Shp1 regulates T cell homeostasis by limiting IL-4 signals

Dylan J. Johnson,[1,3] Lily I. Pao,[5] Salim Dhanji,[1] Kiichi Murakami,[1] Pamela S. Ohashi,[1,3,4] and Benjamin G. Neel[2,4,5]

[1]Campbell Family Institute for Breast Cancer Research and [2]Campbell Family Cancer Research Institute, Ontario Cancer Institute, Princess Margaret Cancer Center, University Health Network, Toronto, ON M5G 2C1, Canada
[3]Department of Immunology and [4]Department of Medical Biophysics, University of Toronto, Toronto, ON M5S 1C1, Canada
[5]Beth Israel Deaconess Medical Center, Boston, MA 02215

The protein-tyrosine phosphatase Shp1 is expressed ubiquitously in hematopoietic cells and is generally viewed as a negative regulatory molecule. Mutations in *Ptpn6*, which encodes Shp1, result in widespread inflammation and premature death, known as the *motheaten* (*me*) phenotype. Previous studies identified Shp1 as a negative regulator of TCR signaling, but the severe systemic inflammation in *me* mice may have confounded our understanding of Shp1 function in T cell biology. To define the T cell–intrinsic role of Shp1, we characterized mice with a T cell–specific Shp1 deletion ($Shp1^{fl/fl}$ CD4-cre). Surprisingly, thymocyte selection and peripheral TCR sensitivity were unaltered in the absence of Shp1. Instead, $Shp1^{fl/fl}$ CD4-cre mice had increased frequencies of memory phenotype T cells that expressed elevated levels of CD44. Activation of Shp1-deficient $CD4^+$ T cells also resulted in skewing to the Th2 lineage and increased IL-4 production. After IL-4 stimulation of Shp1-deficient T cells, Stat 6 activation was sustained, leading to enhanced Th2 skewing. Accordingly, we observed elevated serum IgE in the steady state. Blocking or genetic deletion of IL-4 in the absence of Shp1 resulted in a marked reduction of the $CD44^{hi}$ population. Therefore, Shp1 is an essential negative regulator of IL-4 signaling in T lymphocytes.

CORRESPONDENCE
Pamela Ohashi:
pohashi@uhnresearch.ca
OR
Benjamin G. Neel:
bneel@uhnresearch.ca

Abbreviations used: ITIM, immunoreceptor tyrosine-based inhibitory motif; ITSM, immunoreceptor tyrosine-based switch motif; KLF2, Krüppel-like factor 2; me, motheaten; me^v, motheaten viable; SP, single positive.

T cells are characterized by their ability to expand dramatically in an antigen-specific manner during an immune challenge. After an initial immune response, a small proportion of responding T cells survive and give rise to memory cells (Bruno et al., 1996). Memory T cells express elevated levels of CD44 and can be divided further into central-memory ($CD62L^{hi}$ $CCR7^{hi}$) and effector-memory ($CD62L^{lo}$ $CCR7^{lo}$) compartments. However, not all T cells that display the phenotype of memory cells are the product of a classical antigen-specific immune response (Sprent and Surh, 2011). For example, such cells are found in unimmunized mice, including those raised in germ-free and antigen-free conditions (Dobber et al., 1992; Vos et al., 1992). The precise ontogeny of such cells remains elusive, although several mechanisms by which naive cells can adopt a memory phenotype have been characterized. Naive T cells introduced into lymphopenic environments adopt a memory phenotype through a process of homeostatic proliferation in response to IL-7 and MHC (Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000). Additionally, increased production of IL-4 has been linked to the development of memory phenotype–innate T cell populations in studies of several knockout mouse models (Lee et al., 2011).

The T cell response is tightly regulated by the balance of phosphorylation and dephosphorylation of intracellular signaling molecules. Shp1 (encoded by *Ptpn6*) is a protein tyrosine phosphatase expressed ubiquitously in hematopoietic cells and has been broadly characterized as a negative regulator of immune cell activation (Pao et al., 2007a; Lorenz, 2009). The physiological relevance of Shp1 as a key negative regulator of the immune response is illustrated by

Lily I. Pao's present address is FivePrime Therapeutics, San Francisco, CA.

S. Dhanji's present address is Qu Biologics, Vancouver, BC V5T 4T5, Canada.

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the *motheaten* (*me*) and *motheaten viable* (me^v) mutations, which ablate Shp1 expression or greatly reduce Shp1 activity, respectively (Shultz et al., 1993; Tsui et al., 1993). Homozygous *me/me* or me^v/me^v mice (hereafter, referred to collectively as me mice) suffer from severe systemic inflammation and autoimmunity, which result in retarded growth, myeloid hyperplasia, hypergammaglobulinemia, skin lesions, interstitial pneumonia, and premature death. More recently, a study has identified a third allele of *Ptpn6*, named *spin*, which encodes a hypomorphic form of Shp1 (Croker et al., 2008). Mice homozygous for *spin* develop a milder autoimmune/inflammatory disease that is ablated in germ-free conditions.

Shp1 has been implicated in signaling from many immune cell surface receptors (Zhang et al., 2000; Neel et al., 2003), including the TCR (Plas et al., 1996; Lorenz, 2009), BCR (Cyster and Goodnow, 1995; Pani et al., 1995), NK cell receptors (Burshtyn et al., 1996; Nakamura et al., 1997), chemokine receptors (Kim et al., 1999), FAS (Su et al., 1995; Takayama et al., 1996; Koncz et al., 2008), and integrins (Roach et al., 1998; Burshtyn et al., 2000). Shp1 also has been demonstrated to regulate signaling from multiple cytokine receptors by dephosphorylating various Jak (Klingmüller et al., 1995; Jiao et al., 1996; Minoo et al., 2004) and/or Stat (Kashiwada et al., 2001; Xiao et al., 2009) molecules. Several of these cytokines are pertinent to T cell biology. For example, Stat 5 is an essential mediator of signals from IL-2 and IL-7 (Rochman et al., 2009). IL-4 signaling results in Stat 6 phosphorylation and has potent Th2 skewing effects. Additionally, IL-4 has mitogenic effects on $CD8^+$ T cells (Rochman et al., 2009). Notably, mutation of the immunoreceptor tyrosine-based inhibitory motif (ITIM) in IL-4Rα results in ablation of Shp1 binding and hypersensitivity to IL-4 stimulation (Kashiwada et al., 2001), implicating Shp1 as a regulator of this cytokine receptor.

Although development of the me phenotype does not require T cells (Shultz, 1988; Yu et al., 1996), several aspects of T cell biology reportedly are controlled by Shp1 (Lorenz, 2009). Most previous studies that examined the role of Shp1 in T cells used cells derived from *me/me* or me^v/me^v mice (Carter et al., 1999; Johnson et al., 1999; Zhang et al., 1999; Su et al., 2001) or cells expressing a dominant-negative allele of Shp1 (Plas et al., 1996, 1999; Zhang et al., 1999). Several such reports have concluded that Shp1 negatively regulates the strength of TCR signaling during thymocyte development and/or peripheral activation (Carter et al., 1999; Johnson et al., 1999; Plas et al., 1999; Zhang et al., 1999; Su et al., 2001). Despite the large number of studies that implicate Shp1 in control of TCR signaling, there is no consensus on which component of the TCR signaling cascade is targeted by the catalytic activity of Shp1. Suggested Shp1 targets downstream of T cell activation include TCR-ζ (Chen et al., 2008), Lck (Lorenz et al., 1996; Stefanová et al., 2003), Fyn (Lorenz et al., 1996), ZAP-70 (Plas et al., 1996; Chen et al., 2008), and SLP-76 (Mizuno et al., 2005). Shp1 also is implicated in signal transduction downstream of several immune inhibitory receptors that negatively regulate T cell activity, such as PD-1 (Chemnitz et al., 2004), IL-10R (Taylor et al., 2007), CEACAM1 (Lee et al., 2008), and CD5 (Perez-Villar et al., 1999).

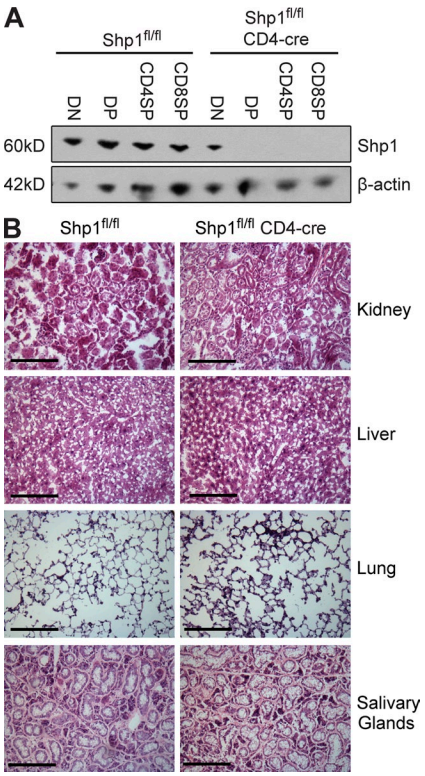

Figure 1. T cell specific deletion of Shp1. (A) The indicated thymocyte populations were FACS sorted and Shp1 expression was determined by immunoblot. (B) Frozen sections of organs from $Shp1^{fl/fl}$ and $Shp1^{fl/fl}$ CD4-cre littermates were stained with hematoxylin and eosin. Images are representative of four littermate pairs. Bars, 250 µm.

The severe inflammation characteristic of the me phenotype might have confounded studies examining the cell-intrinsic role of Shp1 in various hematopoietic cell types. We previously generated a floxed Shp1 allele that facilitates analysis of the role of Shp1 in various lineages (Pao et al., 2007b). Previous studies have used this approach to study the role of Shp1 in T cells during antiviral and antitumor immune responses, respectively (Fowler et al., 2010; Stromnes et al., 2012). However, a more fundamental analysis of the cell-intrinsic role of Shp1 during T cell development, homeostasis, and activation has not been reported. Here, we provide evidence that a major role for Shp1 in T cells is to maintain normal T cell homeostasis through negative regulation of IL-4 signaling.

RESULTS

T cell–specific deletion of Shp1

To examine the cell-intrinsic role of Shp1 in T cells, we generated mice homozygous for a floxed allele of *Ptpn6* that also expressed CD4-cre. As expected, absence of Shp1 expression was detected in double-positive (DP) thymocytes and their progeny (Fig. 1 A). $Shp1^{fl/fl}$ CD4-cre mice did not develop

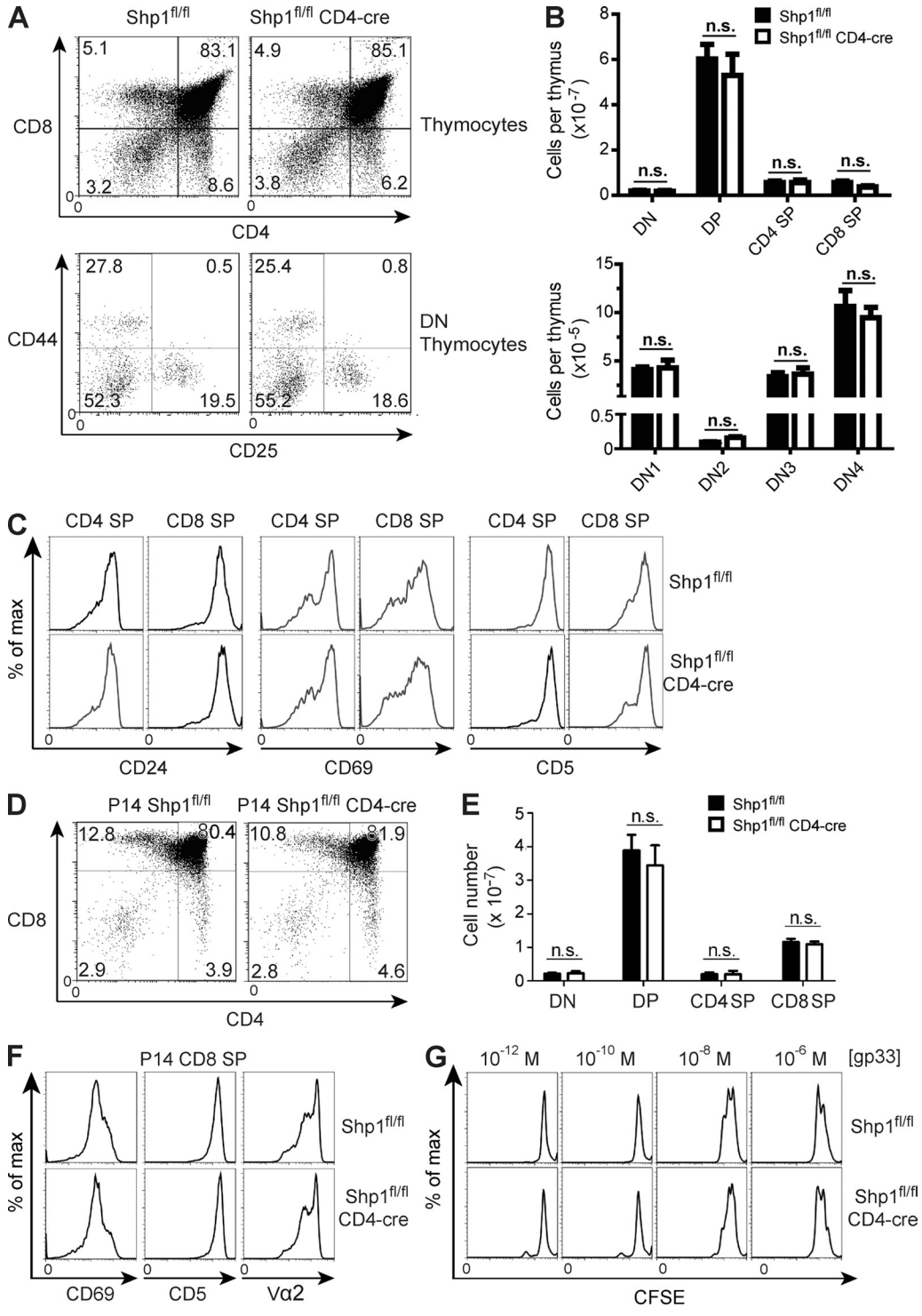

Figure 2. Thymocytes develop normally in the absence of Shp1. (A and B) Thymocytes from Shp1$^{fl/fl}$ and Shp1$^{fl/fl}$ CD4-cre mice were stained with antibodies specific for various surface markers. CD8 and CD4 profiles for total thymocytes and CD44 and CD25 expression for gated DN thymocytes are shown in A. Total cell numbers for each population are shown in B; $n = 4$. (C) Mature CD4$^+$ and CD8$^+$ SP thymocytes were evaluated by flow cytometry for the expression of the surface markers CD24, CD69, and CD5. Thymocytes from P14 Shp1$^{fl/fl}$ and P14 Shp1$^{fl/fl}$ CD4-cre mice were evaluated using a similar panel of antibodies. (D and E) Thymocytes were stained with α-CD4 and α-CD8 antibodies (D), and absolute number of the various subsets are shown in E; $n = 3$. (F) CD8 SP P14 thymocytes were gated and CD24, CD69, CD5, and Vα2 expression was assessed by flow cytometry. (G) CD8 SP P14 thymocytes stained with CFSE were cultured for 2 d with irradiated splenocytes and the indicated concentration of gp33. Statistical analyses were performed by one-way ANOVA (B) or Student's *t* test (E); ns, $P \geq 0.05$. Values for B and E are displayed as ± standard error.

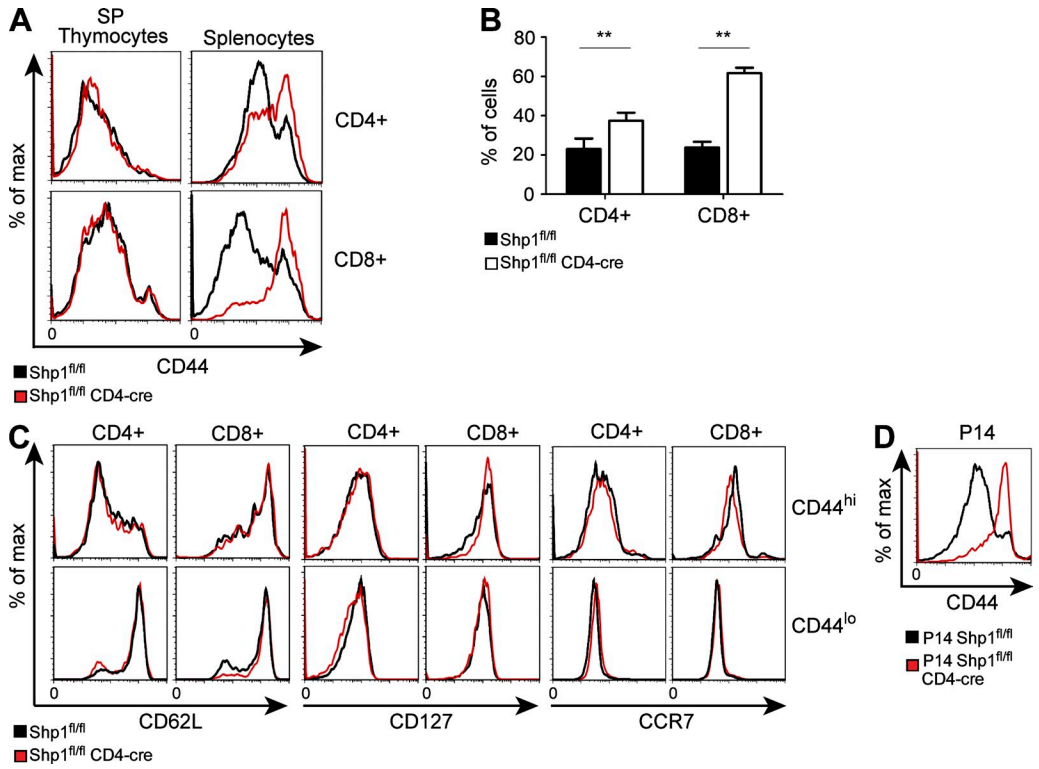

Figure 3. Shp1 restricts the development of memory phenotype T cells. T cells from Shp1$^{fl/fl}$ or Shp1$^{fl/fl}$ CD4-cre mice were stained with monoclonal antibodies against the indicated cell surface molecules. (A) Mature SP thymocytes and splenic T cells were assayed for CD44 expression by flow cytometry. (B) Percentage of splenic T cells with CD44^{hi} phenotype, displayed as ± standard error; $n = 8$. (C) Splenic T cells, gated based on expression of CD4, CD8, and CD44, were stained with antibodies against the indicated markers. (D) Expression of CD44 on splenic T cells from P14$^+$ mice. Cells are gated on the CD8$^+$ Vα2$^+$ population. Statistical analysis of data in B was performed by Student's *t* test; **, $P < 0.01$.

overt autoimmunity or inflammation (Fig. 1 B), consistent with prior work showing that the *me* phenotype is T cell independent (Yu et al., 1996).

Thymocytes develop normally in the absence of Shp1

Next, we asked if Shp1 plays a role in thymocyte development. Shp1 conditional knockout mice contained normal proportions of double negative (DN1→DN4), DP, and CD4$^+$ and CD8$^+$ single-positive (SP) thymocyte populations (Fig. 2 A). There also was no change in the absolute number of cells contained within each developmental compartment of the thymus (Fig. 2 B), nor did we detect any differences in the staining of CD4$^+$ or CD8$^+$ SP thymocytes for the markers CD5, CD69, or CD24 (Fig. 2 C). Together, these data suggest that Shp1 is dispensable for thymocyte development.

Previous studies of mice expressing TCR transgenes identified a role for Shp1 in thymocyte selection (Carter et al., 1999; Johnson et al., 1999; Plas et al., 1999; Zhang et al., 1999). We crossed our T cell–specific Shp1 conditional knockout mice with mice expressing the P14 TCR transgene, an MHC-I restricted TCR composed of Vα2 and Vβ8.1, which recognizes the gp33-41 epitope of lymphocytic choriomeningitis virus in the context of H-2D^b (Pircher et al., 1989). Surprisingly, Shp1-deficient P14 transgenic mice had normal proportions and numbers of thymocyte subsets, including the positively selected CD8$^+$ SP population (Fig. 2, D and E). The CD8$^+$ SP population also expressed similar levels of CD5, CD69, and Vα2, suggesting that positive selection was unaltered by the absence of Shp1 (Fig. 2 F). Shp1 deficiency also did not influence the selection of the OT-I or OT-II TCR transgenes (unpublished data). To verify that Shp1 does not regulate thymocyte selection, we performed in vitro thymocyte stimulations. Wild-type and Shp1-deficient P14 thymocytes required an equivalent concentration of gp33 to induce proliferation. Furthermore, Shp1 had no impact on the extent of proliferation induced by gp33 (Fig. 2 G). Together, these results suggest that Shp1 is not essential for regulating thymocyte positive selection or TCR signaling threshold.

Memory phenotype T cells accumulate in Shp1 conditional knockout mice

Next, we examined the phenotype of peripheral T cells from Shp1$^{fl/fl}$ CD4-Cre mice compared with control Shp1$^{fl/fl}$ mice. We found that spleens from Shp1$^{fl/fl}$ CD4-cre mice were enriched for T cells expressing elevated levels of CD44 (Fig. 3, A and B). This increase was observed in the CD4$^+$ and the CD8$^+$ T cell compartments, although the increase in the CD8$^+$ CD44^{hi} population was more prominent. Shp1-deficient lymph node and blood T cells also displayed a CD44^{hi} phenotype (unpublished data). In contrast, CD44 levels were normal in mature SP thymocytes, indicating that Shp1-deficient

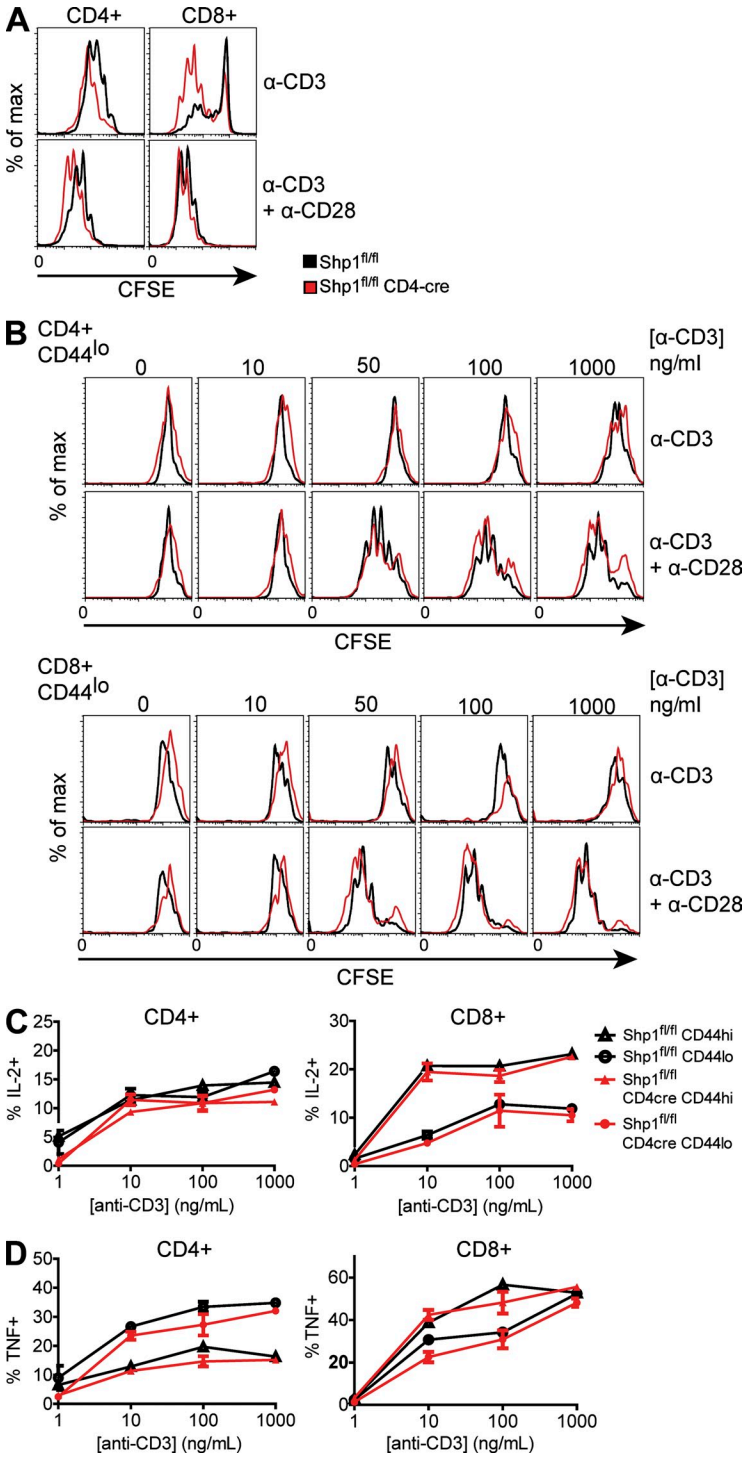

Figure 4. Shp1-deficient T cells exhibit normal responses to TCR stimulation. (A) T cells were isolated from spleens, labeled with CFSE, and cultured on a 96-well plate coated with cross-linked antibodies against CD3 ± CD28. Cells were harvested 3 d later, stained for CD4 and CD8, and analyzed by flow cytometry. (B) Splenic T cells were sorted into $CD44^{hi}$ and $CD44^{lo}$ populations by FACS, labeled with CFSE, and cultured on a 96-well plate coated with cross-linked antibodies against CD3 and CD28 at the indicated concentrations. Cells were analyzed as in A. (C and D) Cells were treated as in A, harvested 6 h after stimulation, and analyzed for IL-2 (C) and TNF (D) expression by intracellular staining; $n = 2$. Data are representative of three independent experiments. Values for C and D are displayed as ± standard error.

T cells become $CD44^{hi}$ after thymic egress (Fig. 3 A). A previous study of me mice implicated Shp1 in control of regulatory T cell (T reg cell) development (Carter et al., 2005). However, we found no difference in the frequency of T reg cells in T cell–conditional Shp1 knockout mice (unpublished data).

Elevated CD44 expression is associated with activated and memory T cells. The $CD44^{hi}$ population in our mice appeared to have a memory phenotype, as they did not express the activation markers CD25 or CD69 (unpublished data). Memory T cells can be divided further into central memory ($CD44^{hi}$ $CD62L^{hi}$ $CD127^{hi}$ $CCR7^{hi}$) and effector memory ($CD44^{hi}$ $CD62L^{lo}$ $CD127^{lo}$ $CCR7^{lo}$) populations. The $CD4^{+}$ $CD44^{hi}$ T cell populations from wild-type and T cell conditional Shp1 knockout mice were composed of a mixture of $CD62L^{hi}$ and $CD62L^{lo}$ cells (Fig. 3 C). The $CD4^{+}$ $CD44^{hi}$ populations also displayed heterogeneous expression of CD127

and CCR7. Together, these findings suggest that the $CD4^+$ memory phenotype compartment contained a mixture of effector and memory phenotype cells. In contrast, the $CD8^+$ $CD44^{hi}$ populations contained a high proportion of $CD62L^{hi}$ $CD127^{hi}$ $CCR7^{hi}$ cells, suggesting a prominent central memory phenotype.

The expression of these markers was identical in Shp1-deficient memory phenotype cells and the naturally occurring memory phenotype population present in wild-type mice. This finding suggests that the Shp1-deficient $CD44^{hi}$ population reflects an expansion of normally occurring memory phenotype T cells. Notably, the central memory phenotype of the $CD44^{hi}$ populations is consistent with the phenotype of naive T cells that have undergone homeostatic expansion (Sprent and Surh, 2011). To test if the memory phenotype population was a consequence of an antigen-specific T cell expansion, we examined the expression of CD44 on P14 TCR transgenic T cells. However, CD44 expression also was elevated in Shp1-deficient P14 T cells, demonstrating that the accumulation of memory phenotype cells is not driven by a response to a specific endogenous antigen (Fig. 3 D).

T cells respond normally to TCR stimulation in the absence of Shp1

To test whether Shp1 plays a role in regulating TCR sensitivity, T cells from Shp1fl/fl and $Shp1^{fl/fl}$ CD4-cre mice were stimulated in vitro with α-CD3 ± α-CD28. Total $CD4^+$ T cells from Shp1 conditional knockout animals displayed increased proliferation, compared with wild-type $CD4^+$ T cells, when stimulated with α-CD3 or α-CD3 + α-CD28. (Fig. 4 A). Shp1-deficient $CD8^+$ T cells stimulated with α-CD3 displayed dramatic hyperproliferation, although this difference was eliminated upon addition of α-CD28. To determine if the enriched memory-like population in Shp1 conditional knockout animals was responsible for the enhanced proliferation, T cells were sorted for CD44, and the $CD44^{lo}$ population was then stimulated with α-CD3 ± α-CD28 (Fig. 4 B). Notably, the enhanced proliferation of Shp1-deficient (total) T cells was eliminated when the large memory phenotype population was removed. This result indicates that the apparent hyperproliferative response of Shp1-deficient T cells reflects the intrinsically more robust response of memory phenotype cells, rather than the effects of Shp1 on TCR responsiveness per se.

We also examined the capacity of Shp1-deficient T cells to produce cytokines in response to TCR stimulation. When cells were gated based on their CD44 expression, there was no difference in the ability of wild-type or Shp1-knockout T cells to produce IL-2 (Fig. 4 C) or TNF (Fig. 4 D) in response to stimulation with α-CD3 and α-CD28. In contrast to previous studies (Johnson et al., 1999; Fowler et al., 2010), these data suggest that Shp1 does not have a role in regulating TCR sensitivity in peripheral T cells.

T cells skew to Th2 in the absence of Shp1

We next investigated if Shp1 has a role in controlling the differentiation of $CD4^+$ Th cells. Naive $CD4^+$ T cells were stimulated in vitro with α-CD3 and α-CD28, followed by 3 d of culture in the presence of IL-2. Upon restimulation, a significantly lower proportion of Shp1-deficient T cells expressed IFN-γ compared with controls (Fig. 5, A and B). Additionally, there was a significant increase in the number of IL-4–producing cells. In contrast, Shp1-deficient T cells stimulated immediately after their isolation exhibited normal production of IL-4 (unpublished data), suggesting that Shp1-deficient T cells are not preprogrammed for IL-4 production. Together, these findings suggest that Shp1 negatively regulates Th2 differentiation in vitro. To examine whether the in vitro Th2 bias is also seen in vivo, we examined serum antibody levels. Indeed, the serum concentration of IgE, the prototypic Th2 antibody isotype, was ∼50-fold higher in knockout mice compared with controls (Fig. 5 C). In contrast, knockout and wild-type mice had similar levels of serum IgG2a, a Th1-driven isotype. Therefore, Shp1 regulates Th2 skewing in vitro and in vivo.

Th2 differentiation is regulated by a transcriptional network that includes IL-4R–induced, pStat6-directed transactivation of the master Th2 transcription factor GATA-3 (Zhou and Ouyang, 2003). To determine if Shp1 regulates signaling downstream of IL-4R in T cells, we stimulated wild-type and Shp1-deficient T cells with IL-4 and measured Stat 6 tyrosyl phosphorylation. Shp1 deficiency did not affect the dose–response curve for IL4-evoked Stat 6 phosphorylation (Fig. 5 D). To measure the kinetics of pStat6 dephosphorylation, T cells were pulsed with IL-4 for 30 min, followed by three washes to remove residual cytokine. Wild-type T cells showed robust Stat6 tyrosyl phosphorylation upon IL4 stimulation, followed by a loss of the pStat6 signal 2 h later. In contrast, Shp1-deficient T cells maintained high levels of Stat6 phosphorylation at 2 and 3 h after cytokine withdrawal (Fig. 5 E), indicating that Shp1 is required for the efficient dephosphorylation of Stat6 after IL-4 stimulation.

Memory phenotype cells in Shp1 conditional knockout mice are dependent on IL-4

We sought to determine if cell-intrinsic or -extrinsic forces were driving the formation of memory phenotype T cells within Shp1 conditional knockout mice. Toward this aim, we generated mixed bone marrow chimeras. Irradiated, congenically marked (CD45.1) hosts were reconstituted with bone marrow cells from wild-type mice (Thy1.1), conditional knockout mice ($Shp1^{fl/fl}$ CD4cre), or a 1:1 mixture of the two. In mice reconstituted with conditional knockout bone marrow, there was a significant enrichment for both $CD4^+$ and $CD8^+$ memory phenotype T cells in comparison with mice reconstituted with wild-type bone marrow (Fig. 6, A and B). Mixed bone marrow chimeras contained wild-type T cells with a predominantly naive phenotype and knockout T cells with an enriched memory phenotype population. This finding indicates that Shp1 knockout T cell phenotype is cell intrinsic and not a response to altered cell-extrinsic factors.

Previous studies have linked IL-4 to the abnormal expansion of memory phenotype $CD8^+$ T cells (Lee et al., 2011).

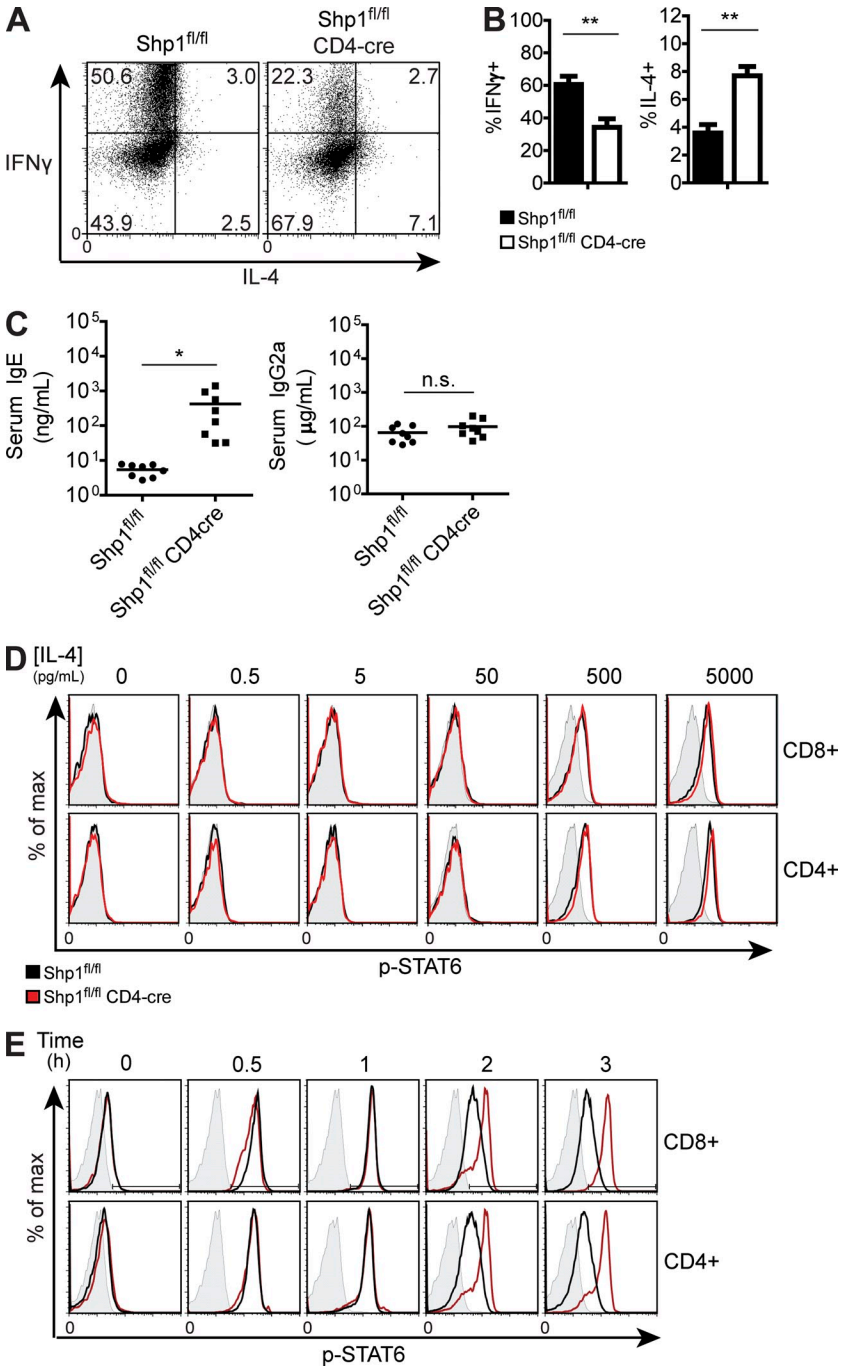

Figure 5. T cells skew to Th2 in the absence of Shp1. (A) Naive ($CD44^{lo}$) $CD4^+$ T cells were isolated, stimulated for 3 d with α-CD3/CD28, and then re-stimulated with PMA/Ionomycin 4 d later. Cells were harvested and stained 6 h after restimulation. (B) Cells were stimulated and analyzed as in A. Percentages of cells staining positive for IFN γ or IL-4. Data are representative of three independent experiments and are displayed as ± standard error; $n = 4$. (C) Concentration of IgE and IgG2a in sera from the indicated unmanipulated age-matched mice. Horizontal bars represent sample means; $n = 8$. (D) T cells were stimulated with the indicated concentrations of IL-4 for 30 min and then fixed and stained for p-Stat6. Histograms are gated on $CD44^{lo}$ T cells. (E) T cells were stimulated with 10 ng/ml IL-4 for 30 min and were then washed three times with media. Cells were harvested at the indicated times and analyzed as in D**.** Data are representative of three independent experiments. Statistical analyses of data in B and C were performed by Student's *t* test; ns, $P \geq 0.05$; *, $P < 0.05$; **, $P < 0.01$.

To test if the enhanced sensitivity of Shp1 deficient T cells to IL-4 was driving the accumulation of memory phenotype cells, we crossed our Shp1 conditional knockout mice to $IL\text{-}4^{-/-}$ mice. The absence of IL-4 had no effect on the frequency of peripheral blood $CD44^{hi}$ cells in mice expressing Shp1 (Fig. 6, C and D). However, lowering ($IL4^{+/-}$) or eliminating ($IL4^{-/-}$) IL-4 expression in Shp1 conditional knockout mice resulted in a reduced percentage of $CD4^+$ and $CD8^+$ $CD44^{hi}$ cells in blood (Fig. 6 D). The percentage of $CD4^+$ $CD44^{hi}$ T cells returned to wild-type levels in double knockout mice, suggesting that IL-4 is essential for the development of excess $CD4^+$ $CD44^{hi}$ T cells caused by the absence of Shp1. Decreasing IL-4 levels also lowered the proportion of $CD8^+$ $CD44^{hi}$ T cells, although the percentage of these cells consistently remained above wild-type levels in all organs, suggesting that additional factors contribute to $CD8^+$ memory phenotype T cell development in the absence of Shp1. Serum IgE was undetectable in double knockout mice (unpublished data), indicating that IL-4 is critical for the elevated IgE levels detected in Shp1 conditional knockout mice.

To ask whether IL-4 also is required to maintain the increased memory phenotype population, mice were treated

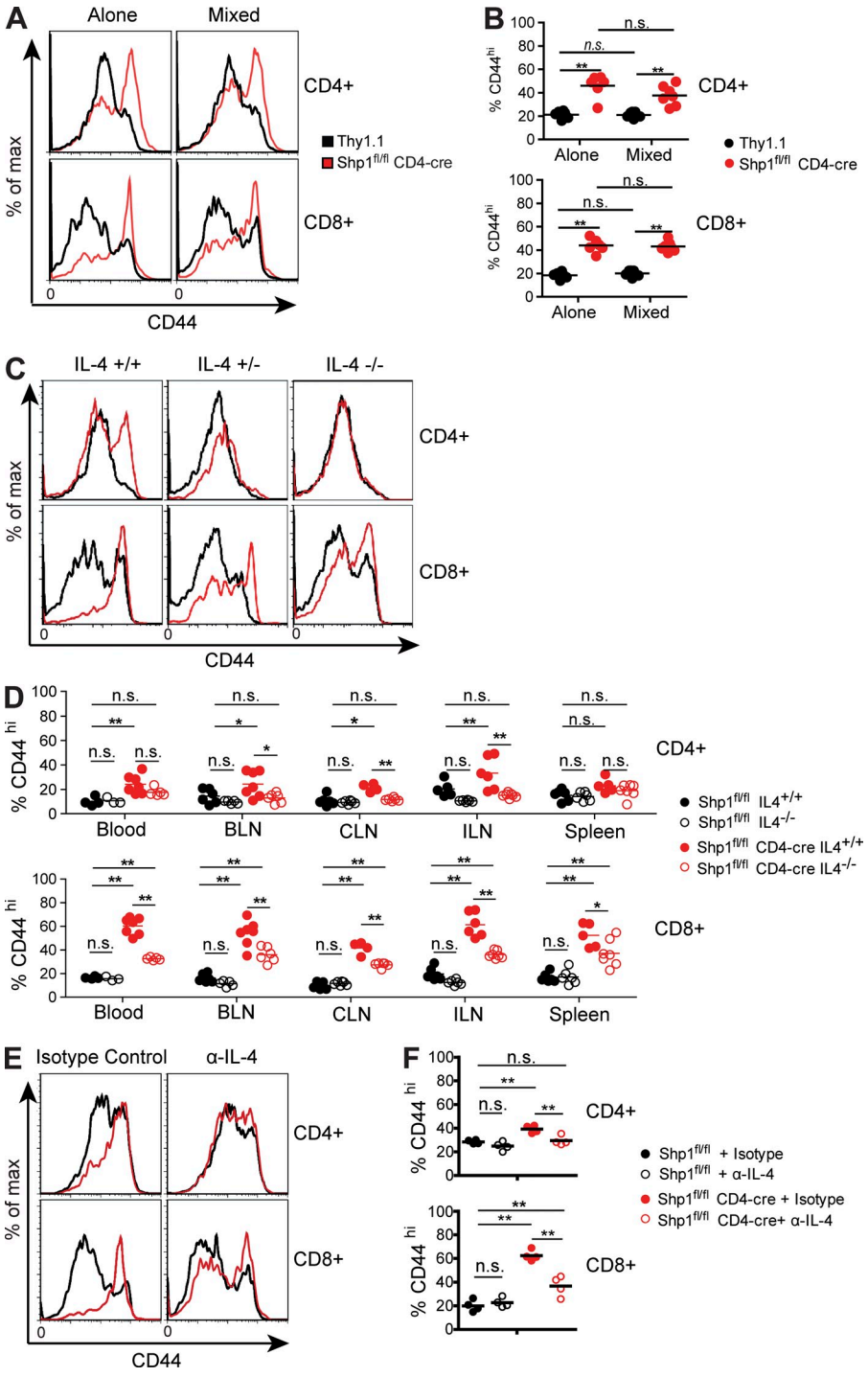

Figure 6. IL-4 is required for the accumulation of CD44^{hi} T cells in Shp1 conditional knockout mice. (A) Bone marrow from Thy1.1 and/or Shp1$^{fl/fl}$ CD4-cre mice was transferred into irradiated CD45.1 host animals to generate mixed bone marrow chimeras. CD44 expression on splenic T cells was analyzed by flow cytometry. Flow plots are gated on CD45.2^{+}Thy1.1^{+}Thy1.2^{-} (Thy1.1) or CD45.2^{+}Thy1.1^{-}Thy1.2^{+} (Shp1 $^{fl/fl}$ CD4-cre) populations, as well as CD4^{+} or CD8^{+}. (B) Percentage of splenic T cells with a CD44^{hi} phenotype in bone marrow chimeras from A.; n = 6–7. (C) CD44 expression on T cells in the blood of mice of the indicated genotypes. (D) Percentage of T cells with a CD44^{hi} phenotype in the indicated tissues of IL-4 and Shp1-deficient mice; n = 6–7. BLN, brachial LN; CLN, cervical LN; ILN, inguinal LN. (E) CD44 expression of T cells in blood of mice given 200 µg α-IL-4 or an isotype control 5 d before sacrifice. (F) Percentage of T cells with a CD44^{hi} phenotype in the blood of mice treated as in E. Data are representative of two independent experiments; n = 4. Statistical analyses of data in B, D, and F were performed by two-way ANOVA and Bonferroni's post-test analysis; ns, $P \geq 0.05$; *, $P < 0.05$; **, $P < 0.01$. Horizontal bars for B, D, and F represent sample means.

with a neutralizing α-IL-4 antibody (Ohara and Paul, 1985). Administration of α-IL-4 to Shp1 conditional knockout mice resulted in a significant reduction in the levels of CD4^{+}CD44^{hi} T cells, which reached wild-type levels, and CD8^{+}CD44^{hi} T cells, although this population remained elevated compared with controls (Fig. 6, E and F). These data further suggest that the enriched CD4^{+} memory phenotype population is completely IL-4 dependent, whereas other factors contribute to the expanded CD8^{+} population. In sum, IL-4 is required for both the development and maintenance of the enriched population of memory phenotype T cells found within mice with Shp1-deficient T cells.

DISCUSSION

The severe and complex phenotype of Shp1 mutant mice has hindered attempts at determining the cell-autonomous role of Shp1 in various hematopoietic cell types. Floxed Shp1 mice

provide the best tool available for analyzing the cell-intrinsic consequences of Shp1 deficiency. Therefore, we generated and analyzed mice with Shp1-deficient T cells. These mice lack the overt autoimmunity of me mice, confirming that the absence of Shp1 in T cells alone is insufficient for the development autoimmunity (Shultz, 1988; Yu et al., 1996).

Absence of Shp1 in T cells does not phenocopy the T cell phenotype of me mice

Shp1 has been identified as a negative regulator of T reg cell development in me mice (Carter et al., 2005). In contrast, our results demonstrate that T reg cell development is normal in T cells specifically lacking Shp1. Furthermore, in contrast to the results of studies of *me/me*- and me^v/me^v-derived T cells, we found that Shp1 deficiency has no effect on sensitivity to TCR stimulation. Several groups have reported enhanced positive selection of me thymocytes expressing transgenic TCRs (Carter et al., 1999; Johnson et al., 1999; Zhang et al., 1999). In contrast, we find no change in the selection of thymocytes expressing either MHCI (P14, OT-I)– or MHCII (OT-II)–restricted transgenic TCRs. The P14, OTI, and OTII transgenes are all "strongly" selected TCRs; consequently, Shp1 deficiency might affect the selection of TCRs with lower affinity (Azzam et al., 1998; Atherly et al., 2006; Hu et al., 2010). However, previous data implicated Shp1 in regulating the selection of the DO11.10 TCR (Carter et al., 1999), which is believed to receive a stronger selecting signal than OT-II (Hu et al., 2010). Together, our findings and the previous reports suggest that the selection defect found in me mice is not the result of a cell-autonomous effect of Shp1 deficiency but rather is a consequence of Shp1 deficiency in another cell type (e.g., thymic DCs) and/or the systemic inflammatory signals in these mice.

Likewise, the reported TCR hypersensitivity of peripheral T cells from me mice (Johnson et al., 1999) also is likely to be the result of systemic inflammation and the enhanced activation state of the cells. Mice expressing a putative dominant-negative (phosphatase inactive) Shp1 allele in T cells also were reported to show enhanced TCR sensitivity for thymocyte selection (Plas et al., 1999; Zhang et al., 1999) and activation (Plas et al., 1996). These mice lack the severe systemic inflammation of me mice yet still display TCR hypersensitivity. The discrepancy between these results and our study might be explained by the ability of the catalytically impaired Shp1 mutant to interfere with the binding of other SH2 domain–containing negative regulators to proteins with ITIMs and immunoreceptor tyrosine-based switch motifs (ITSMs; Lorenz, 2009). Overexpression of dominant-negative Shp1 not only might outcompete wild-type Shp1 for binding to ITIM- and ITSM-containing proteins but also could block the binding of Ship and/or Shp2 to these motifs, thereby inhibiting their roles in antagonizing TCR signaling as well. In Shp1 conditional knockout T cells, competition for ITIM and ITSM binding is, if anything, decreased, allowing other factors to interact with these proteins. Regardless, our results establish that Shp1 is not essential for the negative regulation of TCR signaling. This study underscores the value in separating cell-extrinsic and -intrinsic effects of Shp1 activity.

Shp1 restricts the development of memory phenotype T cells

Memory phenotype T cells, also known as innate T cells, are characterized by their surface memory phenotype and ability to rapidly produce cytokines upon TCR stimulation. Such cells have been hypothesized to play an important role in the early stages of immune responses. IL-4 has been demonstrated to be essential for the accumulation of memory phenotype T cells in several other knockout mice. For example, mice deficient for inducible T cell kinase (Itk) have an accumulation of memory phenotype T cells in the thymus (Atherly et al., 2006), and the development of these $CD44^{hi}$ thymocytes subsequently was shown to be dependent on the production of IL-4 by NKT cells (Weinreich et al., 2010). Mice deficient for Krüppel-like factor 2 (KLF2) or inhibitor of DNA binding 3 (Id3) also develop prominent $CD44^{hi}$ populations in the thymus that are dependent on IL-4 (Weinreich et al., 2009, 2010; Verykokakis et al., 2010). Like these memory-like or innate T cells, the Shp1-deficient memory population expresses high levels of CD44 and has an increased capacity to quickly produce cytokines after TCR stimulation. However, the memory-like populations in Itk-, KLF2-, and Id3-deficient mice all arise during thymocyte development, whereas the $CD44^{hi}$ population in Shp1 conditional knockouts is restricted to the periphery, suggesting that a distinct developmental pathway is involved. Additionally, mixed bone marrow chimera experiments revealed that loss of KLF2 in the T cell lineage results in the development of memory phenotype T cells of both wild-type and cKO origin. The KLF2 phenotype therefore is a result of elevated extracellular IL-4. In contrast, we found that the development memory phenotype cells in Shp1 cKO mice is cell intrinsic; wild-type bystander cells in mixed bone marrow chimeras maintain normal homeostasis. T cell protein tyrosine phosphatase (TCPTP) T cell–conditional knockout mice also accumulate $CD4^+$ $CD44^{hi}$ and $CD8^+$ $CD44^{hi}$ populations in the periphery (Wiede et al., 2011). Unlike Shp1-deficient T cells, however, the T cells in TCPTP conditional knockout mice predominantly have an activated/effector-memory phenotype, and these mice develop systemic inflammation and autoimmunity, including lymphoid infiltrates in the liver and lungs, elevated antinuclear antibodies, and increased germinal center formation. T cells from Shp1 conditional knockout mice had a mixed effector/central memory phenotype, did not infiltrate tertiary tissues, and displayed normal germinal center formation (unpublished data).

We identified IL-4 as a critical factor driving the development and survival of $CD4^+$ $CD44^{hi}$ cells in $Shp1^{fl/fl}$ CD4-cre mice. Elimination of IL-4 resulted in wild-type levels of $CD4^+$ $CD44^{hi}$ cells in all lymphoid organs examined. IL-4 also promoted the development and survival of $CD8^+CD44^{hi}$ cells, as this population was reduced substantially upon elimination of IL-4. In contrast to the normalization of the number of $CD4^+CD44^{hi}$ cells, we observed only a partial reduction

of $CD8^+$ $CD44^{hi}$ cells in the blood, spleen, and lymph nodes of $Shp1^{fl/fl}$ CD4-cre $IL4^{-/-}$ mice. This finding indicates that there are other factors promoting the accumulation of $CD8^+$ $CD44^{hi}$ cells. The identity of these factors and their relative contributions to memory phenotype T cell development in various lymphoid organs remain to be elucidated.

Shp1 negatively regulates Th2 skewing

Previous studies of me-derived cells had identified Shp1 as a negative regulator of Th1 (Park et al., 2005; Yu et al., 2005) and Th2 differentiation (Kamata et al., 2003), but it remained unclear if Shp1 has a cell-intrinsic role in regulating $CD4^+$ T cell differentiation. Our results show that Shp1 restricts the development of Th2 (but not Th1) cells in a cell-autonomous manner. This finding is congruent with the autoimmune lung disease to which me mice succumb, which is characterized by excessive type 2 inflammation and can be partially limited by the elimination of IL-4, IL-13, or Stat6 (Oh et al., 2009). However, given that $CD4^+$ T cells are nonessential for the me lung phenotype (Shultz, 1988; Yu et al., 1996), additional sources of IL-4 and IL-13 must drive lung inflammation in these mice.

A critical step in the differentiation of Th2 cells is the IL-4–mediated activation of Stat 6 (Zhou and Ouyang, 2003), and Shp1 has been reported to antagonize IL-4/Stat 6 signaling. The IL-4Rα chain contains an ITIM that can interact with Shp1, Shp2, and Ship, and mutation of the ITIM results in a hyperproliferative response to IL-4 in a myeloid cell line (Kashiwada et al., 2001). Additionally, B cells harboring the ITIM mutation have impaired dephosphorylation of Stat 6 (Hanson et al., 2003), and various hematopoietic cells types from me mice show enhanced Stat 6 phosphorylation in response to IL-4 or IL-13 (Haque et al., 1998; Hanson et al., 2003). However, Shp1 deficiency has been reported to have no impact on the IL-4–induced phosphorylation of Stat 6 in $CD4^+$ and $CD8^+$ T cells (Huang et al., 2005). Our results are in direct contrast, and suggest a cell-intrinsic mechanism for the regulation of IL-4 signaling by Shp1. A likely explanation for this discrepancy is that the previous study only examined the induction of Stat 6 phosphorylation, whereas we found that Shp1 primarily controls Stat 6 dephosphorylation. Regulation of Stat 6 phosphorylation likely explains how Shp1 regulates Th2 differentiation.

IL-4 reportedly has diverse effects on peripheral T cells beyond its role in Th2 polarization. For example, IL-4 promotes the survival of resting T cells (Vella et al., 1997). Consistent with Shp1 deficiency having a more dramatic effect on $CD8^+$ homeostasis, IL-4 has been demonstrated to have potent mitogenic effects on $CD8^+$ T cells (Morris et al., 2009) and T cells expressing a constitutively active form of Stat 6 primarily adopt an activated phenotype (Bruns et al., 2003). Yet whether IL-4 has a positive or negative effect on $CD8^+$ activation, cytotoxicity, and memory formation remains controversial (Terabe et al., 2000; Schüler et al., 2001; Carvalho et al., 2002; Bronte et al., 2003). The IL-4 hypersensitivity of Shp1-deficient T cells likely contributes to the development of memory phenotype T cells. However, enhanced IL-4 production by Shp1-deficient T cells is detected only after prolonged stimulation (Fig. 5, A and B) and not directly ex vivo. This finding demonstrates that in Shp1 conditional knockout animals, IL-4 facilitates the accumulation of memory phenotype cells but does not induce IL-4 production. Additionally, our mixed bone marrow chimera experiments demonstrate that IL-4 from Shp1-deficient T cells alone is insufficient to alter the homeostasis of wild-type T cells. Together these data strongly suggest that Shp1-deficient T cells are reacting to homeostatic levels of IL-4, and that cell-intrinsic hypersensitivity to IL-4 signals is crucial for the increase in memory phenotype T cells and Th2 differentiation in the absence of Shp1. In this context, our findings highlight that IL-4, like other IL2-Rγc cytokines, can have powerful regulatory effects on $CD4^+$ and $CD8^+$ T cell homeostasis and differentiation.

Concluding remarks

By analyzing T cell–conditional Shp1 knockout mice, we have delineated the cell-autonomous role of Shp1 in T cell development, homeostasis, and activation. What remains to be determined is the precise role Shp1 plays in regulating T cells during various immune responses. We have demonstrated that, in contrast to reports using me mice, Shp1 deficiency in T cells does not have a major impact on thymocyte development. Rather, we have established that Shp1 is an important regulator of peripheral T cell homeostasis. Through regulation of IL-4 signals in T cells, Shp1 limits Th2 differentiation, IgE production, and critically limits the development of memory phenotype T cells.

MATERIALS AND METHODS

Mice. Shp1 floxed ($Shp1^{fl/fl}$; Pao et al., 2007b), P14 (Pircher et al., 1989), and CD4cre (Lee et al., 2001) mice have been previously described. OT-I, CD45.1, Thy1.1, and IL-4 knockout mice ($IL\text{-}4^{-/-}$) were obtained from The Jackson Laboratory. All mice were maintained on C57BL/6 background. Mice were housed in the Ontario Cancer Institute animal facility in accordance with institutional regulations. Animal protocols were approved by the Ontario Cancer Institute Animal Care Committee. For IL-4 neutralization experiments, 200 μg α-IL-4 IgG1 (11B11; BioXCell) or an isotype control (HRPN; BioXCell) were administered to mice by i.p. injection. Mice were sacrificed for analysis 5 d after injection. Bone marrow chimeras were generated by i.v. injection of 5×10^6 bone marrow cells into irradiated mice. Recipient mice received 900 cGy of radiation delivered by an X-RAD320 (PXi) 2 h before reconstitution. Chimeras were analyzed 3 mo after their generation.

Western blots. Sorted thymocyte populations were lysed in NP-40 lysis buffer (Roche). Equal amounts of protein were resolved on NuPAGE 4–12% Bis Tris gels (Invitrogen) and transferred onto PVDF membranes using an iBlot (Invitrogen). Membranes were blocked with 5% milk in TBS containing Tween-20 (Sigma-Aldrich), stained with antibodies against Shp1 (EMD Millipore) and actin (Sigma-Aldrich), and subsequently developed using ECL Plus (GE Healthcare).

ELISAs. Sera were isolated from 12–16-wk-old littermate pairs using microtainer serum separator tubes (BD), and duplicate samples were analyzed by ELISA for IgE (BioLegend) and IgG2a (eBioscience), according to the manufacturer's instructions.

Flow cytometry and FACS. Splenic T cells were isolated using the magnetic Pan T Isolation kit II (Miltenyi Biotec). Where indicated, cells were

stained with α-CD44 (eBioscience) in preparation for cell sorting. Thymocytes were stained with α-CD4 (eBioscience) and α-CD8 (BD). Cell sorting was performed on a MoFlo (Beckman Coulter), and the purities of target populations were routinely >90%. For analytical flow cytometry of T cell subsets, cells were stained with antibodies against CD5, CD8α, CD25, CD45.2, CD69, Thy1.1, Thy1.2, TNF, Vα2, and pStat6 (BD), and CCR7, CD4, CD24, CD44, CD62L, CD127, IFN-γ, IL-2, IL-4, and IL-5 (eBioscience). Data were collected on a FACSCalibur or FACSCanto (BD) and analyzed with FlowJo software (Tree Star).

In vitro assays. Cells were cultured in RPMI-1640 supplemented with 10% FBS, L-glutamine, β-mercaptoethanol, penicillin, and streptomycin. For thymocyte assays, P14 $CD8^+$ SP thymocytes were co-cultured in round-bottom 96-well plates at a 1:10 ratio with irradiated splenocytes from C57BL/6 mice pulsed with gp33 (KAVYNFATM). T cell stimulation assays were performed in flat-bottom 96-well plates containing α-CD3 and α-CD28 (eBioscience), cross-linked with 10 µg/ml α-hamster IgG (Jackson ImmunoResearch Laboratories). For proliferation assays, cells were incubated with 2.5 µM CFSE (Invitrogen) before culture. Cytokine production assays were performed by adding GolgiPlug (BD) to cultures 1 h after stimulation, and then harvesting cells for staining 5 h later. $CD4^+$ T cell stimulation assays were performed by culturing naive $CD4^+$ T cells with α-CD3 and α-CD28 for 3 d, as above, with the addition of 50 U/ml IL-2 (eBioscience) for an additional 3 d. Cells were then restimulated with PMA and ionomycin (eBioscience). To assess Stat 6 activation, cells were stimulated with IL-4 (eBioscience) in 96-well round-bottom plates under the indicated conditions, before fixation with Lyse/Fix Buffer (BD), permeabilization with PermBuffer III (BD), and analysis of pStat6 by flow cytometry.

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