-γ receptor knockout mice, which develop accelerated and more severe CIA (Manoury-Schwartz et al. 1997; Vermeire et al. 1997), exhibit less potent suppressive activity *in vitro* and express lower levels of Foxp3 mRNA, akin to the effects of TNF-α on Treg cells seen in RA patients (Kelchtermans et al. 2005). Additional work suggested that in the absence of IFN-γ, other cytokines such as IL-17 are unchecked and contribute to exacerbated disease (Chu et al. 2007), suggesting that the altered and perhaps more severe inflammatory environment in the IFN-γ receptor knockout mice is affecting the phenotype and function of $CD4^+CD25^+$ Treg cells.

Work examining the impact of $CD4^+CD25^+$ Treg cell deficiency on arthritis development, achieved by genetic means or antibody depletion, has provided evidence that $CD4^+CD25^+$ Treg cells indeed modulate the autoimmune response in inflammatory arthritis. K/BxN mice, which develop spontaneous inflammatory arthritis initiated by a $CD4^+$ T cell response to a GPI peptide, have been crossed to Foxp3-*scurfy* (Foxp3-*sf*) mice to determine how a congenital absence of

$CD4^+CD25^+Foxp3^+$ Treg cells affects disease development. K/BxN.Foxp3-*sf* mice were found to develop an accelerated and more severe disease than K/BxN mice containing $CD4^+CD25^+$ Treg cells, suggesting that while the Treg cells do not ultimately prevent arthritis, their activity is affecting disease pathogenesis and severity (Nguyen et al. 2007). These mice did not suffer from the multi-organ autoimmunity associated with Foxp3-*sf* mice, presumably because the TCR repertoire was restricted by expression of the transgenic TCR. In contrast to the effects of a genetic deficiency in $CD4^+CD25^+$ Treg cells, $CD4^+CD25^+$ T cell depletion by antibody treatment did not appear to affect arthritis development in K/BxN mice, as neither disease onset nor severity were affected (Kang et al. 2008). However, $CD4^+CD25^+$ T cell depleted K/BxN mice exhibited more extensive lymphocyte infiltration into other organs and also an increase in serum anti-dsDNA antibody levels, indicating that the endogenous $CD4^+CD25^+$ Treg cells are able to modulate other aspects of an autoimmune response even as arthritis develops (Kang et al. 2008). In the CIA model, depletion of $CD4^+CD25^+$ T cells by antibody treatment did lead to increased disease severity (Morgan et al. 2003; Kelchtermans et al. 2005). The effect on arthritis development could be reversed by transferring $CD4^+CD25^+$ T cells into Treg cell depleted mice, suggesting that while the Treg cells are not able to prevent disease development, they are reducing severity (Morgan et al. 2003).

Complementary studies involving the transfer of exogenous $CD4^+CD25^+$ T cells into pre-arthritic mice also suggest that Treg cells are capable of modifying arthritis development. Transferring pre-activated $CD4^+CD25^+$ T cells from healthy mice or Foxp3-transduced $CD4^+$ T cells ameliorated disease in the CIA model (Morgan et al. 2005; Ohata et al. 2007; Kelchtermans et al. 2009). However, there appeared to be a limited time frame in which transferred Treg cells could modulate arthritis severity. $CD4^+Foxp3^+$ T cells were best at ameliorating CIA when they were transferred prior to the primary immunization with collagen. At later time points, such as after a secondary immunization, a greater number of transferred Treg cells was required to achieve a comparable reduction in disease severity, and if transferred after the booster immunization the Treg cells had no effect on arthritis development (Ohata et al. 2007). It has been shown that as soon as one day post transfer, $CD4^+CD25^+$ T cells that have been injected systemically can be found in the draining lymph node, synovial fluid, and synovial tissue, indicating that the cells are trafficking to the disease site (Morgan et al. 2005). Interestingly $CD4^+CD25^+$ T cell transfers were more effective at modifying disease severity when the cells were injected systemically rather than directly into the joints, raising questions about how and where the Treg cells are acting to modulate arthritis (Ohata et al. 2007). Together, the results of depletion and addition of Treg cells in multiple mouse models of inflammatory arthritis suggest that functional $CD4^+CD25^+$ Treg cells are present

and able to modulate disease severity and other aspects of the immune response (such as lymphocyte infiltration into different tissues and the generation of autoantibodies), but that their activity can fail to prevent the development of autoimmune arthritis.

There is conflicting data as to whether $CD4^+CD25^+$ Treg cells are modifying the immune response in an antigen-specific manner, and the mechanisms by which Treg cells affect arthritis development remain unclear. In K/BxN.Foxp3-*sf* mice, there is an accelerated accumulation of anti-GPI antibodies in the absence of $CD4^+CD25^+$ Treg cells (Nguyen et al. 2007). There is additional evidence for antigen-specific Treg cell-mediated changes in the CIA model, with observations of increased collagen-specific T and B cell responses after Treg cell depletion, as well as a reduction in collagen-specific antibodies after transfer of exogenous Treg cells (Morgan et al. 2003; Ohata et al. 2007). However, there is also data showing no alterations in the collagen-specific immune response upon manipulation of $CD4^+CD25^+$ Treg cells in CIA (Morgan et al. 2005; Kelchtermans et al. 2009). The same study did however find decreased amounts of TNF-α and IL-6 in the serum of treated mice, which are two cytokines that can contribute to arthritis development and have been shown to interfere with Treg cell function (Keffer et al. 1991; Pasare and Medzhitov 2003; Ehrenstein et al. 2004; Hata et al. 2004; Sawa et al. 2006; Valencia et al. 2006;

Nadkarni et al. 2007). Thus another potential mechanism by which $CD4^+CD25^+$ Treg cells may affect arthritis development is by altering the inflammatory environment. Indeed it appears that the balance of cytokines can play an important role in arthritis development and pathogenesis. Recently, IL-35 has been identified as an effector cytokine of $CD4^+CD25^+$ Treg cells, and separate work has shown that IL-35 treatment reduces disease severity in CIA, possibly by reducing IL-17 levels (Collison et al. 2007; Niedbala et al. 2007). Thus, IL-35 production by Treg cells may be able to modulate arthritis development by altering the inflammatory environment. Other work also suggests that $CD4^+CD25^+$ Treg cells may induce changes in the cytokine environment that lead to inhibition of osteoclastogenesis. In an *in vitro* culture system, high levels of cytokines that can inhibit osteoclastogenesis were found and accompanied by reduced osteoclast formation when $CD4^+CD25^+$ Treg cells were present (Kelchtermans et al. 2009). Given that endogenous $CD4^+CD25^+$ Treg cells are present in the joints, and exogenously administered $CD4^+CD25^+$ Treg cells can traffic to the disease site one to two days post transfer, it is possible then that $CD4^+CD25^+$ Treg cells may reduce osteoclastogenesis in the joints and thereby modify disease severity.

The studies presented in the following chapters further examine the role of $CD4^+CD25^+$ Treg cells in a mouse model of spontaneous arthritis in which

disease is driven by $CD4^+$ T cell recognition of a single systemically presented peptide. We also examine how specificity for the target antigen affects the ability of $CD4^+CD25^+$ Treg cells to suppress arthritis development.

Chapter 2: Materials and Methods

2.1 Transgenic mice

TS1 mice express a transgenic TCR (Vα4, Vβ8.1/2) that is specific for the S1 determinant of influenza virus A/PR/8/34 (PR8) HA presented in the context of MHC Class II I-E^d and can be identified by the monoclonal antibody 6.5 (Kirberg et al. 1994). TS1(SW) mice express a transgenic TCR (Vα8.3, Vβ10) that is specific for an analog of S1 peptide that is derived from the influenza virus A/SW/33 strain (Jordan et al. 2001). TS2 mice encode a transgenic TCR (Vβ8.3) that is specific for the S2 determinant of PR8 HA presented in the context of MHC Class II I-A^d (Scott et al. 1994).

HACII mice express the full length PR8 HA transgene driven by an MHC Class II I-Eα promoter (Kouskoff et al. 1993; Reed et al. 2003). 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).

PevSWHA mice (hereafter referred to as PevSW mice) were created by linking DNA encoding the HA protein from influenza virus A/SW/33 (SWHA) to the human β-globin locus control region. The SWHA protein contains an analog

of the S1(110-120) determinant of PR8 HA, described as the S1(SW) peptide. S1(SW) peptide contains two amino acid substitutions relative to the S1 peptide from PR8 HA: (1) at position 113 R → K and (2) at position 120 E → T.

C.Cg-*Foxp3*$^{tm2(EGFP)}$Tch/J mice (Haribhai et al. 2007), hereafter referred to as Foxp3^{EGFP} mice, were purchased from Jackson Laboratories (Bar Harbor, ME). Foxp3^{EGFP} mice were bred to TS1 or HA28 transgenic mice to generate TS1.Foxp3^{EGFP} and HA28.Foxp3^{EGFP} mice. Expression of the EGFP allele was determined by PCR and confirmed by flow cytometry.

TCR$\alpha^{-/-}$ mice (Mombaerts et al. 1992) were purchased from Jackson Laboratories and bred to homozygosity for the H-2^{d} haplotype. TCR$\alpha^{-/-}$ mice were then backcrossed four generations with BALB/c mice before breeding with HA transgenic mice.

All mice were bred and maintained on a BALB/c background. BALB/c mice were purchased from Taconic (Germantown, NY). Mice were maintained in specific pathogen free conditions in the Wistar Institute Animal Facility (Philadelphia, PA), and all experiments were conducted under protocols approved by the Wistar Institute Institutional Animal Care and Use Committee (IACUC).

2.2 Genotyping of transgenic mice

All transgenic mice were genotyped upon weaning at three to four weeks of age. Tail samples (approximately 1 to 2 mm) were digested overnight at 55° C in 180 μl of tail lysis buffer. Digestion was stopped by heat inactivation at 95° C for 10 minutes. Samples were then used as template DNA for PCR. PCR was performed in 50 μl reactions containing 5 μl 10X PCR amplification buffer, 50 ng of appropriate primers, 2 μl of 5 mM dNTPs, 1 unit of Taq polymerase, and 3 μl of tail DNA. PCR reactions were run in a thermocycler for 35 cycles. For HA and TCR transgene tests each cycle consisted of a 1 minute annealing period at 50° C (for HA transgene tests) and 65° C (for TCR transgene tests), a 2 minute elongation period at 72° C, and a 1 minute denaturation period at 94° C. An additional annealing and elongation step was performed prior to the end of the amplification cycles. For EGFP transgene tests, each cycle consisted of a 3 minute denaturation period at 94° C, a 1 minute annealing period at 60° C, and a 1 minute elongation period at 72° C. An additional elongation step was performed prior to the end of amplification cycles. PCR products were visualized on a 1 % agarose gel containing ethidium bromide.

Primers for genotyping were purchased from Integrated DNA Technologies (Coralville, IA). Primer sequences are shown below.

HA

HA uni-5’	5’-	CCA GCA GAT TTC ATC GAC TAT G -3’
HA uni-3’	3’-	CCA GTA ATA GTT CAT CCT CCC -3’

TS1

V2.1 CDR3	5’-	GAA CTG CTC AGC ATA ACT CCC -3’
VβCROSS5’	5’-	GAG GCT GCA GTC ACC CAA AG -3’

TS1K

TS1K β JUNCTION	5’-	CTC AGC ACC CCT GCC AGC CTG -3’
TS1K β Vgene	5’-	ATG GGC TGT AGG CTC CTA AGC -3’

TS2

T2-5-5 CDR3	5’-	CGA AGT ACT GTT CAG CCC AGT -3’
Vβ8 CROSS5’	5’-	GAG GCT GCA GTC ACC CAA AG -3’

GFP

FORWARD	5’-	GCG TAA GCA GGG CAA TAG AGG -3’
REVERSE	5’-	GCA TGA GGT CAA GGG TGA TG -3’

2.3 Arthritis assessment in TS1xHACII mice

TS1xHACII mice were assessed weekly for visible swelling in the four distal joints from post-weaning at 5 to 6 weeks of age until 14 to 15 weeks of age. Limbs were also measured with a digital caliper (Fisher Scientific). Mice were assigned a score based on the following index: 0 – no swollen limbs, 1 – one swollen limb, 2 – two swollen limbs, 3 – three swollen limbs, and 4 – swelling in all four limbs.

2.4 Flow cytometry

Flow cytometry was performed on single cell suspensions prepared from the lymph nodes (axillary, brachial, cervical, inguinal, mesenteric, and popliteal) or spleens. $1x10^6$ to $3x10^6$ cells were plated in V bottom plates and washed with FACS buffer and stained in 100 to 200 μl of FACS buffer with diluted antibodies at 4° C for 30 minutes. The following antibodies were used: anti-CD4 PerCpCy5.5, PECy7, APC, APCeF780 (L3T4); anti-CD25 PE, PerCpCy5.5 (PC61); anti-CD44 Alexa700 (IM7); anti-CD62L PerCpCy5.5 (MEL14); anti-CD69 PE (H1.2F3); anti-CTLA PE (UC10-4B9); anti-GITR PECy7 (DTA-1); anti-IFN-γ PE, PECy7, APC (XMG1.2); anti-IL-17 PE, APC (eBio17B7); anti-Thy1.1 PerCpCy5.5 (HIS51); anti-Vα8.3 FITC (B21.14), anti-Vβ10 PE (B21.5); and anti-

Vβ8.3 PE (1B3.3). Anti 6.5-biotin (Kirberg et al 1994) was detected using Streptavidin-APC (eBioscience, San Diego, CA) or Streptavidin-Qdot655 (Invitrogen, Carlsbad CA). Intracellular Foxp3 was detected using anti-Foxp3 PE or eF450 (FJK-16s) according to the manufacturer's protocols (eBioscience). In some experiments dead cells were identified using the LIVE⁄DEAD® Fixable Aqua Dead Cell Stain Kit from Invitrogen. Greater than 100,000 events per sample were collected on FACS Calibur or FACS LSR II flow cytometers (BD Biosciences, San Jose, CA), and data was analyzed using FlowJo software (Treestar, Ashland, OR).

2.5 Cell sorting

Single cell suspensions were prepared from lymph nodes (axillary, brachial, cervical, inguinal, mesenteric) or spleens. Spleens were subjected to red blood cell (RBC) lysis prior to antibody staining. Cells were resuspended at $1x10^7$ cells/ml in FACS buffer (NaN_3 deficient) and stained with the appropriate antibodies at 4° C for 30 minutes. Cells were washed twice. When necessary the antibody staining step was repeated with secondary reagents. Cells were resuspended at $5x10^7$ cells/ml in preparation for cell sorting. Sorted cells were collected into a 1:1 mixture of Iscove's Modified Dulbecco's Medium (IMDM; Mediatech, Inc., Herndon, VA) and fetal bovine serum (FBS; Gemini Bio-

products, West Sacramento, CA). Cells were sorted at the Wistar Institute's cell sorting facility using a DakoCytomation MoFlo (DakoCytomation) or a BD FACSAria cell sorter (BD Biosciences).

2.6 anti-IL-17 antibody treatment of TS1xHACII mice

TS1xHACII mice were treated with an anti-IL17A antibody (clone M210, Amgen), hereafter referred to as anti-IL17, or a Rat IgG2a isotype control antibody. The antibody treatment regimen was three intraperitoneal injections of 150 μg of either anti-IL17 or the isotype control antibody per mouse three times per week (Monday, Wednesday, Friday). All mice were treated from weaning for a duration of nine weeks.

2.7 MACS purification of $CD4^+$ cells

Single cell suspensions were prepared from lymph nodes (axillary, brachial, cervical, inguinal, mesenteric) or spleens. Cells were resuspended at $1x10^7$ cells/ml in FACS buffer (NaN_3 deficient) and stained with biotinylated antibodies to B220, CD8, and MHC Class II at 4° C for 30 minutes. Cells were washed twice, resuspended at $2x10^8$ cells/ml, and then incubated with streptavidin bound microbeads (Miltenyi Biotec, Auburn, CA) at 4° C for an additional 30 minutes. Cell suspensions were run through a CS column (Miltenyi

Biotec, Auburn, CA) attached to a magnet. The purity of cells collected in the flow through were analyzed by flow cytometry. $CD4^+$ cell purity was consistently ≥ 90 percent.

2.8 CFSE labeling

Cells were resuspended at $1x10^7$ cells/ml into sterile serum-free Dulbecco's Phosphate Buffered Saline (DPBS) (Mediatech, Manassas, VA). A 1:1000 dilution of CFSE (Invitrogen) was added to the cell suspension and incubated at room temperature for approximately 3.5 to 4 minutes. At end of the incubation, an equal volume of FBS was added to the cell suspension to quench the CFSE-labeling reaction. Cells were then washed and resuspended into either sterile serum-free DPBS for adoptive transfer experiments, or into supplemented IMDM with 10% FBS and 100 μl/ml gentamycin for *in vitro* culture experiments.

2.9 Adoptive transfers

Cells purified by cell sorting were washed once and then resuspended at $1x10^7$ cells/ml in sterile DPBS and injected into the tail veins of recipient mice. In

some experiments donor cells were labeled with CFSE prior to the adoptive transfers.

2.10 Analysis of *in vivo* $CD4^+CD25^+$ Treg cell proliferation

Sorted $CD4^+CD25^+$ cells were CFSE-labeled and adoptively transferred into recipient mice. Three to ten days post-transfer systemic LNs (axillary, brachial, cervical, inguinal, mesenteric) were isolated and single cell suspensions were prepared and stained with the appropriate antibodies. Cells were analyzed by flow cytometry to determine proliferation as defined by CFSE dilution.

2.11 Intracellular cytokine staining

Cells were plated at $1x10^6$ to $4x10^6$ cells/ml in supplemented IMDM with 10% FBS and 100 μl/ml gentamycin and stimulated with 50 ng of PMA (Sigma-Aldrich, St. Louis, MO), 1μM ionomycin (Sigma-Aldrich), and a 1:1000 dilution of Brefeldin-A (eBiosciences) for four hours. At the end of incubation, cells were isolated and stained for appropriate antibodies as described previously. Cytokine production was analyzed by flow cytometry.

2.12 Unfractionated LN cultures

Systemic LN (axillary, brachial, cervical, inguinal, mesenteric) cells were plated at $2x10^6$ to $5x10^6$ cells/ml in supplemented IMDM with 10% FBS and 100 μl/ml gentamycin and cultured for three to four days at 72° C. Culture supernatants were collected for analysis by ELISA or Luminex.

2.13 *In vitro* $CD4^+CD25^+$ Treg cell suppression assays

CFSE-labeled MACS-purified $CD4^+$ T cells from TS1 or TS2 mice were cultured with either 0.1 μg/ml of soluble anti-CD3 (145-2C11), 0.3 or 1 μM of S1 peptide (110-120; SFERFEIFPKE), 1 μM of S2 peptide (HNTNGVTAACSHE), or HACII splenocytes. Irradiated BALB/c splenocytes were used as feeder cells when soluble anti-CD3 or peptide was used. Purified $CD4^+CD25^+$ cells from TS1xHA28, TS1xHACII, or TS1(SW)xPevSW mice were added at a 1:2 Treg cell to responder cell ratio. $CD4^+CD25^+$ cells were stimulated with either 0.1 μg/ml of soluble anti-CD3, titrated doses of S1 peptide (0.3 or 1 μM), 1 μM of S1(SW) peptide (SFEKFEIFPKT), or HACII splenocytes. Cells were resuspended in supplemented IMDM with 10% FBS and 100 μl/ml gentamycin in 96- or 24-well flat bottom plates and cultured for three to four days at 36° C. Cells were plated at a final volume of $4x10^6$ to $6x10^6$ cells/ml. At the end of the culture period

responder $CD4^+$ T cell proliferation (based on CFSE profiles) and/or cytokine production was analyzed by flow cytometry.

2.14 ELISA

ELISAs were performed on supernatants collected from unfractionated LN cultures. Cytokine antibodies and standards were purchased from eBioscience and ELISAs were conducted according to the manufacturer's instructions. Purified anti-IL17A (clone eBioTC11-18H10.1) was used as the capture antibody and biotinylated anti-IL17A (eBio17B7) was used as the detection antibody. ExtrAvidin® alkaline phosphatase was purchased from Sigma-Aldrich.

2.15 Luminex

Luminex analysis was run on unfractionated LN cell culture supernatants using Milliplex kits (Millipore, Billerica, MA). Assays were conducted by the University of Pennsylvania Human Immunology Core.

2.16 Statistical analyses

Statistical analyses were conducted using unpaired two-tailed Student's t-tests assuming unequal variances. Results of arthritis incidence were analyzed using two-tailed Fisher's exact test. $p \leq 0.05$ was considered significant.

2.17 Solutions

Phosphate buffered saline (PBS): 2.2 mM NaH_2PO_4 [Anhydrous], 12.6 mM Na_2HPO_4 [Anydrous], 6.2 mM KCl, and 170mM NaCl (solution pH 7.4)

FACS buffer: PBS, 2 % fetal bovine serum (FBS; Gemini Bio-products, West Sacramento, CA), 0.08 NaN_3

Supplemented Iscove's Modified Dulbecco's Medium (IMDM; Mediatech, Inc., Herndon, VA), 10% FBS, $5x10^{-5}$ M 2-mercaptoethanol, 5 μg/ml iron saturated human transferrin, and 100 μl/ml gentamycin

RBC lysis buffer: 0.15 M NH_4Cl and 0.01 M Tris (pH 7.5)

ELISA developing buffer: 0.1 M $NaHCO_3$, 0.001 M $MgCl_2$ (pH 9.8) and 1 mg/ml PNPP (Sigma-Aldrich)

Tail lysis buffer: 50 mM Tris (pH 8.0), 50 mM KCl, 2.5 mM EDTA, 0.45 % Tween 20, 0.45% NP-40

10x PCR amplification buffer: 0.5M KCl, 0.1M Tris (pH 8.4), 25mM $MgCl_2$-$6H_2O$

TE: 0.01 M Tris-HCl (pH 8.0) and 0.01 M EDTA

5 mM dNTPs: 5 mM dATP, 5 mM dTTP, 5 mM, dCTP, 5 mM dGTP in TE

Chapter 3: Polyclonal $CD4^+CD25^+$ Treg cells can prevent arthritis development in TS1xHACII mice

3.1 Introduction

TS1 mice express a TCR transgene that recognizes the major I-E^d-restricted T cell determinant from the influenza virus PR8 HA (termed S1) and can be detected using the anti-clonotypic mAb 6.5 (Kirberg et al. 1994). HACII mice express a transgene encoding the PR8 HA under the control of a MHC class II I-Eα promoter (Reed et al. 2003). The majority of TS1xHACII mice, which co-express S1-specific 6.5^+CD4^+ T cells and HA as a self-antigen, develop spontaneous inflammatory arthritis that is driven by recognition of the S1 peptide by 6.5^+CD4^+ T cells, since TS1xHACII mice on a $RAG^{-/-}$ background develop disease (Rankin et al. 2008). Moreover, TS1xHACII mice on a $JH^{-/-}$ background, which lack mature circulating B cells, also develop arthritis, demonstrating that neither antibody production nor antigen presentation by B cells is required for disease development. Despite extensive deletion of 6.5^+ thymocytes, some 6.5^+CD4^+ T cells reach the periphery of TS1xHACII mice, interact with S1 peptide presented by antigen presenting cells, and gradually accumulate in number. TS1xHACII mice develop enlarged popliteal LNs, which drain the lower joints, and increased cytokine (e.g. IL-17, IFN-γ, IL-6, TNF-α) production by systemic

LN cells (Rankin et al. 2008). Additionally, arthritic TS1xHACII mice contain $CD4^+CD25^+Foxp3^+$ cells, including a population that is specific for the target antigen S1 peptide that is recognized by the pathogenic 6.5^+CD4^+ T cells (Rankin et al. 2008). A schematic summarizing the key aspects of arthritis development in TS1xHACII mice is presented in Figure 3-1.

Finding that arthritis develops in TS1xHACII mice despite the presence of $CD4^+CD25^+Foxp3^+$ cells is consistent with observations of normal, and even increased, frequencies of $CD4^+CD25^+$ Treg cells in human arthritis patients (de Kleer et al. 2004; Mottonen et al. 2005; Ruprecht et al. 2005; Cao et al. 2006; Lawson et al. 2006), and in other mouse models of arthritis (Morgan et al. 2005; Gonzalez-Rey et al. 2007; Nguyen et al. 2007). Although the representation and function of $CD4^+CD25^+$ Treg cells has been analyzed in human and murine arthritis, the role of $CD4^+CD25^+$ Treg cells in disease development, and how the antigen-specificity of the Treg cell population can affect this, remains unclear. TS1xHACII mice are a powerful system in which to study how $CD4^+CD25^+$ Treg cells can affect arthritis pathogenesis as identification of the disease initiating target antigen (S1 peptide) allows us to more carefully examine whether and how $CD4^+CD25^+$ Treg cells affect the pathogenic S1-specific 6.5^+CD4^+ T cells, other $CD4^+$ T cells (not specific for the disease initiating target antigen), or innate immune cells that may contribute to arthritis pathogenesis.

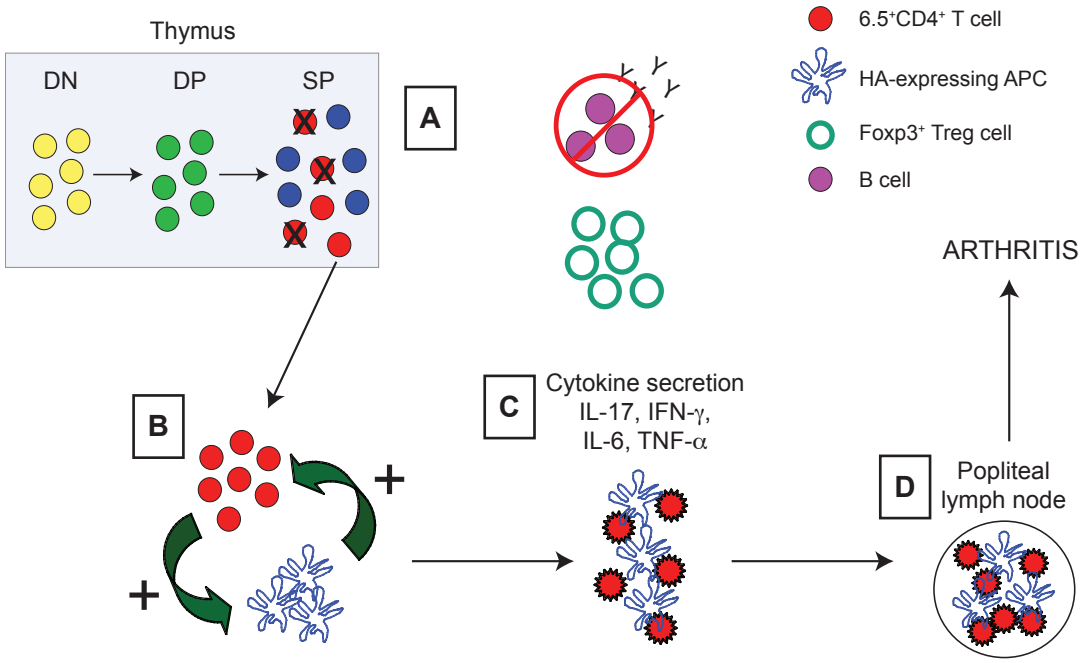

Figure 3-1 Key steps in the development of spontaneous inflammatory arthritis in TS1xHACII mice. TS1xHACII.JH$^{-/-}$ mice, which lack mature circulating B cells develop arthritis, indicating that antibody production and/or antigen presentation by B cells is not required for disease development. Moreover, TS1xHACII.RAG$^{-/-}$ mice also develop arthritis, demonstrating that recognition of the S1 peptide by 6.5^{+}CD4^{+} T cells is sufficient to mediate disease. Although CD4^{+}CD25^{+}Foxp3^{+} Treg cells are present in arthritic mice, whether and how they participate in arthritis development remains unclear. (**A**) 6.5^{+} thymocytes undergo extensive deletion in response to S1 peptide (**B**) 6.5^{+}CD4^{+} T cells that have evaded negative selection accumulate in the periphery of TS1xHACII mice. Interactions between 6.5^{+}CD4^{+} T cells and HA-expressing APCs results in reciprocal activation of the cell populations. (**C**) CD4^{+} T cells differentiate to produce inflammatory cytokines. (**D**) An enhanced immune response develops in the joint-draining popliteal LNs of TS1xHACII mice.

We show here that pre-arthritic TS1xHACII mice contain expected frequencies of $CD4^+CD25^+Foxp3^+$ cells, indicating that arthritis development cannot be attributed to an absence of $CD4^+CD25^+Foxp3^+$ cells in young TS1xHACII mice. The endogenous $CD4^+CD25^+Foxp3^+$ cells in TS1xHACII mice appear activated and are present in the joint-draining LNs. Additionally, purified $CD4^+CD25^+$cells from TS1xHACII mice exhibit *in vitro* regulatory function. Although these observations suggest that the arthritis that develops in TS1xHACII mice may be refractory to $CD4^+CD25^+$ Treg cell activity, we show that the administration of exogenous polyclonal $CD4^+CD25^+$ cells can prevent arthritis development in TS1xHACII mice, most likely by inhibiting the pathogenic Th-17 response. Moreover, specificity for a critical disease target antigen within the $CD4^+CD25^+$ Treg cell population is not required for disease suppression, as BALB/c $CD4^+CD25^+$ cells, which have not developed in the presence of the HA protein from which the S1 peptide is derived, can also prevent arthritis development in TS1xHACII mice. Strikingly, transfer of TS1xHACII $CD4^+CD25^+$ cells that have been depleted of $6.5^+CD4^+CD25^+$ cells (which comprise $Foxp3^+$ and $Foxp3^-$ cells) reduces arthritis incidence in TS1xHACII mice, demonstrating that endogenous $CD4^+CD25^+$ cells from TS1xHACII mice do possess *in vivo* regulatory function and suggesting that $CD4^+CD25^+$ Treg cell function can be remarkably sensitive to the balance of Treg and effector cells.

3.2 Results

3.2.1 Arthritis development in TS1xHACII mice is accompanied by the accumulation of peripheral 6.5^+CD4^+ T cells

The majority of adult TS1xHACII mice develop spontaneous inflammatory arthritis (Rankin et al. 2008). To better characterize the kinetics of disease, a cohort of TS1xHACII mice were assessed weekly for arthritis development. Mice with at least one affected limb (defined by visible swelling) were designated as arthritic. The majority of mice did not exhibit disease symptoms until 11 weeks of age, at which point over 50 percent of the cohort was arthritic (Figure 3-2 A). Disease incidence increased in older mice, with 73 percent of TS1xHACII mice developing arthritis by 14 weeks of age (Figure 3-2 A). Analysis of 16 week old TS1xHACII mice showed that the majority (62.5 percent) of arthritic animals developed disease in all four limbs, and no arthritic mice possessed fewer than two affected limbs (Figure 3-2 B). Thus, the majority of TS1xHACII mice develop spontaneous inflammatory arthritis in which all four limbs are affected.

The recognition of S1 peptide by 6.5^+CD4^+ T cells is a critical initiating step in arthritis development in TS1xHACII mice (Rankin et al. 2008). We examined the frequency of 6.5^+CD4^+ cells in unfractionated systemic LN (axillary, brachial,

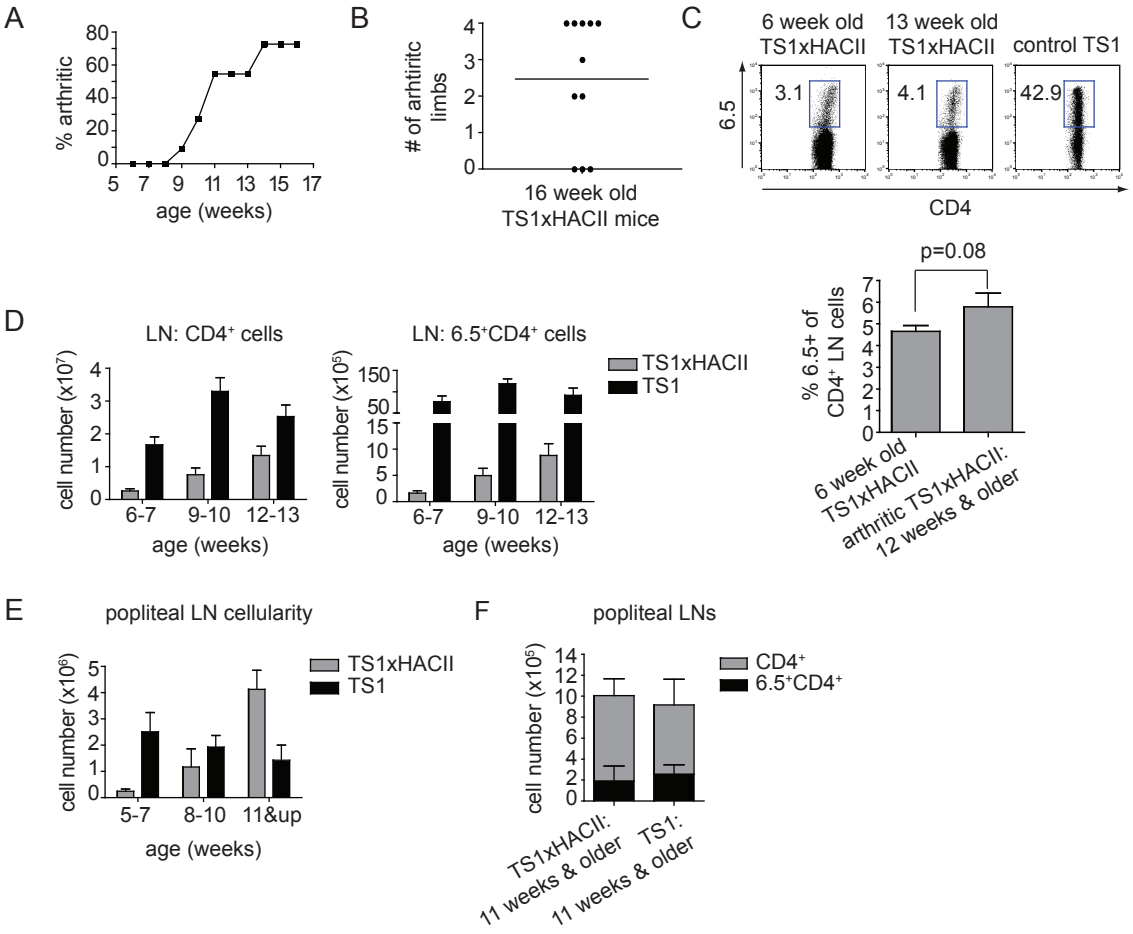

Figure 3-2 6.5$^+$CD4$^+$ T cells accumulate in the periphery of arthritic TS1xHACII mice (**A**) Arthritis incidence over time in TS1xHACII mice. n=11. (**B**) Numbers of affected limbs per mouse in 16 week old TS1xHACII mice. Each circle represents an individual mouse. Line represents the average number of arthritic limbs. (**C**) Dot plots show 6.5$^+$CD4$^+$ cells in the LNs (axillary, brachial, cervical, inguinal, and mesenteric) of pre-arthritic TS1xHACII, arthritic TS1xHACII, and control TS1 mice. Numbers indicate the percentage of 6.5$^+$ cells among CD4$^+$ cells. Graph shows the average percentage of 6.5$^+$ cells in pre-arthritic and arthritic TS1xHACII mice. n ≥ 7 mice per age group. Statistical significance was calculated using the Student's t-test. (**D**) Numbers of total CD4$^+$ (left graph) and 6.5$^+$CD4$^+$ (right graph) cells in TS1xHACII (gray bars) and TS1 (black bars) mice of different ages. n ≥ 3 mice per age group. (**E**) Numbers of total popliteal LN cells in TS1xHACII (gray bars) and TS1 (black bars) mice of different ages. (**F**) The number of total CD4$^+$ (gray bars) and 6.5$^+$CD4$^+$ (black bars) in 11 weeks and older TS1xHACII and TS1 mice. For (**E**) and (**F**) n ≥ 4 mice per age group. Bar graphs show average values ± SEM.

cervical, inguinal, and mesenteric) cells from 6 week old TS1xHACII, 13 week old TS1xHACII, and control single transgenic TS1 mice. In comparison to TS1 mice, both pre-arthritic and arthritic TS1xHACII mice contained markedly reduced percentages of $6.5^{+}CD4^{+}$ cells (Figure 3-2 C, left panel). The decrease in $6.5^{+}CD4^{+}$ T cells in TS1xHACII mice when compared to TS1 mice reflects their development in the presence of HA as a self-antigen. The representation of $6.5^{+}CD4^{+}$ cells appeared to be slightly higher in arthritic TS1xHACII mice in comparison to pre-arthritic TS1xHACII mice (Figure 3-2 C, right panel), though the difference was not statistically significant ($p=0.08$, Student's t-test).

We next analyzed the numbers of $CD4^{+}$ cells in the systemic LNs of TS1xHACII and TS1 mice at different ages. At 6 to 7 weeks of age, TS1xHACII mice possessed dramatically fewer total $CD4^{+}$ and $6.5^{+}CD4^{+}$ cells in comparison to TS1 mice (Figure 3-2 D). Over time, total $CD4^{+}$ and $6.5^{+}CD4^{+}$ cell numbers increased in TS1xHACII mice, with 12 to 13 week old arthritic mice containing the highest numbers of both populations, although the cellularities did not equal those of age-matched TS1 mice (Figure 3-2 D). We also examined the popliteal LNs, which drain the lower joints, in TS1xHACII and TS1 mice at different ages. Although the total cellularity of popliteal LNs was much lower in young TS1xHACII mice in comparison to young TS1 mice, as TS1xHACII mice aged popliteal LN cellularities increased dramatically, and arthritic mice (11 weeks and

older) contained larger popliteal LNs than age-matched TS1 mice (Figure 3-2 E, left panel). In contrast to what was observed in systemic LNs (Figure 3-2 D), the popliteal LNs of older TS1xHACII and TS1 mice contained comparable numbers of total $CD4^+$ cells and of 6.5^+CD4^+ cells (Figure 3-2 E, right panel).

Collectively, these data demonstrate that 6.5^+CD4^+ T cells, which are specific for the S1 autoantigen, accumulate in the periphery of TS1xHACII mice.

3.2.2 TS1xHACII mice contain $CD4^+CD25^+Foxp3^+$ cells, including cells that are specific for the target autoantigen that initiates disease development

To examine the representation of $CD4^+CD25^+Foxp3^+$ cells within TS1xHACII and TS1 mice of different ages, LN cells were stained for expression of CD4, CD25, and the transcription factor Foxp3. The percentage of $CD25^+Foxp3^+$ cells in $CD4^+$ LN cells of young (6 to 7 week old) TS1xHACII mice fell within the expected range of 5 to 10 percent of peripheral $CD4^+CD25^+$ Treg cells (Figure 3-3 A). Additionally, TS1xHACII mice of all ages contained similar frequencies of $CD4^+CD25^+Foxp3^+$ cells, and within each age group the percentages were higher than those in age-matched TS1 mice (Figure 3-3 A). These data show that young, pre-arthritic TS1xHACII mice contain a normal

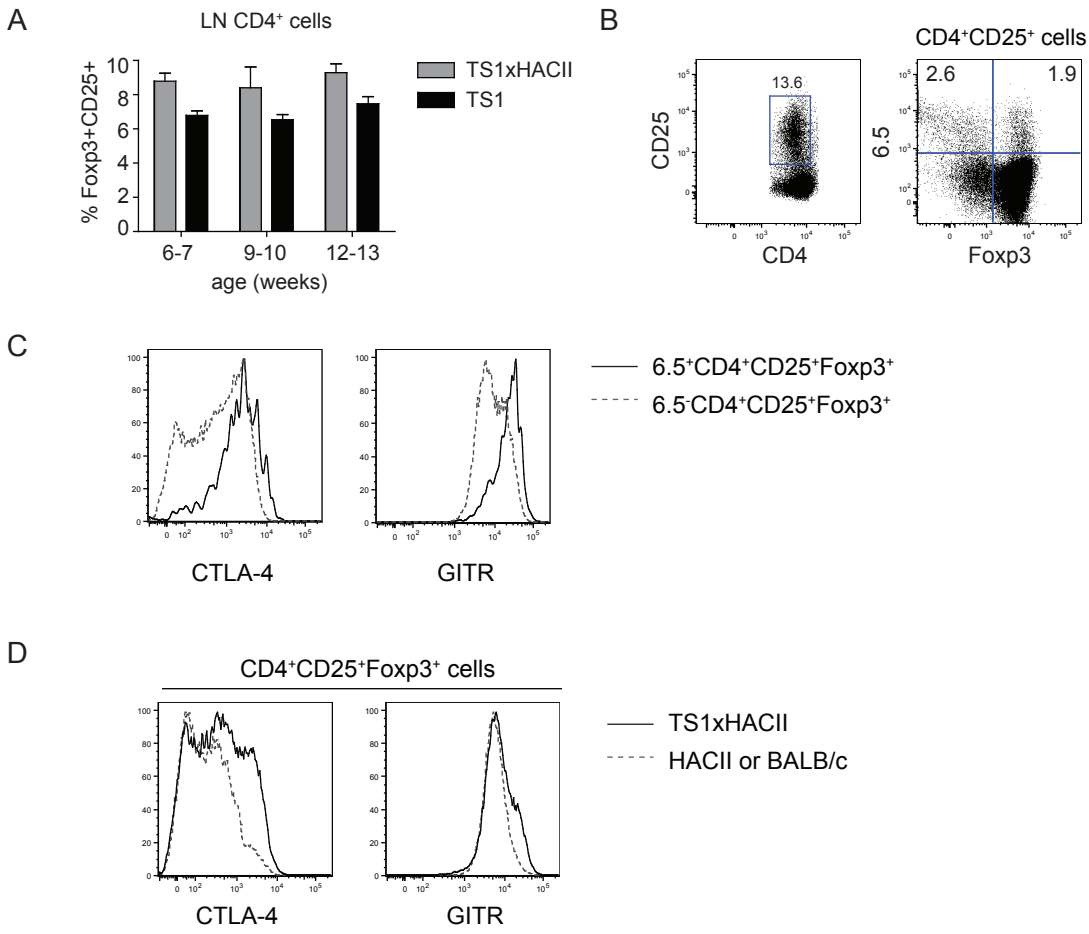

Figure 3-3 TS1xHACII mice contain S1-specific $CD4^+CD25^+Foxp3^+$ cells (**A**) Graph shows the percentage of $CD25^+Foxp3^+$ cells among LN (axillary, brachial, cervical, inguinal, mesenteric) $CD4^+$ cells from TS1xHACII (gray bars) and TS1 (black bars) mice of different ages. n ≥ 3 mice group. Bar graphs show average values ± SEM. (**B**) LN cells from arthritic TS1xHACII mice were stained for 6.5, CD4, CD25, and Foxp3. Dot plot on right shows 6.5 and Foxp3 expression within $CD4^+CD25^+$ cells in TS1xHACII mice. Numbers indicate the percentages of cells within the respective gates. (**C**) CTLA-4 and GITR expression on 6.5^+ (solid line) and 6.5^- (dotted line) $CD4^+CD25^+Foxp3^+$ LN cells from TS1xHACII mice. (**D**) CTLA-4 and GITR expression on $CD4^+CD25^+Foxp3^+$ LN cells from TS1xHACII (solid line) or control (HACII or BALB/c) mice.

representation of $CD4^+CD25^+Foxp3^+$ cells that is similar to the frequency found in arthritic TS1xHACII mice.

To determine whether TS1xHACII mice contain $CD4^+CD25^+Foxp3^+$ cells specific for the disease-initiating antigen (S1 peptide), we analyzed systemic LN cells for expression of 6.5, CD4, CD25, and Foxp3. $CD4^+CD25^+$ LN cells from TS1xHACII mice contained a 6.5^+ population, which comprised both $Foxp3^+$ and $Foxp3^-$ cells (Figure 3-3 B). The majority of the cells within the $CD4^+CD25^+$ pool that did not express the 6.5 TCR did express Foxp3 (Figure 3-3 B). To examine the phenotype of 6.5^+ and 6.5^- $CD4^+CD25^+Foxp3^+$ cells in TS1xHACII mice, we analyzed their expression of the Treg cell markers CTLA-4 and GITR. Whereas the $6.5^-CD4^+CD25^+Foxp3^+$ cells exhibited more diffuse distribution of CTLA-4 and GITR expression, $6.5^+CD4^+CD25^+Foxp3^+$ cells had more uniform expression of high levels of both CTLA-4 and GITR (Figure 3-3 C). The unfractionated (including both 6.5^+ and 6.5^- cells) population of $CD4^+CD25^+Foxp3^+$ cells from TS1xHACII mice contained a larger population of cells that expressed high levels of CTLA-4 and GITR than $CD4^+CD25^+Foxp3^+$ cells from healthy mice (single transgenic HACII or BALB/c) (Figure 3-3 D).

Together, these data show that TS1xHACII mice of all ages contain normal frequencies of $CD4^+CD25^+Foxp3^+$ cells. Moreover, a subset of the

$CD4^+CD25^+Foxp3^+$ cells expresses the 6.5 TCR, which confers specificity for the target autoantigen that initiates arthritis development in TS1xHACII mice, and appeared activated in comparison to $6.5^-CD4^+CD25^+Foxp3^+$ cells.

3.2.3 $CD4^+CD25^+$ cells from TS1xHACII mice possess *in vitro* suppressor function

We conducted *in vitro* suppression assays to determine whether $CD4^+CD25^+$ cells from TS1xHACII mice possessed regulatory function. CFSE-labeled $CD4^+$ T cells were cultured alone or with FACS-purified $CD4^+CD25^+$cells from TS1xHACII mice at a 1:2 Treg:responder cell ratio and stimulated with soluble anti-CD3. Three days later we examined the CFSE division profile and cytokine production of the responder $CD4^+$ T cells. In the presence of TS1xHACII $CD4^+CD25^+$ cells, a greater percentage of responder $CD4^+$ T cells remained undivided, and the cells that did proliferate underwent fewer rounds of division (Figure 3-4 A, left panel). Additionally, when cultured alone, 48.6 percent of responder $CD4^+$ T cells produced IFN-γ, in contrast to 13.2 percent when co-cultured with $CD4^+CD25^+$ cells from TS1xHACII mice (Figure 3-4 A, right panel). These data indicate that $CD4^+CD25^+$ cells from TS1xHACII mice can suppress proliferation and cytokine production by responder $CD4^+$ T cells.

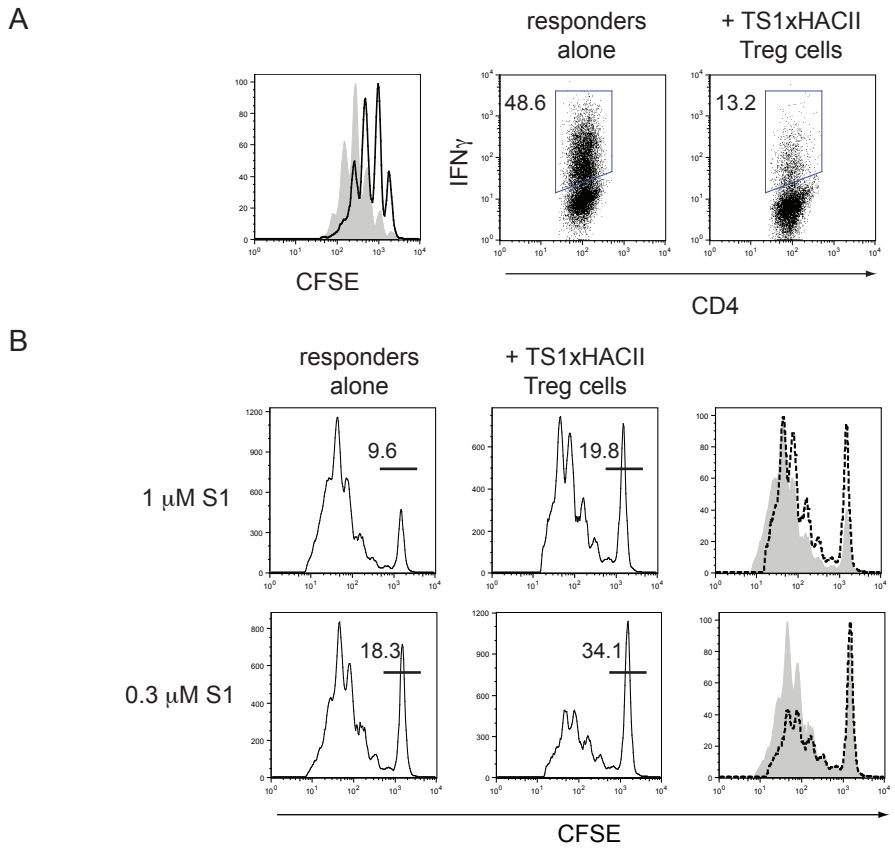

Figure 3-4 $CD4^+CD25^+$ cells in TS1xHACII mice possess *in vitro* suppressor function CFSE-labeled $CD4^+$ TS1 T cells were cultured with $CD4^+CD25^+$ T cells purified from TS1xHACII mice at a 1:2 Treg:responder cell ratio and stimulated with (**A**) soluble anti-CD3 (0.1 μg/ml) or (**B**) with a high (1 μM) or low (0.3 μM) dose of S1 peptide. (**A**) Three days later responder $CD4^+$ T cell proliferation and cytokine production were analyzed by flow cytometric analysis. Histogram shows the CFSE profiles of responder $CD4^+$ T cells when cultured alone (solid histogram) or in the presence of TS1xHACII $CD4^+CD25^+$ cells (black line). Dot plots show IFN-γ production by responder $CD4^+$ T cells cultured alone (left) or with TS1xHACII $CD4^+CD25^+$ cells (right). Numbers indicate the percentages of IFN-γ^+ cells. (**B**) Histograms show the CFSE profiles of responder $CD4^+$ T cells after three days of culture in the absence (left plots) or presence (middle plots) of TS1xHACII $CD4^+CD25^+$ cells. Numbers indicate the percentage of undivided cells. Histograms on the right show overlaid CFSE profiles of responder $CD4^+$ T cells cultured alone (solid gray) or with TS1xHACII $CD4^+CD25^+$ cells (dotted black line).

To examine whether $CD4^+CD25^+$ cells from TS1xHACII mice could exert suppressor function in response to stimulation by S1 peptide, we conducted *in vitro* suppression assays using S1 peptide to stimulate the cells. CFSE-labeled $CD4^+$ cells from TS1 mice were cultured with or without $CD4^+CD25^+$ cells from TS1xHACII mice with a high (1 μM) or low (0.3 μM) dose of S1 peptide as a stimulus. At the high dose of S1 peptide, the presence of $CD4^+CD25^+$ cells from TS1xHACII mice resulted in an increased percentage of undivided responder $CD4^+$ T cells (19.8 percent versus 9.6 percent when responder cells were cultured alone), but had little effect on the extent of division of proliferating cells (Figure 3-4 B, upper panel). In contrast, at the low dose of S1 peptide, co-culture of TS1xHACII $CD4^+CD25^+$ cells not only resulted in a higher representation of undivided cells (34.1 percent versus 18.3 percent), but also less accumulation of responder $CD4^+$ T cells at later cell divisions (Figure 3-4 B, lower panel).

These data show that $CD4^+CD25^+$ cells from TS1xHACII mice possess *in vitro* suppressor function. Moreover, regulatory activity (as defined by inhibition of responder $CD4^+$ T cell proliferation) of the $CD4^+CD25^+$ cells can be activated by interactions with S1 peptide.

3.2.4 $CD4^+CD25^+Foxp3^+$ cells are present in the joint-draining LNs of TS1xHACII mice

Since alterations in $CD4^+CD25^+$ Treg cell distribution can contribute to the development of autoimmune disease (Siegmund et al. 2005; Sather et al. 2007), we examined whether $CD4^+CD25^+Foxp3^+$ cells were equally distributed throughout the lymphoid organs of TS1xHACII mice. To assess this we isolated LNs that drain the lower joints (popliteal), upper limbs (axillary and brachial), as well as the spleen and other LNs (cervical, inguinal, and mesenteric) that are not primary joint-draining sites, and stained the cells for CD4, CD25, and Foxp3 expression. The different lymphoid organs contained comparable percentages of $CD4^+CD25^+Foxp3^+$ cells (Figure 3-5 A), indicating that the joint-draining LNs were not deficient in $CD4^+CD25^+Foxp3^+$ cells. Work in other autoimmune disease systems suggests that draining LNs are enriched for target organ antigen-specific $CD4^+CD25^+$ Treg cells (Samy et al. 2005; Samy et al. 2008; Wheeler et al. 2009), and we wanted to examine whether the representation of $6.5^+CD4^+CD25^+Foxp3^+$ cells (which are specific for the arthritis initiating antigen) was enhanced in the popliteal LNs of TS1xHACII mice. To address this, we compared the distribution of total $CD4^+CD25^+Foxp3^+$ and $6.5^+CD4^+CD25^+Foxp3^+$ cells in the popliteal and non-draining LNs of TS1xHACII mice. Interestingly, while the distribution of total $CD4^+CD25^+Foxp3^+$ cells was similar in draining and

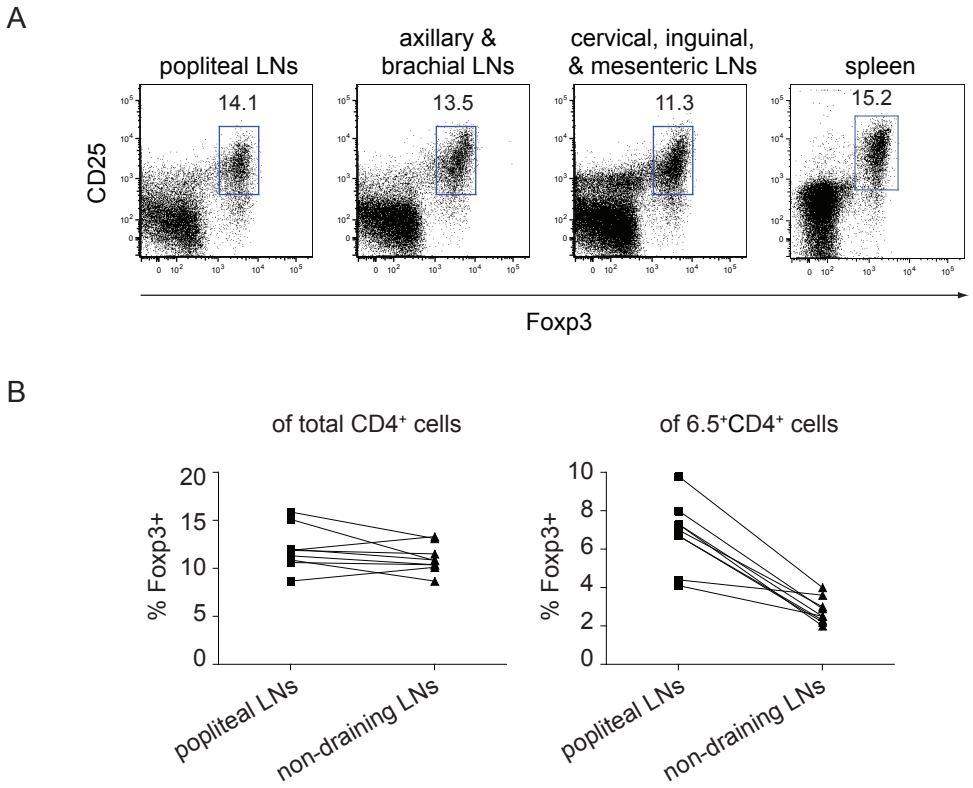

Figure 3-5 6.5$^+$CD4$^+$CD25$^+$Foxp3$^+$ cells are enriched in the popliteal LNs of TS1xHACII mice (**A**) Foxp3 and CD25 expression in CD4$^+$ cells from joint-draining and non-draining lymphoid organs in TS1xHACII mice. Numbers indicate the percentage of CD25$^+$Foxp3$^+$ cells within the CD4$^+$ gate. (**B**) Graphs show the percentages of Foxp3$^+$ cells within total CD4$^+$ (left graph) or 6.5$^+$CD4$^+$ (right graph) cells in the popliteal and non-draining (cervical, inguinal, and mesenteric) LNs in TS1xHACII mice. Two symbols connected by a line represent data from one mouse.

non-draining LNs (Figure 3-5, left panel), $6.5^+CD4^+CD25^+Foxp3^+$ cells were enriched in the popliteal LNs (Figure 3-5, right panel).

Together, these data demonstrate that unfractionated (including both 6.5^+ and 6.5^- cells) $CD4^+CD25^+Foxp3^+$ cells are present at similar frequencies in draining and non-draining LNs in TS1xHACII mice. However, the presence of $6.5^+CD4^+CD25^+Foxp3^+$ cells, which are specific for the disease initiating target antigen, is enriched in the popliteal LNs.

3.2.5 Polyclonal $CD4^+CD25^+$ Treg cells can prevent arthritis development in TS1xHACII mice

We have shown that TS1xHACII mice develop arthritis despite the presence of endogenous $CD4^+CD25^+Foxp3^+$ cells (including a population that is specific for the disease initiating target antigen) that are present in the joint draining LNs and possess *in vitro* suppressor function. It is possible that the arthritis that develops in TS1xHACII mice is refractory to Treg cell activity, and to address this we examined whether arthritis could be modified by the administration of exogenous $CD4^+CD25^+$ Treg cells prior to disease onset. To test this, we purified $CD4^+CD25^+$ cells from HACII mice, which express the HA transgene (under the same MHC II I-Eα promoter as TS1xHACII mice) without

any additional transgenic modifications to the TCR repertoire, and are isogenic with respect to the self peptides that are present in TS1xHACII mice. Thus, although $CD4^+CD25^+$ cells from HACII mice do not contain any 6.5^+Foxp3^+ cells (Figure 3-6 A), the $CD4^+CD25^+Foxp3^+$ cells present in HACII mice have developed in the presence of HA as a self-antigen.

We adoptively transferred $1x10^6$ $CD4^+CD25^+$ cells purified from HACII mice into pre-arthritic TS1xHACII mice and tracked the recipients weekly for disease development. In comparison to a cohort of unmanipulated TS1xHACII mice, mice that received HACII $CD4^+CD25^+$ cells showed a marked reduction in arthritis development (Figure 3-6 B). In 15 week old TS1xHACII mice, arthritis incidence was significantly lower in TS1xHACII mice that received HACII $CD4^+CD25^+$ cells (18.2 percent) than in unmanipulated TS1xHACII mice (80 percent) (*p=0.0089, Fisher's exact test) (Figure 3-6 C). Moreover, among arthritic mice, the number of affected limbs was lower in TS1xHACII mice that received HACII $CD4^+CD25^+$ cells (one affected limb) in comparison to unmanipulated TS1xHACII mice (which had three to four affected limbs) (Figure 3-6 D). Therefore, the average number of affected limbs per mouse was significantly lower in TS1xHACII mice that received HACII $CD4^+CD25^+$ cells than in unmanipulated TS1xHACII mice (***p<0.0001, Mann-Whitney).

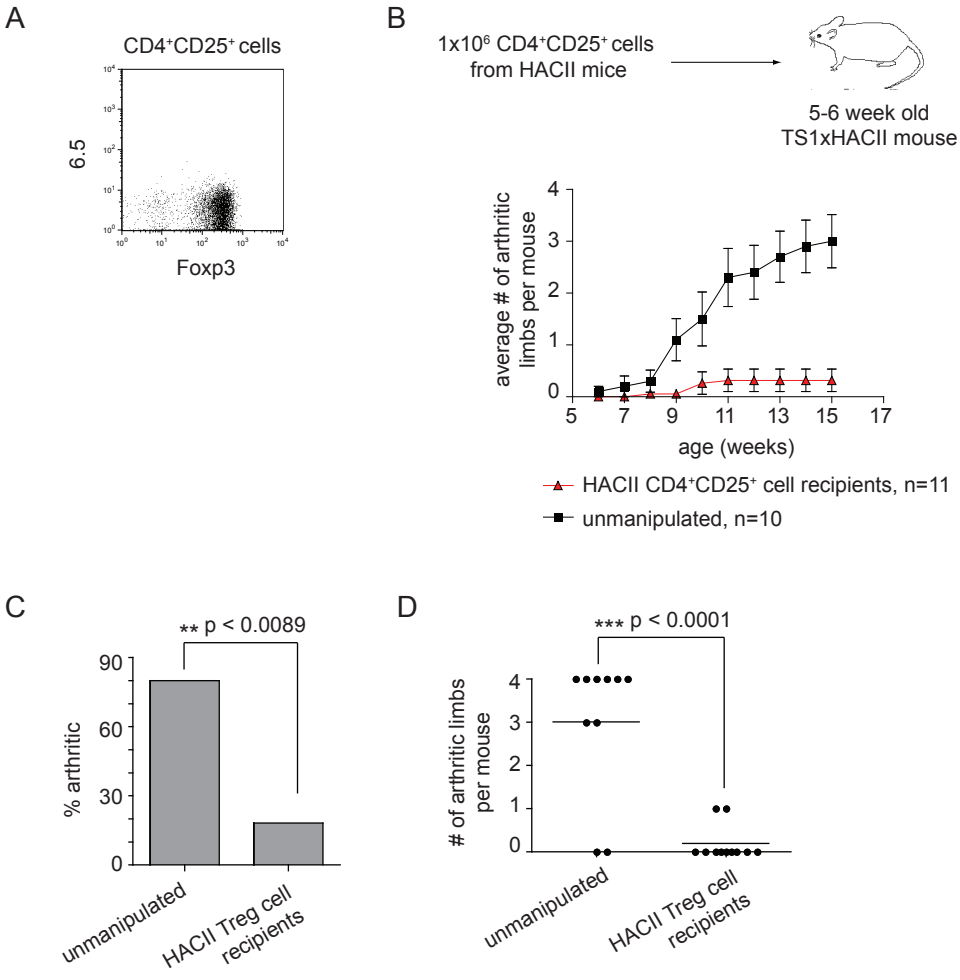

Figure 3-6 The adoptive transfer of CD4$^+$CD25$^+$ cells from HACII mice can prevent arthritis development in TS1xHACII mice (**A**) Dot plots show 6.5 and Foxp3 staining of CD4$^+$CD25$^+$ LN cells from HACII mice. (**B**) 1x10^6 HACII CD4$^+$CD25$^+$ cells were adoptively transferred into 5 to 6 week old TS1xHACII mice. Graph shows the average number of arthritic limbs per mouse (±SEM) over time of unmanipulated (■, n=10) and HACII CD4$^+$CD25$^+$ cell recipient (▲, n=11) TS1xHACII mice. (**C**) Arthritis incidence in 15 week old unmanipulated or HACII CD4$^+$CD25$^+$ cell recipient TS1xHACII mice. Statistical significance was calculated using Fisher's exact test. (**D**) The number of arthritic limbs per mouse in cohorts of unmanipulated and HACII CD4$^+$CD25$^+$ cell recipient TS1xHACII mice. Each circle represents an individual mouse. Statistical significance was calculated by the Mann-Whitney test.

These data show that the administration of exogenous $CD4^+CD25^+$ cells can prevent arthritis in TS1xHACII mice, demonstrating that the arthritis that develops in TS1xHACII mice can be modified by $CD4^+CD25^+$ Treg cell activity.

3.2.6 Protective $CD4^+CD25^+$ Treg cells do not alter the accumulation or activation of 6.5^+CD4^+ T cells in TS1xHACII mice

To determine whether the accumulation and activation of 6.5^+CD4^+ T cells were altered in TS1xHACII mice that received protective $CD4^+CD25^+$ cells from HACII mice (from here on referred to as protected TS1xHACII mice), we examined the number and phenotype of $CD4^+$ T cells in unfractionated systemic LNs (axillary, brachial, cervical, inguinal, and mesenteric) of unmanipulated TS1xHACII mice, and of mice that received HACII $CD4^+CD25^+$ cells. There was little difference in the total cellularity of in the numbers of $CD4^+$ cells in the LNs of arthritic versus protected TS1xHACII mice (Figure 3-7 A). Additionally, the administration of $CD4^+CD25^+$ cells from HACII mice did not affect the accumulation of peripheral 6.5^+CD4^+ T cells, as unmanipulated arthritic and protected TS1xHACII mice contained comparable numbers of 6.5^+CD4^+ cells (Figure 3-7 B). To examine whether HACII $CD4^+CD25^+$ Treg cells altered the activation phenotype of $CD4^+$ T cells in TS1xHACII mice, we analyzed the expression of CD62L, CD44, and CD69 on both 6.5^+CD4^+ and 6.5^-CD4^+ T cells.

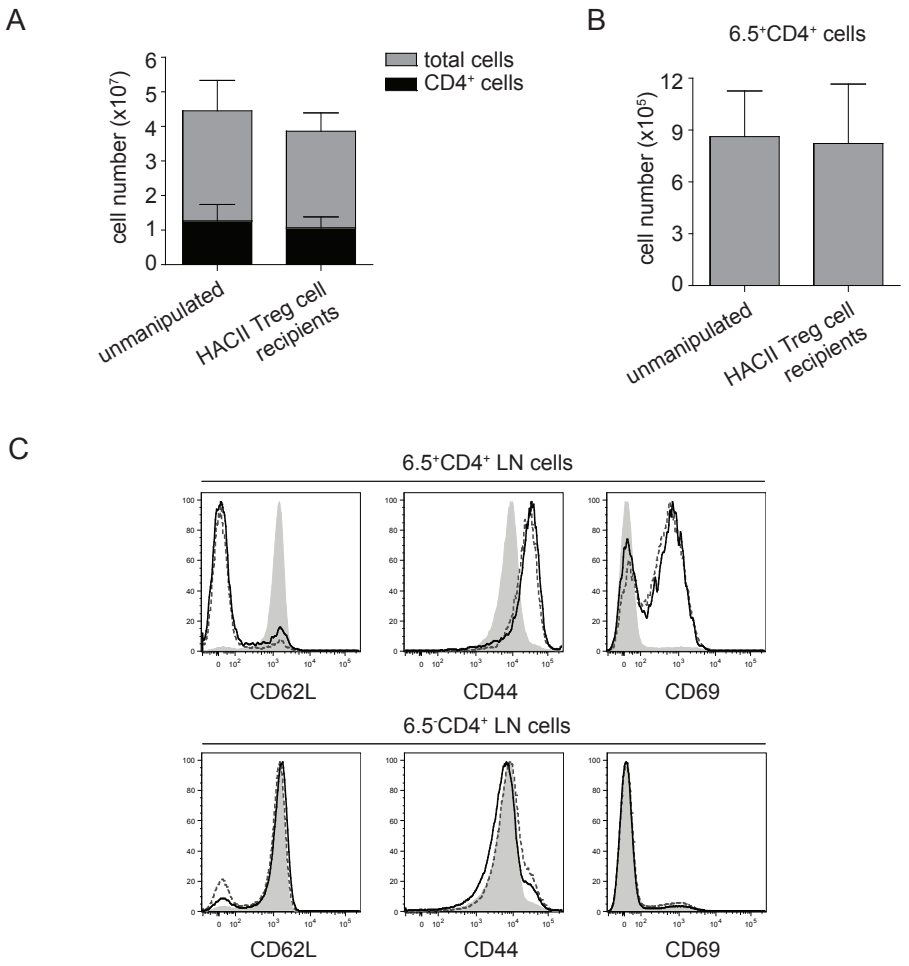

Figure 3-7 Protective HACII CD4$^+$CD25$^+$ Treg cells do not inhibit the accumulation or activation of 6.5$^+$CD4$^+$ T cells in TS1xHACII mice. (**A**) Numbers of total (gray bar) and CD4$^+$ (black bar) cells in the systemic LNs (axillary, brachial, cervical, inguinal, mesenteric) of unmanipulated and HACII CD4$^+$CD25$^+$ Treg cell recipient TS1xHACII mice. n=11 per group. (**B**) Numbers of 6.5$^+$CD4$^+$ T cells in the systemic LNs of unmanipulated and HACII CD4$^+$CD25$^+$ Treg cell recipient TS1xHACII mice. n=11 per group. (**C**) CD62L, CD44, and CD69 expression on 6.5$^+$ (upper panel) and 6.5$^-$ (lower panel) CD4$^+$ cells from unmanipulated TS1xHACII (dashed line), HACII CD4$^+$CD25$^+$ Treg cell recipient TS1xHACII (solid line), or control TS1 (solid histogram) mice. Bar graphs show average values ± SEM.

In comparison to control $6.5^{+}CD4^{+}$ T cells from TS1 mice (which are not exposed to HA as a self-antigen), $6.5^{+}CD4^{+}$ T cells from both arthritic and protected TS1xHACII mice appeared activated, and possessed very similar profiles of CD62L, CD44, and CD69 expression (Figure 3-7 C, upper panel). In contrast, the expression patterns of CD62L, CD44, and CD69 on $6.5^{-}CD4^{+}$ T cells from arthritic and protected TS1xHACII mice were nearly identical to those of $6.5^{-}CD4^{+}$ T cells from single transgenic TS1 mice (Figure 3-7 C, lower panel), and reflected a phenotype consistent with that of naïve cells.

Taken together, these data indicate that HACII $CD4^{+}CD25^{+}$ Treg cells do not prevent arthritis development by inhibiting the accumulation or activation of peripheral 6.5^{+} and 6.5^{-} $CD4^{+}$ T cells in TS1xHACII mice.

3.2.7 Preferential reduction of IL-17 in the popliteal LNs of TS1xHACII mice that received protective $CD4^{+}CD25^{+}$ Treg cells

We analyzed protected TS1xHACII mice to determine whether HACII $CD4^{+}CD25^{+}$ Treg cells modified the accumulation of popliteal LN cells that normally occurs in TS1xHACII mice (Figure 3-2 E). TS1xHACII mice that received HACII $CD4^{+}CD25^{+}$ cells contained significantly lower numbers of total popliteal LN cells (*p=0.018, Student's t-test), as well as lower, though not

statistically significant (p=0.06, Student's t-test), numbers of $CD4^+$ cells (Figure 3-8 A). Although the numbers of popliteal LN $CD4^+$ cells were not significantly reduced in TS1xHACII mice that received HACII $CD4^+CD25^+$ cells, $CD4^+$ cells in protected TS1xHACII mice could possess altered effector properties, and to examine this we analyzed intracellular cytokine production by popliteal LN $CD4^+$ T cells in arthritic and protected TS1xHACII mice. Popliteal LN cells were stimulated for four hours with PMA and ionomycin, and then stained for CD4 and intracellular IL-17 and IFN-γ expression. Strikingly, TS1xHACII mice that received HACII $CD4^+CD25^+$ cells exhibited a reduction in the representation of IL-17, but not IFN-γ, producing $CD4^+$ popliteal LN cells when compared to unmanipulated TS1xHACII mice (Figure 3-8 B). Whereas the difference in the percentages of IL-17^+CD4^+ popliteal LN cells was significantly lower in TS1xHACII mice that received HACII $CD4^+CD25^+$ cells than in unmanipulated TS1xHACII mice (*p=0.04, Student's t-test), the representation of IFN-γ producing cells was comparable between the two groups (Figure 3-8 C). However, numbers of both IFN-γ^+CD4^+ (*p=0.04, Student's t-test) and IL-17^+CD4^+ (*p=0.02, Student's t-test) cells were significantly lower in TS1xHACII mice that received HACII $CD4^+CD25^+$ cells than in unmanipulated TS1xHACII mice, consistent with the reduced total cellularity of the popliteal LNs.

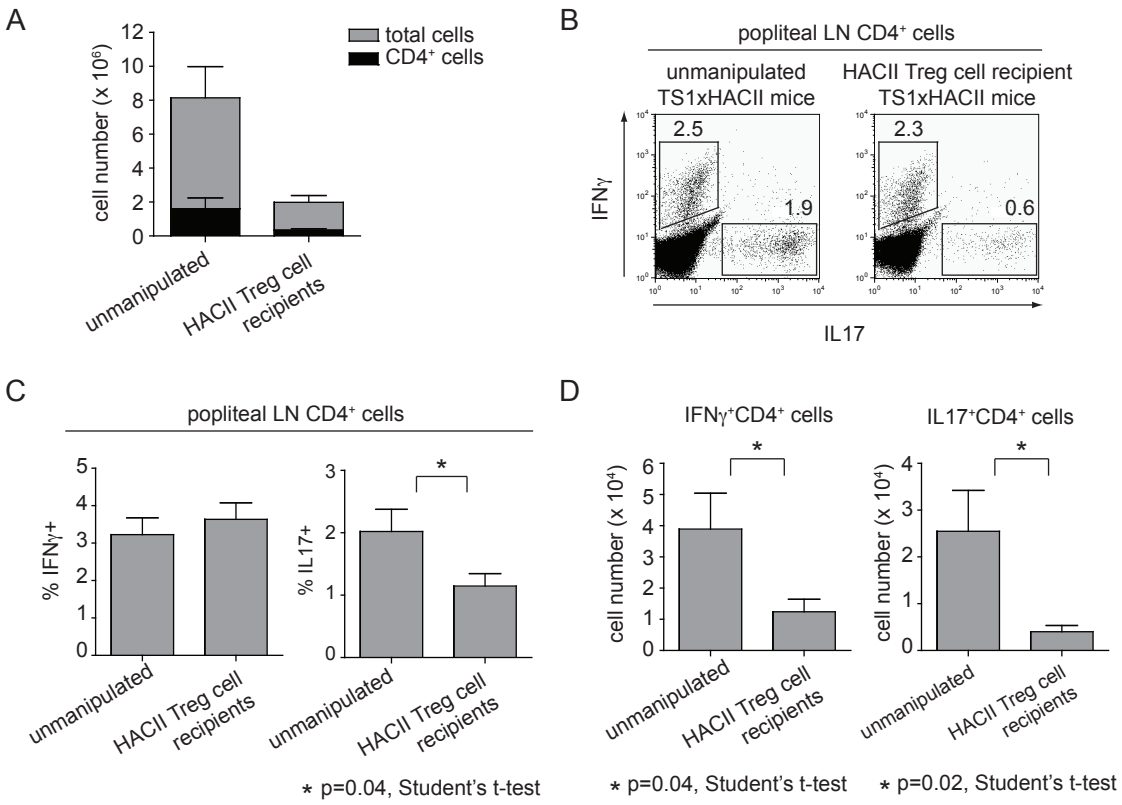

Figure 3-8 Protective HACII CD4$^+$CD25$^+$ Treg cells inhibit Th-17 cells in the popliteal LNs of TS1xHACII mice (**A**) Numbers of total (gray bar) and CD4$^+$ (black bar) cells in the popliteal LNs of unmanipulated and HACII CD4$^+$CD25$^+$ Treg cell recipient TS1xHACII mice. (total cells, *p=0.018; CD4$^+$ cells, p=0.06). (**B**) Popliteal LN cells were stimulated for 4 hours with PMA and ionomycin and stained for intracellular IL-17 and IFN-γ. Dot plots show IL-17 and IFN-γ production by CD4$^+$ T cells from unmanipulated and HACII CD4$^+$CD25$^+$ Treg cell recipient mice. Numbers indicate the percentage of cytokine-producing cells in each gate. (**C**) Average percentages of IFN-γ and IL-17 producing CD4$^+$ T cells in the popliteal LNs of unmanipulated and HACII CD4$^+$CD25$^+$ Treg cell recipient TS1xHACII mice. (**D**) Numbers of IFN-γ$^+$CD4$^+$ and IL-17$^+$CD4$^+$ cells in the popliteal LNs of unmanipulated and HACII CD4$^+$CD25$^+$ Treg cell recipient TS1xHACII mice. Bar graphs show average values ± SEM. Statistical significance was calculated using the Student's t-test. n=11 per group for all data.

Together, these data show that the administration of HACII $CD4^+CD25^+$ Treg cells limits the accumulation of cells in the popliteal LNs of TS1xHACII mice. Interestingly, the protective HACII $CD4^+CD25^+$ Treg cells appear to preferentially inhibit Th-17 cell formation, which has been associated with several autoimmune diseases, including arthritis (Afzali et al. 2007; Korn et al. 2009).

3.2.8 Protective $CD4^+CD25^+$ Treg cells suppress the systemic Th-17 response in TS1xHACII mice

To examine whether the transfer of HACII $CD4^+CD25^+$ cells suppressed systemic IL-17 production in TS1xHACII mice, we set up unfractionated LN (axillary, brachial, cervical, inguinal, and mesenteric) cultures with cells from arthritic and protected TS1xHACII mice. Three days later, supernatants were collected and analyzed for IL-17 production by ELISA. Unfractionated LN cultures from unmanipulated arthritic TS1xHACII mice produced significantly higher concentrations of IL-17 than LN cultures from healthy control TS1 mice (*p=0.025, Student's t-test) (Figure 3-9 A), indicating that increased levels of IL-17 accompany arthritis development. Interestingly, protected TS1xHACII mice exhibited reduced systemic IL-17 production, as unfractionated LN cultures from TS1xHACII mice that received HACII $CD4^+CD25^+$ cells produced significantly lower amounts of IL-17 in comparison to unmanipulated arthritic TS1xHACII mice

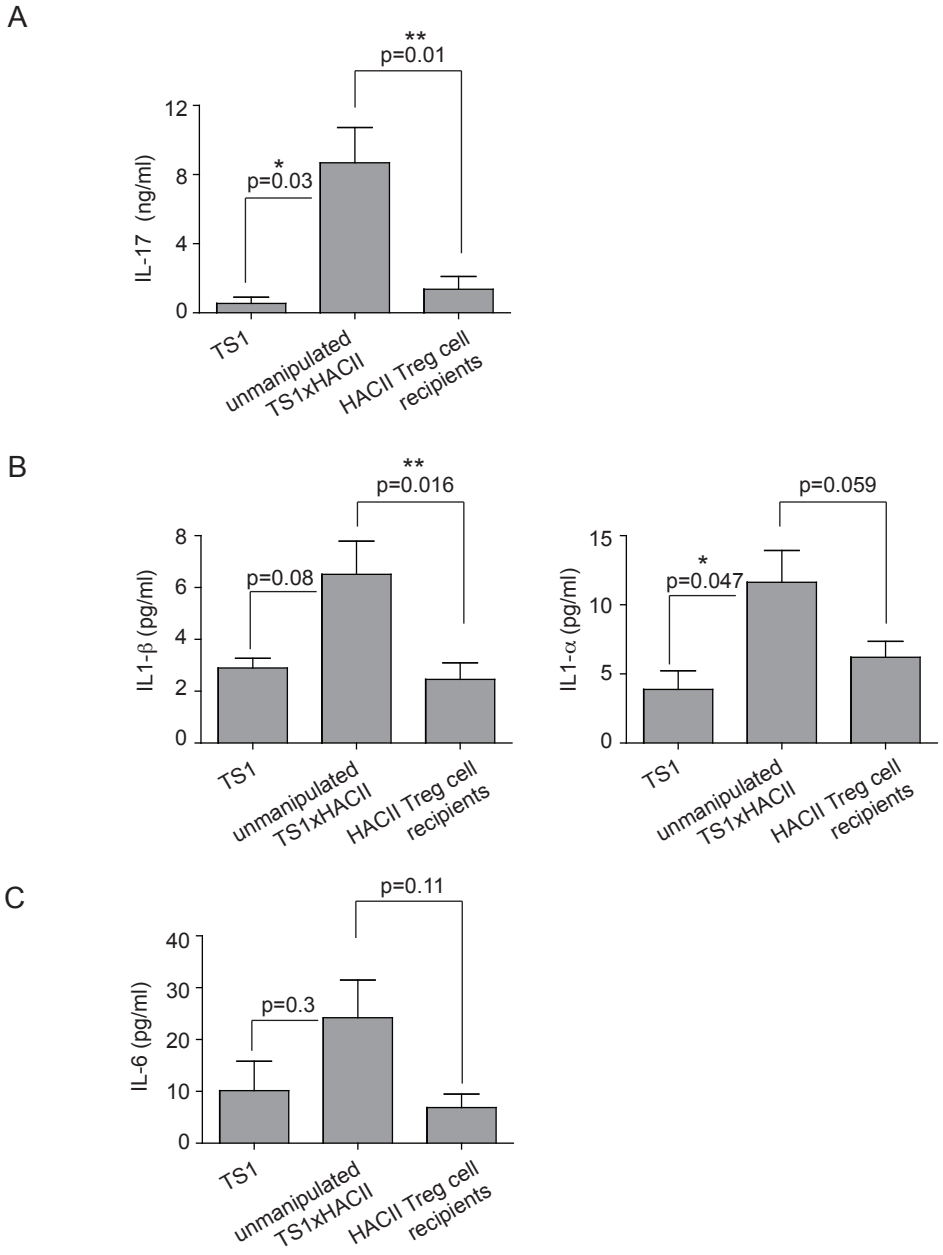

Figure 3-9 Protective HACII CD4$^+$CD25$^+$ Treg cells inhibit systemic IL-17 production in TS1xHACII mice. Unfractionated LN (axillary, brachial, cervical, inguinal, and mesenteric) cells from unmanipulated TS1xHACII, HACII CD4$^+$CD25$^+$ Treg cell recipient TS1xHACII, or control TS1 mice were cultured for three days, and supernatants were collected and analyzed for (**A**) IL-17, (**B**) IL-1β and IL-1α, and (**C**) IL-6. Statistical signficance was calculated using the Student's t-test. All TS1xHACII mice, n=5 per group. TS1 mice, n=3 per group.

(*p=0.01, Student's t-test) (Figure 3-9 A), again suggesting that IL-17 production by LN cells serves as a correlate for disease status. Thus, HACII $CD4^+CD25^+$ cells not only reduce IL-17 production in the popliteal LNs (Figure 3-8 C), but also reduce systemic IL-17 production in TS1xHACII mice.

To examine whether HACII $CD4^+CD25^+$ cells could also suppress cytokines involved in the differentiation of IL-17 producing cells, we also analyzed unfractionated LN culture supernatants for cytokines associated with Th-17 development (i.e. IL-1 and IL-6). LN cultures from protected TS1xHACII mice produced significantly lower levels of IL-1β than LN cultures from arthritic TS1xHACII mice (*p=0.016, Student's t-test), and also lower, but not statistically significant, levels of IL-1α (p=0.059, Student's t-test) (Figure 3-9 B). Whereas IL-1α concentrations were significantly higher in LN cultures from unmanipulated TS1xHACII mice than LN cultures from control TS1 mice (*p=0.047, Student's t-test), the difference in IL-1β levels was not significantly increased (p=0.08, Student's t-test). Therefore, although administration of HACII $CD4^+CD25^+$ cells results in significant downregulation of IL-1β levels in unfractionated LN cultures from TS1xHACII mice, unlike IL-17, production of IL-1β may not correlate with disease status. Unfractionated LN cultures from TS1xHACII mice that received HACII $CD4^+CD25^+$ cells did not produce significantly lower levels of IL-6 than LN cultures from unmanipulated TS1xHACII mice (p=0.11, Student's t-test) (Figure

3-9 C). Additionally, the difference in IL-6 concentrations between LN cultures from arthritic TS1xHACII and control TS1 mice did not reach statistical significance (p=0.3, Student's t-test).

Collectively, these data demonstrate that the administration of HACII $CD4^+CD25^+$ cells inhibits systemic production of IL-17 and also IL-1β, which has been linked to Th-17 differentiation (Sutton et al. 2006; Acosta-Rodriguez et al. 2007; Koenders et al. 2008). The amount of IL-17 produced by LN cultures appeared to be a direct correlate to the disease status of TS1xHACII mice, suggesting that this cytokine may be important for the development of arthritis.

3.2.9 IL-17 is critical for the development of spontaneous autoimmune arthritis in TS1xHACII mice

The finding that arthritic TS1xHACII mice produced significantly higher amounts of IL-17 than both healthy control TS1 mice (Figure 3-9 A) and protected TS1xHACII mice that received HACII $CD4^+CD25^+$ Treg cells (Figure 3-8 C and Figure 3-9 A), suggested that increased IL-17 production correlates with arthritis in TS1xHACII mice. To determine whether IL-17 is critical for arthritis development in TS1xHACII mice, we treated TS1xHACII mice with either anti-IL17 (blocks IL-17A, clone M210, Amgen) or an isotype control antibody and

tracked the mice weekly for disease development. Blockade of IL-17 dramatically reduced arthritis incidence in TS1xHACII mice, as only 8.3 percent (1 of 12) of anti-IL17 antibody treated TS1xHACII mice developed disease in comparison to 100 percent of isotype control treated TS1xHACII mice (**p=0.004, Fisher's exact test) (Figure 3-10 A). Given that all of the isotype control treated TS1xHACII mice had three or four arthritic limbs, whereas only one anti-IL17 antibody treated TS1xHACII mouse developed arthritis (Figure 3-10 B), the average number of arthritic limbs per mouse in anti-IL17 treated TS1xHACII mice was significantly lower than in unmanipulated TS1xHACII mice (**p=0.001, Mann-Whitney).

These data clearly demonstrate that IL-17 activity is critical for arthritis development in TS1xHACII mice. Moreover, this finding indicates that the reduction in regional and systemic IL-17 production observed in TS1xHACII mice that received HACII $CD4^+CD25^+$ cells (Figure 3-8 C and 3-9 A), may be the key mechanism by which protective $CD4^+CD25^+$ Treg cells can prevent arthritis development in TS1xHACII mice.

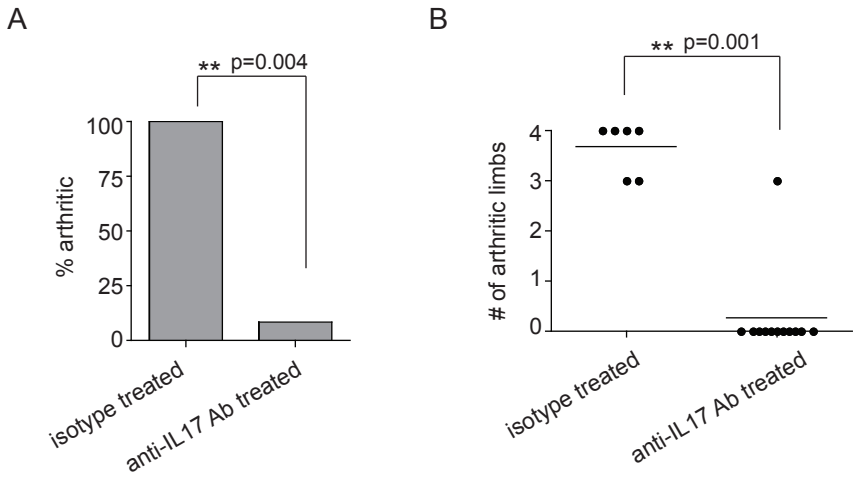

Figure 3-10 IL-17 is critical for the development of arthritis in TS1xHACII mice (**A**) Arthritis incidence in 14 week old TS1xHACII mice that were treated with anti-IL17 antibody (n=12) or an isotype control antibody (n=6). Statistical significance was calculated using Fisher's exact test. (**B**) The numbers ofarthritic limbs per mouse in 14 week old TS1xHACII mice treated with anti-IL17 or isotype control antibodies. Each circle represents an individual mouse. Line represents the average number of arthritic limbs. Statistical significance was calculated using the Mann-Whitney test.

3.2.10 $CD4^+CD25^+$ cells from TS1xHACII mice can modify arthritis development if $6.5^+CD4^+CD25^+$ cells are depleted

The ability of exogenously administered HACII $CD4^+CD25^+$ cells to prevent arthritis development in TS1xHACII mice (Figure 3-6) demonstrates that arthritis in TS1xHACII mice can be modulated by $CD4^+CD25^+$ Treg cell activity, and raises the question of why the endogenous $CD4^+CD25^+Foxp3^+$ cells in TS1xHACII mice fail to prevent arthritis. One possibility is that despite containing a normal percentage of $CD4^+CD25^+Foxp3^+$ cells in arthritic TS1xHACII mice (Figure 3-3 A), pre-arthritic TS1xHACII mice may not contain sufficient numbers of the cells to prevent arthritis from developing (Figure 3-2 D). Thus, we examined whether increasing the $CD4^+CD25^+$ Treg cell population by administering $CD4^+CD25^+$ cells isolated from other TS1xHACII mice to pre-arthritic TS1xHACII mice would be able to prevent arthritis development. However, $CD4^+CD25^+$ cells from TS1xHACII mice contain a population of 6.5^+ cells, which comprise $Foxp3^+$ and $Foxp3^-$ cells (Figure 3-3 B). Given that recognition of S1 peptide by 6.5^+CD4^+ T cells is critical for disease initiation in TS1xHACII mice, we decided to examine the effects of administering both unfractionated (i.e. contains both 6.5^+ and 6.5^- cells) and 6.5-depleted TS1xHACII $CD4^+CD25^+$ cells on arthritis development in TS1xHACII mice.

To address this we purified $CD4^+CD25^+$ cells from TS1xHACII mice that were either unfractionated or were depleted of 6.5^+ cells (Figure 3-11 A, left panel). We adoptively transferred $1x10^6$ of these unfractionated or 6.5-depleted TS1xHACII $CD4^+CD25^+$ cells into pre-arthritic TS1xHACII mice and tracked the recipients for arthritis development. Unmanipulated TS1xHACII mice, and mice that received unfractionated $CD4^+CD25^+$ TS1xHACII cells averaged a similar number of arthritic limbs per mouse at each time point during the nine week period of analysis. However, TS1xHACII mice that received 6.5-depleted $CD4^+CD25^+$ TS1xHACII cells averaged fewer affected limbs per mouse (Figure 3-11 A, right panel). This lower average number of arthritic limbs could reflect either an overall reduction in arthritis incidence within the population, or similar disease incidence that is accompanied by arthritic TS1xHACII mice possessing fewer numbers of affected limbs, which is indicative of reduced disease severity in individual arthritic mice.

To further examine this we assessed the kinetics of arthritis incidence in unmanipulated, unfractionated TS1xHACII $CD4^+CD25^+$ cell recipient, and 6.5-depleted TS1xHACII $CD4^+CD25^+$ cell recipient TS1xHACII mice. Unmanipulated TS1xHACII mice and those that received unfractionated $CD4^+CD25^+$ TS1xHACII cells exhibited comparable kinetics in the development of disease incidence, and by 15 weeks of age over 90 percent of unmanipulated and 100 percent of

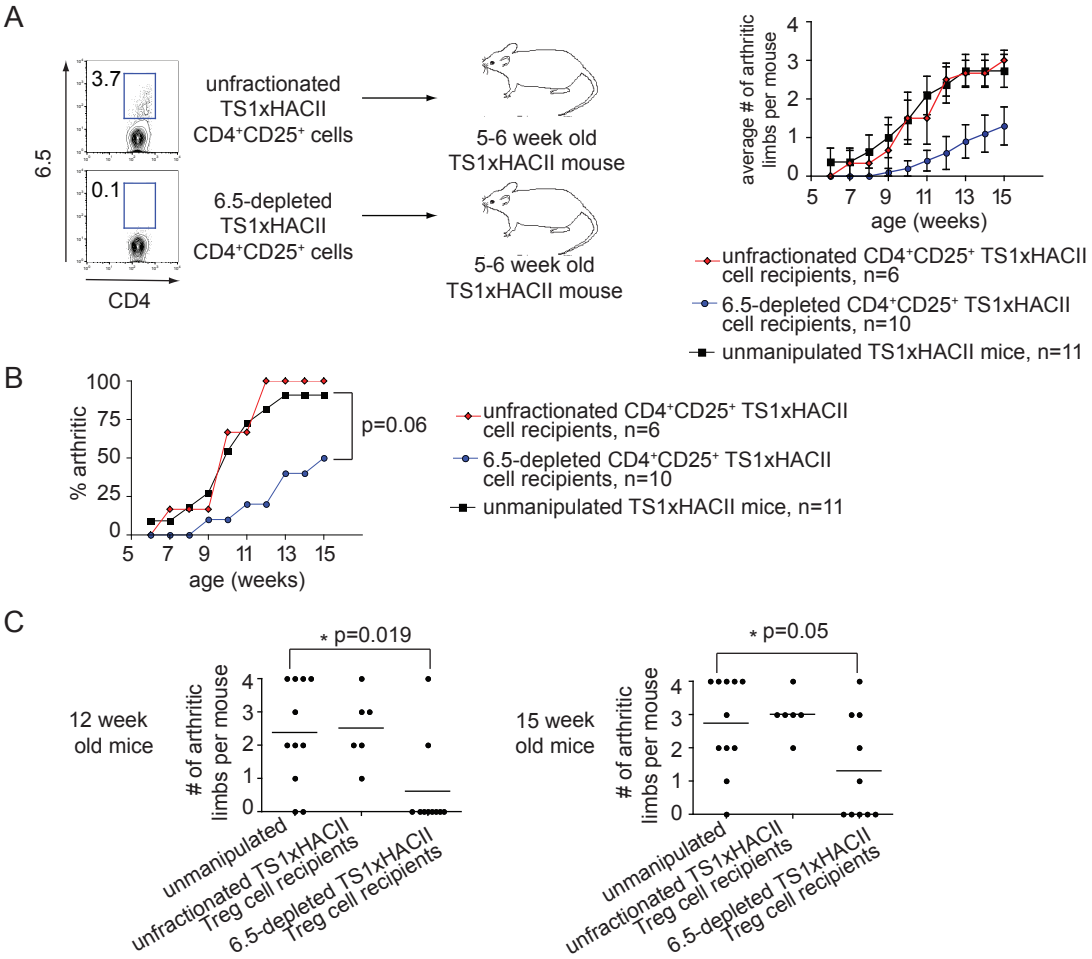

Figure 3-11 The adoptive transfer 6.5-depleted TS1xHACII CD4$^+$CD25$^+$ cells can modify arthritis in TS1xHACII mice (**A**) 1x10^6 unfractionated CD4$^+$CD25$^+$cells or 6.5$^-$CD4$^+$CD25$^+$ cells from TS1xHACII mice were adoptively transferred into pre-arthritic TS1xHACII mice. Dot plots show 6.5 staining in sorted CD4$^+$CD25$^+$ cells that were or were not depleted of 6.5$^+$ cells. Number indicates the percentage of 6.5$^+$ cells. Graph shows the average number of arthritic limbs per mouse (±SEM) over time of unmanipulated (■, n=11), unfractionated TS1xHACII CD4$^+$CD25$^+$cell recipient (▲, n=6), and 6.5-depleted TS1xHACII CD4$^+$CD25$^+$ cell recipient (●, n=10) TS1xHACII mice. (**B**) Arthritis incidence over time in unmanipulated, unfractionated TS1xHACII CD4$^+$CD25$^+$ cell recipient, and 6.5-depleted TS1xHACII CD4$^+$CD25$^+$ cell recipient TS1xHACII mice. Statistical significance was calculated using Fisher's exact test. (**C**) Graphs show the numbers of arthritic limbs per mouse in 12 week old (top) and 15 week old (bottom) unmanipulated, unfractionated TS1xHACII CD4$^+$CD25$^+$ cell recipient, and 6.5-depleted TS1xHACII CD4$^+$CD25$^+$ cell recipient TS1xHACII mice. Each circle represents an individual mouse. Line represents the average number of arthritic limbs per mouse. Statistical significance was calculated using the Mann-Whitney test.

unfractionated TS1xHACII $CD4^+CD25^+$ cell recipient mice had developed arthritis (Figure 3-11 B). In contrast, TS1xHACII mice that received 6.5-depleted TS1xHACII $CD4^+CD25^+$ cells showed much lower arthritis incidence at each time point when compared to unmanipulated TS1xHACII mice, and by 15 weeks of age only 50 percent of the mice had developed arthritis (Figure 3-11 B). However, the difference in arthritis incidence between unmanipulated TS1xHACII mice and TS1xHACII mice that received 6.5-depleted TS1xHACII $CD4^+CD25^+$ cells did not reach statistical significance ($p=0.06$, Fisher's exact test).

Moreover, the administration of unfractionated $CD4^+CD25^+$ TS1xHACII cells to TS1xHACII mice did not affect the number of affected limbs in arthritic mice. Among 12 week old arthritic TS1xHACII mice, 55.6 percent (5 of 9) of unmanipulated TS1xHACII mice and 50 percent (3 of 6) of unfractionated $CD4^+CD25^+$ TS1xHACII cell recipient TS1xHACII mice had three or four affected limbs (Figure 3-11 C, upper panel). By 15 weeks of age 60 percent (6 of 10) of arthritic unmanipulated TS1xHACII mice and 83.3 percent (5 of 6) of arthritic unfractionated $CD4^+CD25^+$ TS1xHACII cell recipient mice had three or four affected limbs (Figure 3-11 C, lower panel). Thus, there was little difference in the degree of arthritis incidence or in the numbers of affected limbs between unmanipulated TS1xHACII mice and mice that received unfractionated $CD4^+CD25^+$ TS1xHACII cells.

Among TS1xHACII mice that received 6.5-depleted $CD4^+CD25^+$ TS1xHACII cells, only 20 percent (2 of 10) were arthritic, with two or four affected limbs, by 12 weeks of age (Figure 3-11 C, upper panel). Furthermore, since the majority of 12 week old 6.5-depleted $CD4^+CD25^+$ TS1xHACII cell recipient mice were not arthritic, the average number of affected limbs was significantly lower than in unmanipulated TS1xHACII mice (*p=0.019, Mann-Whitney). By 15 weeks of age, 5 of 10 (50 percent) TS1xHACII mice that received 6.5-depleted $CD4^+CD25^+$ TS1xHACII cells were arthritic, and among arthritic animals 60 percent (3 of 5) had three or four affected limbs (Figure 3-11 C, lower panel). Although the average number of affected limbs in 15 week old TS1xHACII mice that received 6.5-depleted TS1xHACII $CD4^+CD25^+$ cells was greater than in 12 week old mice, the difference in the average number of arthritic limbs between 15 week old 6.5-depleted $CD4^+CD25^+$ TS1xHACII cell recipient and unmanipulated TS1xHACII mice remained significant (*p=0.05, Mann-Whitney).

Thus, the administration of $CD4^+CD25^+$ cells from TS1xHACII mice that were depleted of the 6.5^+ pool, but not unfractionated TS1xHACII $CD4^+CD25^+$ cells, could reduce arthritis incidence in TS1xHACII mice. Collectively, these data demonstrate that endogenous $CD4^+CD25^+$ cells from TS1xHACII mice possess *in vivo* regulatory activity and can modulate arthritis development in TS1xHACII

mice, but that their efficacy is contingent upon depletion of the $6.5^+CD4^+CD25^+$ population, which is a mixture of $Foxp3^+$ and $Foxp3^-$ cells.

3.2.11 Suppressor function of $CD4^+CD25^+$ Treg cells is highly sensitive to balance of Treg and effector cells

Depletion of 6.5 TCR expressing cells allows endogenous $CD4^+CD25^+$ cells from TS1xHACII mice to modulate arthritis development (Figure 3-11). Since the elimination of $6.5^+CD4^+CD25^+$ cells removes both $Foxp3^+$ and $Foxp3^-$ cells (Figure 3-3 B), the enhanced ability of 6.5-depleted TS1xHACII $CD4^+CD25^+$ cells to suppress arthritis could be explained by the absence of $6.5^+CD4^+CD25^+Foxp3^-$ cells specific for the disease initiating target antigen (S1 peptide). This result suggests that the efficacy of $CD4^+CD25^+$ Treg cell activity could be highly sensitive to the balance of Treg and effector cells. To further address this, we examined whether introducing a small population of conventional 6.5^+CD4^+ T cells to HACII $CD4^+CD25^+$ Treg cells could affect the ability of the protective Treg cells to suppress arthritis in TS1xHACII mice. We purified $CD4^+CD25^-$ cells from TS1xHA28 mice, which co-express the 6.5 TCR and the HA transgene under an SV40 promoter (Jordan et al. 2001), to obtain a source of potentially autoreactive 6.5^+CD4^+ T cells that have developed in the presence of HA, as $6.5^+CD4^+CD25^-$ cells in TS1xHA28 mice have evaded

negative selection despite exposure to HA in the thymus (Jordan et al. 2001). Approximately 30 percent of $CD4^+CD25^-$ cells from TS1xHA28 mice are 6.5^+ and $Foxp3^-$ (Figure 3-12 A).

We adoptively transferred a mixture of $1x10^6$ cells that contained 95 percent protective HACII $CD4^+CD25^+$ cells and 5 percent TS1xHA28 $CD4^+CD25^-$ cells (mixed cell) into pre-arthritic TS1xHACII mice and tracked the recipients for arthritis development. Strikingly, the incidence of arthritis was the same (62.5 percent) in 15 week old unmanipulated TS1xHACII mice and mice that received the mixture of HACII $CD4^+CD25^+$ cells and TS1xHA28 $CD4^+CD25^-$ cells (Figure 3-12 B). Notably, arthritis incidence was higher in TS1xHACII mice that received the mixture of protective HACII $CD4^+CD25^+$ cells and TS1xHA28 $CD4^+CD25^-$ cells than in TS1xHACII mice that received HACII $CD4^+CD25^+$ cells (Figure 3-12 B), although the difference between the two cohorts did not reach statistical significance ($p=0.07$, Fisher's exact test). The average number of arthritic limbs per mouse was also higher, though not statistically significant ($p=0.08$, Mann-Whitney), among TS1xHACII mice that received the mixture of protective HACII $CD4^+CD25^+$ cells and TS1xHA28 $CD4^+CD25^-$ cells than in TS1xHACII mice that received only HACII $CD4^+CD25^+$ cells (Figure 3-12 C).

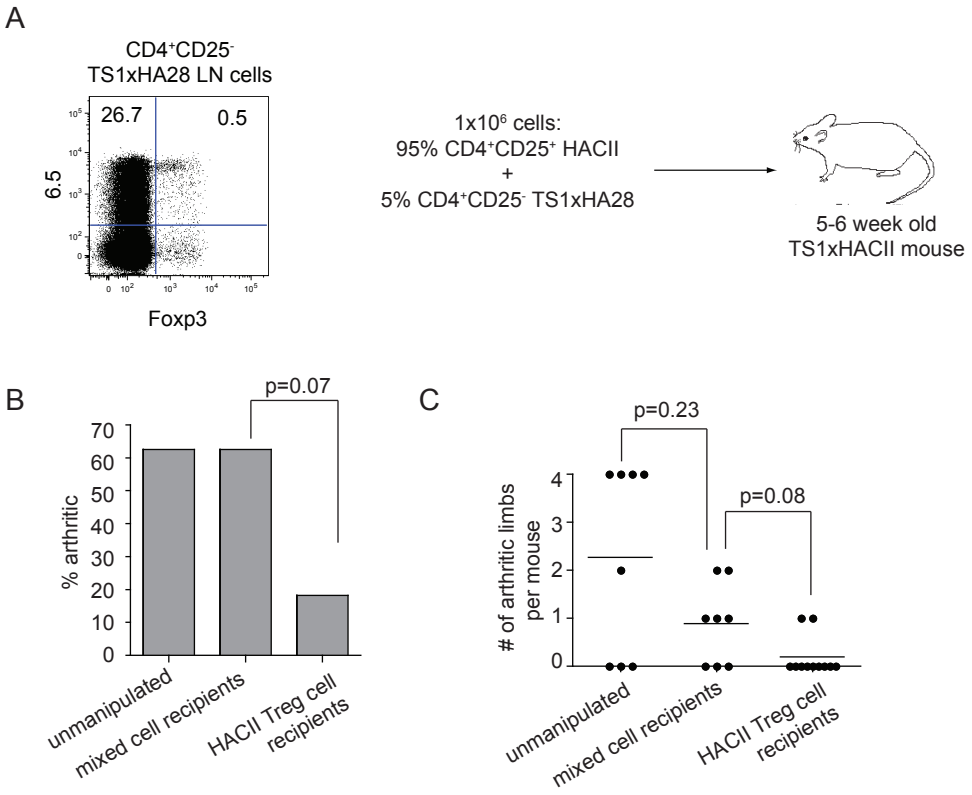

Figure 3-12 $CD4^+CD25^+$ Treg cell activity is sensitive to the balance of Treg and effector cells (**A**) 6.5 and Foxp3 staining in $CD4^+CD25^-$ LN cells from TS1xHA28 mice. Numbers indicate the percentage of cells in the respective quadrants. $1x10^6$ cells from a mixture of 95 percent HACII $CD4^+CD25^+$ and 5 percent TS1xHA28 $CD4^+CD25^-$ cells were adoptively transferred into pre-arthritic TS1xHACII mice. (**B**) Arthritis incidence in unmanipulated TS1xHACII mice, TS1xHACII mice that received the mixture of HACII and TS1xHA28 cells, and TS1xHACII mice that received only HACII $CD4^+CD25^+$ cells. Statistical significance was calculated using Fisher's exact test. (**C**) Graphs show the numbers of arthritic limbs per mouse in unmanipulated, HACII and TS1xHA28 mixed cell recipient, and HACII $CD4^+CD25^+$ Treg cell recipient TS1xHACII mice. Each circle represents an individual mouse. Line represents the average number of arthritic limbs per mouse. Statistical significance was calculated using the Mann-Whitney test.

Collectively, these data show that the presence of a small population of $CD4^+CD25^-$ cells enriched for expression of the 6.5 TCR can alter the ability of protective HACII $CD4^+CD25^+$ Treg cells to suppress arthritis in TS1xHACII mice. These results suggest that the efficacy of $CD4^+CD25^+$ Treg cells is remarkably sensitive to the balance of Treg and effector cells.

3.2.12 Polyclonal $CD4^+CD25^+$ Treg cells that have not developed in the presence of HA can prevent arthritis development in TS1xHACII mice

We have shown that the transfer of $CD4^+CD25^+$ cells purified from HACII mice to pre-arthritic TS1xHACII mice can prevent arthritis development in TS1xHACII mice (Figure 3-6). Furthermore, the adoptive transfer of endogenous $CD4^+CD25^+$ cells from TS1xHACII mice that have been depleted of $6.5^+CD4^+CD25^+$ cells can also ameliorate arthritis in TS1xHACII mice (Figure 3-11). Together, these data demonstrate that $CD4^+CD25^+$ cells that do not express the 6.5 TCR (which is specific for the disease initiating target antigen) can modify arthritis development in TS1xHACII. However, although the $CD4^+CD25^+$ cells from HACII or TS1xHACII mice do not express the 6.5 TCR, both cell populations have developed in the presence of S1 peptide as a self antigen. Therefore, it is possible that $CD4^+CD25^+$ cells from HACII and TS1xHACII mice contain a

population of S1-reactive cells, and that specificity for S1 peptide is required for Treg cell modulation of arthritis in TS1xHACII mice.

To examine whether $CD4^+CD25^+$ cells that have not developed in the presence of S1 peptide would also be able to prevent arthritis development in TS1xHACII mice, we purified $CD4^+CD25^+$ cells from BALB/c mice, which have the same genetic background as TS1xHACII mice, but contain no modifications to the TCR repertoire and do not express HA as a self-peptide. Thus, BALB/c $CD4^+CD25^+$ cells have not experienced the S1 peptide, which drives initiation of arthritis in TS1xHACII mice. As expected $CD4^+CD25^+$ LN cells from BALB/c mice express Foxp3 but no 6.5 (Figure 3-13 A). We adoptively transferred $1x10^6$ BALB/c $CD4^+CD25^+$ cells into pre-arthritic TS1xHACII mice and tracked the recipients for disease development. TS1xHACII mice that received BALB/c $CD4^+CD25^+$ cells exhibited a marked reduction in arthritis development in comparison to unmanipulated TS1xHACII mice (Figure 3-13 B). In contrast to the 80 percent (8 of 10) of unmanipulated TS1xHACII mice that developed arthritis, a significantly lower percentage (22 percent, 2 of 9) of BALB/c $CD4^+CD25^+$ cell recipient TS1xHACII mice were arthritic by 15 weeks of age

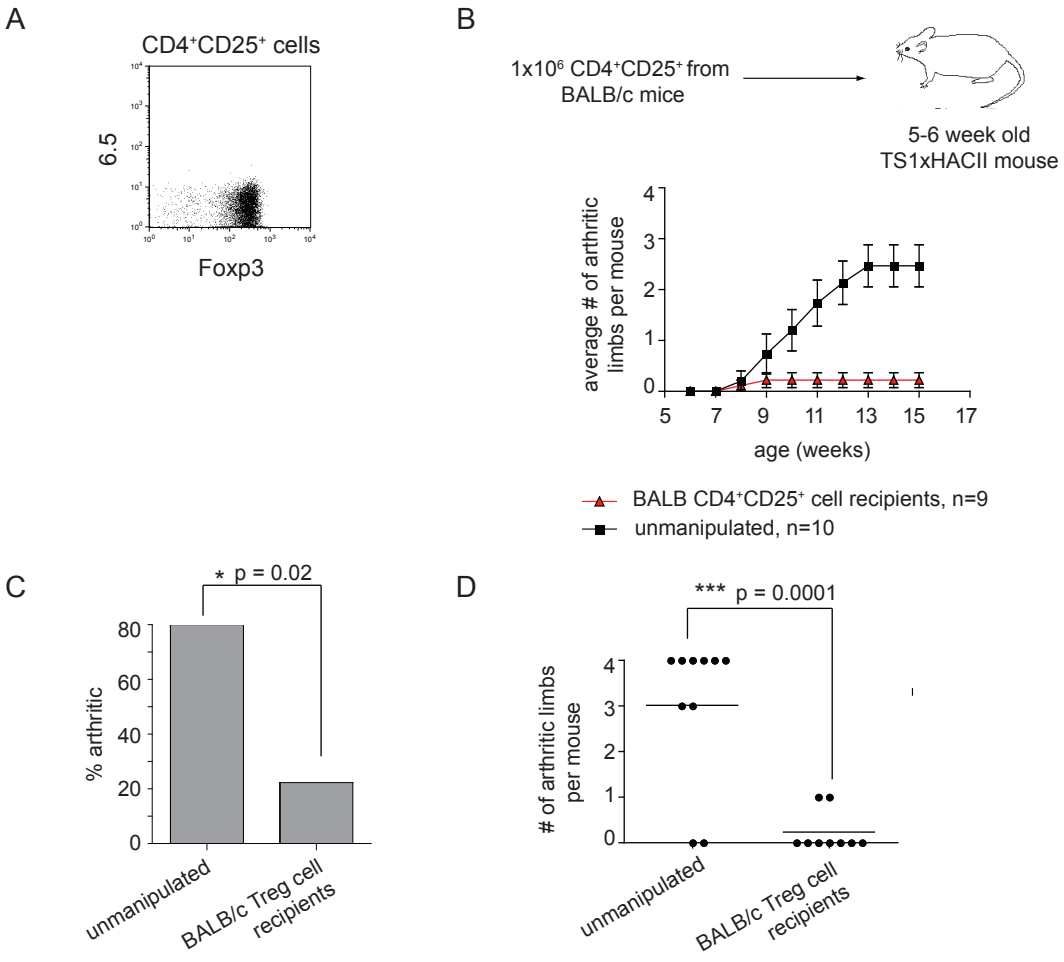

Figure 3-13 The adoptive transfer of $CD4^+CD25^+$ cells from BALB/c mice can prevent arthritis in TS1xHACII mice (**A**) Dot plots show 6.5 and Foxp3 staining of $CD4^+CD25^+$ LN cells from BALB/c mice. (**B**) $1x10^6$ BALB/c $CD4^+CD25^+$ cells were adoptively transferred into 5 to 6 week old TS1xHACII mice. Graph shows the average number of arthritic limbs per mouse (±SEM) over time of unmanipulated (■, n=10) and BALB/c $CD4^+CD25^+$ cell recipient (▲, n=9) TS1xHACII mice. (**C**) Arthritis incidence in 15 week old unmanipulated or BALB/c $CD4^+CD25^+$ cell recipient TS1xHACII mice. Statistical significance was calculated using Fisher's exact test. (**D**) The number of arthritic limbs per mouse in cohorts of unmanipulated and BALB/c $CD4^+CD25^+$ cell receipient TS1xHACII mice. Each circle represents an individual mouse. Line represents the average number of arthritic limbs per mouse. Statistical significance was calculated using the Mann-Whitney test.

(*p=0.02, Fisher's exact test) (Figure 3-13 C). Furthermore, similar to the observations in TS1xHACII mice that received HACII $CD4^+CD25^+$ cells, arthritic TS1xHACII mice that received BALB/c $CD4^+CD25^+$ cells had only one affected limb in comparison to arthritic unmanipulated TS1xHACII mice, which developed disease in three to four limbs (Figure 3-13 D). The reduction in the number of affected limbs among arthritic TS1xHACII mice coupled with the decrease in disease incidence, resulted in a significantly lower average number of arthritic limbs per mouse in TS1xHACII mice that received BALB/c $CD4^+CD25^+$ cells in comparison to unmanipulated TS1xHACII mice (***p=0.0001, Mann-Whitney).

These data demonstrate that $CD4^+CD25^+$ Treg cells that have not developed in the presence of the disease initiating target antigen can effectively suppress arthritis development in TS1xHACII mice.

3.3 Summary of Results and Discussion

3.3.1 TS1xHACII mice contain endogenous $CD4^+CD25^+Foxp3^+$ cells, including a population that expresses the 6.5 TCR and recognizes the disease initiating target antigen

A key feature of the TS1xHACII arthritis model is that we have defined the disease-initiating antigen. TS1xHACII mice on a $RAG^{-/-}$ background develop arthritis, demonstrating that recognition of the S1 peptide by 6.5^+CD4^+ T cells alone is sufficient to mediate disease (Rankin et al. 2008). Arthritis develops in TS1xHACII mice despite profound deletion of HA-specific thymocytes and is accompanied by the gradual accumulation of total $CD4^+$ and 6.5^+CD4^+ T cells in the systemic LNs (Figure 3-2 D). Interestingly, older, arthritic TS1xHACII mice and age-matched TS1 mice contain comparable numbers of total $CD4^+$ cells and 6.5^+CD4^+ cells in the popliteal LNs, which drain the lower joints, but not in systemic LNs (Figure 3-2 D and E). Moreover, the total cellularity of popliteal LNs was also higher in older, arthritic TS1xHACII mice than in age-matched TS1 mice (Figure 3-2 E), suggesting an increased presence of other immune system cells in addition to the accumulation of $CD4^+$ cells. The accumulation of 6.5^+CD4^+ T cells and arthritis development in TS1xHACII mice occurs despite the

presence of a normal frequency of $CD4^+CD25^+Foxp3^+$ cells in TS1xHACII mice of all ages (Figure 3-3 A). Furthermore, TS1xHACII mice also contain $6.5^+CD4^+CD25^+Foxp3^+$ cells, which are specific for the S1 peptide that initiates disease development.

We found that unfractionated (including both 6.5^- and 6.5^+ cells) $CD4^+CD25^+Foxp3^+$ cells from TS1xHACII mice and $CD4^+CD25^+Foxp3^+$ cells from healthy mice (HACII or BALB/c) had comparable patterns of CTLA-4 and GITR expression, indicating that the disease environment does not significantly alter the expression of these $CD4^+CD25^+$ Treg cell markers. CTLA-4 in particular has been shown to be an important mediator of $CD4^+CD25^+$ Treg cell function (Wing et al. 2008), and altered CTLA-4 regulation can be associated with dysfunctional $CD4^+CD25^+$ Treg cell activity in RA patients (Flores-Borja et al. 2008). Indeed, $CD4^+CD25^+Foxp3^+$ cells from TS1xHACII mice appeared to be more activated than their counterparts from healthy mice, as a larger subset of TS1xHACII $CD4^+CD25^+Foxp3^+$ cells expressed higher levels of CTLA-4 and GITR. Interestingly, we found that within the population of TS1xHACII $CD4^+CD25^+Foxp3^+$ cells, 6.5^+ cells expressed higher levels of both CTLA-4 and GITR in comparison to 6.5^- cells (Figure 3-3 C), suggesting that the S1-specific $6.5^+CD4^+CD25^+Foxp3^+$ cells may be more activated in response to interactions with their cognate antigen. Thus $CD4^+CD25^+Foxp3^+$ cells from TS1xHACII mice

are not deficient in classic $CD4^+CD25^+$ Treg cells markers such as CTLA-4 and GITR.

Although $CD4^+CD25^+Foxp3^+$ cells are present in TS1xHACII mice, it was possible that they possessed dysfunctional suppressor activity, as has been reported with $CD4^+CD25^+$ Treg cells isolated from human RA patients (Ehrenstein et al. 2004; Valencia et al. 2006). We found that unfractionated $CD4^+CD25^+$ cells from TS1xHACII mice were able to inhibit both the proliferation and cytokine production of responder $CD4^+$ T cells in response to stimulation with soluble anti-CD3 (Figure 3-4 A), demonstrating that the $CD4^+CD25^+$ cells that are present in TS1xHACII mice possess effective *in vitro* suppressor function. Furthermore, TS1xHACII $CD4^+CD25^+$ cells stimulated with S1 peptide could also inhibit the *in vitro* proliferation of responder $CD4^+$ T cells (Figure 3-4 B), indicating that the $CD4^+CD25^+$ cells could exert suppressor function in response to the disease-initiating target antigen. We found that the $CD4^+CD25^+$ cells from TS1xHACII mice were more effective at inhibiting responder $CD4^+$ T cell proliferation when a low dose (0.3 μM) of S1 peptide was used as a stimulus (Figure 3-4 B). However, it is possible that we are underestimating the regulatory activity of S1-responsive $CD4^+CD25^+$ cells, as we did not isolate $6.5^+CD4^+CD25^+$ cells from TS1xHACII mice for the suppression assays. Therefore, although we set up the assays using a 1:2 unfractionated TS1xHACII $CD4^+CD25^+$ cell to

responder cell ratio, the effective ratio of S1-specific $CD4^+CD25^+$ cells to responder cells is much lower. For a more accurate assessment of the *in vitro* suppressor function of $6.5^+CD4^+CD25^+$ cells from TS1xHACII mice, the suppression assay should be conducted using a 1:2 ratio of TS1xHACII $6.5^+CD4^+CD25^+$ cells to responder cells.

We also examined the presence $CD4^+CD25^+Foxp3^+$ cells in lymphoid organs throughout TS1xHACII mice, since alterations in $CD4^+CD25^+$ Treg cell distribution in lymphoid organs and other tissues can precede the development of autoimmune disease (Siegmund et al. 2005; Sather et al. 2007). Strikingly, while we did not observe differences in the distribution of unfractionated $CD4^+CD25^+Foxp3^+$ cells between the draining and non-draining LNs in TS1xHACII mice, we found that $6.5^+CD4^+CD25^+Foxp3^+$ cells were enriched in the popliteal LNs (which drain the lower joints and are the site of an enhanced immune response in arthritic mice) in comparison to non-draining LNs in TS1xHACII mice (Figure 3-5 B). Interestingly, studies have suggested that draining LNs may be enriched for $CD4^+CD25^+$ Treg cells that are specific for antigens derived from regional organs (Samy et al. 2005; Samy et al. 2008; Wheeler et al. 2009). Indeed, although S1 peptide is presented systemically in TS1xHACII mice, it is possible that there is increased presentation of HA-derived peptides in the popliteal LNs, which could lead to an enrichment of

$6.5^+CD4^+CD25^+Foxp3^+$ cells in the draining LNs. Thus, TS1xHACII mice develop spontaneous inflammatory arthritis despite the presence of $CD4^+CD25^+Foxp3^+$ cells (including a population of $6.5^+CD4^+CD25^+Foxp3^+$ cells that recognize the disease initiating target antigen) that are present in the LNs draining the joints, which are a primary site of autoimmune pathology.

3.3.2 Exogenous $CD4^+CD25^+$ Treg cells can inhibit the pathogenic Th-17 response in TS1xHACII mice

We have shown that the administration of exogenous $CD4^+CD25^+$ cells from HACII or BALB/c mice can prevent arthritis, demonstrating that the endogenous effector $CD4^+$ T cells (and other immune cells) that contribute to arthritis pathogenesis in TS1xHACII mice are not refractory to $CD4^+CD25^+$ Treg cell activity (Figure 3-6 and 3-13). The adoptive transfers of $CD4^+CD25^+$ cells from HACII and BALB/c mice not only dramatically reduced arthritis incidence in TS1xHACII mice, but also appeared to reduce disease severity because TS1xHACII mice that received HACII or BALB/c $CD4^+CD25^+$ cells possessed significantly fewer arthritic limbs than unmanipulated TS1xHACII mice. Thus, HACII and BALB/c $CD4^+CD25^+$ Treg cells could modify the degree of disease in an individual TS1xHACII mouse in addition to affecting arthritis incidence on a population level.

HACII $CD4^+CD25^+$ Treg cells did not mediate arthritis prevention by inhibiting the accumulation of disease initiating S1-specific 6.5^+CD4^+ T cells in TS1xHACII mice, as arthritic unmanipulated TS1xHACII mice and protected TS1xHACII mice that received HACII $CD4^+CD25^+$ cells contained similar numbers of total $CD4^+$ and 6.5^+CD4^+ T cells in the systemic LNs. Furthermore, protective HACII $CD4^+CD25^+$ Treg cells did not alter expression of CD62L, CD44, and CD69 on 6.5^+ or 6.5^- $CD4^+$ T cells in TS1xHACII mice (Figure 3-7 C), as 6.5^+CD4^+ T cells appeared activated and antigen-experienced in both arthritic and protected TS1xHACII mice. Thus, HACII $CD4^+CD25^+$ Treg cells do not prevent arthritis development by suppressing the accumulation or activation of the disease initiating 6.5^+CD4^+ T cells in TS1xHACII mice. Instead, HACII $CD4^+CD25^+$ Treg cells appear to suppress arthritis development by inhibiting the pathogenic Th-17 response. In comparison to arthritic unmanipulated TS1xHACII mice, TS1xHACII mice that received protective HACII $CD4^+CD25^+$ cells exhibited a preferential reduction in the representation of $IL\text{-}17^+CD4^+$ cells in the popliteal LNs (Figure 3-8 B and C), as well as a significant decrease in IL-17 production by systemic LNs (Figure 3-9 A). Work in the SKG model of arthritis has suggested that homeostatic expansion of $CD4^+$ T cells in a lymphopenic environment can contribute to the development of Th-17 cells (Hirota et al. 2007). Given the lymphopenia in young TS1xHACII mice, it is possible that the accumulation of peripheral $CD4^+$ T cells is a contributing factor to the

development of IL-17 producing $CD4^+$ T cells. However, as described previously, protective HACII $CD4^+CD25^+$ Treg cells did not alter the accumulation of 6.5^+ or 6.5^- $CD4^+$ T cells in TS1xHACII mice (Figure 3-7 A and B), suggesting that they are inhibiting Th-17 differentiation by a different mechanism.

Interestingly, TS1xHACII mice that received protective HACII $CD4^+CD25^+$ cells also produced significantly lower amounts of systemic IL-1β (Figure 3-9 B), a cytokine that is associated with promoting IL-17 production (Sutton et al. 2006; Acosta-Rodriguez et al. 2007) and arthritis pathogenesis (Koenders et al. 2006; Koenders et al. 2008). The reduction in IL-1β suggests that protective $CD4^+CD25^+$ Treg cells may suppress IL-17^+CD4^+ cells by inhibiting other cytokines that are required for Th-17 differentiation. While much work has been done to identify the parameters defining *in vitro* Th-17 differentiation (Mangan et al. 2006; Veldhoen et al. 2006; Korn et al. 2009), the key players in *in vivo* IL-17 induction remain unclear, though there is speculation that an as yet unidentified innate immune system cell may play a role in *in vivo* Th-17 development (Korn et al. 2009). $CD4^+CD25^+$ Treg cells can suppress the innate immune response (Maloy et al. 2003), and a potential explanation for decreased IL-17 production in protected TS1xHACII mice is that the exogenous $CD4^+CD25^+$ Treg cells are acting upon an innate immune system cell that is instrumental in inducing the differentiation of Th-17 cells. Moreover, IL-1β has also been shown to induce

synoviocyte production of CCL20 (Hirota et al. 2007), the chemokine that binds CCR6, which is preferentially expressed on Th-17 cells (Acosta-Rodriguez et al. 2007; Annunziato et al. 2007). Thus, it is possible that a decrease in IL-1β production reduces not only Th-17 cell differentiation, but also the trafficking of IL-17 producing $CD4^+$ cells to the joints or joint-draining LNs. However, IL-17 can also act to induce IL-1 expression (Koenders et al. 2006), and it is possible that lower levels of IL-1β result from direct $CD4^+CD25^+$ Treg cell suppression of $IL\text{-}17^+CD4^+$ cells in protected TS1xHACII mice.

In addition to the reduction of regional and systemic cytokine responses, protected TS1xHACII mice contained significantly smaller popliteal LNs than unmanipulated arthritic TS1xHACII mice, indicating that the $CD4^+CD25^+$ Treg cells may also inhibit the accumulation of popliteal LN cells that normally occurs in TS1xHACII mice (Figure 3-2 E). Work in other murine autoimmune disease models has illustrated the importance of the regional LN immune response in the development of autoimmunity (Hoglund et al. 1999; Samy et al. 2005). In the NOD diabetes model, excision of the pancreatic LNs in young mice prevented diabetes onset, demonstrating that the local immune response is required for disease pathogenesis (Gagnerault et al. 2002). Thus, although the disease initiating target autoantigen is presented systemically in TS1xHACII mice, it is possible that the popliteal LNs present elevated levels of S1 peptide that

effectively prime the pathogenic $6.5^{+}CD4^{+}$ T cells, and that inhibition of this regional response by exogenous $CD4^{+}CD25^{+}$ Treg cells is sufficient to prevent arthritis development. Indeed, recent work in other murine arthritis models suggests that $CD4^{+}$ T cells play a more prominent role in the joint-draining LNs than in the joints themselves (Angyal et al. 2010).

It is also possible that $CD4^{+}CD25^{+}$ Treg cell suppression of regional IL-17 production prevents the accumulation of popliteal LN cells in TS1xHACII mice. IL-17 can induce innate immune cell migration (Lundy et al. 2007; Pelletier et al. 2010), and the reduction of the local Th-17 response could inhibit the recruitment of innate immune and antigen presenting cells, which make up the majority of the cells in the popliteal LNs of TS1xHACII mice (Rankin et al. 2008). Indeed, IL-17 has been shown to induce monocyte trafficking in arthritis (Shahrara et al. 2009), and additional work has demonstrated that monocytes isolated from human RA patients can induce Th-17 differentiation of $CD4^{+}$ T cells (Evans et al. 2009). This relationship suggests the possibility of a feedback loop where IL-17 recruits innate immune cells such as monocytes, which can further enhance local IL-17 production by promoting Th-17 cell differentiation. Thus, the effects of exogenous $CD4^{+}CD25^{+}$ Treg cell inhibition on the Th-17 response would be amplified by breaking this cycle.

3.3.3 Specificity for the disease initiating target antigen is not required for $CD4^+CD25^+$ Treg cell suppression of arthritis in TS1xHACII mice

The $CD4^+CD25^+$ Treg cells that most effectively suppressed arthritis development in TS1xHACII mice were purified from single transgenic HACII or BALB/c mice, which provide a polyclonal population of $CD4^+CD25^+$ Treg cells without transgenic modifications to their endogenous T cell repertoires. HACII mice express the HA transgene (under the same MHC Class II I-Eα promoter as in TS1xHACII mice), but not the transgenic 6.5 TCR. Accordingly, $CD4^+CD25^+$ cells in HACII mice are the appropriate control population for $6.5^-CD4^+CD25^+$ cells from TS1xHACII mice in that they also develop in the presence of the HA protein from which the disease target antigen (S1 peptide) is derived. Therefore, while $CD4^+CD25^+$ cells from HACII mice do not express the 6.5 TCR that is specific for S1 peptide, it is possible that there are other S1 peptide or HA reactive cells within the population of $CD4^+CD25^+$ cells because they developed in the presence of HA as a self antigen. Thus, it is possible that $CD4^+CD25^+$ Treg cells from HACII mice are capable of preventing arthritis development in TS1xHACII mice because a subset of cells can recognize and respond to the S1 peptide or other HA-derived peptides. Indeed eight $CD4^+$ T cell epitopes have been identified in PR8 HA (Gerhard et al. 1991). However, BALB/c $CD4^+CD25^+$ Treg cells, which have not experienced the S1 peptide or the HA protein from

which S1 peptide is derived, are also able to suppress arthritis in TS1xHACII mice. This result clearly demonstrates that a population of $CD4^+CD25^+$ Treg cells without any enrichment for S1-specific or other HA reactive cells can effectively shut down S1 peptide initiated autoimmune arthritis in TS1xHACII mice. How might $CD4^+CD25^+$ Treg cells from BALB/c mice suppress arthritis development in TS1xHACII mice? One possibility is that $CD4^+$ T cells of other specificities have been recruited to the effector phase of arthritis, and that $CD4^+CD25^+$ Treg cells from BALB/c mice are able to suppress the pathogenic $CD4^+$ T cells that are not S1-peptide or HA-specific.

3.3.4 $CD4^+CD25^+$ Treg cell activity is highly sensitive to the balance of Treg and effector cells

Strikingly, whereas the administration of unfractionated (includes 6.5^- and 6.5^+ cells) TS1xHACII $CD4^+CD25^+$ cells could not suppress arthritis, TS1xHACII $CD4^+CD25^+$ cells that had been depleted of 6.5^+ cells could modulate arthritis development in TS1xHACII mice. Since $6.5^+CD4^+CD25^+$ cells present in TS1xHACII mice contain both $Foxp3^+$ and $Foxp3^-$ cells, the enhanced ability of TS1xHACII $6.5^-CD4^+CD25^+$ cells to reduce arthritis incidence in TS1xHACII mice could be attributed to the absence of an activated population of $6.5^+CD4^+CD25^+Foxp3^-$ cells (which are specific for the disease initiating target

antigen), suggesting that $CD4^+CD25^+$ Treg cell activity is sensitive to the balance of Treg and effector cells. Indeed, the introduction of a small population of $6.5^+CD4^+CD25^-$ cells (obtained from TS1xHA28 mice) to protective HACII $CD4^+CD25^+$ cells altered the efficacy of the HACII $CD4^+CD25^+$ cells in preventing arthritis development. The same percentage of unmanipulated TS1xHACII mice and TS1xHACII mice that received the mixture of HACII $CD4^+CD25^+$ cells and TS1xHA28 $6.5^+CD4^+CD25^-$ cells developed arthritis, whereas administration of HACII $CD4^+CD25^+$ cells alone dramatically reduced arthritis incidence in recipient TS1xHACII mice. Collectively, these data indicate that the balance of $CD4^+CD25^+$ Treg and effector $CD4^+$ T cells is an important determinant of successful Treg cell activity. Moreover, the marked effects of either depleting or introducing a small population of 6.5^+CD4^+ T cells in the adoptive Treg cell transfers to pre-arthritic TS1xHACII mice further emphasizes the particular importance of 6.5^+CD4^+ T cells for arthritis pathogenesis. Indeed, the development of arthritis in TS1xHACII.$RAG^{-/-}$ mice demonstrates that 6.5^+CD4^+ T cells alone are sufficient to mediate disease (Rankin et al. 2008), indicating that $CD4^+$ T cells of other specificities are not required in later stages of disease.

Although the administration of TS1xHACII $6.5^-CD4^+CD25^+$ cells reduced arthritis incidence in TS1xHACII mice, the cells were not as effective as HACII or BALB/c $CD4^+CD25^+$ cells at preventing arthritis development. The effect of the

transferred 6.5^{-}CD4^{+}CD25^{+} cells appeared to wane over time, as the percentage of arthritic mice among TS1xHACII mice that received the 6.5^{-}CD4^{+}CD25^{+} cells continued to increase during the nine week period of analysis (Figure 3-11 B). It is possible that TS1xHACII 6.5^{-}CD4^{+}CD25^{+} cells merely delay arthritis onset in recipient in TS1xHACII mice, and that eventually disease incidence in TS1xHACII mice that received TS1xHACII 6.5^{-}CD4^{+}CD25^{+} cells would be comparable to that in unmanipulated TS1xHACII mice. Despite using expression of the 6.5 TCR to deplete 6.5^{+}CD4^{+}CD25^{+} cells (which contain both Foxp3^{+} and Foxp3^{-} cells) from the endogenous CD4^{+}CD25^{+} pool in TS1xHACII mice, it is possible that some S1-specific 6.5^{+}CD4^{+}CD25^{+} remain in the depleted population of TS1xHACII CD4^{+}CD25^{+} cells. The 6.5^{+}CD4^{+}CD25^{+} cells may have downregulated expression of the 6.5 TCR in response to interactions with the S1 peptide, or may express two TCRs (due to secondary TCR alpha chain rearrangements) resulting in lower levels of 6.5 expression that are not detected by staining with the mAb 6.5. Thus, it is possible that the presence of a contaminating population of S1-specific CD4^{+}CD25^{+} cells interferes with the *in vivo* suppressor function of endogenous 6.5^{-}CD4^{+}CD25^{+} cells from TS1xHACII mice.

Another explanation for the difference in efficacy of TS1xHACII 6.5^{-} CD4^{+}CD25^{+} cells and HACII or BALB/c CD4^{+}CD25^{+} cells in preventing arthritis

development in TS1xHACII mice is that the 6.5^{-}CD4^{+}CD25^{+} cells from TS1xHACII mice are less functional than their counterparts in healthy mice. This is consistent with reports that CD4^{+}CD25^{+} Treg cells from human RA patients are dysfunctional due to their exposure to the inflammatory cytokines (e.g. TNF-α) in the disease setting (Ehrenstein et al. 2004; Valencia et al. 2006; Nadkarni et al. 2007). However, it is clear that CD4^{+}CD25^{+} cells from HACII or BALB/c mice are able to maintain their regulatory effects for up to nine to ten weeks post transfer in TS1xHACII mice, indicating that CD4^{+}CD25^{+} Treg cell activity can occur in the context of high levels of inflammatory cytokines. Still, the CD4^{+}CD25^{+} T cells in TS1xHACII mice might have unique properties that render them susceptible to these effects. Thus, although we examined expression of CD4^{+}CD25^{+} Treg cell markers such as CTLA-4 and GITR on CD4^{+}CD25^{+} cells from TS1xHACII and HACII or BALB/c mice (Figure 3-3 D), we have not analyzed expression of cytokine receptors on the respective CD4^{+}CD25^{+} cell populations. CD4^{+}CD25^{+} cells from TS1xHACII mice may express higher levels of certain cytokine receptors than CD4^{+}CD25^{+} cells from HACII or BALB/c mice, making them more susceptible to the inhibitory effects of cytokines, such as IL-6 (Pasare and Medzhitov 2003) or TNF-α (Ehrenstein et al. 2004; Valencia et al. 2006; Nadkarni et al. 2007), on Treg cell regulatory activity. Indeed the impact of the presence of S1-specific CD4^{+}CD25^{+} cells described earlier, could be exacerbated if the

TS1xHACII $CD4^+CD25^+$ cells possess dysfunctional, or less functional, regulatory activity.

The data presented in this chapter show that $CD4^+CD25^+$ Treg cells from HACII and BALB/c mice can effectively suppress arthritis development in TS1xHACII mice. Interestingly, a fundamental difference between the two populations is the antigen specificity of the $CD4^+CD25^+$ Treg cells. In contrast to $CD4^+CD25^+$ cells in HACII mice, $CD4^+CD25^+$ cells in BALB/c mice have not developed in the presence of HA, and thus could not be selected on S1 peptide or any other HA-derived peptides. These results have interesting implications for how the antigen specificity of $CD4^+CD25^+$ Treg cells can affect their ability to suppress autoimmune disease. In the following chapter we address the role of antigen specificity in $CD4^+CD25^+$ Treg cell function by examining how enrichment in specificity for a target autoantigen (S1 peptide) affects the ability of $CD4^+CD25^+$ Treg cells to suppress arthritis development in TS1xHACII mice.

Chapter 4: $CD4^+CD25^+$ Treg cells that are enriched in specificity for a disease target antigen fail to suppress arthritis in TS1xHACII mice

4.1 Introduction

Whereas a role for TCR specificity in the thymic development of $CD4^+CD25^+$ Treg cells has been established (Jordan et al. 2001; Apostolou et al. 2002; Hori et al. 2002; Walker et al. 2003), how TCR specificity influences the phenotype and function of peripheral $CD4^+CD25^+$ Treg cells is less clear. *In vitro* studies have shown that while $CD4^+CD25^+$ Treg cells require antigen-specific TCR engagement to activate their regulatory function, they are able to suppress responder $CD4^+$ T cells of different TCR specificities (Takahashi et al. 1998; Thornton and Shevach 1998), indicating that the suppression itself is not antigen-specific. However, work in several different models of murine autoimmune disease indicates that the activation of antigen- or disease-specific $CD4^+CD25^+$ Treg cells can be critical for the effective suppression of organ-specific autoimmunity (Hori et al 2002; Reddy et al. 2004; Tarbell et al. 2004; Samy et al. 2005). In the EAE system, disease development has been linked to an absence of $CD4^+CD25^+$ Treg cells that are specific for the EAE-associated peptides that drive disease, demonstrating that $CD4^+CD25^+$ Treg cell recognition of the target autoantigen is critical for EAE prevention (Hori et al. 2002; Reddy et al. 2004).

However, while studies in both human arthritis patients and mouse models of arthritis indicate that $CD4^+CD25^+$ Treg cells do modify the immune response during arthritis (Morgan et al. 2003; de Kleer et al. 2004; Kelchtermans et al. 2005; Nguyen et al. 2007; Kang et al. 2008), whether and how the TCR specificity for disease target antigen(s) affects $CD4^+CD25^+$ Treg cell function is unknown.

TS1xHACII mice, in which arthritis development is driven by 6.5^+CD4^+ T cell recognition of the systemically presented S1 peptide, provide a powerful system with which to examine whether TCR specificity influences $CD4^+CD25^+$ Treg cell suppression of arthritis. In the previous chapter, we showed that $CD4^+CD25^+$ Treg cells from BALB/c mice, which have not developed in the presence of S1 peptide (or any other HA-derived peptide), could prevent arthritis development in TS1xHACII mice. Here we examine whether $CD4^+CD25^+$ Treg cells specific for the disease target antigen S1 peptide can also suppress arthritis in TS1xHACII mice. TS1xHA28 mice are a rich source of $6.5^+CD4^+CD25^+Foxp3^+$ Treg cells that are generated intrathymically based on the specificity of the 6.5 TCR for the S1 peptide (which is expressed at low levels driven by an SV40 promoter enhancer) (Jordan et al. 2001). Moreover, $CD4^+CD25^+$ cells from TS1xHA28 mice possess *in vivo* regulatory function, as they have been shown to suppress anti-double-stranded DNA B cell responses in an antigen-specific

manner (Seo et al. 2002). Thus, use of TS1xHA28 $CD4^+CD25^+$ cells allows us to examine whether expression of a TCR that possesses high reactivity for a disease target antigen affects the ability of $CD4^+CD25^+$ Treg cells (which are capable of suppressing other *in vivo* immune responses) to prevent arthritis development in TS1xHACII mice.

The data presented in this chapter show that $CD4^+CD25^+$ Treg cells that are enriched in specificity for a critical disease target antigen fail to prevent arthritis development in TS1xHACII mice. $CD4^+CD25^+$ Treg cells from TS1xHA28 mice, which contain a high frequency of 6.5^+Foxp3^+ cells that possess high reactivity for the S1 peptide, do not reduce either arthritis incidence or severity in TS1xHACII mice. This result is recapitulated by the adoptive transfer of $GFP^+CD4^+CD25^+$ cells from TS1xHA28.*Foxp3*^{EGFP} reporter mice, confirming that a highly pure population of $CD4^+CD25^+Foxp3^+$ Treg cells enriched for 6.5 expression cannot suppress a disease that is initiated by recognition of S1 peptide by the endogenous 6.5^+CD4^+ T cells in TS1xHACII mice. Interestingly, $CD4^+CD25^+$ Treg cells from TS1(SW)xPevSW mice, which are enriched for expression of a transgenic TCR (TS1(SW) TCR) with low reactivity for S1 peptide are able to modulate arthritis development in TS1xHACII mice, although these cells are not as effective as $CD4^+CD25^+$ Treg cells obtained from HACII or BALB/c mice. In addition, we show that signals through the TCR can shape the

phenotype and differentiation of $CD4^+CD25^+$ Treg cells, as interactions with high levels of cognate antigen can induce cytokine production and CD25 downregulation by $CD4^+CD25^+$ Treg cells. Collectively, we demonstrate that the presence of S1 peptide-reactive $CD4^+CD25^+$ Treg cells is detrimental to $CD4^+CD25^+$ Treg cell mediated suppression of arthritis development in TS1xHACII mice.

4.2 Results

4.2.1 *In vitro* suppressor function of $CD4^+CD25^+$ cells from TS1xHA28 mice can be activated by S1 peptide

In the previous chapter we showed that the prospective administration of exogenous polyclonal $CD4^+CD25^+$ Treg cells could prevent arthritis in TS1xHACII mice. We next addressed whether $CD4^+CD25^+$ Treg cells that express the 6.5 TCR (which recognizes S1 peptide and is also expressed by the endogenous pathogenic $CD4^+$ T cells) would also be able to suppress arthritis development in TS1xHACII mice. To examine this, we made use of TS1xHA28 mice, which produce a high frequency of $6.5^+CD4^+CD25^+Foxp3^+$ Treg cells (Jordan et al. 2001). Antibody staining and flow cytometric analysis of LN cells from TS1xHA28 mice showed that the majority of $CD4^+CD25^+$cells co-express high levels of the 6.5 TCR and the transcription factor Foxp3 (Figure 4-1 A). To demonstrate that the regulatory activity of TS1xHA28 $CD4^+CD25^+$ cells could be activated in an antigen-specific manner, we conducted *in vitro* suppression assays using S1 peptide as a stimulus. CFSE-labeled $CD4^+$ T cells were cultured with or without TS1xHA28 $CD4^+CD25^+$ cells at a 1:2 Treg:responder cell

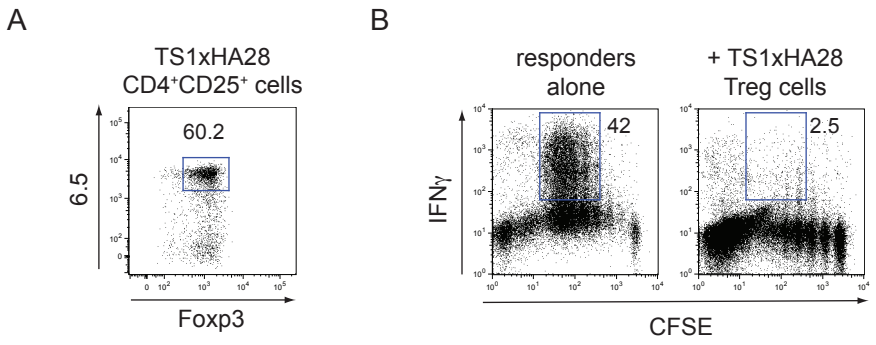

Figure 4-1 $CD4^+CD25^+$ cells from TS1xHA28 mice are enriched in 6.5^+Foxp3^+ cells (**A**) LN cells from TS1xHA28 mice were stained with the mAb 6.5, CD4, CD25, and Foxp3. Number indicates the percentage of 6.5^+Foxp3^+ cells among $CD4^+CD25^+$ cells from TS1xHA28 mice. (**B**) CFSE-labeled $CD4^+$ T cells were cultured alone or in the presence of $CD4^+CD25^+$ cells from TS1xHA28 mice at a 1:2 Treg:responder cell ratio. $CD4^+CD25^+$ cells were stimulated with S1 peptide. Dot plots show CFSE versus IFN-γ production after three days of culture. Plots are gated on total $CD4^+$ cells. Numbers in plots indicate the percentage of IFN-γ^+ cells among the responder $CD4^+$ cell population.

ratio. After three days of culture, the cells were stimulated with PMA and ionomycin and stained for intracellular IFN-γ production. In the absence of TS1xHA28 $CD4^+CD25^+$ cells, the responder $CD4^+$ T cells underwent extensive proliferation, and 42 percent of the cells produced IFN-γ (Figure 4-1 B). When co-cultured with $CD4^+CD25^+$ cells from TS1xHA28 mice, responder $CD4^+$ T cells proliferated less, and only 2.5 percent of the cells produced IFN-γ (Figure 4-1 B). Thus, S1 peptide can induce potent regulatory activity in $CD4^+CD25^+$ cells purified from TS1xHA28 mice, which contain a high frequency of 6.5^+Foxp3^+ cells.

4.2.2 $CD4^+CD25^+$ Treg cells enriched in specificity for a disease target antigen fail to suppress arthritis in TS1xHACII mice

To examine whether $CD4^+CD25^+$ cells enriched in specificity for S1 peptide could suppress arthritis in TS1xHACII mice, we purified and adoptively transferred $1x10^6$ $CD4^+CD25^+$ cells from TS1xHA28 mice into young, pre-arthritic TS1xHACII mice and tracked the recipient mice for arthritis development. Unmanipulated TS1xHACII mice and those that received TS1xHA28 $CD4^+CD25^+$ cells displayed similar kinetics of disease development (Figure 4-2 A), and by 14 weeks of age, 80 percent of unmanipulated (8 of 10) and 88.9 percent of TS1xHA28 $CD4^+CD25^+$ cell recipient (8 of 9) TS1xHACII mice were arthritic

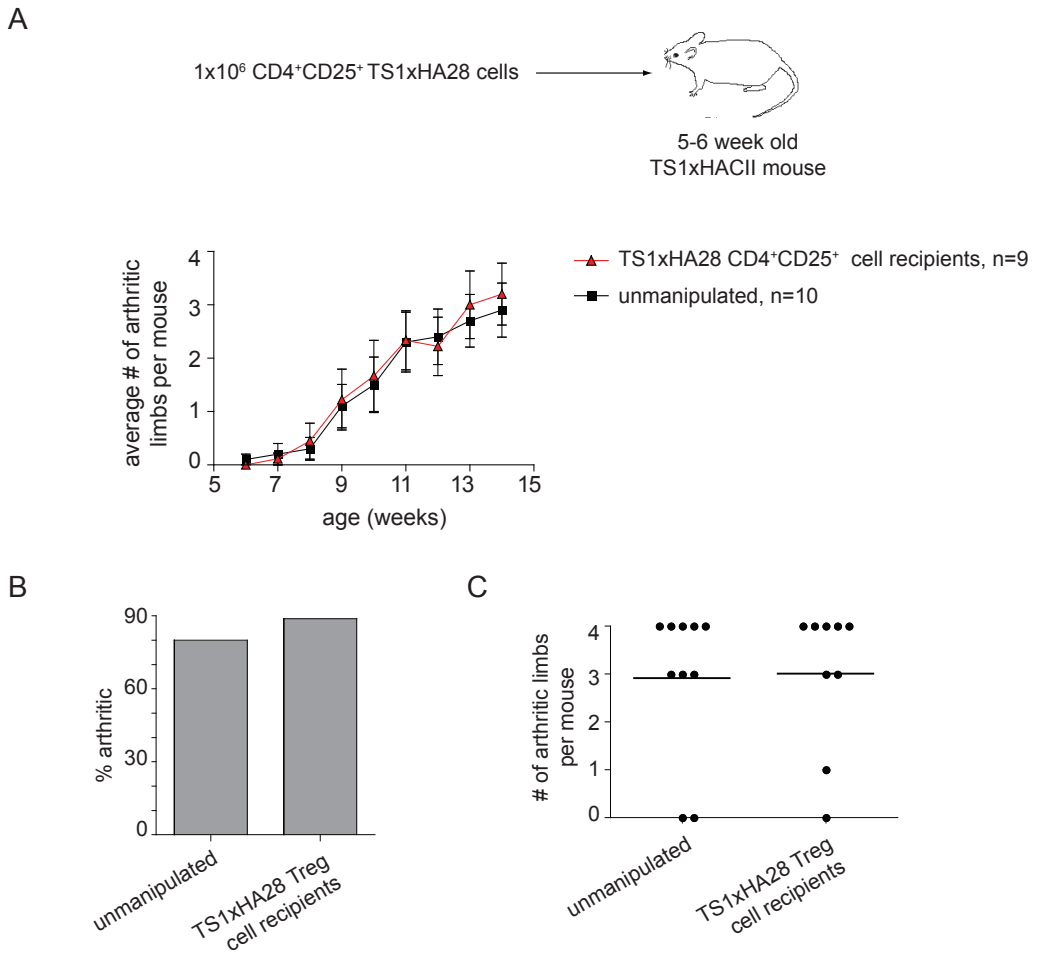

Figure 4-2 TS1xHA28 CD4⁺CD25⁺ cells do not suppress arthritis development in TS1xHACII mice (**A**) 1x10⁶ CD4⁺CD25⁺ cells purified from TS1xHA28 mice were adoptively transferred into pre-arthritic TS1xHACII mice. Graph shows the average number of arthritic limbs per mouse (±SEM) over time for unmanipulated (■, n=10) and TS1xHA28 CD4⁺CD25⁺ cell recipient (▲, n=9) TS1xHACII mice. (**B**) Arthritis incidence in 15 week old unmanipulated TS1xHACII mice and TS1xHACII mice that received TS1xHA28 CD4⁺CD25⁺ cells. (**C**) The number of arthritic limbs per mouse among unmanipulated TS1xHACII mice and TS1xHACII mice that received TS1xHA28 CD4⁺CD25⁺ cells. Each circle represents an individual mouse. Line represents the average number of arthritic limbs per mouse.

(Figure 4-2 B). Furthermore, the majority (5 of 8) of arthritic mice from both the unmanipulated cohort of TS1xHACII mice and the cohort that received TS1xHA28 $CD4^+CD25^+$ cells developed disease in all four limbs (Figure 4-2 C). Thus, in contrast to HACII and BALB/c $CD4^+CD25^+$ cells, which dramatically reduced arthritis incidence in TS1xHACII mice (Figures 3-6 and 3-13), $CD4^+CD25^+$ cells from TS1xHA28 mice were unable to prevent arthritis development in TS1xHACII mice. Moreover, $CD4^+CD25^+$ cells from TS1xHA28 mice did not reduce disease severity in TS1xHACII mice, as arthritic mice that did or did not receive TS1xHA28 $CD4^+CD25^+$ cells exhibited similar numbers of affected limbs.

The use of CD4 and CD25 expression to purify Treg cells is limiting in that it includes activated conventional $CD4^+$ T cells that have upregulated IL2Rα expression. Accordingly, it is possible that the failure of TS1xHA28 $CD4^+CD25^+$ cells to prevent arthritis in TS1xHACII mice is due to the presence of contaminating $6.5^+CD4^+CD25^+Foxp3^-$ T cells. To more rigorously purify $CD4^+CD25^+$ Treg cells based on Foxp3 and CD25 expression rather than on CD25 expression alone, we crossed TS1xHA28 mice with *Foxp3*^{EGFP} mice (Haribhai et al. 2007), to generate TS1xHA28.*Foxp3*^{EGFP} mice. We examined the $CD4^+CD25^+$ Treg cell profile in TS1xHA28.*Foxp3*^{EGFP} mice by staining LN cells for expression of 6.5, CD4, CD25, and Foxp3. GFP^+CD25^+ cells were clearly

identifiable among $CD4^+$ cells, and the majority of $GFP^+CD4^+CD25^+$ cells co-expressed high levels of the 6.5 TCR and Foxp3 (Figure 4-3 A). For adoptive transfers into TS1xHACII mice, we purified $CD4^+CD25^+$ Treg cells from TS1xHA28.*Foxp3*^{EGFP} mice based on expression of CD4, CD25, and GFP.

We transferred $1x10^6$ TS1xHA28.*Foxp3*^{EGFP} $GFP^+CD4^+CD25^+$ cells into pre-arthritic TS1xHACII mice and evaluated the recipient mice for arthritis development. Over the course of nine weeks, there was no significant difference in the kinetics of disease development between unmanipulated TS1xHACII mice and TS1xHACII mice that received $GFP^+CD4^+CD25^+$ cells from TS1xHA28.*Foxp3*^{EGFP} mice (Figure 4-3 A). At 15 weeks of age, 85.7 percent of unmanipulated TS1xHACII mice (6 of 7) and 72.7 percent of $GFP^+CD4^+CD25^+$ cell recipient TS1xHACII mice (8 of 11) had developed arthritis (Figure 4-3 B). Although arthritis incidence was lower in TS1xHACII mice that received $GFP^+CD4^+CD25^+$ cells from TS1xHA28.*Foxp3*^{EGFP} mice, the percentage of arthritic animals is consistent with previous observations of disease incidence in unmanipulated cohorts of TS1xHACII mice (Figure 3-2 A). Furthermore, in a second cohort of TS1xHACII mice that received TS1xHA28.*Foxp3*^{EGFP} $GFP^+CD4^+CD25^+$ cells, approximately 90 percent of TS1xHACII mice developed arthritis (data not shown), indicating that the $GFP^+CD4^+CD25^+$ cells do not reduce disease incidence in TS1xHACII mice. Among TS1xHACII mice that

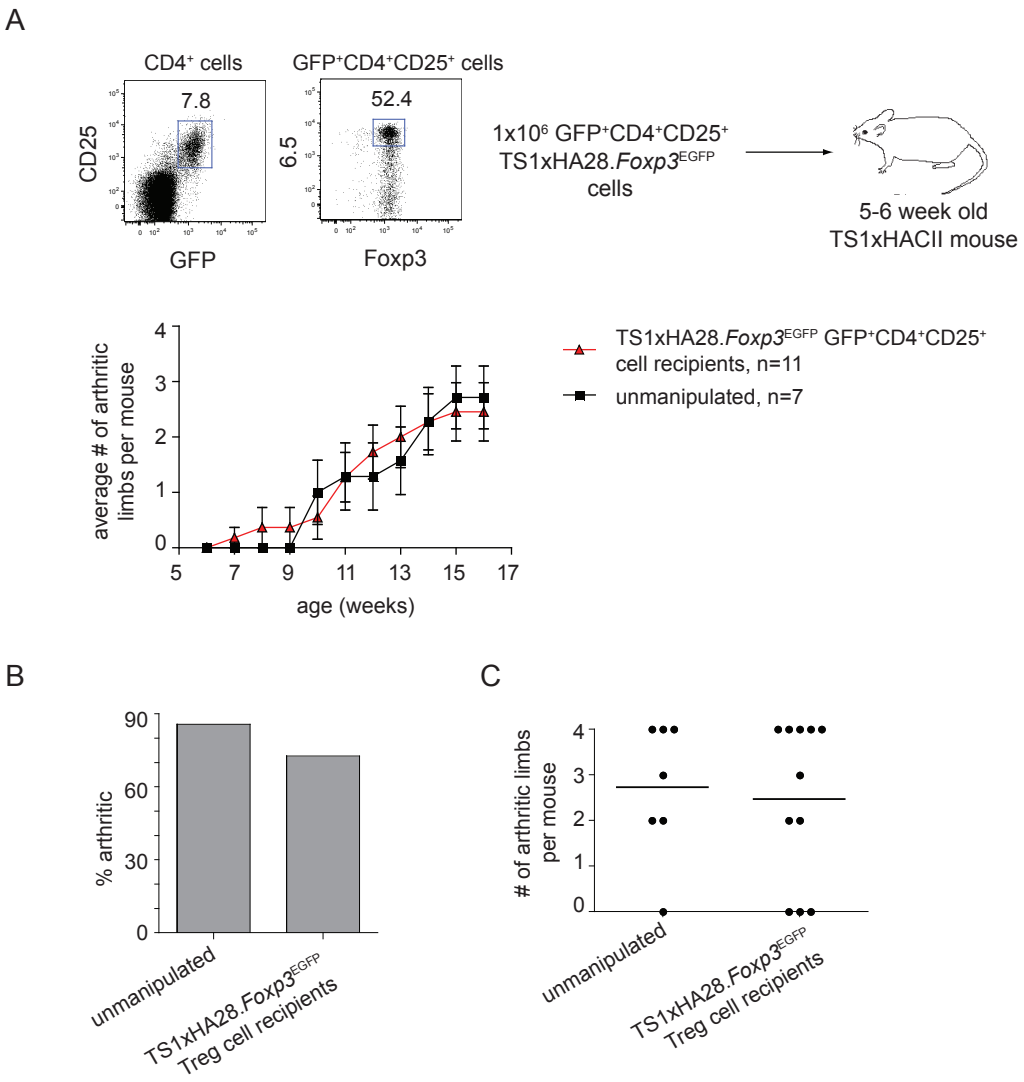

Figure 4-3 TS1xHA28.*Foxp3*^{EGFP} GFP^{+}CD4^{+}CD25^{+} cells do not prevent arthritis in TS1xHACII mice (**A**) 1x10^{6} TS1xHA28.*Foxp3*^{EGFP} GFP^{+}CD4^{+}CD25^{+} cells were adoptively transferred into TS1xHACII mice. Graph shows the average number of arthritic limbs per mouse (±SEM) over time for unmanipulated (■, n=7) TS1xHACII mice and GFP^{+}CD4^{+}CD25^{+} cell recipient (▲, n=11) TS1xHACII mice. Dot plots show CD4, CD25, 6.5, and Foxp3 staining of LN cells from TS1xHA28.*Foxp3*^{EGFP} mice. (**B**) Arthritis incidence in 15 week old unmanipulated TS1xHACII mice and TS1xHACII mice that received GFP^{+}CD4^{+}CD25^{+} cells. (**C**) The number of arthritic limbs per mouse among unmanipulated TS1xHACII mice and TS1xHACII mice that received GFP^{+}CD4^{+}CD25^{+} cells. Each circle represents an individual mouse. Line represents the average number of arthritic limbs per mouse.

developed arthritis, 50 percent of unmanipulated TS1xHACII mice (3 of 6) and 62.5 percent of TS1xHA28.*Foxp3*^{EGFP} GFP^{+}CD4^{+}CD25^{+} cell recipient TS1xHACII mice (5 of 8) developed disease in all four limbs, and in both cohorts of mice no arthritic mouse possessed fewer than two affected limbs (Figure 4-3 C). Thus, GFP^{+}CD4^{+}CD25^{+} cells from TS1xHA28.*Foxp3*^{EGFP} mice, which are heavily enriched for 6.5^{+}Foxp3^{+} cells, fail to suppress arthritis development in TS1xHACII mice.

Taken together, these data indicate that CD4^{+}CD25^{+}Foxp3^{+} Treg cells that are enriched in specificity for the target antigen S1 peptide fail to suppress arthritis development in TS1xHACII mice. Moreover, the adoptive transfer of TS1xHA28.*Foxp3*^{EGFP} GFP^{+}CD4^{+}CD25^{+} cells and TS1xHA28 CD4^{+}CD25^{+} cells appear to have similar effects in TS1xHACII mice, indicating that the failure of TS1xHA28 CD4^{+}CD25^{+} cells to prevent arthritis in TS1xHACII mice cannot be solely attributed to the presence of 6.5^{+}CD4^{+}CD25^{+}Foxp3^{-} cells in the initial transfer population.

4.2.3 Donor $CD4^+CD25^+$ Treg cells that are enriched for expression of the 6.5 TCR persist in TS1xHACII mice

Because $CD4^+CD25^+$ cells from TS1xHA28.*Foxp3*^{EGFP} mice expressed GFP, we could determine whether the donor $GFP^+CD4^+CD25^+$ cells persisted in arthritic TS1xHACII mice. Lymphoid organs from 15 week old TS1xHACII mice were examined for the presence of TS1xHA28.*Foxp3*^{EGFP} cells, which were identified by co-expression of GFP and the Foxp3 antibody. Nine to ten weeks after the initial transfer, the donor $GFP^+CD4^+CD25^+$ cells could be detected in LNs that drain the lower (popliteal) and upper (axillary & brachial) limbs, as well is in the spleen and LNs (cervical, inguinal, and mesenteric) that are not primary joint-draining sites (Figure 4-4 A). GFP^+Foxp3^+ cells were found at the highest percentages in the spleens of TS1xHACII mice that received TS1xHA28.*Foxp3*^{EGFP} $GFP^+CD4^+CD25^+$ cells, and among LNs, the representation of $GFP^+Foxp3^+CD4^+$ cells was comparable between joint-draining and non-draining LNs (Figure 4-4 B). Moreover, the recovered $CD4^+GFP^+Foxp3^+$ cells were enriched for high levels of CTLA-4 expression when compared to endogenous $CD4^+CD25^+Foxp3^+$ cells in TS1xHACII mice (Figure 4-4 C), suggesting that the 6.5^+ cells within the TS1xHA28.*Foxp3*^{EGFP} $GFP^+CD4^+CD25^+$ population were activated upon interactions with their cognate antigen.

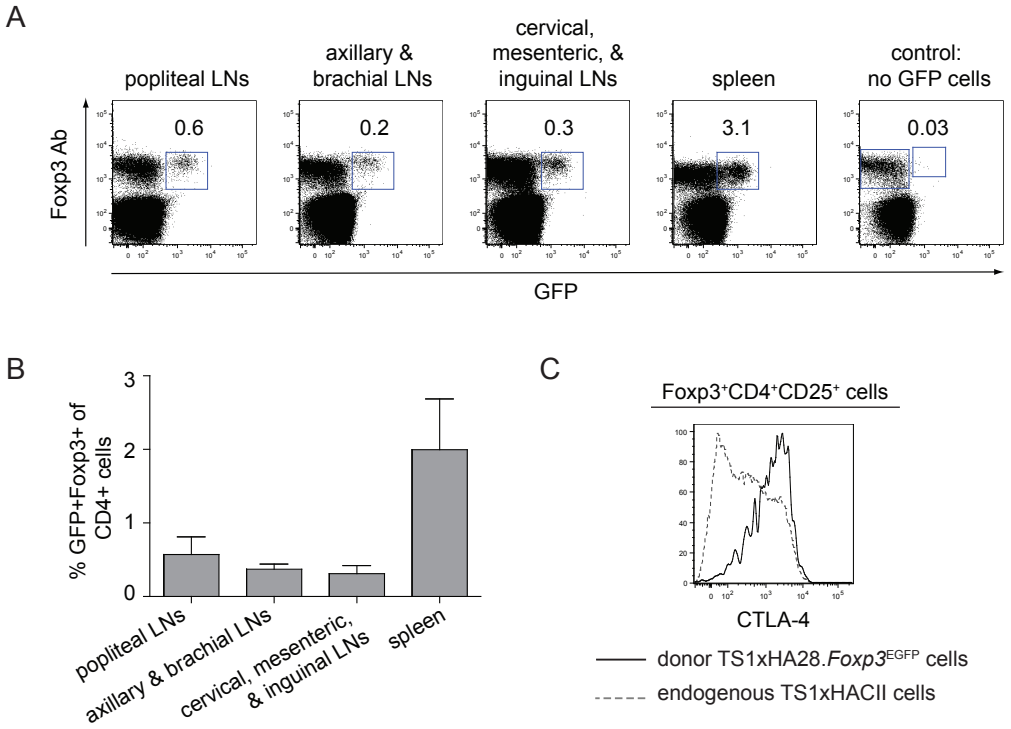

Figure 4-4 TS1xHA28.*Foxp3*^{EGFP} GFP^{+}CD4^{+}CD25^{+} cells persist in TS1xHACII mice (**A**) Dot plots show GFP versus Foxp3 antibody staining in CD4^{+} cells from lymphoid organs of TS1xHACII mice ten weeks post transfer of 1x10^{6} GFP^{+}CD4^{+}CD25^{+} cells. Numbers indicate the percentage of GFP^{+}Foxp3^{+} cells among CD4^{+} cells. (**B**) The percentages of GFP^{+}Foxp3^{+} cells among CD4^{+} cells from lymphoid organs of TS1xHACII mice ten weeks post transfer of 1x10^{6} GFP^{+}CD4^{+}CD25^{+} cells. Bars show average values ± SEM. n=6. (**C**) CTLA-4 staining of endogenous CD4^{+}CD25^{+}Foxp3^{+} cells (dotted line) and donor TS1xHA28.*Foxp3*^{EGFP} GFP^{+}Foxp3^{+}CD4^{+} cells (solid line) at ten weeks post transfer into TS1xHACII mice.

Thus, $GFP^+CD4^+CD25^+$ cells enriched in specificity for the target antigen S1 peptide fail to suppress arthritis in TS1xHACII mice despite persisting in TS1xHACII mice for up to ten weeks post transfer, trafficking to the joint draining LNs, and upregulating functional markers such as CTLA-4.

4.2.4 $CD4^+CD25^+$ Treg cells enriched for expression of the 6.5 TCR do not suppress the Th-17 response in popliteal LNs of TS1xHACII mice

We previously showed that protective HACII $CD4^+CD25^+$ cells could inhibit the regional Th-17 response that develops in the popliteal LNs of TS1xHACII mice (Figure 3-8). To determine whether the inability of TS1xHA28 $CD4^+CD25^+$ cells to suppress arthritis in TS1xHACII mice could be attributed to a failure to inhibit the local Th-17 response, or if disease develops despite TS1xHA28 $CD4^+CD25^+$ cell mediated reduction of IL-17 producing $CD4^+$ T cells in the popliteal LNs in TS1xHACII mice, we analyzed IL-17 and IFN-γ production by $CD4^+$ T cells in the popliteal LNs of unmanipulated TS1xHACII mice, and of TS1xHACII mice that received TS1xHA28 $CD4^+CD25^+$ cells. Popliteal LN cells were stimulated for four hours with PMA and ionomycin, and then stained for CD4 and intracellular IL-17 and IFN-γ. $CD4^+$ cells in the popliteal LNs of TS1xHACII mice that received TS1xHA28 $CD4^+CD25^+$ cells contained similar percentages of IL-17-producing cells and slightly higher percentages of IFN-γ-

producing cells (ns, p=0.31, Student's t-test) in comparison to unmanipulated arthritic TS1xHACII mice (Figure 4-5 A and B). Thus, $CD4^+CD25^+$ cells from TS1xHA28 mice did not reduce the Th-17 response in the popliteal LNs of TS1xHACII mice.

Whereas the administration of HACII $CD4^+CD25^+$ cells resulted in significantly smaller popliteal LNs in TS1xHACII mice (Figure 3-8 A), TS1xHACII mice that received TS1xHA28 $CD4^+CD25^+$ cells did not exhibit a decrease in popliteal LN cellularity, and actually averaged higher popliteal LN cellularities than unmanipulated TS1xHACII mice, although the difference was not statistically significant (p=0.16, Student's t-test) (Figure 4-5 C). Correspondingly, TS1xHA28 $CD4^+CD25^+$ cell recipient TS1xHACII mice also contained higher numbers of IFN-γ^+CD4^+ (ns, p=0.09, Student's t-test) and IL-17^+CD4^+ (ns, p=0.18, Student's t-test) cells when compared to unmanipulated TS1xHACII mice (Figure 4-5 D). Although the difference in cell numbers did not reach statistical significance, it is clear that unlike protective HACII $CD4^+CD25^+$ cells, $CD4^+CD25^+$ cells from TS1xHA28 mice were unable to inhibit the immune response that develops in the popliteal LN cells of TS1xHACII mice.

Although we demonstrated that $GFP^+CD4^+CD25^+$ cells from TS1xHA28.*Foxp3*^{EGFP} mice failed to suppress arthritis in TS1xHACII mice (Figure

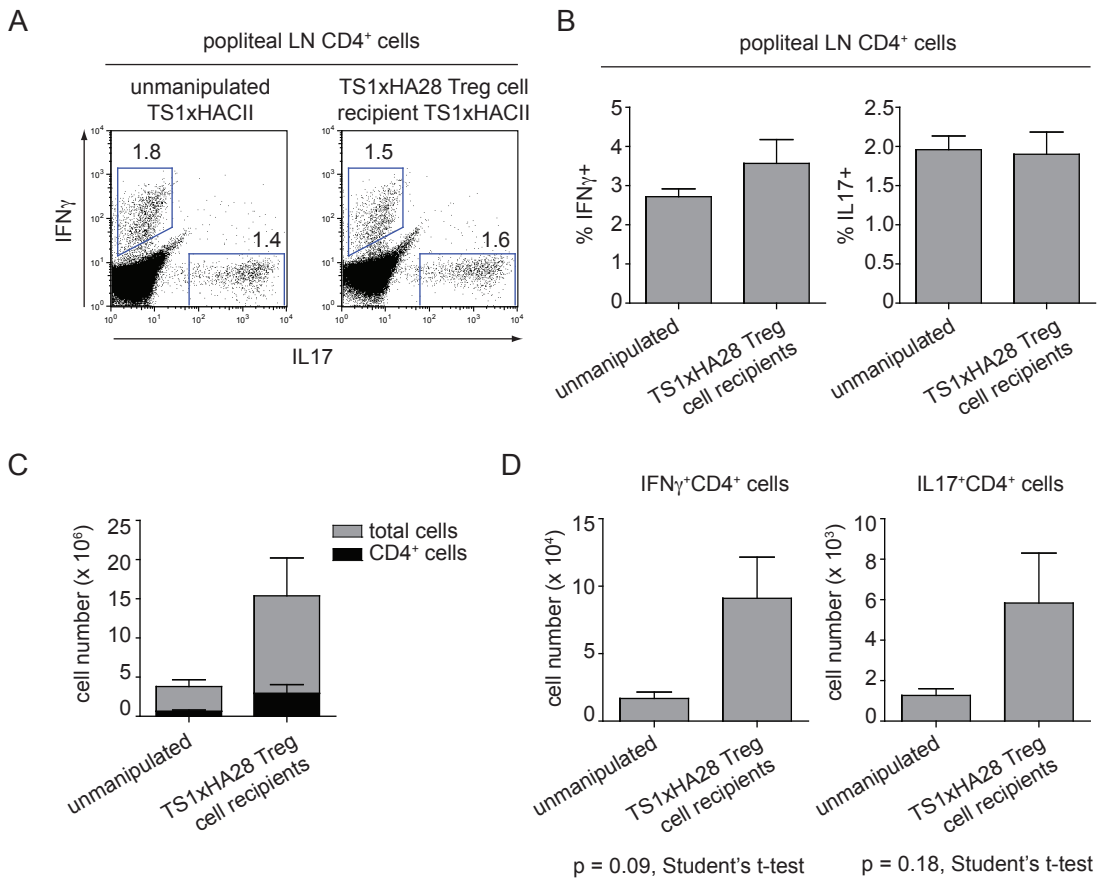

Figure 4-5 TS1xHA28 CD4$^+$CD25$^+$ cells do not suppress the Th-17 response in popliteal LNs of TS1xHACII mice (**A**) Popliteal LN cells were stimulated for 4 hours with PMA and ionomycin and then stained for intracellular IL-17 and IFN-γ. Dot plots show IL-17 and IFN-γ production by CD4$^+$ T cells from unmanipulated and TS1xHA28 CD4$^+$CD25$^+$ cell recipient TS1xHACII mice. Numbers indicate the percentage of cytokine-producing cells in each gate. (**B**) Percentages of IL-17 and IFN-γ producing popliteal LN CD4$^+$ T cells in unmanipulated and TS1xHA28 CD4$^+$CD25$^+$ cell recipient TS1xHACII mice. (**C**) Numbers of total (gray bar) and CD4$^+$ (black bar) cells in the popliteal LNs of unmanipulated and TS1xHA28 CD4$^+$CD25$^+$ cell recipient TS1xHACII mice. (total cells, p=0.16) (**D**) Numbers of IFN-γ$^+$CD4$^+$ and IL-17$^+$CD4$^+$ cells in the popliteal LNs of unmanipulated and TS1xHA28 CD4$^+$CD25$^+$ cell recipient TS1xHACII mice. Statistical significance was calculated using the Student's t-test. Bar graphs show average values ± SEM. unmanipulated TS1xHACII mice, n=5; TS1xHA28 CD4$^+$CD25$^+$ cell recipient TS1xHACII mice, n=8

4-3), we wanted to examine whether a more rigorously purified population of $CD4^+CD25^+Foxp3^+$ Treg cells enriched in specificity for S1 peptide could inhibit the regional immune response that develops in the popliteal LNs of TS1xHACII mice. Popliteal LN cells from unmanipulated TS1xHACII mice and TS1xHACII mice that received TS1xHA28.*Foxp3*^{EGFP} $GFP^+CD4^+CD25^+$ cells were stimulated for four hours with PMA and ionomycin, and then stained for CD4 and intracellular IL-17 and IFN-γ. In comparison to unmanipulated TS1xHACII mice, TS1xHACII mice that received TS1xHA28.*Foxp3*^{EGFP} $GFP^+CD4^+CD25^+$ cells contained slightly higher percentages of IFN-γ producing $CD4^+$ popliteal LN cells and lower percentages of IL-17 producing $CD4^+$ popliteal LN cells (Figure 4-6 A and B). However, the changes in the representation of IFN-γ (ns, p=0.5, Student's t-test) and IL-17 (ns, p=0.25, Student's t-test) producing $CD4^+$ cells in the popliteal LNs of TS1xHACII mice that received TS1xHA28.*Foxp3*^{EGFP} $GFP^+CD4^+CD25^+$ cells were not statistically significant when compared to unmanipulated TS1xHACII mice.

TS1xHACII mice that received $GFP^+CD4^+CD25^+$ cells averaged higher, though not statistically significant (p=0.13, Student's t-test), numbers of popliteal LN cells when compared to unmanipulated TS1xHACII mice (Figure 4-6 C). Thus, despite the reduction in the percentage of IL-17-producing $CD4^+$ cells in the popliteal LNs, TS1xHACII mice that received TS1xHA28.*Foxp3*^{EGFP}

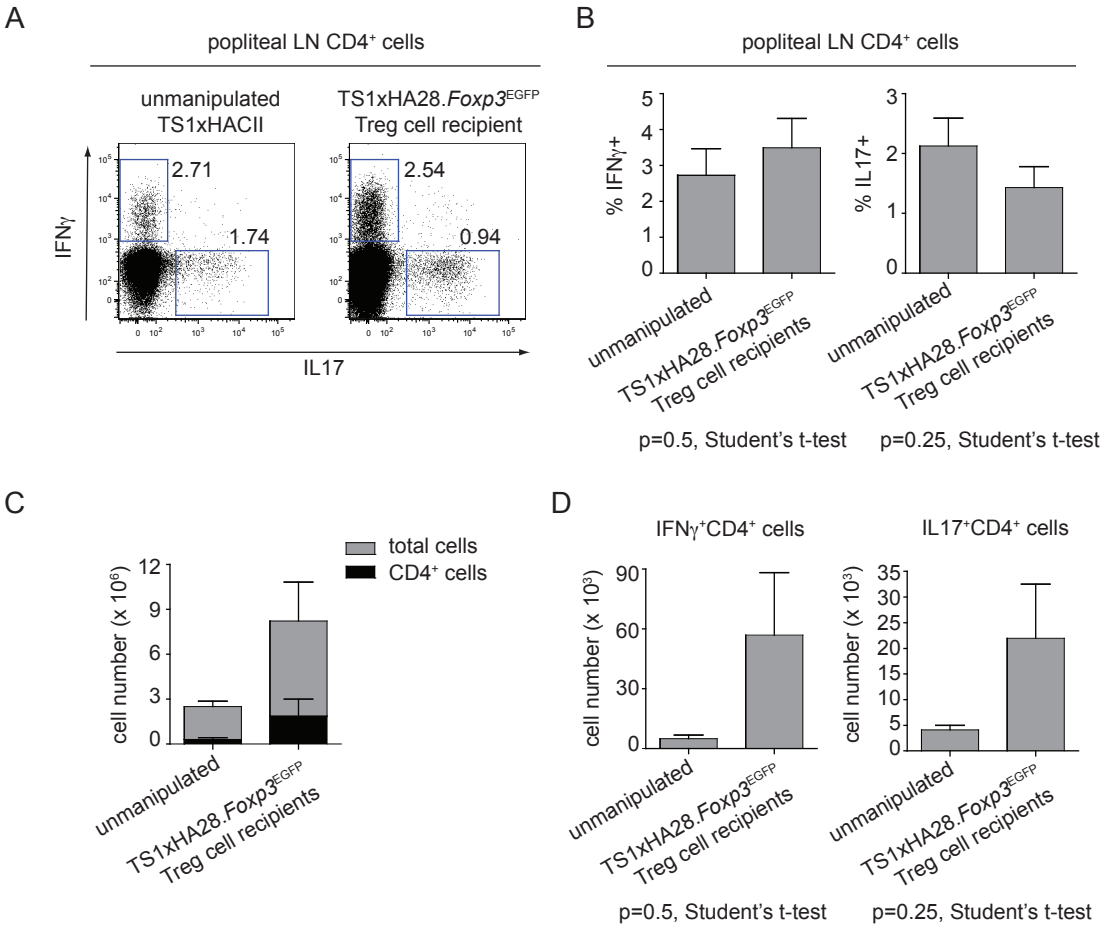

Figure 4-6 TS1xHA28.*Foxp3*^{EGFP} GFP$^+$CD4$^+$CD25$^+$ cells do not suppress the Th-17 response in the popliteal LNs of TS1xHACII mice (**A**) Popliteal LN cells were stimulated for 4 hours with PMA and ionomycin and then stained for intracellular IL-17 and IFN-γ. Dot plots show IL-17 and IFN-γ production by CD4$^+$ T cells from unmanipulated and GFP$^+$CD4$^+$CD25$^+$ cell recipient TS1xHACII mice. Numbers indicate the percentage of cytokine-producing cells in each gate. (**B**) Percentages of IL-17 and IFN-γ producing popliteal LN CD4$^+$ T cells in unmanipulated TS1xHACII mice and GFP$^+$CD4$^+$CD25$^+$ cell recipient TS1xHACII mice. (**C**) Numbers of total (gray bar) and CD4$^+$ (black bar) cells in the popliteal LNs of unmanipulated and GFP$^+$CD4$^+$CD25$^+$ cell recipient TS1xHACII mice. (total cells, p=0.13) (**D**) Numbers of IFN-γ$^+$CD4$^+$ and IL-17$^+$CD4$^+$ cells in the popliteal LNs of unmanipulated and GFP$^+$CD4$^+$CD25$^+$ cell recipient TS1xHACII mice. Statistical significance was calculated using the Student's t-test. Bar graphs show average values ± SEM. n=9 mice per group for all analyses.

GFP$^+$CD4$^+$CD25$^+$ cells averaged higher numbers of IL-17$^+$CD4$^+$ popliteal LN cells than unmanipulated TS1xHACII mice (Figure 4-6 D). There was also a corresponding increase in the numbers of IFN-γ^+CD4$^+$ cells in the popliteal LNs of TS1xHA28.*Foxp3*^{EGFP} GFP$^+$CD4$^+$CD25$^+$ cell recipient TS1xHACII mice (Figure 4-6 D). Although the increases in total, IL-17$^+$CD4$^+$ (p=0.11, Student's t-test), and IFN-γ^+CD4$^+$ (p=0.5, Student's t-test) popliteal LN cells of TS1xHACII mice that received TS1xHA28.*Foxp3*^{EGFP} GFP$^+$CD4$^+$CD25$^+$ cells did not reach statistical significance, these data suggest that the TS1xHA28.*Foxp3*^{EGFP} GFP$^+$CD4$^+$CD25$^+$ Treg cells not only fail to inhibit the immune response in the popliteal LNs of TS1xHACII mice, but may even exacerbate the accumulation of immune cells in the joint-draining LNs.

Collectively, these data demonstrate that a highly pure population of CD4$^+$CD25$^+$ Treg cells that are enriched for 6.5$^+$Foxp3$^+$ cells fails to inhibit the regional Th-17 response and the accumulation of immune cells in the popliteal LNs of TS1xHACII mice.

4.2.5 $CD4^+CD25^+$ Treg cells enriched for expression of the 6.5 TCR do not suppress the systemic Th-17 response in TS1xHACII mice

To further analyze IL-17 production in TS1xHACII mice that received $CD4^+CD25^+$ cells enriched for 6.5^+ cells, we set up unfractionated LN (axillary, brachial, cervical, inguinal, and mesenteric) cultures with cells from unmanipulated TS1xHACII mice and TS1xHACII mice that received TS1xHA28 $CD4^+CD25^+$ cells. After three days of culture, supernatants were collected and analyzed for IL-17 production. Unfractionated LN cultures from unmanipulated and TS1xHA28 $CD4^+CD25^+$ cell recipient TS1xHACII mice contained comparable concentrations of IL-17 (Figure 4-7 A). Indeed LN cells from TS1xHACII mice that received TS1xHA28 $CD4^+CD25^+$ cells produced slightly higher amounts of IL-17 than LN cells from unmanipulated TS1xHACII mice, although the difference was not statistically significant ($p=0.16$, Student's t-test). Thus, $CD4^+CD25^+$ cells from TS1xHA28 mice failed to inhibit both the regional and systemic Th-17 response in TS1xHACII mice.

We also examined whether TS1xHA28 $CD4^+CD25^+$ cells affected the production of cytokines associated with Th-17 differentiation (i.e. IL-1 and IL-6) in TS1xHACII mice. Interestingly, LN cultures from TS1xHACII mice that received TS1xHA28 $CD4^+CD25^+$ cells produced significantly higher levels of IL-1α

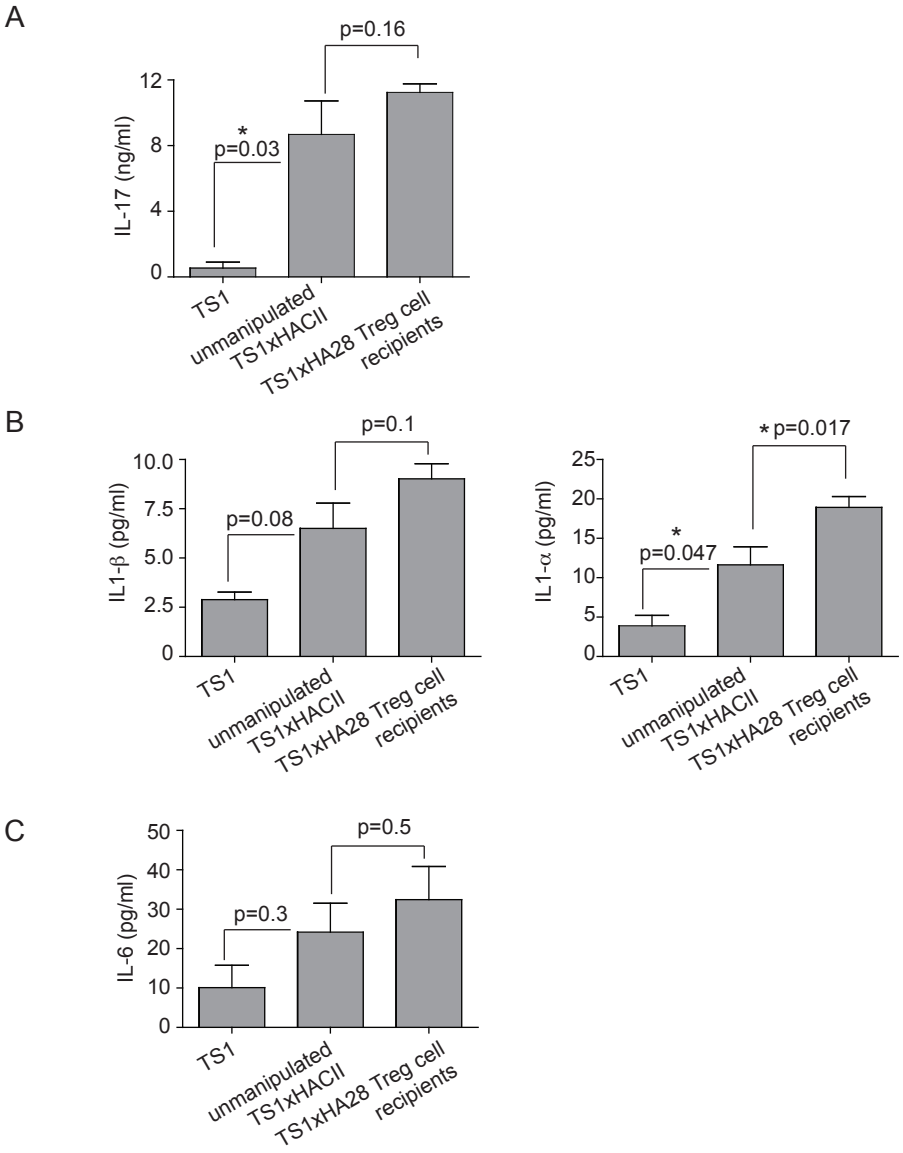

Figure 4-7 TS1xHA28 $CD4^+CD25^+$ cells fail to suppress the systemic Th-17 response in TS1xHACII mice. Unfractionated LN (axillary, brachial, cervical, inguinal, and mesenteric) cells from unmanipulated TS1xHACII, TS1xHA28 $CD4^+CD25^+$ Treg cell recipient TS1xHACII, or control TS1 mice were cultured for three days, and supernatants were collected and analyzed for (**A**) IL-17, (**B**) IL-1β and IL-1α , and (**C**) IL-6. Statistical signficance was calculated using the Student's t-test. Bar graphs show average values ± SEM. unmanipulated TS1xHACII mice, n=5; TS1xHA28 $CD4^+CD25^+$ Treg cell recipient TS1xHACII mice, n=8; control TS1 mice, n=3.

(*p=0.017, Student's t-test) and increased, though not statistically significant (p=0.1, Student's t-test), amounts of IL-1β in comparison to LN cultures from unmanipulated TS1xHACII mice (Figure 4-7 B). The production of IL-6 by unfractionated LN cells from TS1xHACII mice that received TS1xHA28 $CD4^+CD25^+$ cells was also slightly higher than in unmanipulated TS1xHACII mice (Figure 4-7 C), though the difference did not reach statistical significance (p=0.5, Student's t-test). Thus, TS1xHACII mice that received TS1xHA28 $CD4^+CD25^+$ cells produced similar or even increased levels of cytokines associated with Th-17 differentiation in comparison to unmanipulated TS1xHACII mice. This stands in marked contrast to TS1xHACII mice that received HACII $CD4^+CD25^+$ cells, which averaged lower amounts of IL-1 and IL-6 than unmanipulated TS1xHACII mice (Figure 3-9 B and C).

Collectively, these data demonstrate that $CD4^+CD25^+$ Treg cells enriched in specificity for a disease target antigen not only fail to inhibit the pathogenic Th-17 response in TS1xHACII mice, but may also enhance the production of cytokines that can contribute to the differentiation of IL-17 producing cells.

4.2.6 Strong antigenic stimulation coupled with lymphopenia can induce $CD4^+CD25^+Foxp3^+$ Treg cells to produce IL-17

While $CD4^+CD25^+$ Treg cells enriched for 6.5^+ cells that possess high reactivity for S1 peptide are unable to prevent arthritis development in TS1xHACII mice, BALB/c $CD4^+CD25^+$ Treg cells that have no developmental exposure to S1 peptide can suppress arthritis (Figure 3-13). Collectively, these results suggest that expression of the 6.5 TCR, which possesses high reactivity for the S1 peptide, may be detrimental to the ability of $CD4^+CD25^+$ Treg cells to prevent arthritis TS1xHACII mice. Work in humans has shown that activated, "memory" $CD4^+CD25^+Foxp3^+$ Treg cells are capable of producing IL-17 (Ayyoub et al. 2009; Beriou et al. 2009; Voo et al. 2009), and that cytokine production can be preferentially induced by strong stimulation of the $CD4^+CD25^+$ Treg cells (Voo et al. 2009). To examine whether $6.5^+CD4^+CD25^+Foxp3^+$ cells exposed to strong, chronic antigenic stimulation could develop the ability to produce IL-17, we purified and adoptively transferred $6.5^+GFP^+CD4^+CD25^+$ cells from TS1xHA28.*Foxp3*^{EGFP} mice into HACII.Tcr$\alpha^{-/-}$ or Tcr$\alpha^{-/-}$ recipient mice (Figure 4-8 A, upper panel). In both hosts, $6.5^+GFP^+CD4^+CD25^+$ cells would enter a lymphopenic environment (as is also the condition in young TS1xHACII mice), but in HACII.Tcr$\alpha^{-/-}$ mice the donor $CD4^+CD25^+$ Treg cells would also encounter high levels of their cognate antigen S1 peptide.

One week after transfer, LN cells were isolated from HACII.Tcr$\alpha^{-/-}$ and Tcr$\alpha^{-/-}$ mice, stimulated for four hours with PMA and ionomycin, and stained with intracellular IL-17 and IFN-γ to determine cytokine production. Donor $CD4^+CD25^+$ Treg cells, which were identified by co-expression of GFP and Foxp3, were present in the LNs of both HACII.Tcr$\alpha^{-/-}$ and Tcr$\alpha^{-/-}$ mice, though there was a far greater percentage in HACII.Tcr$\alpha^{-/-}$ mice (Figure 4-8 A, lower left panel), most likely due to enhanced expansion of the $6.5^+GFP^+CD4^+CD25^+$ cells in response to S1 peptide. Although donor $6.5^+GFP^+CD4^+CD25^+$ cells could be detected in both HACII.Tcr$\alpha^{-/-}$ and Tcr$\alpha^{-/-}$ mice, we observed notable cytokine production only in GFP^+Foxp3^+ cells recovered from HACII.Tcr$\alpha^{-/-}$ mice (Figure 4-8 A, lower right panel), indicating that TCR interactions with self-peptide can promote cytokine production in $CD4^+CD25^+Foxp3^+$ Treg cells. Interestingly, the GFP^+Foxp3^+ cells in HACII.Tcr$\alpha^{-/-}$ mice preferentially produced IL-17 (7.6 percent) over IFN-γ (2.1 percent).

To examine the effects of exposure to high levels of cognate antigen coupled with lymphopenia on Th-17 differentiation of conventional $CD4^+$ T cells, we purified $CD4^+$ cells from TS1 $Thy1.1^+$ mice (which are enriched for 6.5^+ cells), and adoptively transferred the cells into HACII.Tcr$\alpha^{-/-}$, Tcr$\alpha^{-/-}$, or BALB/c mice which all expressed Thy1.2. One week post transfer LN cells from HACII.Tcr$\alpha^{-/-}$,

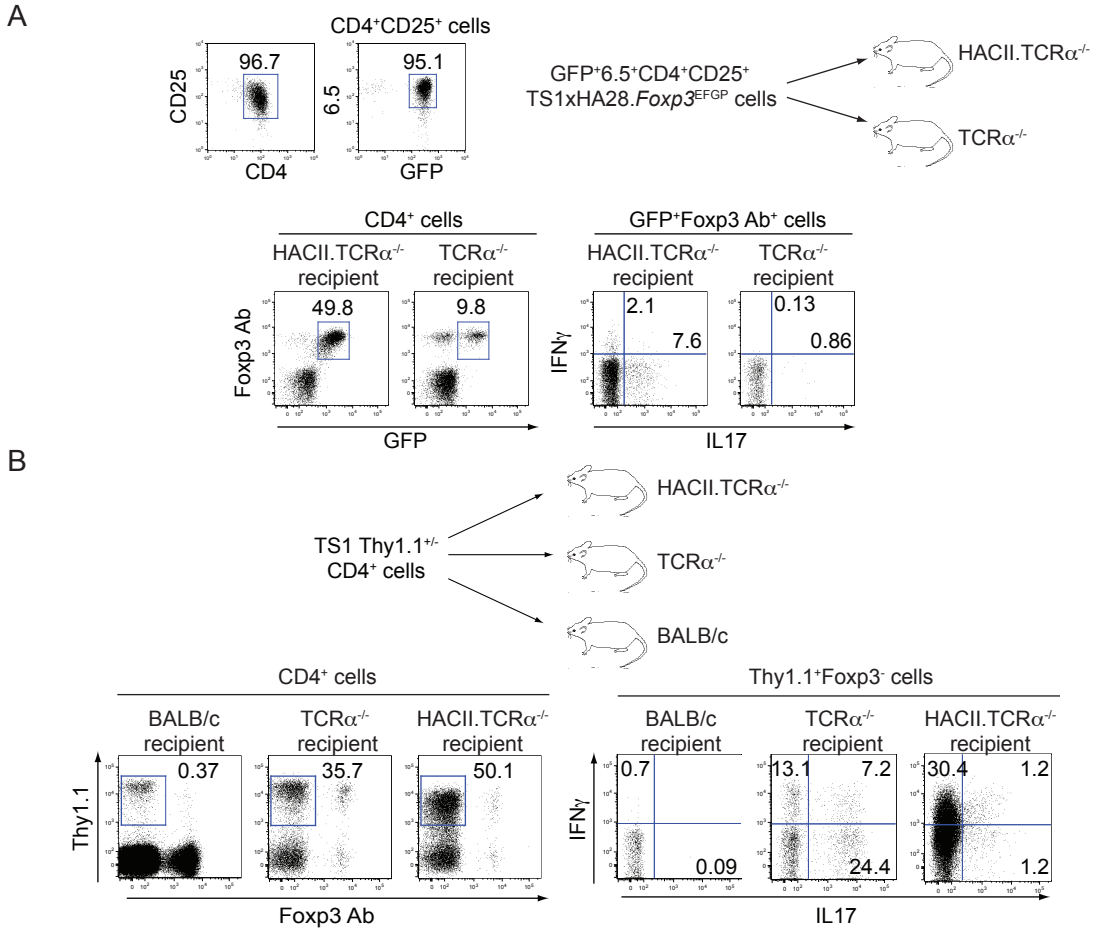

Figure 4-8 6.5+CD4+CD25+Foxp3+ cells produce IL-17 and IFN-γ upon exposure to high levels of cognate antigen in a lymphopenic environment (**A**) 1x10^6 6.5+GFP+ CD4+CD25+ cells from TS1xHA28.*Foxp3*^{EGFP} mice were transferred into HACII.TCRα$^{-/-}$ or TCRα$^{-/-}$ mice. One week later LN cells were stimulated for 4 hours with PMA and ionomycin and stained for CD4, Foxp3, IL-17, and IFN-γ. *Upper panel*, Dot plots show representative purities from cell sorts. Numbers indicate the percentages of CD4+CD25+ cells and 6.5+GFP+ cells. *Lower panel*, Left dot plots show donor GFP+Foxp3+ donor Treg cells within CD4+ cells. Numbers indicate the percentage of GFP+Foxp3+ cells. Right dot plots show IL-17 and IFN-γ staining in GFP+Foxp3+CD4+ cells. Numbers indicate the percentages of the respective cytokines. (**B**) 1x10^6 CD4+ T cells from TS1 Thy1.1 mice were transferred into HACII.TCRα$^{-/-}$, TCRα$^{-/-}$, or BALB/c mice. One week later LN cells were stimulated for 4 hours with PMA and ionomycin and stained for Thy1.1, CD4, Foxp3, IL-17, and IFN-γ. Left dot plots show donor Thy1.1+CD4+ cells among CD4+ cells. Numbers indicate the percentage of Thy1.1+ Foxp3- cells. Right dot plots show IL-17 and IFN-γ staining in Thy1.1+Foxp3-CD4+ cells. Numbers indicate the percentages of the respective cytokines.

Tcr$\alpha^{-/-}$, and BALB/c mice were stimulated with PMA and ionomycin and stained for Thy1.1, CD4, IL-17, and IFN-γ. The donor Thy1.1^{+}CD4^{+} cells were present in all recipient mice (Figure 4-8 B, left panel), albeit at the highest frequency in HACII.Tcr$\alpha^{-/-}$ mice, which most likely reflects the expansion of the 6.5^{+}CD4^{+} T cells in response to S1 peptide. Donor CD4^{+} cells produced cytokines in Tcr$\alpha^{-/-}$ but not BALB/c mice (Figure 4-8 B, right panel), indicating that exposure to lymphopenic environments could induce the differentiation of conventional CD4^{+} T cells. In Tcr$\alpha^{-/-}$ mice, the highest percentage of Thy1.1^{+}CD4^{+}Foxp3^{-} cells produced IL-17 (24.4 percent), followed by 13.1 percent of IFN-γ producing cells, and 7.2 percent of IL-17^{+}IFN-γ^{+} double-producers (Figure 4-8 B). In contrast, in HACII.Tcr$\alpha^{-/-}$ mice, Thy1.1^{+}CD4^{+}Foxp3^{-} cells primarily produced IFN-γ (30.4 percent), with lower percentages of cells producing IL-17 (1.2 percent) or co-producing IL-17 and IFN-γ (1.2 percent) (Figure 5-1 B).

These data show that signals received through the TCR can induce CD4^{+}CD25^{+}Foxp3^{+} Treg cells to differentiate to produce cytokines. Moreover, upon exposure to a lymphopenic environment containing high levels of their cognate antigen CD4^{+}CD25^{+}Foxp3^{+} Treg cells preferentially produced IL-17 rather than IFN-γ. Interestingly, while conventional CD4^{+} T cells preferentially produced IL-17 in Tcr$\alpha^{-/-}$ mice, introduction of high levels of HA skewed the cells towards IFN-γ production. These results suggest that upon exposure to high

levels of cognate antigen in the context of a lymphopenic environment, $CD4^+CD25^+$ Treg cells preferentially produce IL-17, whereas conventional $CD4^+$ T cells preferentially produce IFN-γ.

4.2.7 $6.5^+CD4^+CD25^+$ Treg cells from TS1xHA28.*Foxp3*^{EGFP} mice do not differentiate to produce IL-17 in TS1xHACII mice

We next examined whether $6.5^+CD4^+CD25^+$ Treg cells could also acquire the ability to produce IL-17 or IFN-γ in TS1xHACII mice. To test this, we purified and transferred $1x10^6$ $6.5^+GFP^+CD4^+CD25^+$ cells from TS1xHA28.*Foxp3*^{EGFP} mice into young pre-arthritic TS1xHACII, older arthritic TS1xHACII, or control HACII mice. Pre-arthritic and arthritic TS1xHACII mice were used to determine whether the additional "space" available in young, lymphopenic TS1xHACII mice contributes to the differentiation of $CD4^+CD25^+$ Treg cells. HACII mice were used to examine how strong antigenic stimulation without the inflammation found in TS1xHACII mice would affect cytokine production by $CD4^+CD25^+$ Treg cells. Donor $CD4^+CD25^+$ Treg cells (identified by co-expression of GFP and Foxp3) were present in the LNs of pre-arthritic TS1xHACII, older arthritic TS1xHACII, and single transgenic HACII mice one week post transfer (Figure 4-9 A, upper panel). The increased percentage of GFP^+Foxp3^+ cells in young, pre-arthritic TS1xHACII mice is due to the lower number of $CD4^+$ cells in young TS1xHACII

mice, and GFP^+Foxp3^+ cell recoveries were comparable in the three different recipients (data not shown). However, in contrast to what was observed in HACII.Tcr$\alpha^{-/-}$ mice, the transferred $6.5^+GFP^+CD4^+CD25^+$ cells produced negligible amounts of IL-17 and IFN-γ in all three recipients (Figure 4-9 A, lower panel). Moreover, $6.5^+GFP^+CD4^+CD25^+$ cells did not produce either IL-17 or IFN-γ two or three weeks post transfer into pre-arthritic or arthritic TS1xHACII mice (data not shown).

We next examined whether localization to the popliteal versus non-joint draining LNs might affect the differentiation of $CD4^+CD25^+Foxp3^+$ Treg cells in TS1xHACII mice. To address, this we analyzed the cytokine profile of donor $GFP^+CD4^+CD25^+$ Treg cells from the popliteal and non-draining LNs in 15 week old TS1xHACII mice that had received $1x10^6$ TS1xHA28.*Foxp3*^{EGFP} $GFP^+CD4^+CD25^+$ cells ten weeks earlier. Although GFP^+Foxp3^+ cells were present in both the draining and non-draining LNs (Figure 4-9 B, left panel), the GFP^+Foxp3^+ cells did not produce IL-17 or IFN-γ in either the popliteal or non-draining LNs of TS1xHACII mice (Figure 4-9 B, right panel). These data demonstrate that $GFP^+CD4^+CD25^+$ cells from TS1xHA28.*Foxp3*^{EGFP} mice do not differentiate to produce either IL-17 or IFN-γ even after ten weeks in TS1xHACII mice. Furthermore, the micro-environment of the draining LN did not favor induction of IL-17 or IFN-γ production by the donor $CD4^+CD25^+$ Treg cells, as

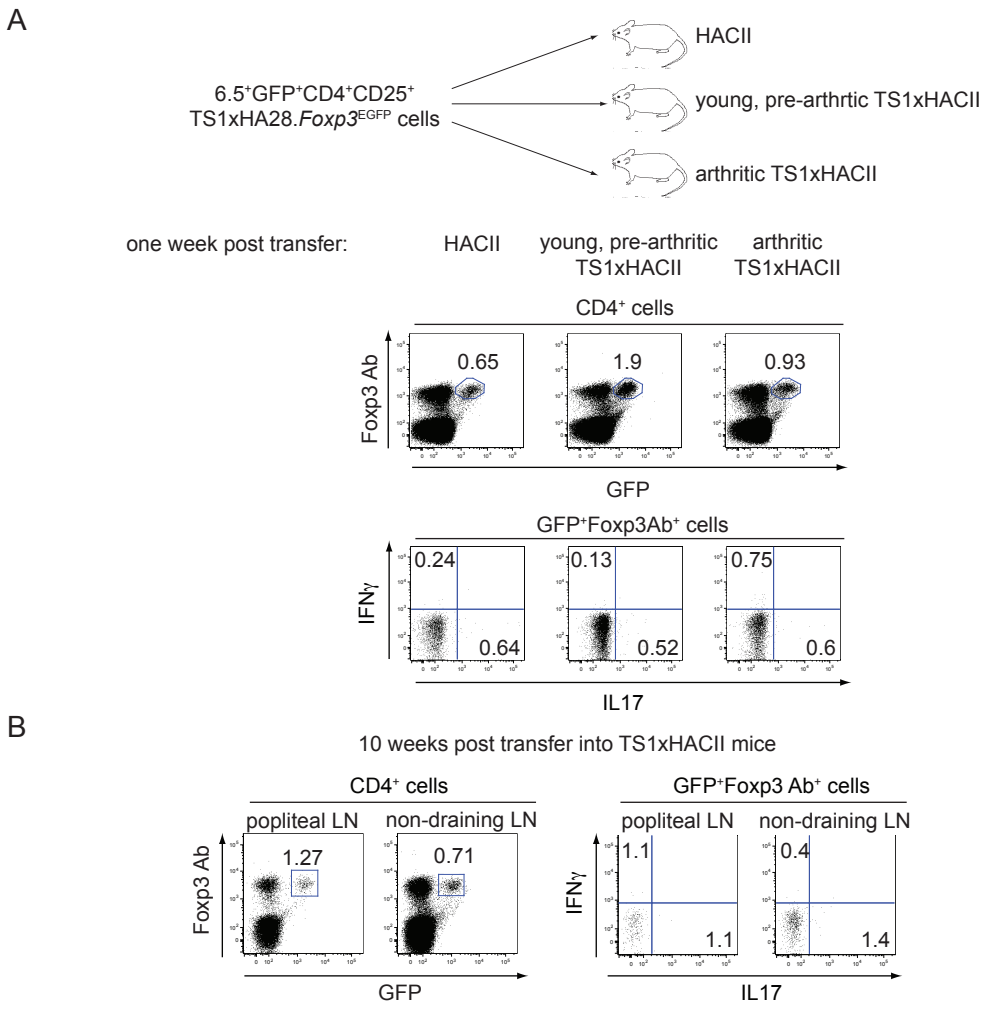

Figure 4-9 TS1xHA28.*Foxp3*^{EGFP} 6.5^{+}CD4^{+}CD25^{+}Foxp3^{+} cells do not produce IL-17 or IFN-γ in TS1xHACII mice (**A**) 1x10^{6} TS1xHA28.*Foxp3*^{EGFP} 6.5^{+}GFP^{+}CD4^{+}CD25^{+} cells were adoptively transferred into HACII, young pre-arthritic TS1xHACII, or arthritic TS1xHACII mice. One week post transfer LN cells from recipient mice were stimulated with PMA and ionomycin, and stained for CD4, Foxp3, IL-17, and IFN-γ. *Upper panel*, dot plots show donor GFP^{+}Foxp3^{+} Treg cells among CD4^{+} cells. Numbers indicate the percentage of GFP^{+}Foxp3^{+} cells. *Lower panel*, dot plots are gated on GFP^{+}Foxp3^{+}CD4^{+} cells. Numbers indicate the percentages of IL-17 and IFN-γ producing cells. (**B**) TS1xHACII mice that received 1x10^{6} TS1xHA28.*Foxp3*^{EGFP} GFP^{+}CD4^{+}CD25^{+} cells (from Figure 4-3) were analyzed 10 weeks post transfer. Popliteal and non-draining (cervical, inguinal, mesenteric) LNs were stimulated as described in part (**A**) to determine IL-17 and IFN-γ production by GFP^{+}Foxp3^{+} cells. Numbers indicate the percentages of GFP^{+}Foxp3^{+} and cytokine-producing cells in the respective dot plots.

GFP$^+$CD4$^+$CD25$^+$ cells in both the popliteal and non-draining LNs in TS1xHACII mice did not produce either cytokine.

Thus, while 6.5$^+$CD4$^+$CD25$^+$Foxp3$^+$ cells can acquire the ability to produce IL-17 in response to strong TCR signals, they do not undergo this phenotypic change upon transfer into TS1xHACII mice, and it is unlikely that IL-17 production by 6.5$^+$CD4$^+$CD25$^+$Foxp3$^+$ cells explains the inability of CD4$^+$CD25$^+$ Treg cells enriched for S1-specificity to prevent arthritis development in TS1xHACII mice.

4.2.8 Strong antigenic stimulation induces CD4$^+$CD25$^+$ Treg cell downregulation of CD25

In addition to observations that CD4$^+$CD25$^+$Foxp3$^+$ Treg cells can produce cytokines (such as IL-17 and IFN-γ) that are associated with activated conventional CD4$^+$ T cells (Ayyoub et al. 2009; Beriou et al. 2009; Oldenhove et al. 2009; Voo et al. 2009), recent work has demonstrated that CD4$^+$CD25$^+$ Treg cells can also lose Foxp3 expression and subsequently acquire effector functions (Xu et al. 2007; Osorio et al. 2008; Yang et al 2008; Duarte et al. 2009; Hoffman et al. 2009; Komatsu et al. 2009; Murai et al. 2009; Zhou et al. 2009). The plasticity of the CD4$^+$CD25$^+$ Treg cell population has been most apparent in

conditions of lymphopenia and inflammation (Komatsu et al. 2009; Murai et al. 2009; Zhou et al. 2009). While we have shown that $6.5^{+}CD4^{+}CD25^{+}Foxp3^{+}$ cells do not differentiate to produce IL-17 or IFN-γ in TS1xHACII mice, we could not eliminate the possibility that a subset of TS1xHA28 $CD4^{+}CD25^{+}$ Treg cells in TS1xHACII mice might undergo phenotypic changes that could contribute to the loss of Foxp3 expression and regulatory function. CD25 has been shown to play an important role not only in the survival of $CD4^{+}CD25^{+}$ Treg cells (D'Cruz and Klein 2005; Fontenot et al. 2005; Setoguchi et al. 2005), but also in the maintenance of Foxp3 expression (Fontenot et al. 2005; Zorn et al. 2006). Indeed, recent work showed $CD4^{+}CD25^{-}Foxp3^{+}$ cells were more susceptible to losing Foxp3 expression than $CD4^{+}CD25^{+}Foxp3^{+}$ cells (Komatsu et al. 2009).

To determine the effects of the the environment in TS1xHACII mice on CD25 expression by $CD4^{+}CD25^{+}$ Treg cells from TS1xHA28 mice upon exposure, we adoptively transferred CFSE-labeled $CD4^{+}CD25^{+}$ cells purified from TS1xHA28 mice into TS1xHACII, HACII, or BALB/c mice and analyzed the donor cells three days post transfer. CFSE-labeling the TS1xHA28 $CD4^{+}CD25^{+}$ cells allows us to examine their proliferation in the respective host mice, and also to distinguish the donor 6.5^{+} cells from endogenous $6.5^{+}CD4^{+}$ cells in TS1xHACII mice. As expected, we observed no CFSE dilution by TS1xHA28 $CD4^{+}CD25^{+}$ cells in BALB/c mice, which do not express HA as a self antigen (Figure 4-10,

upper panel). In contrast, TS1xHA28 $CD4^+CD25^+$ cells underwent extensive proliferation in both TS1xHACII and HACII mice (Figure 4-10, upper panel), since the $6.5^+CD4^+CD25^+$ cells encounter high levels of their cognate antigen (S1 peptide) in both hosts. Proliferation of TS1xHA28 $CD4^+CD25^+$ cells was comparable in TS1xHACII and HACII mice, indicating that competition for S1 peptide with endogenous TS1xHACII 6.5^+CD4^+ T cells was not inhibitory to TS1xHA28 $CD4^+CD25^+$ cell proliferation in TS1xHACII mice.

Three days post transfer, the majority of transferred TS1xHA28 $CD4^+CD25^+$ cells maintained high levels of CD25 expression in BALB/c mice, and only 7.1 percent of the cells were $Foxp3^+CD25^-$ (Figure 4-10, lower panel). In contrast, a larger population of $Foxp3^+CD25^-$ cells was present among donor TS1xHA28 $CD4^+CD25^+$ cells in TS1xHACII and HACII mice. In TS1xHACII mice, 27.3 percent of the transferred TS1xHA28 $CD4^+CD25^+$ cells were $Foxp3^+CD25^-$, and 35.2 percent of donor TS1xHA28 $CD4^+CD25^+$ cells were $Foxp3^+CD25^-$ in HACII mice (Figure 4-10, lower panel). The enhanced loss of CD25 expression by TS1xHA28 $CD4^+CD25^+$ cells in TS1xHACII and HACII mice but not BALB/c mice demonstrates that TCR signals and/or cell proliferation contribute to CD25 downregulation by $CD4^+CD25^+$ Treg cells. Moreover, TS1xHA28 $CD4^+CD25^+$ cells exhibited similar degrees of CD25 downregulation in TS1xHACII and HACII

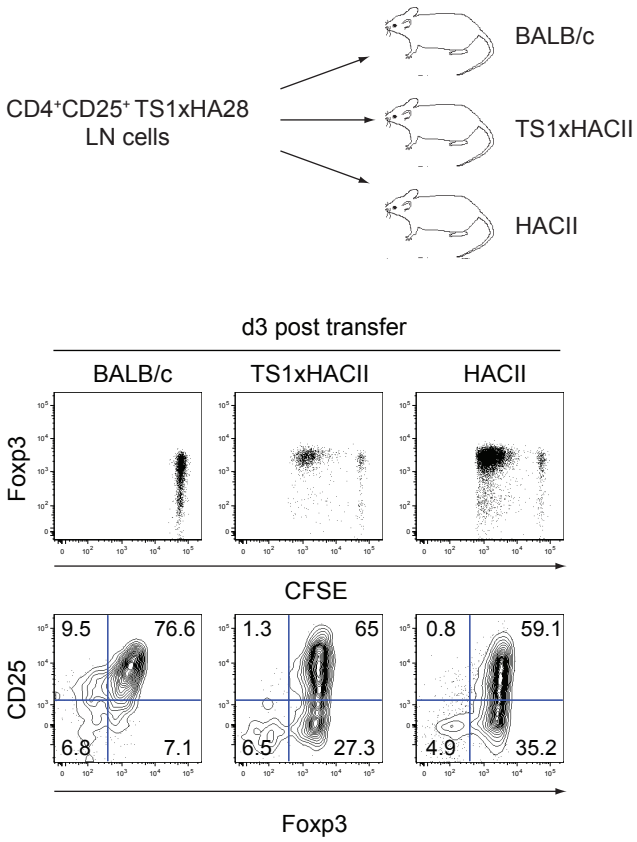

Figure 4-10 Exposure to high levels of cognate antigen induces CD25 downregulation in $CD4^+CD25^+$ Treg cells. $1x10^6$ CFSE-labeled $CD4^+CD25^+$ cells from TS1xHA28 mice were adoptively transferred into BALB/c, TS1xHACII, or HACII mice. Three days post transfer, LN cells from recipient mice were analyzed for the proliferation (upper panel) and the Foxp3 and CD25 expression profile (lower panel) of the donor Treg cells. Flow plots are gated on $CFSE^+$ cells. *Lower panel*, Numbers indicate the percentage of cells in each quadrant.

mice, indicating that a strong antigenic stimulus alone, rather than high levels of antigen in addition to inflammation can induce CD25 downregulation.

Together, these data show $CD4^+CD25^+$ cells from TS1xHA28 mice can undergo *in vivo* proliferation in response to S1 peptide, and that a subset of cells loses CD25 expression upon exposure to high levels of their cognate antigen in TS1xHACII and HACII mice.

4.2.9 $CD4^+CD25^+$ Treg cells can maintain CD25 expression in the presence of low levels of their cognate antigen

We next examined whether the maintenance of CD25 expression by TS1xHA28 $CD4^+CD25^+$ cells differed in conditions that induced strong (defined as high levels of HA) versus weak (defined as low levels of HA) proliferation by transferring CFSE-labeled TS1xHA28 $CD4^+CD25^+$ cells into HACII or HA28 mice, which contain high and low levels of HA respectively. Three and ten days post transfer LN cells were isolated from HACII and HA28 recipient mice and stained for 6.5, CD4, CD25, and Foxp3 expression. At three days post transfer, the majority of TS1xHA28 $CD4^+CD25^+$ cells in HACII mice had undergone several rounds of division, whereas in HA28 mice most of the TS1xHA28 $CD4^+CD25^+$ cells remained CFSE bright and only a small subset of cells had

begun to proliferate (Figure 4-11 A, left panel). By ten days post transfer, TS1xHA28 $CD4^+CD25^+$ cells had proliferated further in HACII mice, and some cells had completely diluted their CFSE (Figure 4-11 A, right panel). TS1xHA28 $CD4^+CD25^+$ cells in HA28 mice also proliferated more by day ten in comparison to day three post transfer, with some cells undergoing three or four cell divisions (Figure 4-11 A, right panel). Consistent with our previous observations (Figure 4-10), by three days after the initial transfer a subset of TS1xHA28 $CD4^+CD25^+$ cells in HACII mice downregulated CD25 expression (Figure 4-11 B, left panel), and by ten days post transfer into HACII mice the representation of both $Foxp3^+CD25^-$ and $Foxp3^-CD25^-$ cells was increased within the donor TS1xHA28 $CD4^+CD25^+$ cell population (Figure 4-11 B, right panel). In contrast, TS1xHA28 $CD4^+CD25^+$ cells maintained high levels of CD25 expression at both three and ten days post transfer into HA28 mice (Figure 4-11 B, left panel).

Taken together, these data demonstrate that $CD4^+CD25^+$ Treg cells can maintain high levels of CD25 expression when exposed to low, but not high, levels of their cognate antigen. Moreover, maintenance of CD25 expression appears to be inversely related to the extent of proliferation by the $CD4^+CD25^+$ Treg cells.

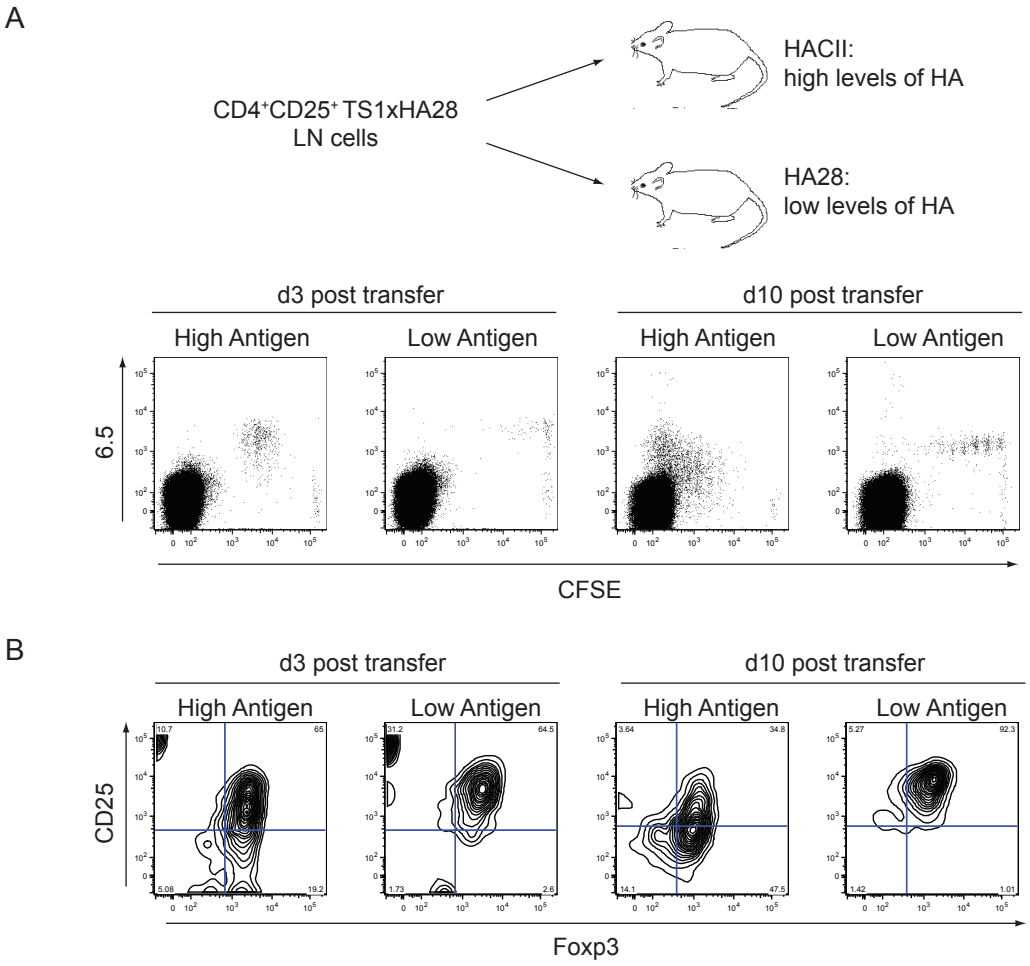

Figure 4-11 $CD4^+CD25^+$ Treg cells can maintain high levels of CD25 expression upon exposure to low, but not high, levels of cognate antigen (**A**) $1x10^6$ CFSE-labeled TS1xHA28 $CD4^+CD25^+$ cells were adoptively transferred into HACII (high HA levels) or HA28 (low HA levels) mice. Proliferation of the donor cells (identified by CFSE and the mAb 6.5) was analyzed at days 3 (left panel) and 10 (right panel) post transfer. Dot plots are gated on $CD4^+$ cells. (**B**) Dot plots show Foxp3 and CD25 staining of donor TS1xHA28 $CD4^+CD25^+$ Treg cells in HACII and HA28 mice at days 3 (left panel) and 10 (right panel) post transfer. Data is representative of three separate experiments.

4.2.10 S1 peptide can activate the *in vitro* suppressor function of $CD4^+CD25^+$ cells expressing a TCR with low reactivity for S1

The finding that BALB/c $CD4^+CD25^+$ Treg cells (which have no exposure to S1 peptide or HA), but not TS1xHA28 $CD4^+CD25^+$ Treg cells (which are enriched for highly reactive S1-specific cells), can prevent arthritis development in TS1xHACII mice strongly suggests that enrichment for cells that recognize S1 peptide is detrimental to $CD4^+CD25^+$ Treg cell suppression of arthritis. Data presented in this chapter also demonstrate that interactions with high levels of cognate antigen can induce phenotypic changes (i.e. cytokine production, CD25 downregulation) in $CD4^+CD25^+$ Treg cells that express the 6.5 TCR, which possesses high reactivity for S1 peptide. However, TS1xHA28 $CD4^+CD25^+$ Treg cells were able to maintain high levels of CD25 expression when exposed to low levels of S1 peptide, suggesting that the strength of signal through the TCR can affect the phenotype of $CD4^+CD25^+$ Treg cells. Thus, we examined whether a population of $CD4^+CD25^+$ Treg cells that was enriched for cells expressing a TCR with low reactivity for S1 peptide would be able to modulate arthritis in TS1xHACII mice by using TS1(SW)xPevSW mice which co-express $CD4^+$ T cells that express the TS1(SW) TCR and the SW virus HA (driven by a β-globin locus control region) as a self-antigen. The TS1(SW) TCR (identified by antibodies to Vα8.3 and Vβ10) is specific for the S1(SW) peptide, which differs from the PR8

S1 peptide by two amino acid substitutions, and possesses much lower reactivity for the S1 peptide (Jordan et al. 2001). TS1(SW)xPevSW mice generate a high frequency of $CD4^+CD25^+Foxp3^+$ cells that are enriched for expression of the TS1(SW) TCR (Figure 4-12 A).

To confirm that suppressor function in $CD4^+CD25^+$ cells from TS1(SW)xPevSW mice can be triggered in an antigen-specific manner, we conducted *in vitro* assays using S1(SW) peptide to stimulate the $CD4^+CD25^+$ cells. CFSE-labeled responder $CD4^+$ T cells were cultured alone or with TS1(SW)xPevSW $CD4^+CD25^+$ cells at a 1:2 Treg:responder cell ratio. Three days later we examined proliferation and cytokine production by responder $CD4^+$ T cells. When cultured alone responder $CD4^+$ T cells underwent extensive proliferation and approximately 75 percent of the cells produced IFN-γ (Figure 4-12 B, left panel). In contrast, when $CD4^+CD25^+$ cells from TS1(SW)xPevSW mice were present, responder $CD4^+$ T cells proliferated less and only 10.4 percent of the cells differentiated to produce IFN-γ (Figure 4-12 B, left panel). This inhibition was also reflected in the recoveries of total and IFN-γ^+ responder $CD4^+$ T cells, which were far lower in the presence of TS1(SW)xPev(SW) $CD4^+CD25^+$ cells than when responder $CD4^+$ T cells were cultured alone (Figure 4-12 B, right panel).

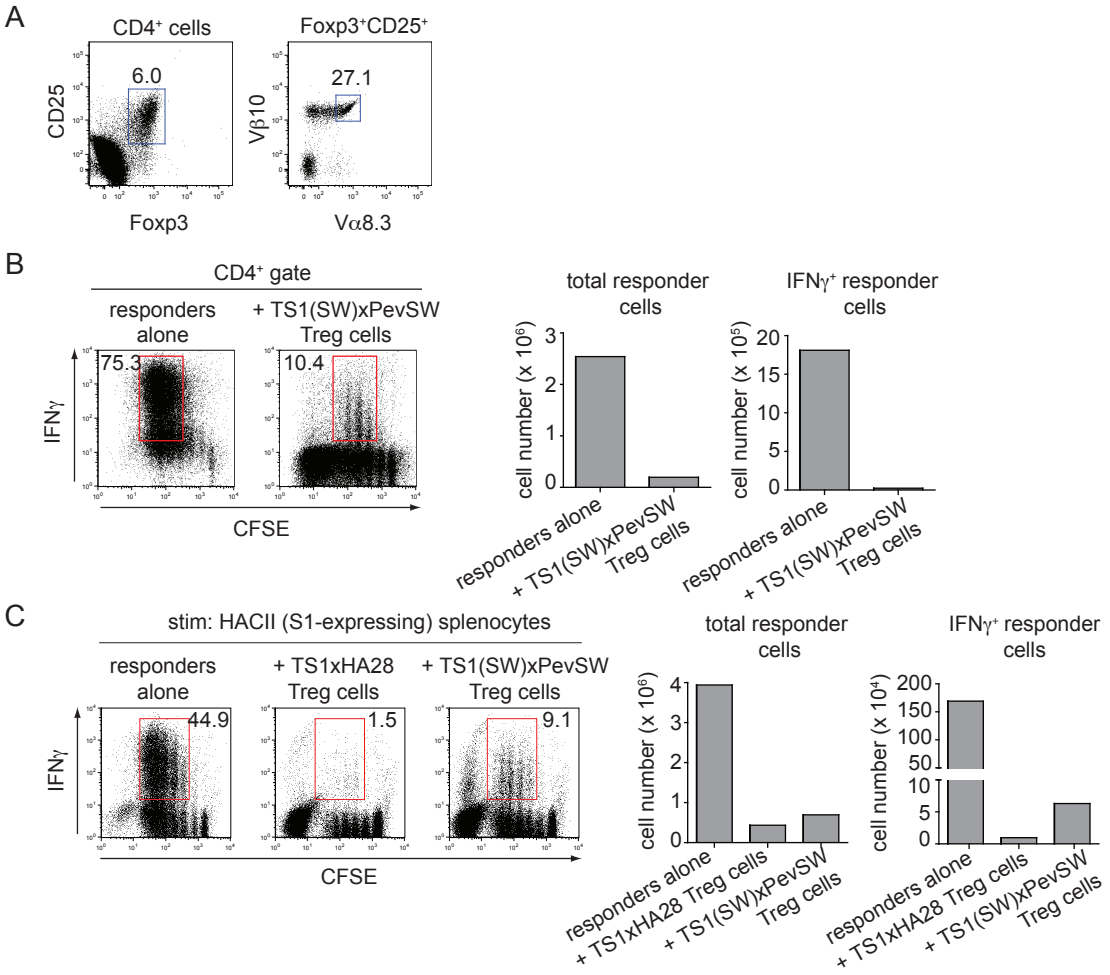

Figure 4-12 The suppressor function of CD4+CD25+ cells expressing the TS1(SW) TCR can be activated by non-cognate interactions with S1 peptide (**A**) Vα8.3+Vβ10+ cells in CD4+CD25+Foxp3+ LN cells from TS1(SW)xPevSW mice. Numbers indicate the percentage of cells in the respective gates. (**B**) CFSE-labeled CD4+ T cells were cultured alone or with TS1(SW)xPevSW CD4+CD25+ cells at a 1:2 Treg:responder cell ratio. CD4+CD25+ cells were stimulated with S1(SW) peptide. *Left panel*, Dot plots show CFSE versus IFN-γ production after three days of culture. Numbers indicate the percentage of IFN-γ+ cells among the responder CD4+ cells. *Right panel*, Graphs show the numbers of total and IFN-γ+ responder cells in the respective culture conditions. (**C**) CFSE-labeled CD4+ T cells were cultured alone or in the presence of CD4+CD25+ cells from TS1xHA28 or TS1(SW)xPevSW mice at 1:2 Treg:responder cell ratio. HACII splenocytes (which present S1 peptide) were used to stimulate the cultures. *Left panel*, Dot plots show CFSE versus IFN-γ production after three days of culture. Numbers indicate the percentage of IFN-γ+ cells among the responder CD4+ cells. *Right panel*, Graphs show the numbers of total and IFN-γ+ responder cells in the respective culture conditions.

We next determined whether $CD4^+CD25^+$ cells from TS1(SW)xPevSW mice could exert *in vitro* regulatory function when activated by S1 peptide, which is less reactive with the TS1(SW) TCR. CFSE-labeled responder $CD4^+$ T cells were co-cultured with $CD4^+CD25^+$ cells from TS1(SW)xPevSW mice, or as a control for $6.5^+CD4^+CD25^+$ Treg cells, TS1xHA28 mice. HACII splenocytes, which present S1 peptide, were used to stimulate the cultured cells. After three days of culture, we examined proliferation and cytokine production by responder $CD4^+$ T cells. When cultured alone, responder $CD4^+$ T cells underwent robust proliferation, and approximately 45 percent of the cells produced IFN-γ (Figure 4-12 C, left panel). The addition of TS1xHA28 $CD4^+CD25^+$ cells reduced responder $CD4^+$ T cell proliferation and cytokine production, as only 1.5 percent of the cells produced IFN-γ (Figure 4-12 C, left panel). When co-cultured with $CD4^+CD25^+$ cells from TS1(SW)xPevSW mice, responder $CD4^+$ T cells also proliferated less and only 9.1 percent of the cells produced IFN-γ (Figure 4-12 C, left panel).

$CD4^+CD25^+$ Treg cell suppression of responder $CD4^+$ T cells was also evident in the numbers of total responder $CD4^+$ T cells in the cultures, which were much lower in the presence of either set of $CD4^+CD25^+$ cells than when the responder $CD4^+$ T cells were cultured alone (Figure 4-12 C, lower panel). Moreover, the numbers of total responder $CD4^+$ T cells were comparable in

cultures with either TS1xHA28 or TS1(SW)xPevSW $CD4^+CD25^+$ cells. In comparison to when responder $CD4^+$ T cells were cultured alone, co-culture of $CD4^+CD25^+$ cells also dramatically reduced the numbers of IFN-γ^+ responder $CD4^+$ T cells. However, reflecting the difference in percentages of IFN-γ^+ cells in cultures with TS1xHA28 or TS1(SW)xPevSW $CD4^+CD25^+$ cells (Figure 4-12 C, upper panel), more IFN-γ producing responder $CD4^+$ T cells were recovered from cultures with TS1(SW)xPevSW $CD4^+CD25^+$ cells than TS1xHA28 $CD4^+CD25^+$ cells (Figure 4-12 C, lower panel).

Thus, the *in vitro* suppressor function of $CD4^+CD25^+$ Treg cells enriched for expression of the TS1(SW) TCR can be activated by both cognate (S1(SW) peptide) and non-cognate interactions (S1 peptide). Moreover, although the TS1(SW) TCR has much lower reactivity for the S1 peptide than the 6.5 TCR, stimulation with S1 peptide induced comparable suppression of responder $CD4^+$ T cells by both TS1(SW)xPevSW and TS1xHA28 $CD4^+CD25^+$ cells.

4.2.11 $CD4^+CD25^+$ Treg cells expressing a TCR with low reactivity for S1 peptide can modulate arthritis development in TS1xHACII mice

We have established that $CD4^+CD25^+$ Treg cells expressing the TS1(SW) TCR can be activated S1 peptide, and to examine the ability of

TS1(SW)xPevSW $CD4^+CD25^+$ cells to modulate arthritis development in TS1xHACII mice we purified and transferred $1x10^6$ $CD4^+CD25^+$ cells from TS1(SW)xPevSW mice into pre-arthritic TS1xHACII mice and tracked the recipients for disease development. At each time point during the nine week period of analysis, TS1xHACII mice that received TS1(SW)xPevSW $CD4^+CD25^+$ cells averaged fewer arthritic limbs per mouse than unmanipulated TS1xHACII mice (Figure 4-13 A). To determine whether this was a reflection of reduced disease incidence or lower numbers of affected limbs per arthritic mouse, we examined the kinetics of arthritis incidence in both cohorts of TS1xHACII mice. TS1xHACII mice that received TS1(SW)xPevSW $CD4^+CD25^+$ cells exhibited a slightly delayed kinetics in disease development, but by 15 weeks of age 75 percent of both TS1(SW)xPevSW $CD4^+CD25^+$ cell recipient and unmanipulated TS1xHACII mice had developed arthritis (Figure 4-13 B).

We also examined 12 and 15 week old TS1xHACII mice to determine whether the administration of TS1(SW)xPevSW $CD4^+CD25^+$ cells reduced the number of affected limbs in arthritic TS1xHACII mice. Among 12 week old arthritic mice, the majority (6 of 9, 66.7 percent) of unmanipulated TS1xHACII mice had three or four affected limbs, where as the majority (4 of 6, 66.7 percent) of TS1xHACII mice that received TS1(SW)xPevSW $CD4^+CD25^+$ cells had two or one affected limbs (Figure 4-13 C, upper left panel). Thus, in 12 week old mice,

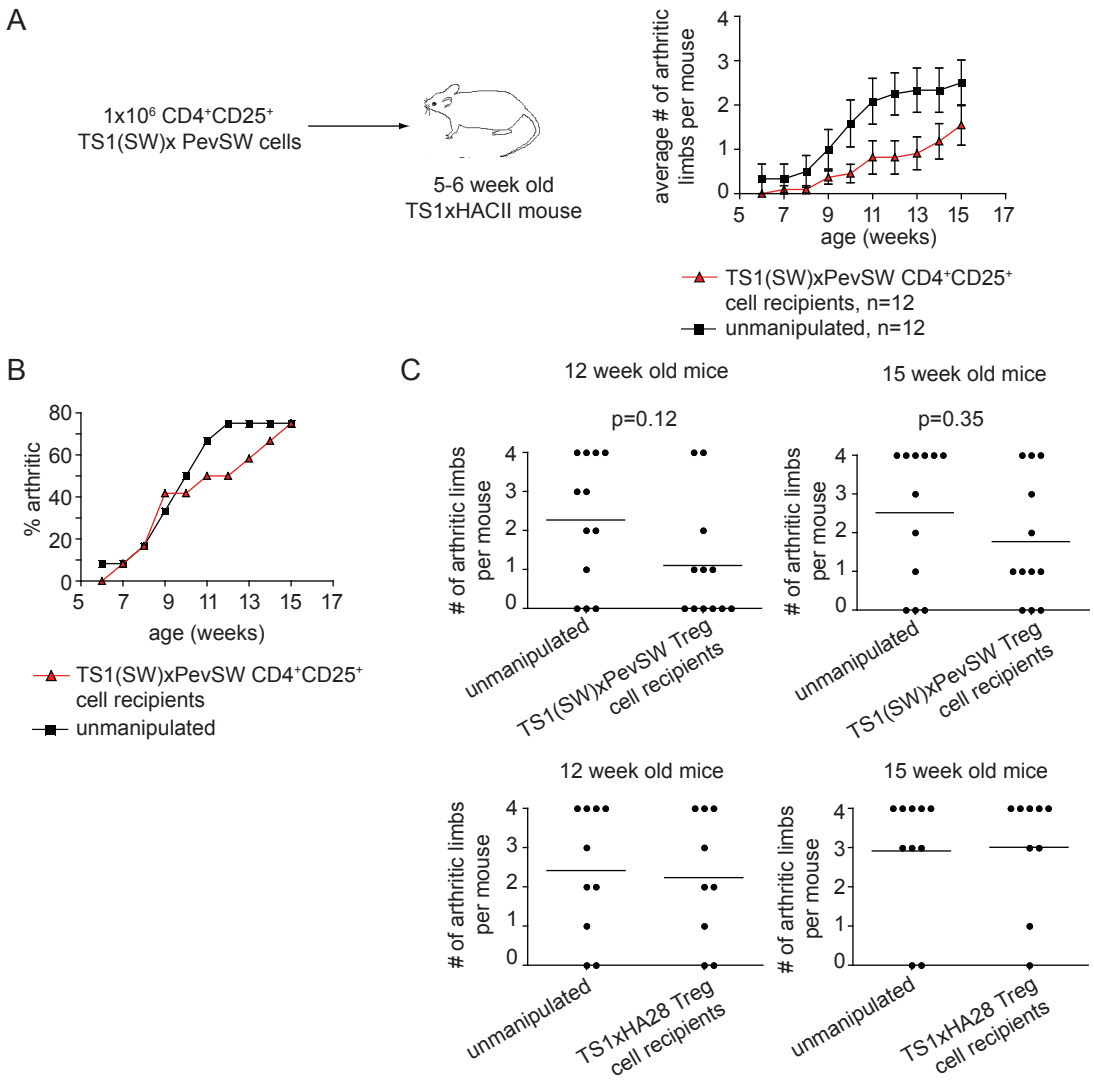

Figure 4-13 TS1(SW)xPevSW CD4⁺CD25⁺ cells modify arthritis development in TS1xHACII mice (**A**) 1x10⁶ TS1(SW)xPevSW CD4⁺CD25⁺ cells were adoptively transferred into TS1xHACII mice. Graph shows the average number of arthritic limbs per mouse (±SEM) over time for unmanipulated TS1xHACII mice (■, n=12) and TS1xHACII mice that received TS1(SW)xPevSW CD4⁺CD25⁺ cells (▲, n=12) (**B**) Arthritis incidence over time in cohorts of unmanipulated TS1xHACII mice and TS1(SW)xPevSW CD4⁺CD25⁺ cell recipient TS1xHACII mice. (**C**) Graphs show the number of arthritic limbs per mouse in 12 week old (left) and 15 week old (right) cohorts of unmanipulated TS1xHACII mice and TS1xHACII mice that received TS1(SW)xPevSW CD4⁺CD25⁺ cells (upper panel) or TS1xHA28 CD4⁺CD25⁺ cells (lower panel). Each circle represents an individual mouse. Line represents the average number of arthritic limbs per mouse. Statistical significance was calculated using the Mann-Whitney test.

the reduction in arthritis incidence in TS1xHACII mice that received TS1(SW)xPevSW $CD4^+CD25^+$ cells was accompanied by a decrease in the number of affected limbs per arthritic TS1xHACII mouse. By 15 weeks of age despite having the same incidence of arthritis development, 77.8 percent (7 of 9) of arthritic unmanipulated TS1xHACII mice possessed three or four affected limbs, in comparison to 44.4 percent (4 of 9) of TS1xHACII mice that received TS1(SW)xPevSW $CD4^+CD25^+$ cells (Figure 4-13 C, right graph). Moreover, 44.4 percent (4 of 9) of arthritic TS1xHACII mice that received TS1(SW)xPevSW $CD4^+CD25^+$ cells had only one affected limb, whereas only 11.1 percent (1 of 9) of arthritic unmanipulated TS1xHACII mice had one affected limb.

In contrast, the transfer of TS1xHA28 $CD4^+CD25^+$ cells into TS1xHACII mice (Figure 4-2) did not result in a reduction in the number of affected limbs in arthritic mice. Among 12 week old arthritic mice, 50 percent of unmanipulated TS1xHACII mice and 57.1 percent of TS1xHACII mice that received TS1xHA28 $CD4^+CD25^+$ cells had three or four affected limbs (Figure 4-13 C, lower left panel). Moreover, by 15 weeks of age, the representation of arthritic mice with three or four affected limbs had increased to 87.5 percent in TS1xHACII mice that received TS1xHA28 $CD4^+CD25^+$ cells (Figure 4-13 C, lower right panel). These data suggest that $CD4^+CD25^+$ Treg cells possessing low reactivity for S1 peptide can ameliorate arthritis severity in TS1xHACII mice.

Collectively, these data show that $CD4^+CD25^+$ Treg cells enriched for expression of the TS1(SW) TCR, which possesses low reactivity for the disease target antigen S1 peptide, can modulate arthritis development in TS1xHACII mice by delaying the kinetics of disease incidence and reducing arthritis severity.

4.2.12 $CD4^+CD25^+$ Treg cells enriched for the TS1(SW) TCR proliferate less and can maintain CD25 expression upon exposure to high levels of the mis-matched S1 peptide

We previously showed that $CD4^+CD25^+$ cells from TS1xHA28 mice downregulate CD25 in response to high levels of HA present in TS1xHACII or HACII mice (Figure 4-10). Given the low reactivity of the TS1(SW) TCR for S1 peptide, we hypothesized that $CD4^+CD25^+$ Treg cells from TS1(SW)xPevSW mice might be less susceptible to CD25 downregulation upon exposure to the high levels of HA present in TS1xHACII mice. Since TS1xHACII mice do not contain endogenous $CD4^+$ cells that express the TS1(SW) TCR, we could identify the donor $CD4^+CD25^+$ cells from TS1(SW)xPevSW mice by staining for co-expression of $V\alpha8.3$ and $V\beta10$, and examine the Foxp3 and CD25 profiles of the donor $V\alpha8.3^+V\beta10^+CD4^+$ cells. LN and spleen cells from unmanipulated TS1xHACII mice and mice that received TS1(SW)xPevSW $CD4^+CD25^+$ cells

were stained for Vα8.3, Vβ10, CD4, Foxp3, and CD25. Nine weeks after transfer, donor Vα8.3$^+$Vβ10$^+$CD4$^+$ cells could be detected in the LNs and spleens of TS1xHACII mice that received TS1(SW)xPevSW CD4$^+$CD25$^+$ cells (Figure 4-14 A, upper panel). Among Vα8.3$^+$Vβ10$^+$CD4$^+$ cells recovered from the LNs of TS1xHACII mice, approximately 50 percent of the cells were Foxp3$^+$CD25$^+$, and 36.9 percent expressed neither Foxp3 nor CD25 (Figure 4-14 A, lower left panel). In Vα8.3$^+$Vβ10$^+$CD4$^+$ cells recovered from the spleens of TS1xHACII mice, up to 72.6 percent of cells retained Foxp3 and CD25 expression, 13.9 percent of cells were Foxp3$^+$CD25$^-$, and 5.8 percent of the cells expressed neither Foxp3 nor CD25 (Figure 4-14 A, lower right panel).

The maintenance of Foxp3 and CD25 expression in Vα8.3$^+$Vβ10$^+$CD4$^+$ cells recovered from TS1xHACII mice was striking when compared to TS1xHA28 CD4$^+$CD25$^+$ cells, which had dramatically downregulated CD25 expression after just three days in TS1xHACII or HACII mice (Figure 4-10). Indeed, after ten days in HACII mice, the majority of TS1xHA28 CD4$^+$CD25$^+$ cells were either Foxp3$^+$CD25$^-$ or Foxp3$^-$CD25$^-$ (Figure 4-11). To examine whether CD4$^+$CD25$^+$ cells from TS1(SW)xPevSW mice could better maintain CD25 expression in response to high levels of HA we purified and adoptively transferred CFSE-labeled CD4$^+$CD25$^+$ cells from TS1(SW)xPevSW mice into PevSW (which contain low levels of the cognate S1(SW) peptide) or HACII (which contain high

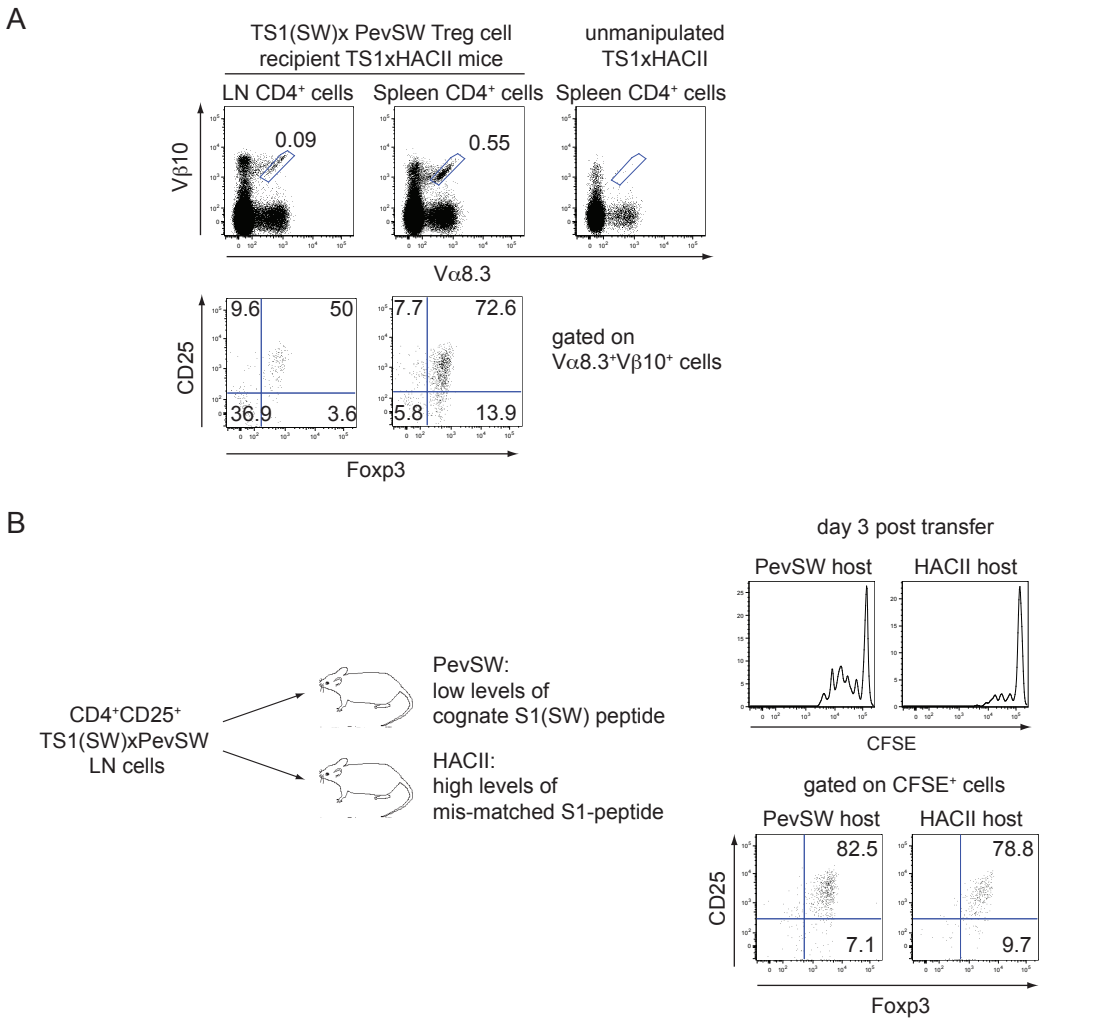

Figure 4-14 Foxp3 and CD25 profile of TS1(SW)xPevSW CD4$^+$CD25$^+$ cells exposed to high levels of the mis-matched S1 peptide (**A**) LN cells from TS1xHACII mice that received 1x10^6 TS1(SW)xPevSW CD4$^+$CD25$^+$ cells were stained for CD4, CD25, Foxp3, Vα8.3, and Vβ10 expression ten weeks post transfer. *Upper panel*, Dot plots show Vα8.3$^+$Vβ10$^+$ cells in the LNs and spleen of TS1xHACII mice. Right plot shows representative staining from a control TS1xHACII mouse that did not receive cells. *Lower panel*, Foxp3 and CD25 profile of recovered Vα8.3$^+$Vβ10$^+$CD4$^+$ cells. (**B**) 1x10^6 CFSE-labeled TS1(SW)xPevSW CD4$^+$CD25$^+$ cells were adoptively transferred into PevSW or HACII mice. Three days later donor cells were analyzed for their CFSE and Foxp3 and CD25 expression profiles. *Upper panel*, histograms show CFSE profiles of donor cells in PevSW and HACII mice. *Lower panel*, Foxp3 and CD25 profiles of CFSE$^+$ cells from PevSW and HACII mice. Numbers indicate the percentage of cells in the respective quadrants.

levels of the mis-matched S1 peptide) mice. Three days post transfer we examined the CFSE and Foxp3 and CD25 expression profiles of the donor cells.

At three days post transfer, a subset of TS1(SW)xPevSW $CD4^+CD25^+$ cells had proliferated in PevSW mice, with some cells undergoing up to five divisions (Figure 4-14 B, upper panel). The dividing cells are most likely the $V\alpha8.3^+V\beta10^+$ population responding to the presence of their cognate antigen S1(SW) peptide in PevSW mice. In contrast, TS1(SW)xPevSW $CD4^+CD25^+$ cells proliferated less in HACII mice, and the cells that did expand underwent fewer cell divisions than proliferating cells in PevSW mice (Figure 4-14 B, upper panel). Additionally, the majority of TS1(SW)xPevSW $CD4^+CD25^+$ cells recovered from PevSW mice maintained high levels of CD25 expression (Figure 4-14 B, lower panel), similar to what was observed with TS1xHA28 $CD4^+CD25^+$ cells in HA28 mice (Figure 4-11). Interestingly, the majority of TS1(SW)xPevSW $CD4^+CD25^+$ cells recovered from HACII mice also maintained high levels of CD25 expression (Figure 4-14, lower panel), which is in marked contrast to the phenotype of TS1xHA28 $CD4^+CD25^+$ cells after transfer into HACII mice.

Collectively these data demonstrate that $CD4^+CD25^+$ cells from TS1(SW)xPevSW mice proliferate more extensively in response to lower levels of the cognate antigen S1(SW) peptide in PevSW mice than to high levels of the

mis-matched S1 peptide (which can activate their suppressor function) present in HACII mice. Moreover, although a subset of TS1(SW)xPevSW $CD4^+CD25^+$ cells lost CD25 expression upon transfer into TS1xHACII mice, our data suggest that the phenotypic changes may occur more slowly than in $6.5^+CD4^+CD25^+$ Treg cells, since TS1(SW)xPevSW $CD4^+CD25^+$ cells but not TS1xHA28 $CD4^+CD25^+$ cells could maintain high levels of CD25 expression after three days in HACII mice. These data suggest that the low reactivity of the TS1(SW) TCR for S1 peptide counters the high levels of HA present in TS1xHACII or HACII mice. Therefore, although the mis-matched S1 peptide can activate comparable *in vitro* suppressor function in TS1xHA28 and TS1(SW)xPevSW $CD4^+CD25^+$ Treg cells, it has disparate effects on the *in vivo* proliferation and CD25 maintenance of $CD4^+CD25^+$ Treg cells enriched for expression of the 6.5 TCR (cognate interaction) versus the TS1(SW) TCR (non-cognate interaction).

4.3 Summary of Results and Discussion

4.3.1 $CD4^+CD25^+$ Treg cells that are enriched in specificity for a disease target antigen fail to suppress arthritis development in TS1xHACII mice

The recognition of S1 peptide by 6.5^+CD4^+ T cells is required for arthritis development in TS1xHACII mice. Strikingly, we found that $CD4^+CD25^+$ Treg cells enriched in specificity for S1 peptide were unable to modulate arthritis development in TS1xHACII mice, as the administration of $CD4^+CD25^+$ cells from TS1xHA28 mice, which generate a high frequency of 6.5^+Foxp3^+ cells (Jordan et al. 2001), did not reduce arthritis incidence or disease severity (as defined by the number of affected limbs per arthritic mouse) in TS1xHACII mice. $GFP^+CD4^+CD25^+$ cells from TS1xHA28.*Foxp3*^{EGFP} mice, a more stringently isolated $CD4^+CD25^+$ Treg cell population (since GFP was used as a surrogate for Foxp3 expression), also failed to suppress arthritis development in TS1xHACII mice. Collectively, these data demonstrate that $CD4^+CD25^+$ Treg cells with high reactivity for a critical disease target antigen fail to prevent arthritis in TS1xHACII mice. Moreover, the failure of TS1xHA28.*Foxp3*^{EGFP} $GFP^+CD4^+CD25^+$ cells to suppress arthritis development in TS1xHACII mice indicates that the inability of TS1xHA28 $CD4^+CD25^+$ cells (in which GFP expression was not used as a

parameter for $CD4^+CD25^+$ Treg cell purification) to prevent disease in TS1xHACII mice is not solely due to the presence of $6.5^+CD4^+CD25^+Foxp3^-$ cells in the initial transfer population.

Donor TS1xHA28.*Foxp3*^{EGFP} $GFP^+CD4^+CD25^+$ cells could be detected in lymphoid organs throughout TS1xHACII mice (including the popliteal LNs) for up to ten weeks post transfer, indicating that the failure of $CD4^+CD25^+$ Treg cells that are enriched for 6.5 TCR expression to prevent arthritis in TS1xHACII mice could not be attributed to deletion of the donor $CD4^+CD25^+$ Treg cells or an inability to traffic to the joint-draining LNs, which are a primary site of autoimmune pathology. However despite accessing the joint draining LNs, $CD4^+CD25^+$ Treg cells from TS1xHA28 and TS1xHA28.*Foxp3*^{EGFP} mice could not suppress the accumulation of immune cells or the Th-17 response that develops in the popliteal LNs of TS1xHACII mice, whereas both responses were significantly diminished in TS1xHACII mice that received protective HACII $CD4^+CD25^+$ cells. Furthermore, TS1xHA28 $CD4^+CD25^+$ cells were also unable to suppress IL-17 production by systemic LN cells in TS1xHACII mice. Strikingly, beyond an inability to suppress the systemic Th-17 response in TS1xHACII mice, $CD4^+CD25^+$ cells from TS1xHA28 mice appeared to enhance production of other cytokines that could contribute to Th-17 differentiation, as unfractionated LN cultures from TS1xHACII mice that received TS1xHA28 $CD4^+CD25^+$ cells

contained significantly higher levels of IL-1β, and also slightly higher levels of IL-1α and IL-6 in comparison to unmanipulated TS1xHACII mice (Figure 4-7 B and C). Thus, $CD4^+CD25^+$ Treg cells that are enriched in specificity for a disease target antigen not only fail to suppress a cytokine that is critical for arthritis development in TS1xHACII mice, but also appear to enhance the production of other cytokines that not only contribute to Th-17 differentiation, but also have roles in arthritis pathogenesis.

4.3.2 Interactions with high levels of cognate antigen can induce phenotypic changes in $CD4^+CD25^+$ Treg cells

Recent work has shown that activated human $CD4^+CD25^+Foxp3^+$ Treg cells can produce IL-17 (Ayyoub et al. 2009; Beriou et al. 2009; Voo et al. 2009), and interestingly, there is some indication that strong TCR signals are more effective at inducing IL-17 production by $CD4^+CD25^+Foxp3^+$ Treg cells (Voo et al. 2009). We found that $6.5^+GFP^+CD4^+CD25^+$ cells from TS1xHA28.*Foxp3*^{EGFP} mice could differentiate to produce both IL-17 and IFN-γ in HACII.Tcrα$^{-/-}$ but not Tcrα$^{-/-}$ mice (Figure 4-8), indicating that high levels of cognate antigen coupled with lymphopenia but not lymphopenia alone could induce $CD4^+CD25^+Foxp3^+$ Treg cells to produce cytokines. Moreover, we also observed that $6.5^+CD4^+CD25^+Foxp3^+$ Treg cells in HACII.Tcrα$^{-/-}$ mice preferentially produced

IL-17 over IFN-γ, whereas conventional $CD4^+$ T cells that were enriched for 6.5 expression produced more IFN-γ than IL-17 upon transfer into HACII.Tcr$\alpha^{-/-}$ mice, suggesting that $CD4^+CD25^+$ Treg cells may be predisposed towards IL-17 production. Studies in human $CD4^+CD25^+$ Treg cells have shown that memory $CD4^+CD25^+$ Treg cells produce more IL-17 than IFN-γ (Ayyoub et al. 2009), and attempts to induce *in vitro* cytokine production by murine $CD4^+CD25^+Foxp3^+$ Treg cells suggest that $CD4^+CD25^+$ Treg cells may favor IL-17 production (Xu et al. 2007; Osorio et al. 2008).

However, despite our observation that $6.5^+CD4^+CD25^+Foxp3^+$ Treg cells could acquire the ability to produce cytokines in response to strong TCR signals, TS1xHA28.*Foxp3*^{EGFP} $6.5^+GFP^+CD4^+CD25^+$ cells did not produce IL-17 or IFN-γ upon transfer into TS1xHACII mice regardless of the duration of exposure (up to ten weeks post transfer) or localization (popliteal versus non-draining LNs) in TS1xHACII mice. These data indicate that the inability of $CD4^+CD25^+$ Treg cells enriched for S1-specificity to prevent arthritis in TS1xHACII mice is not due to IL-17 production by the donor $CD4^+CD25^+Foxp3^+$ Treg cells. It is interesting that $6.5^+CD4^+CD25^+Foxp3^+$ Treg cells differentiate to produce IL-17 and IFN-γ in HACII.Tcr$\alpha^{-/-}$, but not TS1xHACII mice, which not only contain high levels of their cognate antigen, but also an inflammatory environment that supports the differentiation of endogenous pathogenic IL-17 producing cells. Indeed, work in

both humans and mice have demonstrated the important role that cytokines (e.g. IL-6, IL-1β, IL-23) play in inducing IL-17 production by $CD4^+CD25^+Foxp3^+$ Treg cells (Koenen et al. 2008; Beriou et al. 2009; Voo et al. 2009). One possibility is that competition for cytokines between donor $6.5^+CD4^+CD25^+$ Treg cells and endogenous $CD4^+$ T cells in TS1xHACII mice prevents the donor $CD4^+CD25^+$ Treg cells from differentiating to produce IL-17. However, it is also possible that $CD4^+CD25^+$ Treg cells must be exposed to high levels of cognate antigen coupled with extreme lymphopenia for *in vivo* differentiation to produce cytokines and although young TS1xHACII mice are lymphopenic, they do not recapitulate the condition in HACII.Tcr$\alpha^{-/-}$ mice, which completely lack αβ TCR T cells.

In addition to the observation that $Foxp3^+$ Treg cells are capable of producing Th-1 or Th-17 cytokines, recent work has further highlighted the plasticity of the $CD4^+CD25^+$ Treg cell population, as multiple groups have shown that $CD4^+CD25^+$ Treg cells can lose Foxp3 expression and undergo differentiation to acquire effector T cell properties (Komatsu et al. 2009; Murai et al. 2009; Zhou et al. 2009). Thus, although $GFP^+CD4^+CD25^+Foxp3^+$ cells from TS1xHA28.*Foxp3*^{EGFP} mice did not differentiate to produce IL-17 in TS1xHACII mice, we could not eliminate the possibility that a subset of $6.5^+CD4^+CD25^+$ Treg cells had lost Foxp3 expression and acquired effector functions that could contribute to arthritis pathogenesis in TS1xHACII mice. Indeed work in the NOD

diabetes model has shown that $CD4^+CD25^+$ Treg cells that have lost Foxp3 expression can become pathogenic and promote diabetes development (Zhou et al. 2009). In the absence of a congenic mismatch between TS1xHA28 $CD4^+CD25^+$ Treg cells and TS1xHACII cells, we could not distinguish between the donor and endogenous 6.5^+CD4^+ cells to analyze whether a subset of $6.5^+CD4^+CD25^+$ Treg cells lost Foxp3 expression after long term exposure to the environment in TS1xHACII mice. However, to begin to address the potential instability of $6.5^+CD4^+CD25^+$ Treg cells we conducted short term experiments with CFSE-labeled TS1xHA28 $CD4^+CD25^+$ cells in TS1xHACII mice, and extended analyses in single HA-transgenic mice that did contain any endogenous 6.5^+CD4^+ cells to examine whether TS1xHA28 $CD4^+CD25^+$ cell might undergo phenotypic changes that could lead to loss of Foxp3 expression.

Interestingly, we observed that $CD4^+CD25^+$ cells from TS1xHA28 mice downregulated CD25 expression upon transfer into arthritic TS1xHACII or healthy HACII mice, but not in BALB/c mice. This demonstrated that strong TCR signals (via high levels of HA) alone could induce the loss of CD25 expression, and that the inflammatory environment present in TS1xHACII mice was not required for this change. CD25 has been shown to be critical for $CD4^+CD25^+$ Treg cell survival and also for maintenance of Foxp3 expression (D'Cruz et al. 2005; Fontenot et al. 2005; Setoguchi et al. 2005; Zorn et al. 2006), as signaling

through the IL2Rα chain has been shown to potentiate both CD25 and Foxp3 expression (Fontenot et al. 2005). Since TS1xHA28 $CD4^+CD25^+$ cells downregulate CD25 upon exposure to high levels of HA in TS1xHACII mice, it is possible that with time some of the $Foxp3^+CD25^-$ cells will also lose Foxp3 expression. This would generate a population of $6.5^+CD4^+CD25^-Foxp3^-$ cells that possess high reactivity for S1 peptide in TS1xHACII mice, and the potential to differentiate and develop effector functions that could contribute to arthritis development.

Interestingly, whereas TS1xHA28 $CD4^+CD25^+$ cells downregulated CD25 after transfer into HACII mice, the cells were able to maintain high levels of CD25 expression in HA28 mice, which express low levels of HA, suggesting that the amount of cognate antigen correlates with the degree of CD25 downregulation. However, it is also possible that the differences in maintenance of CD25 expression by TS1xHA28 $CD4^+CD25^+$ cells in HACII and HA28 mice are due to the more extensive proliferation of TS1xHA28 $CD4^+CD25^+$ cells in HACII mice. Indeed, TS1xHA28 $CD4^+CD25^+$ cells at day ten post transfer in HA28 mice had undergone fewer rounds of division than TS1xHA28 $CD4^+CD25^+$ cells in HACII mice at day three post transfer. Accordingly, it is possible that TS1xHA28 $CD4^+CD25^+$ cells would also begin to downregulate CD25 expression in HA28 mice after undergoing additional rounds of proliferation. Therefore, while

exposure to high levels of cognate antigen can induce rapid downregulation of CD25 by $CD4^+CD25^+$ Treg cells, low levels of the same antigen either do not induce or significantly delay CD25 downregulation.

4.3.3 $CD4^+CD25^+$ Treg cells enriched for cells with low reactivity for S1 peptide can modify arthritis in TS1xHACII mice

We have shown that interactions with high levels of cognate interaction can induce phenotypic changes in TS1xHA28 $CD4^+CD25^+$ Treg cells that could contribute to their failure in preventing arthritis development in TS1xHACII mice. Furthermore, the ability of $CD4^+CD25^+$ Treg cells from HACII or BALB/c mice, neither of which contain 6.5^+ cells, to suppress arthritis development in TS1xHACII mice demonstrates that $CD4^+CD25^+$ Treg cells that are not enriched for cells with high reactivity for S1 peptide can prevent disease. Taken together, these data suggest that interactions with high levels of cognate antigen are detrimental to the ability of $6.5^+CD4^+CD25^+$ Treg cells to suppress arthritis in TS1xHACII mice. Indeed, interactions with high levels of cognate antigen can promote cytokine production and CD25 downregulation by $CD4^+CD25^+$ Treg cells (Figures 4-8 and 4-11). However, $CD4^+CD25^+$ Treg cells were able to maintain high levels of CD25 expression when exposed to lower levels of their cognate

antigen, indicating that adjusting the strength of TCR signaling could alter the $CD4^+CD25^+$ Treg cell response to the same S1 peptide.

Thus, we examined whether reducing the reactivity of the $CD4^+CD25^+$ Treg cell TCR for S1 peptide would allow $CD4^+CD25^+$ Treg cells that were enriched for S1 peptide reactive cells to modulate arthritis development in TS1xHACII mice. We found that $CD4^+CD25^+$ Treg cells from TS1(SW)xPevSW mice, which are enriched for cells expressing the TS1(SW) TCR (identified by $V\alpha8.3^+V\beta10^+$ staining) that possesses low reactivity for S1 peptide, could delay the kinetics of arthritis development in TS1xHACII mice and also appeared to ameliorate disease severity among arthritic TS1xHACII mice. We found that TS1(SW)xPevSW $CD4^+CD25^+$ cells proliferated better in response to low levels of their cognate S1(SW) peptide than to high levels of the mis-matched S1 peptide in HACII mice, indicating that increased amounts of antigen cannot overcome the low reactivity of the TS1(SW) TCR to induce robust proliferation of the $CD4^+CD25^+$ cells. However, the poor proliferation of TS1(SW)xPevSW $CD4^+CD25^+$ cells in HACII mice may explain the interesting result that TS1(SW)xPevSW $CD4^+CD25^+$ cells were able to maintain high levels of CD25 expression in the presence of both low levels of their cognate antigen S1(SW) peptide (in PevSW mice) and high levels of the mis-matched S1 peptide (in HACII mice). These data suggest that the lower reactivity of the TS1(SW) TCR

for S1 peptide counteracts the effects of high HA levels in HACII mice, similar to the effect of reducing HA levels on CD25 maintenance by TS1xHA28 $CD4^+CD25^+$ cells. However, it is clear that TS1(SW)xPevSW $CD4^+CD25^+$ cells can lose CD25 expression and potentially Foxp3 expression in TS1xHACII mice (Figure 4-14), and it is therefore possible that the lower reactivity TCR simply delays this process.

Although $CD4^+CD25^+$ cells from TS1(SW)xPevSW mice were more effective than $CD4^+CD25^+$ cells from TS1xHA28 mice at modulating arthritis in TS1xHACII mice, they were not as successful as HACII or BALB/c $CD4^+CD25^+$ cells, suggesting that the presence of $CD4^+CD25^+$ Treg cells that recognize the S1 peptide is detrimental to $CD4^+CD25^+$ Treg cell suppression of arthritis in TS1xHACII mice, regardless of whether the $CD4^+CD25^+$ Treg cells express TCRs possessing high or low reactivity for the S1 peptide. Indeed the different abilities of TS1(SW)xPevSW $CD4^+CD25^+$ cells and TS1xHA28 $CD4^+CD25^+$ cells to modulate arthritis in TS1xHACII mice could be due to differences in the representation of cells that can recognize the S1 peptide rather than differences in the respective TCR reactivities for S1 peptide. Whereas approximately 30 percent of TS1(SW)xPevSW $CD4^+CD25^+$ cells express the TS1(SW) TCR, upwards of 60 percent of $CD4^+CD25^+$ cells from TS1xHA28 mice express the 6.5 TCR. Thus, if $CD4^+CD25^+$ Treg cells that are enriched for cells that recognize S1

peptide are unable to prevent arthritis development in TS1xHACII mice because of the de-differentiation of $CD4^+CD25^+Foxp3^+$ Treg cells to $Foxp3^-$ cells, it is likely that the transfer of TS1xHA28 $CD4^+CD25^+$ cells into TS1xHACII mice would result in a larger population of effector cells originating from former $CD4^+CD25^+$ Treg cells, due to both the increased size of the starting 6.5^+ population and also enhanced proliferation of $6.5^+CD4^+CD25^+$ cells in response to S1 peptide. The most accurate way to assess how reactivity alone affects the ability of $CD4^+CD25^+$ Treg cells to suppress arthritis in TS1xHACII mice would be to use clonotype in addition to CD4 and CD25 to sort $6.5^+CD4^+CD25^+$ cells from TS1xHA28 mice and $V\alpha8.3^+V\beta10^+CD4^+CD25^+$ cells from TS1(SW)xPevSW mice.

4.3.4 $CD4^+CD25^+$ Treg cells enriched in specificity for S1 peptide may be unable to suppress arthritis development in TS1xHACII mice

While we have considered whether TCR specificity for S1 peptide could lead to dysfunctional $CD4^+CD25^+$ Treg cell activity that renders $CD4^+CD25^+$ Treg cells unable to suppress arthritis in TS1xHACII mice, another possibility is that $CD4^+CD25^+$ Treg cells that are enriched for S1-specificity function normally in TS1xHACII mice, but do not prevent disease development. Activated TS1xHA28 $CD4^+CD25^+$ Treg cells could promote the differentiation of pathogenic IL-17-

producing $CD4^+$ cells by producing high levels of the regulatory cytokines TGF-β, which in the context of cytokines such as IL-6 and IL-1 (which are present in TS1xHACII mice) can induce Th-17 differentiation. Indeed, early studies examining Th-17 differentiation identified TGF-β as a key factor in IL-17 production because of the presence of $CD4^+CD25^+$ Treg cells in the *in vitro* cultures (Veldhoen et al. 2006). Thus, it is possible that upon transfer into TS1xHACII mice, TS1xHA28 $CD4^+CD25^+$ Treg cells become activated, undergo extensive proliferation, produce TGF-β, and thus foster an environment that is even more supportive of the development of pathogenic IL-17 producing cells.

Another possibility is that $CD4^+CD25^+$ Treg cells from TS1xHA28 or TS1(SW)xPevSW mice, while enriched for cells that recognize S1 peptide, do not contain the appropriate specificities to suppress cells that are mediating the effector stage of arthritis in TS1xHACII mice. Although recognition of S1 peptide by 6.5^+CD4^+ T cells is important for arthritis development in TS1xHACII mice, as disease progresses other autoreactive $CD4^+$ T cells may be recruited to participate in subsequent stages of arthritis pathogenesis in TS1xHACII mice. Indeed, epitope spreading has been reported in a variety of murine autoimmune diseases (Vanderlugt and Miller 2002). An intriguing aspect of this possibility is it that it implies that *in vivo* $CD4^+CD25^+$ Treg cell suppression of target cells is antigen specific, as opposed to *in vitro* studies demonstrating that while

$CD4^+CD25^+$ Treg cells require antigen-specific TCR engagement for activation, they are capable of suppressing responder $CD4^+$ T cells regardless of their specificity. Therefore, if $CD4^+CD25^+$ Treg cells from TS1xHA28 or TS1(SW)xPevSW mice can suppress 6.5^+CD4^+ T cells that initiate arthritis development in TS1xHACII mice, it would be interesting to examine whether adoptive transfers of TS1xHA28 or TS1(SW)xPevSW $CD4^+CD25^+$ into TS1xHACII mice that are younger than 5 to 6 weeks of age could prevent arthritis development.

Chapter 5: Discussion and Speculation

5.1 Introduction

It is well established that $CD4^+CD25^+$ Treg cells play a critical role in the prevention of autoimmunity (Bennett et al. 2001; Gambineri et al. 2003; Fontenot and Rudensky 2005; Kim et al. 2007), but how $CD4^+CD25^+$ Treg cells mediate suppression remains unknown. In particular, the role that TCR specificities play in guiding the *in vivo* regulatory function of $CD4^+CD25^+$ Treg cells is unclear. While studies in several different models of murine autoimmune disease (e.g. AOD, diabetes, EAE) indicate that $CD4^+CD25^+$ Treg cells that are specific for disease-associated antigens are more effective at suppressing organ-specific autoimmune diseases than polyclonal $CD4^+CD25^+$ Treg cells (Hori et al. 2002; Tarbell et al. 2004; Samy et al. 2005; Samy et al 2008; Wheeler et al. 2009), how the antigen-specificity of $CD4^+CD25^+$ Treg cells affects their ability to modulate autoimmune arthritis is unknown. We have shown that $CD4^+CD25^+$ Treg cells that are enriched in specificity for a critical disease target antigen fail to prevent arthritis development in TS1xHACII mice, whereas $CD4^+CD25^+$ Treg cells that have not been educated by the disease target antigen can suppress disease. This chapter discusses these results in the context of antigen-specificity and $CD4^+CD25^+$ Treg cell mediated suppression of other autoimmune disease

models, and also examines how expression of a TCR with high reactivity for a target antigen may contribute to the failure of antigen-specific Treg cells to prevent arthritis in TS1xHACII mice.

5.2 The role of $CD4^+CD25^+$ Treg cells in arthritis development

Studies in human arthritis patients and in mouse models of inflammatory arthritis have reached the seemingly paradoxical conclusion that arthritis develops despite the presence of $CD4^+CD25^+Foxp3^+$ Treg cells. The presence of a normal and in some instances increased representation of $CD4^+CD25^+Foxp3^+$ cells in arthritis patients and arthritic mice raises questions about whether $CD4^+CD25^+$ Treg cells are capable of modifying the disease. However, several studies have provided some evidence for $CD4^+CD25^+$ Treg cell activity during arthritis as $CD4^+CD25^+$ cells isolated from human arthritis patients (Ehrenstein et al. 2004; Valencia et al. 2006; Nadkarni et al. 2007) and arthritic mice (Kang et al. 2008; Monte et al. 2008) have been shown to possess *in vitro* suppressor function. More importantly, there are indications that $CD4^+CD25^+$ Treg cells are exerting regulatory effects *in vivo*. Among individuals with JIA, an enrichment of $CD4^+CD25^+$ T cells correlates with persistent oligoarticular JIA, a milder form of the disease (de Kleer et al. 2004), suggesting that $CD4^+CD25^+$ Treg cells may modulate disease severity in human arthritis patients. In mice,

depletion of $CD4^+CD25^+$ Treg cells by anti-CD25 treatment results in more severe CIA (Morgan et al. 2003; Kelchtermans et al. 2005), and in lymphocytic infiltration into otherwise unaffected organs in K/BxN mice (Kang et al. 2008). Moreover, K/BxN.Foxp3-*sf* mice, which lack $CD4^+CD25^+Foxp3^+$ cells, develop an accelerated and more severe disease (Nguyen et al. 2007). Collectively, these data indicate that $CD4^+CD25^+$ Treg cells are active in arthritic patients and in arthritic mice, and while they are unable to fully prevent arthritis, they can ameliorate disease severity and/or modify other aspects of the autoimmune response.

TS1xHACII mice contain $CD4^+CD25^+Foxp3^+$ cells, including a population of $6.5^+CD4^+CD25^+Foxp3^+$ cells that recognize S1 peptide (Figure 3-3 A and B), and similar to observations in the CIA and K/BxN arthritis models, $CD4^+CD25^+Foxp3^+$ cells are present in the joint-draining LNs of TS1xHACII mice (Figure 3-5). Additionally $CD4^+CD25^+$ cells isolated from arthritic TS1xHACII mice possess *in vitro* suppressor function (Figure 3-4). Thus, TS1xHACII mice develop arthritis despite the presence of endogenous $CD4^+CD25^+Foxp3^+$ cells, including a population of cells that are specific for the disease target antigen S1 peptide. However, the arthritis that develops in TS1xHACII mice is susceptible to $CD4^+CD25^+$ Treg cell activity, as the administration of exogenous polyclonal $CD4^+CD25^+$ Treg cells can prevent arthritis development in TS1xHACII mice

(Figures 3-6 and 3-13). Given the ability of exogenous $CD4^+CD25^+$ Treg cells to suppress arthritis in TS1xHACII mice, why do the endogenous $CD4^+CD25^+Foxp3^+$ cells fail to prevent disease? Studies in human arthritis patients have indicated that effector $CD4^+$ T cells isolated from individuals with RA are resistant to $CD4^+CD25^+$ Treg cell mediated suppression (de Kleer et al. 2004; van Amelsfort et al. 2007). Additionally, work in the EAE system has also demonstrated that effector $CD4^+$ T cells can become refractory to $CD4^+CD25^+$ Treg cell activity, as wildtype $CD4^+CD25^+$ Treg cells could not suppress $CD4^+$ T cells isolated from the CNS (which is the primary disease site) of mice with acute EAE (Korn et al. 2007). However, it is clear that in TS1xHACII mice effector $CD4^+$ T cells (or other immune system cells) that contribute to arthritis development are not refractory to $CD4^+CD25^+$ Treg cell activity, since the administration of exogenous $CD4^+CD25^+$ Treg cells can modulate disease.

Another possibility is that $CD4^+CD25^+$ Treg cells are functional in TS1xHACII mice, and are able to suppress autoimmune responses in other organs, but not in the joints, as the joints or draining LNs may be enriched for cytokines such as IL-6 or TNF-α that have been shown to interfere with $CD4^+CD25^+$ Treg cell function (Pasare and Medzhitov 2003; Ehrenstein et al. 2004; Valencia et al. 2006; Nadkarni et al. 2007). Indeed, although HA expression is systemic in TS1xHACII mice, the primary manifestation of

autoimmunity is the development of inflammatory arthritis, with some mice also exhibiting a pulmonary lymphocyte infiltration and/or valvulitis. We do not observe lymphocyte infiltration in most organs (e.g. kidney, liver, salivary glands) (Rankin et al. 2008), and it is possible that the regulatory activity of endogenous $CD4^+CD25^+Foxp3^+$ cells prevents the development of more systemic autoimmunity in TS1xHACII mice. Interestingly, recent work demonstrates that $CD4^+CD25^+$ Treg cell mediated suppression of spontaneous lympophenia induced proliferation, which is thought to favor the expansion of more highly self-reactive T cells (Min et al. 2004; Min et al. 2005), helps to generate a more diverse repertoire of T cells (Winstead et al. 2010). Accordingly, the $CD4^+CD25^+$ Treg cells present in TS1xHACII mice may limit the expansion of other (non S1-specific) $CD4^+$ T cells with high reactivity for different self-antigens in young, lymphopenic TS1xHACII mice and thus prevent a more systemic manifestation of autoimmunity. Moreover, the ability of TS1xHACII $CD4^+CD25^+$ cells that have been depleted of $6.5^+CD4^+CD25^+$ cells (which comprise $Foxp3^+$ and $Foxp3^-$ cells) to modulate arthritis development in TS1xHACII mice (Figure 3-11), clearly demonstrates that $CD4^+CD25^+$ Treg cells from arthritic TS1xHACII mice are functional *in vivo*. In contrast to $CD4^+CD25^+$ cells used in the CIA model, which were obtained from healthy mice, this is a unique demonstration that $CD4^+CD25^+$ cells isolated from arthritic mice are capable of ameliorating disease.

Although TS1xHACII 6.5^{-}CD4^{+}CD25^{+} cells can modulate arthritis in TS1xHACII mice, they are less effective than CD4^{+}CD25^{+} cells obtained from HACII or BALB/c mice. Studies in human arthritis patients have demonstrated that CD4^{+}CD25^{+} cells isolated from RA patients are less effective *in vitro* suppressors than CD4^{+}CD25^{+} cells isolated from healthy individuals (Ehrenstein et al. 2004; Valencia et al. 2006). However, the suppressor function of CD4^{+}CD25^{+} cells from RA patients was improved upon anti-TNF-α treatment (Ehrenstein et al. 2004; Valencia et al. 2006; Nadkarni et al. 2007), suggesting that the inflammatory disease environment negatively impacts CD4^{+}CD25^{+} Treg cells. In the CIA model, CD4^{+}CD25^{+} T cells from IFN-γ receptor knockout mice, which develop accelerated and more severe arthritis (Manoury-Schwartz et al. 1997; Vermeire et al. 1997), exhibit less potent suppressor activity *in vitro*, and express lower levels of Foxp3 mRNA, akin to the effects of TNF-α on CD4^{+}CD25^{+} Treg cells in RA patients (Manoury-Schwartz et al. 1997). Thus, it is possible that CD4^{+}CD25^{+} Treg cells in TS1xHACII mice are less functional due to development in or prolonged exposure to the inflammatory environment associated with autoimmune arthritis. Moreover, the diminished function of CD4^{+}CD25^{+} Treg cells could be a contributing factor to arthritis development in TS1xHACII mice, as the progressive loss of CD4^{+}CD25^{+} Treg cell function could ultimately tip the balance between tolerance and autoimmunity in favor of the pathogenic immune response and arthritis development in TS1xHACII mice. In

NOD mice, a reduction in $CD4^+CD25^+$ Treg cell function and numbers prior to disease onset has been linked to diabetes development (Tang et al. 2008; Tritt et al. 2008).

Thus, the administration of exogenous $CD4^+CD25^+$ cells to TS1xHACII mice may mediate arthritis prevention by increasing the representation of $CD4^+CD25^+$ Treg cells relative to pathogenic effector $CD4^+$ T cells. In NOD mice, the diminished function of $CD4^+CD25^+$ Treg cells mice (Tritt et al. 2008) essentially results in a decrease in the representation of effective $CD4^+CD25^+$ Treg cells in relation to the diabetogenic $CD4^+$ T cells. The striking observation that endogenous TS1xHACII $CD4^+CD25^+$ cells can ameliorate arthritis in TS1xHACII mice upon depletion of $6.5^+CD4^+CD25^+$ cells, which recognize the disease target S1 peptide, also supports the concept that altering the balance between Treg cells and pathogenic effector T cell influences the development of autoimmunity. As described previously, $6.5^+CD4^+CD25^+$ cells in TS1xHACII mice include both $Foxp3^+$ and $Foxp3^-$ cells (Figure 3-3 B), and thus it is possible that elimination of $6.5^+CD4^+CD25^+Foxp3^-$ cells allows the endogenous $6.5^-CD4^+CD25^+$ cells to be more effective suppressors by reducing the representation of pathogenic cells that drive arthritis development in TS1xHACII mice. Furthermore, the concomitant transfer of a population of $CD4^+$ cells enriched for 6.5^+Foxp3^- cells rendered HACII $CD4^+CD25^+$ cells less protective in

TS1xHACII mice (Figure 3-12), indicating that even a subtle increase in the representation of $6.5^{+}CD4^{+}$ T cells could alter $CD4^{+}CD25^{+}$ Treg cell function.

Collectively, these observations suggest that a contributing factor to the development of arthritis (and autoimmunity in general) may be an imbalance of $CD4^{+}CD25^{+}$ Treg cells relative to pathogenic effector cells during the time period that precedes disease development. Moreover, observations in TS1xHACII mice suggest that the presence of certain TCR specificities may play a more critical role in altering the equilibrium between successful $CD4^{+}CD25^{+}$ Treg cell activity and the development of a pathogenic autoimmune response. For the adoptive transfer experiments, endogenous $CD4^{+}CD25^{+}$ cells were purified from arthritic TS1xHACII mice, and it is therefore likely that other $CD4^{+}$ cells (which do not express the 6.5 TCR) within the population have acquired pathogenic properties, such as the ability to produce IL-17. However, the elimination of a very small population of $6.5^{+}CD4^{+}CD25^{+}$ cells rendered the remaining $6.5^{-}CD4^{+}CD25^{+}$ cells better able to modulate arthritis in TS1xHACII mice, demonstrating the particular importance of the representation of $6.5^{+}CD4^{+}$ T cells that recognize S1 peptide for arthritis development in TS1xHACII mice. These data also argue against the idea that the $CD4^{+}CD25^{+}$ Treg cell repertoire itself is somehow compromised in TS1xHACII mice because of the severe thymocyte deletion that occurs in young TS1xHACII mice.

What conditions might contribute to creating an imbalance between $CD4^+CD25^+$ Treg cells and pathogenic autoreactive $CD4^+$ T cells? One possibility is that lymphopenia, which has been associated with the development of autoimmunity (King et al. 2004; Khoruts and Fraser 2005; Marleau and Sarvetnick 2005), has differing effects on distinct T cell populations. Both conventional $CD4^+$ T cells and $CD4^+CD25^+$ Treg cells undergo proliferation in lymphopenic settings (Min et al. 2004; Min et al. 2005; Zhang et al. 2005), but how differences in their responses may contribute to the maintenance of tolerance as opposed to the development of autoimmunity remain unclear. Studies in human patients who have undergone allogeneic hematopoetic stem cell (HSC) transplantation have documented that while both conventional and $CD4^+CD25^+$ Treg cells undergo extensive proliferation in the lymphopenic environment induced by the treatment, $CD4^+CD25^+$ Treg cells appear to be more susceptible to apoptosis than conventional $CD4^+$ T cells (Matsuoka et al. 2010). Moreover, humans that had more severe and prolonged lymphopenia had greater defects in the $CD4^+CD25^+$ Treg cell pool after reconstitution of the immune system, and a greater incidence of graft-versus-host disease (GVHD) (Matsuoka et al. 2010). These findings suggest that the differential responses of conventional $CD4^+$ T cells and $CD4^+CD25^+$ Treg cells in response to

lymphopenia can result in a decreased representation of $CD4^+CD25^+$ Treg cells that may contribute to the development of systemic autoimmunity.

Work in the NOD diabetes model has shown that conventional $CD4^+$ T cells undergo more extensive proliferation than $CD4^+CD25^+$ Treg cells in lymphopenic environments (Le Campion et al. 2009). Moreover, in NOD.scid mice, diabetes development (which is induced by the adoptive transfer of $CD4^+$ cells) was reduced when higher numbers of $CD4^+$ cells were transferred, effectively reducing the degree of lymphopenia in the recipient mice, and protection correlated with a higher percentage of $Foxp3^+$ cells among the transferred $CD4^+$ cells (Le Campion et al. 2009). Interestingly, it appears that lymphopenia induced proliferation of $CD4^+CD25^+$ Treg cells is dependent on interactions with self-peptides (Cozzo et al. 2003; Hsieh et al. 2004), whereas conventional $CD4^+$ T cells can undergo lymphopenia induced proliferation in the absence of their cognate antigen (Cozzo et al. 2003). Moreover, conventional $CD4^+$ T cells expressing the 6.5 TCR underwent more extensive proliferation in response to low levels of S1 peptide in an irradiation induced lymphopenic environment than $CD4^+CD25^+$ Treg cells expressing the same 6.5 TCR (Cozzo et al. 2003), indicating that even in the presence of their cognate antigen, $CD4^+CD25^+$ Treg cells proliferate less than conventional $CD4^+$ T cells in a lymphopenic setting. These observations suggest that TCR specificity for self-

peptides exerts ongoing effects on conventional $CD4^+$ T cells and $CD4^+CD25^+$ Treg cells, and can play an important role in shaping the balance of these respective cell populations in response to lymphopenia and potentially influence the development of autoimmunity.

Accordingly, it is possible that the lymphopenia in young, pre-arthritic TS1xHACII mice promotes the expansion of not only S1-specific 6.5^+CD4^+ T cells, which are exposed to high levels of their cognate antigen, but also 6.5^- $CD4^+$ T cells that can differentiate to acquire pathogenic properties. Moreover it seems likely that 6.5^+CD4^+ T cells (and also other $CD4^+$ T cells with high reactivity for other self-peptides) would undergo more extensive proliferation than $CD4^+CD25^+Foxp3^+$ cells, including the population of $6.5^+CD4^+CD25^+Foxp3^+$ cells. Thus, differences in lymphopenia induced proliferation between conventional $CD4^+$ T cells and $CD4^+CD25^+$ Treg cells may lead to the preferential expansion of 6.5^+ and other highly self reactive $CD4^+$ T cells in TS1xHACII mice. Therefore, while functional $CD4^+CD25^+$ Treg cells are present in pre-arthritic TS1xHACII mice, it is possible that they are not present in sufficient numbers to inhibit the response of 6.5^+CD4^+ T cells (and other pathogenic cells) in young TS1xHACII mice.

Both the studies presented here and work in the CIA system have shown that increasing the representation of $CD4^+CD25^+$ Treg cells by administering exogenous Treg cells to pre-arthritic mice can ameliorate or prevent arthritis. However, many questions remain regarding where and how $CD4^+CD25^+$ Treg cells are acting to modulate the immune response in arthritis. In the CIA model, protective exogenous $CD4^+CD25^+$ T cells that have been injected systemically can be found in the draining lymph node, synovial fluid, and synovial tissue as early as one day post transfer, indicating that the cells are trafficking to the disease site (Morgan et al. 2005). Interestingly, it has also been reported that $CD4^+CD25^+$ T cell transfers were more effective at modifying disease severity when the cells were injected systemically rather than directly into the joints, raising questions about where the $CD4^+CD25^+$ Treg cells are acting to modulate disease (Ohata et al. 2007). Moreover, although $CD4^+$ T cells have been found in the joints of arthritic mice (Hirota et al. 2007; Jacobs et al. 2009), recent work suggests that $CD4^+$ T cells may play a more important role in the joint-draining LNs, as $CD4^+$ T cells that were blocked from exiting the LNs were still able to contribute to arthritis pathogenesis (Angyal et al. 2010).

We found that protective HACII $CD4^+CD25^+$ Treg cells could inhibit the accumulation of cells in the joint draining popliteal LNs that normally occurs in TS1xHACII mice (Figures 3-2 E and 3-8 A). Work in the NOD diabetes model

has illustrated the importance of the regional LN immune response in the development of autoimmunity (Hoglund et al. 1999; Gagnerault et al. 2002). Excision of the pancreatic LNs in young NOD mice prevented diabetes onset, demonstrating that the local immune response is required for disease pathogenesis (Gagnerault et al. 2002). However, while removal of the pancreatic LN could prevent diabetes development in young mice, it was no longer protective in 10 week old mice (Gagnerault et al. 2002), suggesting that once a certain threshold is achieved it becomes more difficult to alter the course of disease. Therefore, it is possible that protective $CD4^+CD25^+$ Treg cells suppress arthritis in TS1xHACII mice by preventing the development of an enhanced immune response in the popliteal LN, which may be a critical step for arthritis pathogenesis.

Studies in other murine arthritis models suggest that another potential mechanism by which $CD4^+CD25^+$ Treg cells may affect arthritis development is by altering the inflammatory environment. In the CIA model, mice that received Treg cells had reduced serum levels of TNF-α and IL-6 (Morgan et al. 2005; Kelchtermans et al. 2009). Additionally, treatment with IL-35, which has been identified as a $CD4^+CD25^+$ Treg cell effector cytokine (Collison et al. 2007), can reduce disease severity in CIA, which is associated with a decrease in IL-17 levels (Niedbala et al. 2007), suggesting that $CD4^+CD25^+$ Treg cell production of

IL-35 could inhibit IL-17 production in arthritic mice. We found that protective HACII $CD4^+CD25^+$ Treg cells inhibit the pathogenic Th-17 response in TS1xHACII mice (Figures 3-8, 3-9, and 3-10). While the mechanism by which the exogenous $CD4^+CD25^+$ Treg cells inhibit IL-17 production in TS1xHACII mice remains unknown, TS1xHACII mice that received protective HACII $CD4^+CD25^+$ Treg cells also produced decreased amounts of IL-1 and IL-6 (Figure 3-10), two cytokines that can contribute to Th-17 cell differentiation (Acosta-Rodriguez et al. 2007). Innate immune cells, which are potential sources of cytokines such as IL-1 and IL-6, may play an important role in the *in vivo* development of IL-17 producing $CD4^+$ T cells (Korn et al. 2009), and recent work has shown that monocytes from arthritic patients can induce Th-17 cell differentiation (Evans et al. 2009). Thus, protective $CD4^+CD25^+$ Treg cells may decrease IL-17 production in TS1xHACII mice by acting upon an innate immune system cell(s) that is instrumental in inducing the differentiation of Th-17 cells.

We have shown that the arthritis that develops in TS1xHACII mice is susceptible to $CD4^+CD25^+$ Treg cell activity, as the administration of exogenous $CD4^+CD25^+$ cells to 5 to 6 week old TS1xHACII mice can ameliorate or completely prevent arthritis. The majority of unmanipulated TS1xHACII mice do not develop arthritis until 10 to 12 weeks of age, and this lag period most likely reflects the time required to establish the pathogenic immune response (e.g.

development of IL-17 producing cells, immune response in the popliteal LNs) that can overcome regulatory mechanisms in TS1xHACII mice. Thus, increasing the representation of $CD4^+CD25^+$ Treg cells during this incipient stage of the autoimmune response in TS1xHACII mice may be critical for effective suppression of disease. Indeed, in the CIA model, there appeared to be a limited time frame in which transferred Treg cells could modulate disease severity. $CD4^+Foxp3^+$ T cells could best ameliorate CIA when they were transferred prior to the primary immunization with collagen. At later time points, such as after a secondary immunization, a greater number of transferred Treg cells was required to achieve a comparable reduction in disease severity, and if transferred after the booster immunization, the Treg cells had no effect on arthritis development (Ohata et al. 2007). It remains to be determined whether the administration of protective $CD4^+CD25^+$ Treg cells would be as effective at later time points in TS1xHACII mice.

5.3 The effect of $CD4^+CD25^+$ Treg cell antigen-specificity in preventing autoimmunity

While studies from our lab and others have shown that interactions with cognate antigens can direct the thymic selection of $CD4^+CD25^+$ Treg cells (Jordan et al. 2001; Apostolou et al. 2002; Hori et al. 2002; Kawahata et al. 2002;

Walker et al. 2003), the importance of these interactions in guiding the *in vivo* suppressor activity of $CD4^+CD25^+$ Treg cells is less clear. Work in multiple murine autoimmune diseases (e.g. AOD, CIA, colitis, diabetes, gastritis) has shown that that the adoptive transfer of polyclonal $CD4^+CD25^+$ Treg cells can prevent or ameliorate disease (Olivares-Villagomez et al. 1998; Van de Keere and Tonegawa 1998; Singh et al. 2001; Sarween et al. 2004; Morgan et al. 2005; Zwar et al. 2006; Kelchtermans et al. 2009), indicating that $CD4^+CD25^+$ Treg cells that are not necessarily enriched for disease-associated antigen specific cells can mediate suppression of an organ specific disease. However, there is also a significant body of work demonstrating that antigen- or disease-specific $CD4^+CD25^+$ Treg cells are more effective than polyclonal $CD4^+CD25^+$ Treg cells at suppressing organ-specific autoimmune diseases (e.g. AOD, diabetes, EAE) (Hori et al. 2002; Reddy et al. 2004; Tarbell et al. 2004; Samy et al. 2005; Samy et al. 2008).

Whereas the role of $CD4^+CD25^+$ Treg cell specificity in disease suppression has been better established in other models of murine autoimmunity, how $CD4^+CD25^+$ Treg cell specificity may affect the suppression of arthritis is unknown. The primary approach to examining the role of antigen-specificity in $CD4^+CD25^+$ Treg cell modulation of arthritis development has been to examine whether modifications to the $CD4^+CD25^+$ Treg cell population (i.e.

depletion or administration of exogenous Treg cells) result in antigen-specific changes in the autoimmune response. K/BxN mice, which develop arthritis due to $CD4^+$ T cell recognition of a GPI peptide and the subsequent activation of GPI-specific B cells, exhibit an accelerated accumulation of anti-GPI antibodies when crossed to Foxp3-*sf* mice (Nguyen et al. 2007), suggesting that endogenous $CD4^+CD25^+$ Treg cells are tempering the GPI-specific immune response. There is additional evidence for antigen-specific $CD4^+CD25^+$ Treg cell activity in the CIA model, with observations of increased collagen-specific T and B cell responses after $CD4^+CD25^+$ Treg cell depletion, as well as a reduction in collagen-specific antibodies after the transfer of exogenous $CD4^+CD25^+$ Treg cells (Morgan et al. 2003; Ohata et al. 2007). However, there are also data showing no alterations in the collagen-specific immune response upon manipulation of $CD4^+CD25^+$ Treg cells in CIA (Morgan et al. 2005; Kelchtermans et al. 2009). In contrast, beyond the observation that K/BxN mice contain $CD4^+CD25^+Foxp3^+$ cells that express Vβ6 (Vβ chain of the KRN Tg TCR) and can mediate *in vitro* suppression in response to the GPI peptide (Kang et al. 2008), there is little information regarding how $CD4^+CD25^+$ Treg cell specificity for a target antigen can affect affect arthritis suppression.

TS1xHACII mice are a useful system to examine how the TCR specificity of $CD4^+CD25^+$ Treg cells can affect their ability to modulate arthritis development

since we have established the identity of a critical disease target antigen (S1 peptide). Similar to the observations in K/BxN mice, the finding that TS1xHACII mice contain 6.5^{+}CD4^{+}CD25^{+}Foxp3^{+} cells (Figure 3-3 B) shows that arthritis can develop despite the presence of CD4^{+}CD25^{+}Foxp3^{+} cells that are specific for a disease target antigen. Moreover, the results from the adoptive transfers of TS1xHA28 CD4^{+}CD25^{+} Treg cells into pre-arthritic TS1xHACII mice demonstrate that CD4^{+}CD25^{+} Treg cells that are enriched in specificity for S1 peptide fail to suppress disease (Figures 4-2 and 4-3), whereas polyclonal CD4^{+}CD25^{+} Treg cells from HACII or BALB/c mice can prevent arthritis in TS1xHACII mice (Figures 3-6 and 3-13). Indeed no enrichment in specificity for the S1 peptide appears to be necessary for Treg cell mediated suppression of arthritis, as BALB/c CD4^{+}CD25^{+} Treg cells have not developed in the presence of S1 peptide or any other HA-derived peptides.

In contrast, studies from the Tung laboratory suggest that CD4^{+}CD25^{+} Treg cells with specificity for disease-associated antigens possess superior ability to prevent organ-specific autoimmune diseases than polyclonal CD4^{+}CD25^{+} Treg cells. Work in thymectomized mice that develop autoimmune ovarian disease (AOD) has shown that the transfer of CD4^{+}CD25^{+} Treg cells isolated from the draining LNs are more effective suppressors of AOD than CD4^{+}CD25^{+} Treg cells from non-draining LNs (Samy et al. 2005; Samy et al.

2008; Wheeler et al. 2009). Draining LNs have been shown to uniquely present organ-specific peptides (Hoglund et al. 1999; Gagnerault et al. 2002; Scheinecker et al. 2002), and $CD4^+CD25^+$ Treg cells isolated from the ovary-draining LNs may be more effective at suppressing AOD because they are enriched for ovarian antigen-specific $CD4^+CD25^+$ Treg cells (Samy et al. 2005; Samy et al. 2008). Moreover, studies in male mice that develop experimental autoimmune prostatis (EAP) have demonstrated that $CD4^+CD25^+$ Treg cells can acquire the ability to suppress organ-specific autoimmune diseases after exposure to organ-derived antigens (Setiady et al. 2006). Male mice subjected to a neonatal orchiectomy do not exhibit normal prostate development due to the absence of testicular androgen, but re-introduction of the hormone into neonatal orchiectomized mice can induce normal prostate development and expression of prostate antigens. Interestingly, $CD4^+CD25^+$ Treg cells isolated from male mice undergoing androgen treatment (and thus experiencing prostate-derived antigens) can better suppress EAP than $CD4^+CD25^+$ Treg cells purified from male mice subjected to only the neonatal orchiectomy, suggesting that exposure to the normal prostate antigens confers the ability to suppress prostate-specific autoimmune disease (Setiady et al. 2006). Thus, it possible that upon transfer into TS1xHACII mice, BALB/c $CD4^+CD25^+$ Treg cells are educated by exposure to the HA protein and gain specificity to HA derived peptides.

Given the ability of polyclonal $CD4^+CD25^+$ Treg cells to suppress arthritis, it was surprising that $CD4^+CD25^+$ Treg cells from TS1xHA28 and TS1xHA28.*Foxp3*^{EGFP} mice, which are enriched for 6.5^+Foxp3^+ cells, failed to prevent arthritis in TS1xHACII mice despite expanding, becoming activated, and trafficking to the joint draining popliteal LNs (Figures 4-4 and 4-10). This finding is at odds with observations in other murine models of autoimmune disease (e.g. diabetes, EAE, pneumonitis) where antigen-specific $CD4^+CD25^+$ Treg cells are able to inhibit disease that is initiated by $CD4^+$ T cells of the same specificity (Hori et al. 2002; Tarbell et al. 2004; Huang et al. 2005). What might explain the discrepancy in our observations of antigen-specificity requirements for $CD4^+CD25^+$ Treg cell suppression of autoimmunity? Although recognition of S1 peptide by 6.5^+CD4^+ T cells is a critical step in arthritis development in TS1xHACII mice, it is possible that other non S1-specific $CD4^+$ T cells are recruited later in the response and become more important during the effector stage of arthritis in TS1xHACII mice. Indeed, epitope spreading can play a role in the pathogenesis of a variety of autoimmune diseases, including arthritis, and is also a mechanism by which viral infections can trigger the development of autoimmunity (Vanderlugt and Miller 2002).

Thus it is possible that the cells that must be suppressed in TS1xHACII mice are not the 6.5^+CD4^+ T cells, and that $CD4^+CD25^+$ cells from HACII or

BALB/c mice, but not TS1xHA28 or TS1xHA28.*Foxp3*^{EGFP} mice, contain the appropriate specificities to mediate suppression, suggesting that *in vivo* suppression of $CD4^+$ T cells by $CD4^+CD25^+$ Treg cells is antigen-specific. This is consistent with the finding of antigen-specific $CD4^+CD25^+$ Treg cell suppression of effector $CD4^+$ T cells in a model of fatal autoimmune pneumonitis (Huang et al. 2005). While the adoptive transfer of 6.5^+CD4^+ T cells into transgenic mice expressing HA protein in the lungs induces pneumonitis, disease does not develop if the cells are transferred into mice that express HA in the lungs but also contain $6.5^+CD4^+CD25^+$ Treg cells. Moreover, transfer of $6.5^+CD4^+CD25^+$ Treg cells but not polyclonal $CD4^+CD25^+$ Treg cells could prevent pneumonitis in single transgenic HA mice. Protection, which is mediated by IL-10 and TGF-β, results in the inhibition of proliferation and cytokine production (IFN-γ) by pathogenic 6.5^+CD4^+ T cells (Huang et al. 2005). Interestingly, $6.5^+CD4^+CD25^+$ Treg cells could suppress proliferation by 6.5^+CD4^+ T cells but not $CD4^+$ T cells of a different specificity (Tanchot et al. 2004; Huang et al. 2005), indicating that $CD4^+CD25^+$ Treg cell suppression of *in vivo* proliferation by responder $CD4^+$ T cells is antigen-specific.

However, the ability of TS1xHACII $CD4^+CD25^+$ cells to ameliorate arthritis in TS1xHACII mice when depleted of 6.5^+ cells argues that 6.5^+CD4^+ T cells continue to play an important role in arthritis development in TS1xHACII mice at

the time of the exogenous $CD4^+CD25^+$ Treg cell transfers (Figure 3-11). In addition, the co-transfer of a population of conventional $6.5^+CD4^+CD25^-$ cells rendered HACII $CD4^+CD25^+$ Treg cells less protective in TS1xHACII mice (Figure 3-12). Furthermore, the development of arthritis in TS1xHACII.$RAG^{-/-}$ mice demonstrates that $CD4^+$ T cells of other TCR specificities are not required for arthritis pathogenesis, and that 6.5^+CD4^+ T cells alone can mediate arthritis (Rankin et al. 2008). Collectively, these observations suggest that S1-specific 6.5^+CD4^+ T cells are a critical pathogenic population not only during disease initiation, but also in other stages of arthritis in TS1xHACII mice, and it remains surprising that the administration of $CD4^+CD25^+$ Treg cells enriched for 6.5^+ cells are unable to suppress or even ameliorate arthritis in TS1×HACII mice.

Moreover, studies in the NOD diabetes and EAE models provide evidence that $CD4^+CD25^+$ Treg cells can suppress $CD4^+$ T cells that possess specificity for different antigens. The majority of $CD4^+CD25^+$ cells from BDC2.5 mice that were expanded *in vitro* with BDC peptide pulsed APCs express the BDC2.5 TCR. However, the adoptive transfer of these BDC peptide expanded $CD4^+CD25^+$ cells could suppress diabetes development in NOD.scid mice, which is induced by the adoptive transfer of splenocytes from diabetic NOD mice (Tarbell et al. 2004). Thus, in this case $CD4^+CD25^+$ Treg cells that are enriched for expression of the BDC2.5 TCR were able to suppress diabetes that is driven by $CD4^+$ T cells with a

diverse array of pathogenic TCRs, although the $CD4^+CD25^+$ Treg cells and diabetogenic $CD4^+$ T cells may recognize the same target antigen(s). Work in the EAE model has shown that pre-activated Ig-proteolipid protein 1 (PLP1) specific $CD4^+CD25^+$ Treg cells could suppress EAE driven by responses to myelin oligodendrocyte glycoprotein (MOG) or myelin basic protein (MBP) peptides (Yu et al. 2005), again demonstrating that $CD4^+CD25^+$ Treg cells could mediate suppression regardless of the specificity of effector $CD4^+$ T cells.

The question of antigen-specificity and $CD4^+CD25^+$ Treg cell function has primarily been considered in relation to suppression of effector $CD4^+$ T cells. However, $CD4^+CD25^+$ Treg cells can also inhibit innate immune cells (Maloy et al. 2003), and how the antigen-specificity of $CD4^+CD25^+$ Treg cells might influence this suppression is less clear. While protective HACII $CD4^+CD25^+$ cells suppressed IL-17 production by $CD4^+$ T cells in the popliteal LNs of TS1xHACII mice (Figure 3-8 B and C), whether the reduction in IL-17, IL-1, and IL-6 production by systemic LN cells (Figure 3-9) is attributed to $CD4^+CD25^+$ Treg cells acting upon $CD4^+$ T cells or other immune cells is unknown. Moreover, in addition to producing cytokines such as IL-1 or IL-6, innate immune cells can also produce IL-17 (Buonocore et al. 2010). Indeed, 6.5^+CD4^+ T cells may promote arthritis development in TS1xHACII.$RAG^{-/-}$ by inducing inflammation that

recruits innate immune cells (e.g. neutrophils, macrophages) that can mediate joint damage (Niki et al. 2001).

Interestingly, S1-specific $CD4^+CD25^+$ cells not only failed to suppress IL-17 production in TS1xHACII mice, but also appeared to increase production of IL-1 and IL-6 (Figure 4-7). It has been shown that T cell mediated inhibition of innate immune cells is MHC dependent (Kim et al. 2007), and more recent work demonstrates that upon interactions with cognate antigen, memory $CD4^+$ T cells can induce the production of inflammatory cytokines and chemokines by innate immune cells, (Strutt et al. 2010). Thus, one can speculate as to whether interactions between $CD4^+CD25^+$ Treg cells and innate immune cells presenting their cognate antigen could induce a similar induction of inflammatory proteins. Given that HA expression is driven by a MHC Class II promoter in TS1xHACII mice, it is likely that innate immune cells expressing MHC Class II can also present HA-derived peptides (such as S1 peptide) and activate TS1xHA28 $CD4^+CD25^+$ cells, which are enriched for expression of the 6.5 TCR. Moreover, activated TS1xHA28 $CD4^+CD25^+$ Treg cells could also promote the differentiation of pathogenic IL-17-producing $CD4^+$ cells by producing high levels of the regulatory cytokines TGF-β, which in the context of cytokines such as IL-6 and IL-1, can induce Th-17 differentiation.

5.4 Plasticity of $CD4^+CD25^+$ Treg cells: implications for suppression of autoimmunity

Recent studies have demonstrated the plasticity of the $CD4^+CD25^+$ Treg cell population, as a subset of $CD4^+CD25^+$ Treg cells can lose Foxp3 when exposed to conditions of lymphopenia or inflammation in an autoimmune setting (Komatsu et al. 2009; Murai et al. 2009; Zhou et al. 2009). Foxp3 expression is critical for the maintenance of the $CD4^+CD25^+$ Treg cell phenotype (Wann and Flavell 2007; Williams and Rudensky 2007), and $CD4^+CD25^+$ Treg cells that lose Foxp3 expression can acquire the ability to produce cytokines such as IFN-γ and IL-17 (Xu et al. 2007; Komatsu et al. 2009; Murai et al. 2009; Zhou et al. 2009). Work from the Bluestone laboratory has shown that ex-$CD4^+CD25^+$ Treg cells can differentiate into pathogenic $CD4^+$ T cells and mediate diabetes development in NOD mice (Zhou et al. 2009). Interestingly, upon transfer into NOD mice, a greater percentage of BDC2.5 TCR expressing $CD4^+CD25^+$ Treg cells lost Foxp3 expression than did $CD4^+CD25^+$ cells that did not express the Tg TCR, suggesting that interactions between the TCR and its cognate antigen could further promote the loss of Foxp3 and the development of effector functions by $CD4^+CD25^+$ Treg cells. Moreover, repetitive *in vitro* stimulation of human $CD4^+CD25^+$ Treg cells can induce loss of Foxp3 (Hoffman et al. 2009), providing

additional evidence that TCR engagement can contribute to phenotypic changes in $CD4^+CD25^+$ Treg cells.

TS1xHA28 $CD4^+CD25^+$ cells, which are enriched for expression of the 6.5 TCR, rapidly downregulated CD25 upon transfer into TS1xHACII or HACII mice, which contain high levels of their cognate antigen (Figure 4-10). In contrast, TS1xHA28 $CD4^+CD25^+$ cells were able to maintain high levels of CD25 expression in HA28 mice, which contain low levels of S1 peptide (Figure 4-11). Interestingly the downregulation of CD25 in response to high levels of S1 peptide appeared to be delayed if $CD4^+CD25^+$ Treg cells expressed a TCR with low reactivity for S1 peptide (Figure 4-14), suggesting that the lower reactivity of the TS1(SW) TCR for S1 peptide counteracts the effects of high levels of S1 peptide in HACII mice, similar to the effect of reducing S1 peptide levels on CD25 maintenance by TS1xHA28 $CD4^+CD25^+$ cells. Taken together these data support a role for strong TCR signals in inducing CD25 downregulation by $CD4^+CD25^+$ Treg cells. Signaling through the IL-2Rα chain has been shown to potentiate both CD25 and Foxp3 expression (Fontenot et al. 2005; Zorn et al. 2006), and recent work has shown that $CD4^+CD25^-Foxp3^+$ cells are more prone to lose Foxp3 expression than $CD4^+CD25^+Foxp3^+$ cells (Komatsu et al. 2009). Thus, it is possible that upon exposure to highly stimulatory levels of S1 peptide, a subset of TS1xHA28 $CD4^+CD25^+$ cells loses CD25 and Foxp3 expression, and

in the context of the inflammatory disease setting, differentiates into pathogenic effector $CD4^+$ T cells in TS1xHACII mice. Certain inflammatory cytokines, such as IL-1β (which was elevated in TS1xHACII mice that received TS1xHA28 $CD4^+CD25^+$ cells), have been shown to contribute to $CD4^+CD25^+$ Treg cell conversion to Th-17 cells (Deknuydt et al. 2009).

Although the findings presented here suggest that TCR signals can induce phenotypic changes in $CD4^+CD25^+$ Treg cells that could lead to loss of Foxp3 and regulatory function, whether a subset of TS1xHA28 $CD4^+CD25^+$ cells loses Foxp3 expression and acquires cytokine production in TS1xHACII mice remains to be determined. The adoptive transfer of $CD4^+CD25^+$ Treg cells purified from TS1xHA28.*Foxp3*^{EGFP} mice into congenically mismatched TS1xHACII mice would allow us to better address whether $6.5^+CD4^+CD25^+$ Treg cells become pathogenic upon exposure to the high levels of S1 peptide and inflammation present in TS1xHACII mice, since the congenic marker could be used to detect all donor $CD4^+CD25^+$ Treg cells rather than the use of GFP which only identifies the cells that have maintained Foxp3 expression. Additionally, recent studies have shown that the methylation status of the Foxp3 promoter plays an important role in Foxp3 stability in $CD4^+CD25^+$ Treg cells (Kim and Leonard 2007; Janson et al 2008). However, the signals and mechanisms involved in establishing and maintaining the demethylation of the Foxp3 promoter remain unknown. It would

be of interest to determine whether the strength of signal through the TCR could affect the methylation status of the Foxp3 promoter and therefore the stability of Foxp3 protein expression in $CD4^+CD25^+$ Treg cells. Recent work has linked the loss of Foxp3 expression in human $CD4^+CD25^+$ Treg cells that have been subjected to repetitive *in vitro* stimulation to changes in the methylation status of the Foxp3 promoter (Hoffman et al. 2009).

5.5 Summary and working model

The studies presented in this dissertation demonstrate that the TCR specificity(s) of $CD4^+CD25^+$ Treg cells can be a significant determinant of their ability to suppress autoimmune disease. Polyclonal $CD4^+CD25^+$ Treg cells, but not $CD4^+CD25^+$ Treg cells that are enriched in specificity for a critical disease target antigen (S1 peptide), are able to prevent arthritis development in TS1xHACII mice, which is driven by recognition of the S1 peptide by 6.5^+CD4^+ T cells (Rankin et al. 2008). Moreover, $CD4^+CD25^+$ Treg cells that have not developed in the presence of S1 peptide or any other HA-derived peptides can prevent arthritis in TS1xHACII mice, demonstrating that specificity for this disease target antigen is not required for $CD4^+CD25^+$ Treg cell mediated suppression of arthritis. What might explain the ability of polyclonal $CD4^+CD25^+$ Treg cells to prevent disease development in TS1xHACII mice? The

administration of exogenous $CD4^+CD25^+$ cells to pre-arthritic TS1xHACII mice increases the representation of $CD4^+CD25^+$ Treg cells relative to pathogenic $CD4^+$ T cells in recipient mice, and it is possible that this increase is enough to shift the balance in favor of regulation and away from autoimmunity.

Yet, if simply altering the balance of $CD4^+CD25^+$ Treg cells and effector cells in favor of the regulatory population is sufficient to prevent arthritis development, $CD4^+CD25^+$ Treg cells that are enriched for 6.5^+ cells should also be able to suppress arthritis in TS1xHACII mice. One possible explanation for the failure of $CD4^+CD25^+$ Treg cells enriched for S1-specificity to prevent arthritis development in TS1xHACII mice is that the $CD4^+$ T cells mediating the effector stage of arthritis are not the 6.5^+CD4^+ T cells, and that $CD4^+CD25^+$ Treg cells that are enriched for S1-specific cells do not possess the appropriate TCR specificities to suppress the effector $CD4^+$ T cells. This would suggest that $CD4^+CD25^+$ Treg cell suppression of $CD4^+$ T cells is antigen-specific, and thus a highly diverse population of polyclonal $CD4^+CD25^+$ Treg cells may contain the necessary TCR specificities to suppress the relevant effector $CD4^+$ T cells in TS1xHACII mice. Indeed, the transfer of exogenous $CD4^+CD25^+$ Treg cells into TS1xHACII mice could serve to increase the diversity of the TCR repertoire of $CD4^+CD25^+$ Treg cells present in TS1xHACII mice, and thereby increase the

probability of recognizing a critical target peptide to mediate suppression of arthritis.

However, the ability of $CD4^+CD25^+$ cells from TS1xHACII mice to modulate arthritis when depleted of a small population of 6.5^+ cells indicates that 6.5^+CD4^+ T cells are still active participants in promoting arthritis development at the time of the exogenous $CD4^+CD25^+$ Treg cell transfers into TS1xHACII mice. Accordingly, if 6.5^+CD4^+ T cells continue to play an important role in arthritis pathogenesis in TS1xHACII mice, increasing the representation of $CD4^+CD25^+$ Treg cells enriched in expression for the 6.5 TCR should be able to modulate arthritis development by suppressing the endogenous 6.5^+CD4^+ T cells. Furthermore, $CD4^+$ T cells of other TCR specificities are not required for arthritis development as TS1xHACII.$RAG^{-/-}$ mice also develop disease (Rankin et al. 2008). Thus, the question of why $CD4^+CD25^+$ Treg cells enriched for S1-specific cells are unable to prevent arthritis in TS1xHACII mice remains. Additional data presented in this dissertation suggest that strong stimulation with cognate antigen can induce effector functions and/or phenotypic changes in $CD4^+CD25^+$ Treg cells that may be detrimental in the context of certain autoimmune diseases.

$CD4^+CD25^+$ Treg cells expressing the 6.5 TCR are strongly reactive to the S1 peptide, and we have shown that the $CD4^+CD25^+$ cells enriched for 6.5^+ cells

undergo extensive proliferation and become activated in response to the high levels of HA present in TS1xHACII mice. $CD4^+CD25^+$ Treg cells undergoing strong stimulation may produce high levels of regulatory cytokines such as TGF-β, which in the context of other inflammatory cytokines (e.g. IL-1, IL-6, IL-23), can promote Th-17 differentiation. Therefore, activated $6.5^+CD4^+CD25^+$ Treg cells may contribute to the development of pathogenic Th-17 cells in TS1xHACII mice. Another possibility is that a subset of $6.5^+CD4^+CD25^+$ Treg cells loses Foxp3 expression upon exposure to high levels of their cognate antigen, and differentiates into pathogenic effector $CD4^+$ T cells in TS1xHACII mice. Indeed, in light of our data suggesting that the ability of $CD4^+CD25^+$ Treg cells to suppress arthritis development in TS1xHACII mice is incredibly sensitive to the presence of effector 6.5^+CD4^+ T cells, a population of de-differentiated $6.5^+CD4^+CD25^+$ Treg cells may alter the balance of Treg cells and effector cells in favor of the pathogenic $CD4^+$ T cells and arthritis development in TS1xHACII mice. A working model of how exogenous $CD4^+CD25^+$ Treg cells can affect arthritis development in TS1xHACII mice is presented in Figure 5-1.

Collectively, these data indicate that $CD4^+CD25^+$ Treg cells have the ability to modulate arthritis, but that the antigen-specificity of the $CD4^+CD25^+$ Treg cells is critical for successful suppression of disease. Therefore, while there is potential for the use of $CD4^+CD25^+$ Treg cells in therapeutic settings to treat

autoimmunity, careful consideration should be given to nature of the autoimmune disease, both from the perspective of predominant disease-associated antigens and the inflammatory environment associated with the disease.

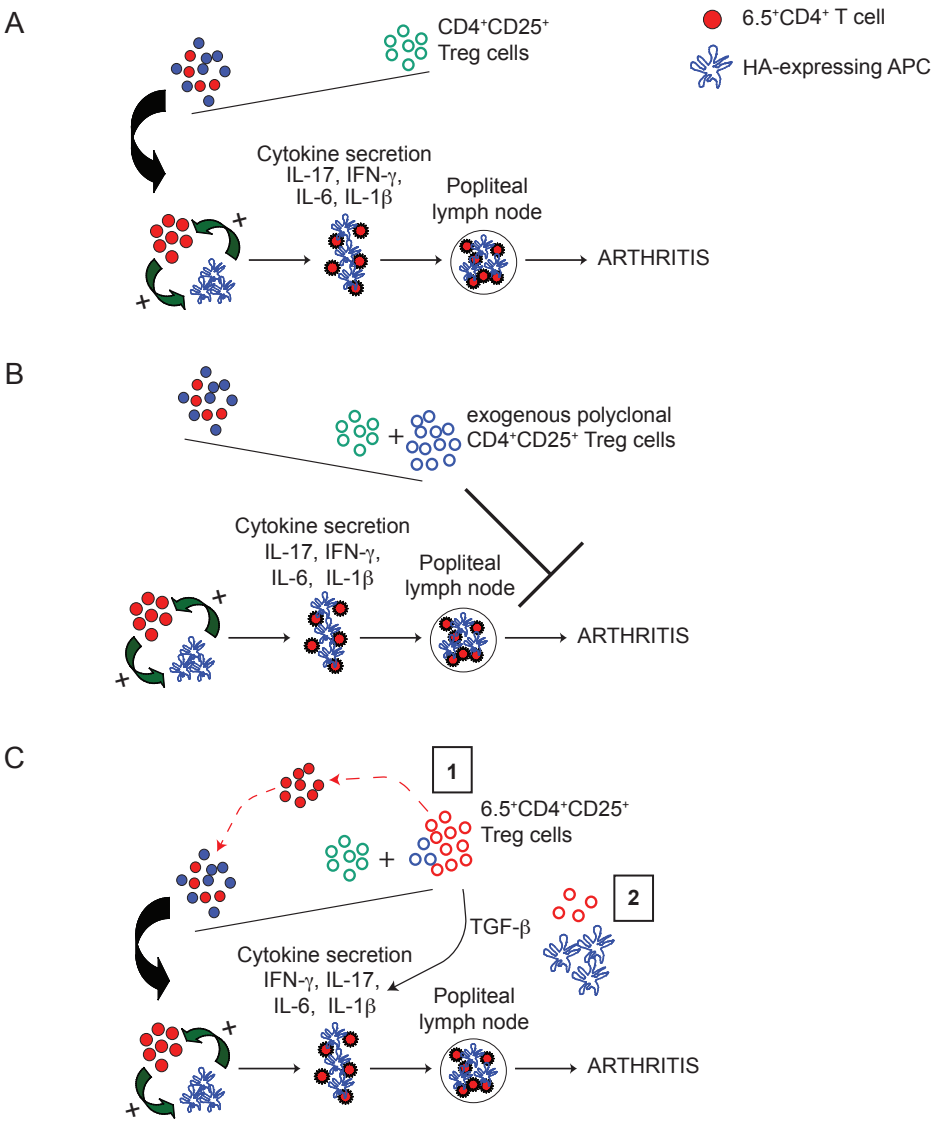

Figure 5-1 Working model: The balance of CD4⁺CD25⁺ Treg cells to pathogenic 6.5⁺CD4⁺ T cells is a critical parameter for arthritis prevention in TS1xHACII mice (**A**) In unmanipulated TS1xHACII mice the balance between CD4⁺CD25⁺ Treg cells and effector CD4⁺ T cells favors pathogenic 6.5⁺CD4⁺ T cells and arthritis development. (**B**) The administration of exogenous polyclonal CD4⁺CD25⁺ Treg cells increases the representation of Treg cells relative to the representation of pathogenic 6.5⁺CD4⁺ T cells in TS1xHACII mice, favoring Treg cell activity and suppression of arthritis. (**C**) The administration of exogenous CD4⁺CD25⁺ Treg cells enriched for 6.5⁺Foxp3⁺ cells are unable to prevent arthritis development in TS1xHACII mice. Potential mechanisms of failure: (1) A subset of 6.5⁺CD4⁺CD25⁺ Treg cells de-differentiates to lose Foxp3 expression and acquire pathogenic effector function. (2) Strongly activated 6.5⁺CD4⁺CD25⁺ Treg cells produce high levels of TGF-β and promote the differentiation of pathogenic Th-17 cells.

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