The Journal of Immunology

RESEARCH ARTICLE | JUNE 15 2007

Agonist-Driven Development of $CD4^+CD25^+Foxp3^+$ Regulatory T Cells Requires a Second Signal Mediated by Stat6[1] FREE

Vanesa Sanchez-Guajardo; ... et. al

J Immunol (2007) 178 (12): 7550–7556.
https://doi.org/10.4049/jimmunol.178.12.7550

Agonist-Driven Development of $CD4^+CD25^+Foxp3^+$ Regulatory T Cells Requires a Second Signal Mediated by Stat6[1]

Vanesa Sanchez-Guajardo,* Corinne Tanchot,† John T. O'Malley,‡ Mark H. Kaplan,‡ Sylvie Garcia,* and Antonio A. Freitas[2]*

The factors that induce Foxp3 expression and regulatory T (Treg) cell development remain unknown. In this study, we investigated the role of STAT4 and STAT6 in agonist-driven generation of Ag-specific Foxp3-expressing Treg cells. Our findings indicate that fully efficient induction of Foxp3 expression and development of Ag-specific Treg cells requires the synergistic action of two signals: a TCR-mediated signal and a second signal mediated by STAT6. Indeed, by comparing the development of wild-type and STAT4- and STAT6-deficient hemagglutinin-specific T cells in the presence of hemagglutinin Ag, we found that the absence of STAT6 impaired the generation of Ag-specific $CD4^+CD25^+Foxp3^+$ cells. Moreover, in transgenic mice expressing a constitutively active form of STAT6, we found that the fraction of $CD4^+Foxp3^+$ cells exceeds that of control wild-type littermates. Overall these findings support a role for the STAT6 pathway in Treg cell development and maintenance. ***The Journal of Immunology,*** **2007, 178: 7550–7556.**

Regulatory $CD4^+CD25^+$ T lymphocytes play a major role in T cell homeostasis and maintenance of tolerance (1, 2). They represent a specific T cell lineage whose development occurs in the thymus and is dependent on the expression of the forkhead family transcription factor Foxp3 (3, 4). However, the pathways that lead to the induction of Foxp3 expression and T regulatory (Treg)[3] cell development in the thymus remain largely unknown.

STATs are a family of transcription factors that regulate a broad range of cellular processes such as T cell differentiation and proliferation. Among the STAT proteins concerned in T cell differentiation, STAT4 is implicated in the induction of $CD4^+$ Th1 T cells (5), whereas STAT6 is involved in the generation of $CD4^+$ Th2 T cells (6, 7). The STAT4 and STAT6 proteins have also been implicated in the regulation of cell cycle progression (8) and T cell expansion in vivo (9). We have shown that the absence of STAT6 results in faster cell cycle progression, which confers to the $STAT6^{-/-}$ cells the capacity to out-compete wild-type (WT) and $STAT4^{-/-}$ cells during competitive lymphopenia-driven proliferation (LDP) (9). In contrast, $STAT4^{-/-}$ $CD4^+$ T cells are outcompeted by WT and STAT4/6 double-deficient T cells, suggesting that the presence of STAT6 alone moderates T cell proliferation (9). The role of STAT6 as a regulator of T cell proliferation is supported by the observation that mice expressing a constitutively active form of STAT6 have reduced T cell numbers (10). In summary, these findings suggest that STAT proteins influence $CD4^+$ T cell proliferation and accumulation and, thus, may participate in the control of peripheral T cell numbers. As we have shown that Treg cells play a major role in T cell homeostasis and in the control of LDP (1), we raised the possibility that STAT4 and STAT6 proteins could also play a yet undetermined function in the development of $CD4^+$ Treg cells. Indeed, although the role of STAT4 and STAT6 in $CD4^+$ T cell differentiation and proliferation has been extensively studied, little is know about their involvement in T cell development.

To evade problems inherent to the study of polyclonal populations, we studied T cell development in a well-characterized experimental model in which monoclonal TCR transgenic $CD4^+$ T cells, specific for the 111–119 peptide of the hemagglutinin of influenza virus (HA), are selected into the $CD4^+CD25^+$ Treg cell lineage by an agonist ligand, leading to the generation of in vivo functional Treg cells (11). We generated $STAT4^{-/-}$ Thy1.1 and $STAT6^{-/-}$ Thy1.2 $Rag2^{-/-}$ mice expressing the transgenic HA-specific TCR (TCR-HA), and followed the development of the Ag-specific $CD4^+6.5^+$ T cells in Ig-HA $Rag2^{-/-}$ hosts expressing the HA peptide under the control of the Igκ promoter (12). Studying the expression of Foxp3, a key marker of the $CD4^+CD25^+$ Treg cell lineage, we found that the absence of STAT6 impaired the generation of Treg cells. We also studied the relative representation of $Foxp3^+$ T cells in mice expressing an active form of STAT6 (Stat6VT) (10). We found that these mice have increased percentages of $CD4^+Foxp3^+$ Treg cells. Our findings directly implicate the STAT6 pathway in the development and survival of Ag-specific $CD4^+CD25^+Foxp3^+$ Treg cells.

*Unité de Biologie des Populations Lymphocytaires, Centre National de la Recherche Scientifique, Institut Pasteur, Paris, France; †Institut National de la Santé et de la Recherche Médicale Unité 591, Institut Necker, Paris, France; and ‡Department of Pediatrics, Microbiology and Immunology, HB Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN 46202

Received for publication December 6, 2006. Accepted for publication March 30, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grant 642-02-0016 from the Danish Research Agency (to V.S.-G.) and by a study loan from El Consejo Nacional de Ciencia y Tecnologia. This work was also supported by the Association pour la Recherche contre le Cancer, Agence Nationale de la Recherche contre le SIDA, Association Française des Myopathies, Centre National de la Recherche Scientifique, Programmes Transversaux de Recherche from the Institiut Pasteur/Necker 114, and Public Health Service Awards from the National Institutes of Health (to M.H.K.).

[2] Address correspondence and reprint requests to Dr. Antonio A. Freitas, l'Unité Biologie des Populations Lymphocytaires, Institut Pasteur, 25 rue du Dr. Roux, 75015 Paris, France. E-mail address: afreitas@pasteur.fr

[3] Abbreviations used in this paper: Treg, T regulatory; HA, hemagglutinin; TCR-HA, HA-specific TCR; LN, lymph node; BM, bone marrow; LDP, lymphopenia-driven proliferation; SP, single positive; WT, wild type.

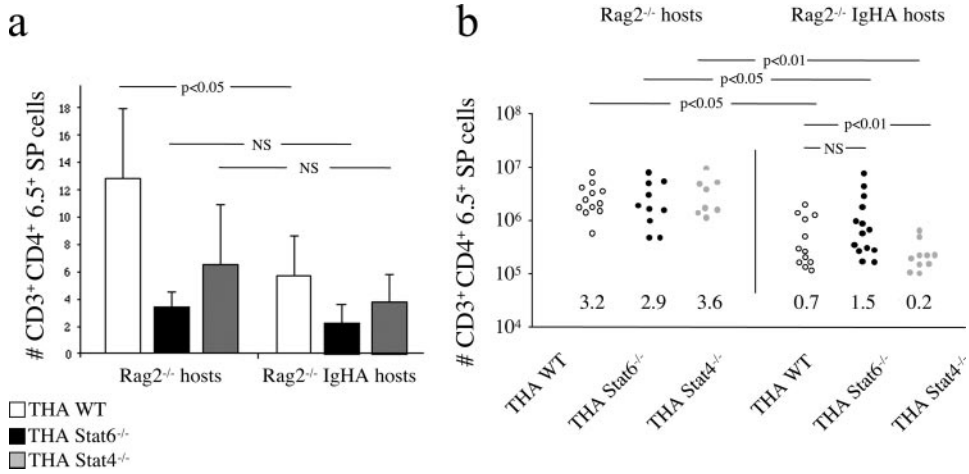

FIGURE 1. Number of TCR-HA CD4$^+$ T cells in HA-free and HA-expressing BM chimeras. Rag2$^{-/-}$ or Ig-HA Rag2$^{-/-}$ hosts were lethally irradiated and reconstituted with T cell-depleted BM from either Rag$^{-/-}$ TCR-HA WT (open symbols), STAT6$^{-/-}$ (filled symbols), or STAT4$^{-/-}$ (gray-filled symbols) donors. Ten weeks later, the mice were killed, and the T cell compartments were analyzed. *a*, Data are mean + SE of SP CD3$^+$CD4$^+$6.5$^+$ cells in the thymus in hosts lacking (*left*) or expressing (*right*) HA. *b*, Shows the number of CD3$^+$CD4$^+$6.5$^+$ T cells recovered in the peripheral pools of Rag2$^{-/-}$ (*left*) and Ig-HA Rag2$^{-/-}$ (*right*) BM chimeras. The average number of cells recovered is shown under each group of symbols. Each circle corresponds to one mouse. Results are from three independent experiments each with $n = 3$–5 mice per group.

Materials and Methods

Mice

STAT6$^{-/-}$ Thy1.2 and STAT4$^{-/-}$ Thy1.2 BALB/c mice were from The Jackson Laboratory, and Rag2$^{-/-}$ BALB/c mice were from the Centre de Développment des Techniques Avancées-Centre National de la Recherche Scientifique (Orléans, France). STAT4$^{-/-}$ Thy 1.2 mice were crossed with BALB/c Thy1.1 to obtain STAT4$^{-/-}$ Thy1.1 mice. TCR-HA BALB/c mice transgenic for a viral HA-specific TCR and BALB/c mice expressing the HA peptide under the Igκ promoter (Ig-HA) (11) were crossed into the BALB/c.Rag2$^{-/-}$ background. The Ig-HA and TCR-HA mice were a gift from Drs. H. von Boehmer (Harvard Medical School, Boston, MA) and A. Sarukhan (Centre Hospitalier Universitaire Necker, Paris, France). The STAT-deficient strains were breed into a BALB/c.Rag2$^{-/-}$ background and crossed with TCR-HA transgenic Rag2$^{-/-}$ mice. Athymic *nu/nu* BALB/c mice were from Charles River Breeding Laboratories. Stat6VT mice, transgenic for a mutant form of STAT6 under the control of the CD2 promoter, were C57BL/6 (10). All mice were subsequently raised in our animal facilities. For each experiment, animals were matched for age and sex. Experiments were performed according to the Experimental Ethics Committee guidelines.

Bone marrow (BM) chimeras

Seven- to 10-wk-old Rag2$^{-/-}$ or Ig-HA Rag2$^{-/-}$ mice were lethally irradiated with a ^{137}Ce source (800–850 rad) and rescued by the i.v. injection of 1×10^6 T cell-depleted (<0.1%) BM cells from different donor mice. T cell depletion was done in an AutoMACS (Dynal Biotech) after incubating the BM cells with anti-CD3-PE followed by PE MACS beads. At 9–10 wk after transplant, thymus, spleen, and lymph node (LN) cell suspensions were prepared and the number and phenotype of the cells from each donor population evaluated. Chimeras were also prepared by reconstituting lethally irradiated *nu/nu* BALB/c mice with BM cells from Ig-HA Rag$^+$ BALB/c donors.

Cell transfers

LN cells from WT, STAT4$^{-/-}$ Thy1.1, and/or STAT6$^{-/-}$ Thy1.2 TCR-HA$^+$ Rag2$^{-/-}$ donor mice were i.v. injected, alone or mixed at a 1:1 ratio, into Rag2$^{-/-}$, Ig-HA Rag2$^{-/-}$, or nude mice reconstituted with BM from Ig-HA Rag$^+$ BALB/c donors. The host mice were sacrificed at different times after cell transfer.

Flow cytometry analysis

Cell surface staining was performed with the appropriate combinations of FITC-, PE-, PE-Cy7-, and allophycocyanin-coupled Abs. TCR-HA$^+$ cells were labeled with the biotinylated clonotype-specific mAb 6.5 produced by F. Vasseur (Institut National de la Santé et de la Recherche Médicale Unité 591, Paris, France) followed by streptavidin-PerCp. Dead cells were excluded according to their light-scattering characteristics. Intracellular staining of Foxp3 was done following the protocol of the PE anti-mouse/anti-rat Foxp3 staining set (eBioscience). All acquisitions and analysis were performed in a LSR (BD Biosciences) interfaced to the Macintosh CellQuest software.

STAT6 activation and IL-4R expression in Treg cells

Splenocytes (1×10^6/ml) were cultured in the presence or absence of 10 ng/ml IL-4 (PeproTech) for 30 min, 1 h, or 1.5 h (for pStat6 staining) or 24 h (for IL-4R staining) in medium as described previously (10). For analysis of STAT6 activation, cells were collected and fixed with 1.5% formaldehyde and permeabilized with 100% methanol for 10 min before staining with anti-pStat6 Alexa Fluor 647, anti-CD4-PE Cy5, and anti-Foxp3 FITC (BD Pharmingen) for 30 min at room temperature. For analysis of IL-4R expression, cells were surface stained with anti-IL-4R-PE and anti-CD4-PE Cy5 at 4°C for 30 min (BD Pharmingen) followed by permeabilizing for Foxp3 staining as described. Histograms are gated on CD4$^+$Foxp3$^-$ or CD4$^+$Foxp3$^+$ as indicated. Percentages were obtained using CellQuest software.

Results

We studied the role of STAT4 and STAT6 proteins in the generation of Ag-specific CD4$^+$CD25$^+$ Treg cells by comparing the development of WT, STAT4-, and STAT6-deficient monoclonal HA-specific T cells in the absence and the presence of the HA-neo self-Ag. For this purpose, lethally irradiated Rag2$^{-/-}$ or Ig-HA Rag2$^{-/-}$ hosts were reconstituted with T cell-depleted BM cells from WT, STAT4$^{-/-}$, or STAT6$^{-/-}$ TCR-HA Rag$^{-/-}$ donors. In the chimeras reconstituted with WT TCR-HA BM, we found that the number of HA-specific CD4$^+$6.5$^+$ single positive (SP) thymus cells was diminished in the HA-expressing thymus when compared with the Ag-free thymus ($p < 0.05$) (Fig. 1*a*), reflecting Ag-induced negative selection. In the chimeras receiving BM from STAT4- or STAT6-deficient donors, the number of CD4$^+$6.5$^+$ SP cells was slightly reduced when compared with the WT TCR-HA BM chimeras (p = NS), but not significantly modified by the presence of HA (Fig. 1*a*). As the number of double positive thymus cells remains similar in the different chimeras (varying from 20 to 80 $\times 10^6$) these findings suggest that, in the absence of Ag, the lack of STAT4 or STAT6 may reduce the efficiency of thymus-positive selection resulting in a lower yield of CD4$^+$ SP mature T cells. This result would allow an increased fraction of STAT-deficient HA-specific transgenic T cells to escape self-Ag-mediated

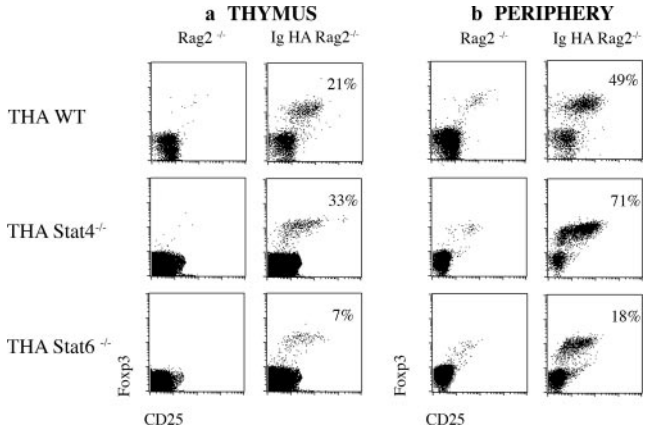

FIGURE 2. The generation of Ag-specific $CD25^+Foxp3^+$ Treg cells varies with the STAT competence of the developing cells. $Rag2^{-/-}$ or Ig-HA $Rag2^{-/-}$ hosts were lethally irradiated and reconstituted with T cell-depleted BM from either $Rag^{-/-}$ TCR-HA WT, $STAT6^{-/-}$, or $STAT4^{-/-}$ mice. Ten weeks after cell transfer, the mice were killed and analyzed. *a*, The expression of CD25 and Foxp3 by gated $CD3^+CD4^+6.5^+$ SP thymus cells developed from TCR-HA WT (*upper*), $STAT4^{-/-}$ (*middle*), and $STAT6^{-/-}$ (*lower*) BM donors in the absence (*left*) or presence (*right*) of HA. *b*, The expression of CD25 and Foxp3 by gated $CD3^+CD4^+6.5^+$ spleen T cells developed from TCR-HA WT (*upper*), $STAT4^{-/-}$ (*middle*), and $STAT6^{-/-}$ (*lower*) BM donors in hosts lacking (*left*) or expressing (*right*) HA. The percentage of $CD25^+Foxp3^+$ is shown. Data are representative of at least three independent experiments each containing $n = 3$–5 mice per group.

deletion in the thymus (Fig. 1*a*). In the peripheral compartments of the HA-free BM chimeras, the number of Ag-specific $CD4^+6.5^+$ T cells was similar (range from 2.9 to 3.6×10^6) whether the mice were reconstituted with WT, STAT4-, or STAT6-deficient TCR-HA BM (Fig. 1*b*), indicating that peripheral homeostatic mechanisms operate to compensate the lower SP compartment of the STAT-deficient HA-specific T cells (13). In the BM chimeras expressing HA, the number of peripheral $CD4^+6.5^+$ T cells was reduced as compared with HA-free chimeras (12). The reduction of HA-specific cell numbers was more marked for $STAT4^{-/-}$ (0.2×10^6; $p < 0.01$) than for WT (0.7×10^6; $p < 0.05$) or $STAT6^{-/-}$ (1.5×10^6; $p < 0.05$) T cells (Fig. 1*b*). Comparing the different HA chimeras, we observed that although the number of $CD4^+6.5^+$ T cells recovered was significantly lower ($p < 0.01$) for STAT4-deficient than WT or STAT6-deficient BM donors, the recovery of $STAT6^{-/-}$ and WT $CD4^+6.5^+$ T cells was not significantly different. These findings indicate that the several HA-specific populations are differently affected by self-Ag during development and peripheral seeding.

Surprisingly, we found that the generation of Ag-specific Treg cells varied with the presence of Ag and the STAT competence of the developing cells. In the absence of HA, the fraction of Ag-specific $CD4^+6.5^+$ T cells expressing CD25 and/or Foxp3 was low for all cell types studied (Fig. 2). In the presence of HA and as described (11), an increased fraction of the WT $CD4^+6.5^+$ SP thymus T cells expressed CD25 and Foxp3 ($18.7 \pm 5.3\%$, average 1×10^6 cells) (Fig. 2*a*). The STAT4-deficient cells followed the same trend and $45.3 \pm 12.6\%$ (average 1.8×10^6 cells) of the $STAT4^{-/-}$ $CD4^+6.5^+$ SP thymocytes were $CD25^+Foxp3^+$ ($p < 0.01$ for $STAT4^{-/-}$ vs WT thymocytes) (Fig. 2*a*). In contrast, in the HA chimeras reconstituted with BM from $STAT6^{-/-}$ donors, the fraction of $CD4^+6.5^+$ SP thymus cells that were $CD25^+Foxp3^+$ ($\sim 6.6 \pm 3.4\%$) and their absolute number (average 0.15×10^6 cells) was significantly lower than in WT and $STAT4^{-/-}$ chimeras ($p = 0.001$ and $p < 0.001$, respectively) (Fig. 2*a*). We conclude that the absence of STAT6 has a direct effect on Treg cells diminishing their generation in the thymus. In the peripheral compartments of the different HA-positive chimeras, $\sim 39.8 \pm 4.9\%$ of the spleen and $41.3 \pm 9.6\%$ of the LN WT $CD4^+6.5^+$ T cells and $52.7 \pm 9.2\%$ of the spleen ($p < 0.01$) and $53.5 \pm 15.3\%$ of the LN (p = NS) $STAT4^{-/-}$ $CD4^+6.5^+$ T cells were $CD25^+Foxp3^+$, whereas the fraction of $CD25^+Foxp3^+$ among the STAT6-deficient $CD4^+6.5^+$ T cells was 2- to 3-fold lower, $\sim 17.7 \pm 5.1\%$ ($p < 0.001$) in the spleen and $18.6 \pm 6.4\%$ ($p < 0.001$) in the LN (Fig. 2*b*). This 3-fold decrease in the fraction of Treg cells could not be simply attributable to an increased expansion of naive $CD4^+$ T cells and subsequent dilution of the Treg fraction because the total number of $CD4^+6.5^+$ T cells recovered in the periphery of the HA chimeras reconstituted with STAT6 BM was not significantly different from the total number reconstituted with WT BM cells (see data above); the decrease represents a genuine diminution of the number of peripheral Treg cells among the $STAT6^{-/-}$ $CD4^+$ T cells. Thus, by comparing the development of WT, STAT4-, and STAT6-deficient cells, we showed that although the presence of STAT6 in the absence of STAT4 increased the fraction of Treg cells, the lack of STAT6 impaired the thymus maturation and peripheral accumulation of HA-specific $CD4^+CD25^+Foxp3^+$ Treg cells. These findings strongly suggest that STAT6 promotes the development and survival of Ag-specific Treg cells.

As TCR-HA STAT4- and STAT6-deficient T cells may generate different Th subsets of HA-specific $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, we investigated whether the divergent Foxp3 expression between STAT6- and STAT4-deficient cells represented an intrinsic property of the cells or whether it was due to different environmental cues generated during their differentiation. For this purpose, we compared the codevelopment of TCR-HA STAT4- and STAT6-deficient T cells in the same mice by reconstituting $Rag2^{-/-}$ or Ig-HA $Rag2^{-/-}$ hosts with a mixture (1:1) of BM from both $STAT4^{-/-}$ and $STAT6^{-/-}$ TCR-HA donors. Differences in Thy1 allotype allowed discriminating the two donor populations in the same host. We compared directly the two $STAT4^{-/-}$ and $STAT6^{-/-}$ populations rather than each STAT-deficient with WT cells first because differences in cytokine environment would be less evident between WT and STAT-deficient cells, and second because WT and STAT6-deficient mice share the same Thy1 allotype, which makes distinction of the two cell types impossible. In the peripheral pools of the chimeras containing both STAT4- and STAT6-deficient cells, the number of cells recovered in the presence of the HA-Ag was variable and on average lower than in the Ag-free mice (Fig. 3*a*). In these mixed chimeras, the number of $CD4^+6.5^+$ T cells derived from $STAT6^{-/-}$ and $STAT4^{-/-}$ origin was similar and kept the 1:1 ratio of the injected donor BM cells (Fig. 3*a*). This contrast with results obtained in the chimeras in which STAT-deficient cells were transferred alone where we recovered a lower number of STAT4-deficient cells (Fig. 1*b*), suggesting that in the mixed chimeras the two populations may interact and mutually modify cell numbers. Notably, we found that in the presence of HA, the fraction of peripheral $CD25^+Foxp3^+$ Treg cells was significantly higher ($p < 0.001$) for $STAT4^{-/-}$ ($55.2 \pm 8.5\%$) than $STAT6^{-/-}$ $CD4^+6.5^+$ T cells ($22 \pm 4\%$) (Fig. 3, *b* and *c*) repeating the results obtained when each population developed alone (Fig. 2). Thus, the differential expression of CD25 and Foxp3 by STAT4- and STAT6-deficient $CD4^+$ T cells is maintained when the two populations codevelop in the same HA^+ hosts. These observations indicate that the differences of Foxp3 and CD25 expression between $STAT4^{-/-}$ and

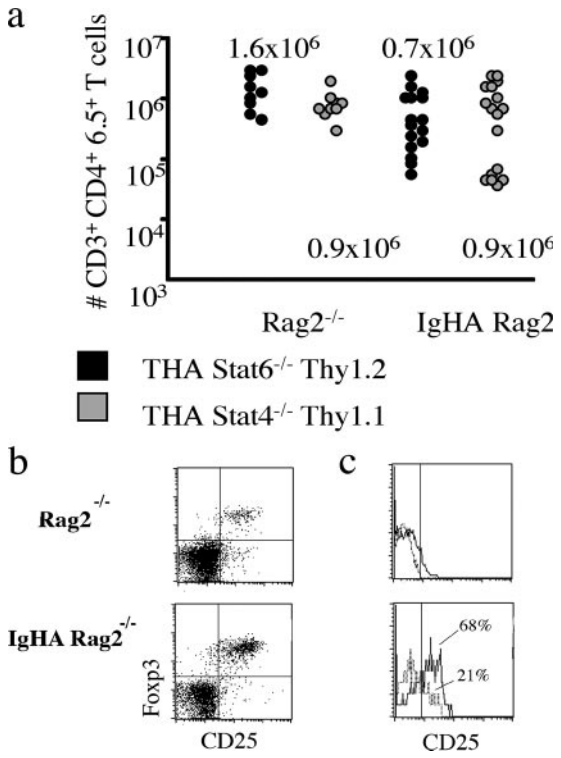

FIGURE 3. The differential expression of CD25 and Foxp3 by STAT4- and STAT6-deficient $CD4^+$ T cells is kept when the two populations co-develop in the same hosts. $Rag2^{-/-}$ or Ig-HA $Rag2^{-/-}$ hosts were lethally irradiated and reconstituted with a 1:1 mix of T cell-depleted BM from $STAT6^{-/-}$ Thy1.2 and $STAT4^{-/-}$ Thy1.1 donors. Ten weeks after transfer, mice were killed and analyzed. *a*, The number of $CD3^+CD4^+6.5^+$ $STAT6^{-/-}$ (filled symbols) or $STAT4^{-/-}$ (gray-filled symbols) cells in the peripheral pool is shown in hosts lacking (*left*) or expressing (*right*) HA. Data pooled are from at least three independent experiments each with $n =$ 3–5 mice per group. The mean number of cells is given for each STAT-deficient T cell population. *b*, The expression of CD25 and Foxp3 by gated $CD3^+CD4^+6.5^+$ spleen T cells in the chimeras lacking (*top*) or expressing (*bottom*) HA. Note the coexpression of the two molecules. *c*, The expression of CD25 by the $CD3^+CD4^+6.5^+$ $STAT6^{-/-}$ Thy 1.2 (dotted line histogram) or $STAT4^{-/-}$ Thy 1.1 (full line histogram) spleen T cells in one of the mixed chimeras studied. The percentage of $CD25^+$ cells for each population is shown. $Rag2^{-/-}$ (*top*) and Ig-HA $Rag2^{-/-}$ (*bottom*) hosts are shown. Similar findings were obtained in two independent experiments with $n = 3$–4 mice per group.

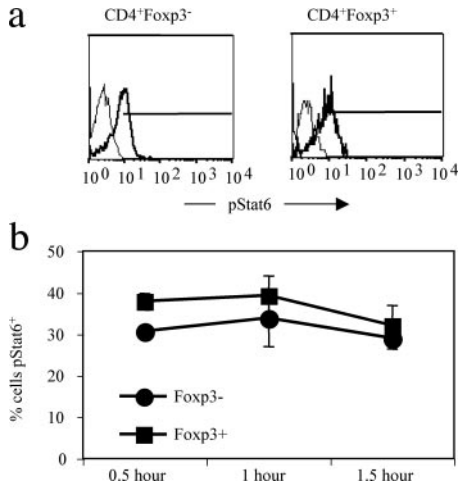

FIGURE 4. STAT6 activation in Treg cells. Spleen cells were stimulated with ± 10 ng/ml IL-4 for 30 min, 1 h, or 1.5 h. At each time point, cells were collected and stained for pStat6 Alexa Fluor 647, CD4-PE Cy5, and Foxp3. *a*, Histograms are either gated on $CD4^+Foxp3^-$ or $CD4^+$ $Foxp3^+$ as denoted. Histograms represent the 1-h time point. *b*, Samples were done in duplicate and were pooled samples from five mice. Error bars represent SD and there was no significant difference between $Foxp3^-$ and $Foxp3^+$ $CD4^+$ T cells using Student's paired *t* test.

$STAT6^{-/-}$ $CD4^+$ T cells are the result of an intrinsic developmental program of the deficient cells and are not related to a different cytokine environment produced by the developing STAT-deficient cells or to putative differences in peripheral T cell numbers.

To investigate whether induction of CD25 and Foxp3 expression and Treg differentiation could result from the exposure of mature T cells to Ag at the periphery we compared the fate of mature peripheral STAT4- and STAT6-deficient $CD4^+6.5^+$ T cells after adoptive transfer into $Rag2^{-/-}$ and Ig-HA $Rag2^{-/-}$ hosts. Five weeks after adoptive T cell transfer, we found that upon in vivo Ag stimulation Ag-specific $STAT6^{-/-}$ out-competed $STAT4^{-/-}$ $CD4^+$ T cells (data not shown) as it was the case during LDP of polyclonal T cells (9). More importantly, we found that the fraction of Treg cells recovered after adoptive transfer of WT, $STAT4^{-/-}$, or $STAT6^{-/-}$ TCR-HA $CD4^+$ T cells was <2% and identical in both Ag-free and HA^+ hosts (data not shown) and indistinguishable from that of the original injected $CD4^+$ T cell population (13). This indicates that the exposure of mature peripheral HA-specific T cells to HA did not result in "de novo" generation of $Foxp3^+$ Treg cells, in contrast to what was observed when these cells developed throughout the thymus of the HA BM chimeras (see Fig. 2). Thus, these findings are in agreement with previous reports demonstrating that the agonist-driven commitment of HA-specific cells to the Treg lineage is stringently dependent on the presence of the thymus (11).

It has been shown that Treg cell development requires high-affinity T cell interactions with agonist peptides during T cell development in the thymus (11, 14). We now report that the absence of STAT6 impairs development of Ag-specific transgenic Treg cells occurring during agonist-driven thymus selection. These findings suggest that fully efficient Foxp3 induction and Treg cell development may require two signals: one agonist-driven TCR signal and a second STAT6-mediated signal. To further corroborate the possible role of STAT6 in Treg cells we had to demonstrate the STAT6 is functionally active in Treg cells. We were able to confirm it by showing that IL-4R is expressed (data not shown) and that pStat6 is induced by IL-4 treatment of the Treg cells (Fig. 4). Next, we studied polyclonal populations of $CD4^+$ T cells from STAT6-deficient and transgenic mice expressing a mutant form of STAT6 (Stat6VT) that is constitutively activated (10). In absence of agonist-driven interactions, the absence of STAT6 did not alter Treg cell number as polyclonal $STAT6^{-/-}$ $CD4^+$ T cells contain normal percentages of $Foxp3^+$ Treg cells (data not shown). We also found that STAT6 was not required for regulatory functions because Treg cells from $STAT6^{-/-}$ mice were able to inhibit the in vitro expansion of naive T cells (data not shown). In contrast, in Stat6VT transgenic mice, we found that an increased fraction of the $CD4^+$ T cells expresses Foxp3 (Fig. 5). The relative increase in Foxp3-expressing cells was, however, not evident in the thymus and more marked in the LNs (27.6 ± 3.7% vs 11.3 ± 2.6%; $p <$ 0.001) than in the spleen (15.2 ± 5.9% vs 8.9 ± 1.7%; $p = 0.07$). The absolute numbers of $Foxp3^+$ cells were similar in WT and Stat6VT transgenic mice (10.7 ± 4.2 vs 8.5 ± 7.1 respectively; $p =$ NS). The reduced proliferation capacities shared by Treg cells and anergic cells and their suppressive capacities may explain the lower number of $CD4^+$ T cells present in both STAT4-deficient

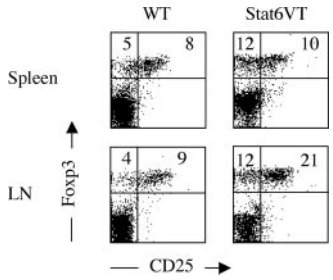

FIGURE 5. Foxp3 expression by the $CD4^+$ T cells from Stat6VT transgenic mice. Expression of CD25 and Foxp3 by gated $CD3^+CD4^+$ spleen (*top*) and LN (*bottom*) T cells from B6 WT littermates (*left*) and Stat6VT (*right*) mice are shown. Similar results were obtained in four to five mice. Foxp3 expression was selective on $CD4^+$ T cells and not observed on other cells.

BM chimeras and Stat6VT Tg mice. These findings suggest that constitutive activation of STAT6 does not compensate for the lack of strong TCR agonist-driven signals. However, in the absence of strong agonist TCR signals, constitutive STAT6 activation seems to favor the survival and enrichment of Treg cells in the peripheral pool. On the whole, these latter findings support the involvement of the STAT6 pathway in Foxp3 induction and Treg cell biology.

Discussion

The processes underlying induction of Foxp3 expression and generation of Treg cells are yet unknown. Maturation of "natural" $CD4^+CD25^+$ Treg cells requires the integrity of the IL-2/IL-2R/STAT5 signaling pathway. Mice genetically deficient in IL-2 (15), IL-2Rα (1), and IL-2Rβ (16) succumb to lymphoproliferation and autoimmune disease around 6–10 wk of age. The lymphoproliferative syndrome of IL-2- and IL-2R-deficient mice is reversed in mixed BM chimeras containing WT T cells and the adoptive transfer of $CD4^+CD25^+$ T cells corrects it, demonstrating that disease is due to the lack of Treg cells, rather than an intrinsic T cell defect (1, 16). Mice lacking isoforms 5A and 5B of STAT5, the IL-2 signaling factor, also develop multiorgan autoimmune disease and have reduced numbers of Treg cells (17–19). In humans STAT5b is also important for the in vivo accumulation of Treg cells (20) and it was recently shown that in vitro IL-2 up-regulates Foxp3 expression through a STAT5-dependent mechanism (21). However, most of the available data suggest that IL-2 signals are not essential for the generation of Treg cells, but rather to their peripheral survival and expansion (13, 22, 23). Indeed, $CD4^+CD25^+$ $Foxp3^+$ cells are strongly reduced, but not totally absent in either IL-2- or STAT5-deficient mice (19, 22). The rare $CD4^+CD25^+$ cells found in the periphery of IL-2-deficient mice are capable of suppressing expansion of naive $CD4^+$ T cells (1) and in absence of IL-2, agonist-driven TCR signals induced Ag-specific Treg cell development (24). Moreover, $CD25^{-/-}$ mice show normal $CD4^+$ $Foxp3^+$ LN cell numbers (Ref. 22 and our unpublished observations), suggesting that IL-2Rα is not required for the induction and development of $Foxp3^+$ cells, but rather to the maturation of Treg lineage cells to full suppressor functions. It is therefore likely that signaling pathways other than IL-2/IL-2R/STAT5 may also play a role in Treg cell lineage development.

The strength of TCR-mediated signal seems to be one of the prerequisites for Treg generation. Development and positive selection of Ag-specific $CD4^+CD25^+Foxp3^+$ Treg cells occurs preferentially upon "high-affinity" interactions between the TCR and MHC-self-peptide complexes in the thymus (11, 14) and requires CD28 costimulation (25). Using a well-established TCR transgenic experimental model (11, 24, 26), we now show that such agonist-driven generation of Ag-specific Treg cells is impaired in the absence of STAT6, directly implicating the STAT6 pathway in the induction of Foxp3 expression. Our results indicate that fully efficient Treg cell development require the synergistic effect of an agonist-driven TCR signal and a second STAT6-mediated signal. This hypothesis is supported by the following observations: 1) STAT6 deficiency reduces considerably the number of monoclonal HA-specific Treg cells in the thymus and periphery of the HA-positive chimeras, indicating that STAT6 is required for fully efficient agonist driven Treg cell development; 2) The presence of Treg cells is, however, not completely abrogated by the absence of STAT6 meaning that STAT6 is not essential for their generation; 3) Polyclonal populations of $STAT6^{-/-}$ mice contain normal percentages of Treg cells (data not shown), suggesting that developing T cells expressing a broad range of TCR affinities can make up for the absence of STAT6 and reconstitute a normal sized Treg cell pool; and 4) In Stat6VT mice the constitutive activation of STAT6 increases the fraction of polyclonal Treg cells, but this relative increase is lower than that observed for TCR-HA WT T cells developing in the HA-positive chimeras and it is only observed at the periphery demonstrating that the constitutive activation of STAT6 alone does not fully compensate for the lack of high-affinity TCR agonist-driven signals. STAT6 has been shown to act upstream of GATA-3, which is involved in "β selection" and is critical for the generation of mature SP $CD4^+$ T cells (27). It was recently shown that the first intron of the *Foxp3* gene contains a highly conserved STAT-binding site (18, 19, 21). Moreover, the thymus is biased toward a pro-Th2 cytokine environment, i.e., IL-4 is present, whereas IL-12 is absent, which favors STAT6 activation (28). We may assume that during T cell development in the thymus, high-affinity TCR ligation (14) and CD28 costimulation (25) preferentially activates STAT6 (29) and that this combination of signals fully induces Foxp3 expression and triggers the generation of Treg lineage cells. However, as the absence of STAT6 does not completely abrogate Treg cell development, it is also possible that STAT6 activation may also act by favoring survival of $Foxp3^+$ cells in the thymus.

The IL-2-STAT5 and IL-4-STAT6 signaling pathways are closely linked. IL-2R signals can induce IL-4 production through either STAT5 (30) or c-Maf (31) activation. Conversely, IL-4 through STAT6 also induces c-Maf, which in turn is able to bind to the CD25 locus (32) and through the cytokine common γ chain may also act on STAT5. The IL-4/STAT6/c-Maf/CD25 and IL-2/CD25/c-Maf/IL-4 pathways may converge to act on $CD25^+$ $Foxp3^+$ Treg cells, but their relative roles remain to be established. It was recently shown that STAT5 is required for Foxp3 induction (18, 19) and Foxp3 may be at the crossroads to Th2 or Th17 differentiation (33, 34). In the thymus, Ag-specific Treg cell development occurs in the absence of IL-2 (24), indicating that the STAT6 pathway may act independently of IL-2 and that TCR and STAT6 signals suffice for efficient Treg cell generation. Whether, in the absence of IL-2, STAT6 acts in association or independently of STAT5 remains to be established. In contrast, at the periphery, survival of functional Treg cells has been shown to be strictly dependent on IL-2 levels (13, 35). Thus, in Stat6VT mice the constitutive STAT6 activation, which relatively enriches the peripheral $CD4^+$ T cell pool with cells of the $Foxp3^+$ Treg lineage, probably favors Treg cell survival by using common downstream elements of the IL-2 pathway. Two independent in vitro observations further support the role of STAT6 in Treg cell survival. First, it was shown that IL-4, whereas sustaining STAT6 activation (36) prevents apoptosis of $CD25^+$ Treg cells and Foxp3 mRNA decline (37). Second, rapamycin, an inhibitor of Jak2 (38) kinase that associates with the IL-12Rβ2 chain to phosphorylate STAT4, favors

expansion of $CD4^+$ Treg cells (39), mimicking our observations in STAT4-deficient cells. We may assume that at the periphery, STAT6 does not replace IL-2 signals, but rather acts as an additional cofactor enhancing Treg cell survival or expansion. These effects may vary according to the tissue and in the course of immune responses by integrating different environmental factors that modulate gene effects affecting T cell differentiation. It would be of interest to investigate whether the constitutive activation of STAT6 could ensure Treg cell survival in IL-2- or STAT5-deficient mice.

Our current observations may shed new light on the possible role of the STAT4/6 pathway in Treg cell intervention and the pathogenesis of autoimmune diseases. The results reported are, however, not consensual and STAT6 has been show to improve, to exacerbate, or to have no effect. STAT6 deficiency did not influence development of autoimmune diabetes in a streptozotocin model of diabetes, (40) and did not increase severity of colitis in $TCR\alpha^{-/-}$ mice (41). At the same time, IL-4 has been reported to be necessary for the induction of regulatory cells that prevent diabetes (42) and $STAT4^{-/-}$ NOD mice were resistant to induction of diabetes and experimental allergic encephalomyelitis (43, 44). $STAT6^{-/-}$ mice were more susceptible to myasthenia gravis (45). In other Th1 models of autoimmunity STAT6 was also shown to exert a protective effect (46). According to our present findings these effects might result from the STAT6 effects increasing Treg cell generation. In contrast, STAT6 deficiency or anti-IL-4 treatment ameliorates kidney disease in lupus nephritis (47), reduces incidence of kidney disease with a dramatic increase in survival in New Zealand mice (48). These findings suggest that the role of STAT6 might vary according to the pathogenesis of disease as it affects both Treg cells, inflammation, and Th2 responses required for autoantibody production. The role of Foxp3 in Th2 or Th17 differentiation further increases the heterogeneity of responses (33).

In summary, our findings demonstrate the involvement of STAT6 in the development and survival of Treg cells. The absence of STAT6 impaired the generation of Ag-specific $CD4^+CD25^+$ $Foxp3^+$ cells in the thymus and the constitutive expression of a transcriptionally active form of STAT6 promoted an increase in the fraction of cells expressing Foxp3, a key marker of the Treg cell lineage in $CD4^+$ T cells. Therefore STAT6 might be a key target molecule in the control of inflammatory autoimmune diseases by inducing either Th2 T cell differentiation with the production of anti-inflammatory cytokines or Foxp3 expression and Treg cell generation.

Acknowledgments

We thank Drs. H. von Boehmer and A. Sarukhan for the gift of Ig-HA and TCR-HA transgenic mice, A. Cumano for help, M.-P. Lembezat for technical assistance, and A. Louise for cell sorting.

Disclosures

The authors have no financial conflict of interest.

References

1. Almeida, A. R., N. Legrand, M. Papiernik, and A. A. Freitas. 2002. Homeostasis of peripheral $CD4^+$ T cells: IL-2Rα and IL-2 shape a population of regulatory cells that controls $CD4^+$ T cell numbers. *J. Immunol.* 169: 4850–4860.
2. Sakaguchi, S. 2005. Naturally arising Foxp3-expressing $CD25^+CD4^+$ regulatory T cells in immunological tolerance to self and non-self. *Nat. Immunol.* 6: 345–352.
3. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. *Nat. Immunol.* 4: 330–336.
4. Khattri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in $CD4^+CD25^+$ T regulatory cells. *Nat. Immunol.* 4: 337–342.
5. Kaplan, M., Y. Sun, T. Hoey, and M. Grusby. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 382: 174–177.
6. Takeda, K., T. Tanaka, W. Shi, M. Matsumoto, M. Minami, S. Kashiwamura, K. Nakanishi, N. Yoshida, T. Kishimoto, and S. Akira. 1996. Essential role of Stat6 in IL-4 signalling. *Nature* 380: 627–630.
7. Kaplan, M., U. Schindler, S. Smiley, and M. Grusby. 1996. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 4: 313–319.
8. Kaplan, M., C. Daniel, U. Schindler, and M. Grusby. 1998. Stat proteins control lymphocyte proliferation by regulating $p27^{Kip1}$ expression. *Mol. Cell. Biol.* 18: 1996–2003.
9. Sanchez-Guajardo, V., J. Borghans, M. Marquez, S. Garcia, and A. Freitas. 2005. Different competitive capacities of Stat4- and Stat6-deficient $CD4^+$ T cells during lymphophenia-driven proliferation. *J. Immunol.* 174: 1178–1187.
10. Bruns, H. A., U. Schindler, and M. H. Kaplan. 2003. Expression of a constitutively active Stat6 in vivo alters lymphocyte homeostasis with distinct effects in T and B cells. *J. Immunol.* 170: 3478–3487.
11. Apostolou, I., A. Sarukhan, L. Klein, and H. von Boehmer. 2002. Origin of regulatory T cells with known specificity for antigen. *Nat. Immunol.* 3: 756–763.
12. Lanoue, A., C. Bona, H. von Boehmer, and A. Sarukhan. 1997. Conditions that induce tolerance in mature $CD4^+$ T cells. *J. Exp. Med.* 185: 405–414.
13. Almeida, A. R., B. Zaragoza, and A. A. Freitas. 2006. Indexation as a novel mechanism of lymphocyte homeostasis: the number of $CD4^+CD25^+$ regulatory T cells is indexed to the number of IL-2-producing cells. *J. Immunol.* 177: 192–200.
14. Jordan, M. S., A. Boesteanu, A. J. Reed, A. L. Petrone, A. E. Holenbeck, M. A. Lerman, A. Naji, and A. J. Caton. 2001. Thymic selection of $CD4^+CD25^+$ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* 2: 301–306.
15. Schorle, H., T. Holtschke, T. Hünig, A. Schimpl, and I. Horak. 1991. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature* 352: 621–624.
16. Malek, T. R., A. Yu, V. Vincek, P. Scibelli, and L. Kong. 2002. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rβ-deficient mice: implications for the nonredundant function of IL-2. *Immunity* 17: 167–178.
17. Snow, J., N. Abraham, M. Ma, B. Herndier, A. Pastuszak, and M. Goldsmith. 2003. Loss of tolerance and autoimmunity affecting multiple organs in STAT5A/5B-deficient mice. *J. Immunol.* 171: 5042–5050.
18. Yao, Z., Y. Kanno, M. Kerenyi, G. Stephens, L. Durant, W. T. Watford, A. Laurence, G. W. Robinson, E. M. Shevach, R. Morrigl, et al. 2007. Nonredundant roles for Stat5a/b in directly regulating *Foxp3*. *Blood*. In press.
19. Burchill, M. A., J. Yang, C. Vogtenhuber, B. R. Blazar, and M. A. Farrar. 2007. IL-2 receptor β-dependent STAT5 activation is required for the development of $Foxp3^+$ regulatory T cells. *J. Immunol.* 178: 280–290.
20. Cohen, A. C., K. C. Nadeau, W. Tu, V. Hwa, K. Dionis, L. Bezrodnik, A. Teper, M. Gaillard, J. Heinrich, A. M. Krensky, et al. 2006. Cutting edge: decreased accumulation and regulatory function of $CD4^+CD25^{high}$ T cells in human STAT5b deficiency. *J. Immunol.* 177: 2770–2774.
21. Zorn, E., E. A. Nelson, M. Mohseni, F. Porcheray, H. Kim, D. Litsa, R. Bellucci, E. Raderschall, C. Canning, R. J. Soiffer, et al. 2006. IL-2 regulates FOXP3 expression in human $CD4^+CD25^+$ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. *Blood* 108: 1571–1579.
22. Fontenot, J. D., J. P. Rasmussen, M. A. Gavin, and A. Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* 6: 1142–1151.
23. Setoguchi, R., S. Hori, T. Takahashi, and S. Sakaguchi. 2005. Homeostatic maintenance of natural $Foxp3^+CD25^+CD4^+$ regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J. Exp. Med.* 201: 723–735.
24. D'Cruz, L., and L. Klein. 2005. Development and function of agonist-induced $CD25^+Foxp3^+$ regulatory T cells in the absence of interleukin 2 signaling. *Nat. Immunol.* 6: 1152–1159.
25. Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12: 431–440.
26. Cabarrocas, J., C. Cassan, F. Magnusson, E. Piaggio, L. Mars, J. Derbinski, B. Kyewski, D. A. Gross, B. L. Salomon, K. Khazaie, et al. 2006. $Foxp3^+CD25^+$ regulatory T cells specific for a neo-self-antigen develop at the double-positive thymic stage. *Proc. Natl. Acad. Sci. USA* 103: 8453–8458.
27. Pai, S. Y., M. L. Truitt, C. N. Ting, J. M. Leiden, L. H. Glimcher, and I. C. Ho. 2003. Critical roles for transcription factor GATA-3 in thymocyte development. *Immunity* 19: 863–875.
28. Bendelac, A., and R. H. Schwartz. 1991. $CD4^+$ and $CD8^+$ T cells acquire specific lymphokine secretion potentials during thymic maturation. *Nature* 353: 68–71.
29. Oki, S., N. Otsuki, T. Kohsaka, and M. Azuma. 2000. Stat6 activation and Th2 cell differentiation [correction of proliferation] driven by CD28 [correction of CD28 signals]. *Eur. J. Immunol.* 30: 1416–1424.
30. Zhu, J., J. Cote-Sierra, L. Guo, and W. Paul. 2003. Stat5 activation plays a critical role in Th2 differentiation. *Immunity* 19: 739–748.
31. Hwang, E., I. White, and I. Ho. 2002. An IL-4-independent and CD25-mediated function of c-maf in promoting the production of Th2 cytokines. *Proc. Natl. Acad. Sci. USA* 99: 13026–13030.
32. Kurata, H., H. Lee, A. O'Garra, and N. Arai. 1999. Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. *Immunity* 11: 677–688.

33. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235–238.
34. Gavin, M. A., J. P. Rasmussen, J. D. Fontenot, V. Vasta, V. C. Manganiello, J. A. Beavo, and A. Y. Rudensky. 2007. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* 445: 771–775.
35. Yu, A., and T. Malek. 2006. Selective availability of IL-2 is a major determinant controlling the production of $CD4^+CD25^+Foxp3^+$ T regulatory cells. *J. Immunol.* 177: 5115–5121.
36. Quelle, F. W., K. Shimoda, W. Thierfelder, C. Fischer, A. Kim, S. M. Ruben, J. L. Cleveland, J. H. Pierce, A. D. Keegan, K. Nelms, et al. 1995. Cloning of murine Stat6 and human Stat6, Stat proteins that are tyrosine phosphorylated in responses to IL-4 and IL-3 but are not required for mitogenesis. *Mol. Cell. Biol.* 15: 3336–3343.
37. Maerten, P., C. Shen, D. M. Bullens, G. Van Assche, S. Van Gool, K. Geboes, P. Rutgeerts, and J. L. Ceuppens. 2005. Effects of interleukin 4 on $CD25^+CD4^+$ regulatory T cell function. *J. Autoimmun.* 25: 112–120.
38. Chiang, P., L. Wang, C. Bonham, X. Liang, J. Fung, L. Lu, and S. Qian. 2004. Mechanistic insights into impaired dendritic cell function by rapamycin: inhibition of Jak2/Stat4 signaling pathway. *J. Immunol.* 172: 1355–1363.
39. Battaglia, M., A. Stabilini, and M. G. Roncarolo. 2005. Rapamycin selectively expands $CD4^+CD25^+Foxp3^+$ regulatory T cells. *Blood* 105: 4743–4748.
40. Cetkovic-Cvrlje, M., and F. M. Uckun. 2005. Effect of targeted disruption of signal transducer and activator of transcription (Stat)4 and Stat6 genes on the autoimmune diabetes development induced by multiple low doses of streptozotocin. *Clin. Immunol.* 114: 299–306.
41. Okuda, Y., I. Takahashi, J. K. Kim, N. Ohta, K. Iwatani, H. Iijima, Y. Kai, H. Tamagawa, T. Hiroi, M. N. Kweon, et al. 2003. Development of colitis in signal transducers and activators of transcription 6-deficient T-cell receptor α-deficient mice: a potential role of signal transducers and activators of transcription 6-independent interleukin-4 signaling for the generation of Th2-biased pathological $CD4^+$ $\beta\beta$T cells. *Am. J. Pathol.* 162: 263–271.
42. Homann, D., A. Holz, A. Bot, B. Coon, T. Wolfe, J. Petersen, T. P. Dyrberg, M. J. Grusby, and M. G. von Herrath. 1999. Autoreactive $CD4^+$ T cells protect from autoimmune diabetes via bystander suppression using the IL-4/Stat6 pathway. *Immunity* 11: 463–472.
43. Anderson, M. S., and J. A. Bluestone. 2005. The NOD mouse: a model of immune dysregulation. *Annu. Rev. Immunol.* 23: 447–485.
44. Kaplan, M. H. 2005. STAT4: a critical regulator of inflammation in vivo. *Immunol. Res.* 31: 231–242.
45. Wang, W., N. S. Ostlie, B. M. Conti-Fine, and M. Milani. 2004. The susceptibility to experimental myasthenia gravis of $STAT6^{-/-}$ and $STAT4^{-/-}$ BALB/c mice suggests a pathogenic role of Th1 cells. *J. Immunol.* 172: 97–103.
46. Yukawa, K., M. Kishino, M. Goda, X. M. Liang, A. Kimura, T. Tanaka, T. Bai, K. Owada-Makabe, Y. Tsubota, T. Ueyama, et al. 2005. STAT6 deficiency inhibits tubulointerstitial fibrosis in obstructive nephropathy. *Int. J. Mol. Med.* 15: 225–230.
47. Singh, R. R., V. Saxena, S. Zang, L. Li, F. D. Finkelman, D. P. Witte, and C. O. Jacob. 2003. Differential contribution of IL-4 and STAT6 vs STAT4 to the development of lupus nephritis. *J. Immunol.* 170: 4818–4825.
48. Jacob, C. O., S. Zang, L. Li, V. Ciobanu, F. Quismorio, A. Mizutani, M. Satoh, and M. Koss. 2003. Pivotal role of Stat4 and Stat6 in the pathogenesis of the lupus-like disease in the New Zealand mixed 2328 mice. *J. Immunol.* 171: 1564–1571.