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Role of STAT3 in $CD^4^+CD^25^+FOXP3^+$ Regulatory Lymphocyte Generation: Implications in Graft-versus-Host Disease and Antitumor Immunity

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Immunological tolerance is maintained by specialized subsets of T cells including $CD^4^+CD^25^+FOXP3^+$ regulatory cells (Treg). Previous studies established that Treg thymic differentiation or peripheral conversion depend on CD28 and Lck signaling. Moreover, $fox_p_3$ gene transfer in murine $CD^4^+CD^25^+T$ lymphocytes results in the acquisition of suppressive functions. However, molecular pathways leading to FOXP3 expression remain to be described. In this study, we investigated the molecular events driving FOXP3 expression. We demonstrated that CD28 activation in $CD^4^+CD^25^+T$ lymphocytes leads to STAT3 Tyr$^{705}$ phosphorylation in an Lck-dependent manner. STAT3 neutralization during naive peripheral $CD^4^+CD^25^+T$ cell conversion into Treg through costimulation with TCR/CD28 and TGF-$\beta$1, decreased FOXP3 expression, prevented the acquisition of suppressive functions and restored the ability of the converted lymphocytes to produce IL-2 and IFN-$\gamma$. Furthermore, we observed that STAT3 ablation using small interfering RNA strategies inhibited FOXP3 expression and suppressive functions among naturally differentiated $CD^4^+CD^25^+T$ lymphocytes, suggesting a direct role of STAT3 in Treg phenotype and function maintenance. $CD^4^+CD^25^+T$ lymphocytes transduced with specific STAT3 small interfering RNA were devoid of suppressive functions and failed to control the occurrence of acute graft-vs-host disease. Finally, STAT3 inhibition in $CD^4^+T$ lymphocytes enhanced the anti-tumor immunity conferred by a lymphocyte adoptive transfer. In summary, our findings determine that STAT3 is critical in the molecular pathway required for FOXP3 expression. STAT3 modulation should be taken into account when assessing how regulatory T cells contribute to inflammatory diseases and tumor immunosurveillance. The Journal of Immunology, 2007, 179: 7593–7604.

Self-tolerance is an important feature in the prevention of autoimmune diseases which relies on both central and peripheral mechanisms. Central tolerance refers to the deletion of developing autoreactive T cells that bind with high-affinity to intrathymic self Ags. Despite this stringent selection, autoreactive T cells can evade the thymus. Peripheral regulatory mechanisms are then required for the prevention of autoimmunity (1–3). $CD^4^+CD^25^+$ regulatory T cells (Treg)¹ have been recognized as a major population of suppressing T cells that maintain peripheral immune tolerance (4). Mature Treg can be identified by their constitutive expression of CD25, CTLA-4, and glucocorticoid-induced TNF receptor family related receptor (5–7). In addition, Treg differentiation requires the expression of the Forkhead box P3 ($fox_p_3$) transcription factor which remains the more specific Treg marker today (8–10).

Although naturally occurring Treg lymphocytes develop in the thymus, several reports have demonstrated that peripheral $CD^4^+CD^25^+$ naïve T cells can be converted into adaptive FOXP3$^+$ Treg (11–14). Adaptive Treg can be induced in the periphery throughout the course of immune responses. Several researchers have compared the TCR-$\gamma$-$\delta$ repertoires in $CD^4^+CD^25^+CD45RO^-$ memory T cells and $CD^4^+CD^25^+T$ subsets to determine what extent both populations express T cell receptors that belong to the same family. These studies highlighted that these two populations are related (15–16). This raises the question of whether Treg might derive from memory $CD^4^+T$ cells. Then, the crucial question to be addressed is what molecular signaling leads to Foxp3 expression and confers regulatory functions to $CD^4^+CD^25^+T$ cells. Walker et al. (17) have shown that TCR and CD28 stimulation of human $CD^4^+CD^25^+T$ cells induces Foxp3 transcription and acquisition of regulatory activity in vitro. Moreover, mice deficient in either CD28 or B7 have a reduced number of Treg in both the thymus and periphery (18–19). Recently, Tai X et al. (20) have provided evidence in a rodent model that the Lck binding activity of CD28 is required for Foxp3 transcription and Treg cell differentiation. However, the precise

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3 Abbreviations used in this paper: Treg, regulatory T cell; GVHD, graft-vs-host disease; aGVHD, acute aGVHD; Foxp3, forkhead box P3; siRNA, small interfering RNA; ALK, anaplastic lymphoma kinase; qRT-PCR, quantitative real-time PCR.

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molecular pathway driven by CD28 costimulation and leading to FOXP3 expression has yet to be described.

Treg have been shown to exert a regulatory function in various disease states in mice and humans. Particularly, Treg mediate immune suppression to prevent autoimmunity, to control inflammation (1–4) and graft-vs-host diseases (GVHD) (21–23). Moreover, increasing evidence indicates that Treg induce tumor specific immune tolerance and Treg expansion has been observed during cancer progression in rodent models and humans (24–30).

Interestingly, constitutive STAT3 has emerged as one of the major molecule involved in the mechanisms governing cancer immune tolerance and inflammatory diseases. STATs are latent cytoplasmic transcription factors activated by the phosphorylation of a conserved tyrosine residue that play a critical role in cytokine and growth factor signaling pathways. Upon ligand stimulation, STAT3 is phosphorylated on Tyr705, dimerizes and translocates to the nucleus to transactivate target genes. STAT3 activation is frequently observed in cancer samples (31–34). Wang et al. (35) showed that activated STAT3 has a potent tolerogenic effect on tumor-infiltrating DC enabling cancer cells to escape from immune system surveillance. Kortylewski et al. (36) demonstrated that STAT3 inhibition in hemopoietic cells elicits an immune response against established tumors. Immune cells from STAT3-deficient mice mounted stronger immune responses against tumor Ag. A direct role for STAT3 in mediating the generation of suppressive functions in CD4+ T cells was raised by a study conducted in mice lacking SOCS3, which is an inhibitor of STAT3. Loss of SOCS3 resulted in reduced immune responses and hyperproduction of cytokines. However, constitutive STAT3 expression is necessary for STAT3 dimerization and DNA binding activity was purchased from Calbiochem. PpYLYKTK-mts were purchased from Calbiochem. STA-21, a selective inhibitor of STAT3 dimerization and transcriptional activity, was previously described (37). These observations prompted us to investigate the precise role of STAT3 in the molecular signaling governing Treg generation and functions.

In this study, we demonstrate that STAT3 phosphorylation is a critical step for FOXP3 induction in CD4+CD25+ T lymphocytes. Blocking STAT3 phosphorylation avoids foxp3 transcription upon CD28 and CD3 stimulation. We show that interfering with STAT3 signaling in vitro inhibits both adaptive Treg conversion and suppressive functions of naturally Treg. In vivo, STAT3 knockdown decreases Treg generation. Furthermore, STAT3 knockdown in CD4+ T lymphocytes promotes the initiation of severe GVHD and enhances anti-tumor immune responses. Therefore, it appears that STAT3 signaling plays a major role in CD4+ T cell-mediated immune tolerance.

**Materials and Methods**

**Mice**

CD45.1+ C57BL/6 mice were obtained from Centre de Développement des Technologies Avancées Centre National de la Recherche Scientifique. CD45.2+ C57BL/6 or BALB/c mice were purchased from Janvier. Male mice were used for our animal experiments at six to nine weeks of age. All mice were housed in autoclaved microisolator environments, and all manipulations were performed in a laminar flow hood. Animals were maintained in our animal facility according to the French National Ethics Committee Guidelines. Abs and reagents

Recombinant human IL-2 was obtained from Chiron. The following Abs were used: anti-human CD3 (B-B11), CD25 (B-B10), CD4 (B-F5) (Diaclon); anti-mouse CD4 (RM4–5), CD25 (3C7), CD3 (14–2) (C11), CD62L (MEL–14), CTLA-4 (UC1–4F10–11), Lag3 (C9B7W), CD45.2 (104), anti-H2-Kb (AF6–88.5), anti-H2-Kb (5F1–1,1) (BD Biosciences); anti-mouse FOXP3 (FJK–161) (eBioscience). Anti-p-tyr-STAT3 (pY705) (3E2) and anti-STAT3 (7D9) were obtained from Cell Signaling Technology. For Western Blot studies, we used anti-mouse peroxidase conjugated and Goat anti-rabbit peroxidase conjugated secondary Abs (Jackson Immunoresearch Laboratories). The selective inhibitor of Src family tyrosine kinase P2 was used at 10 nM to assess Src kinases influence on STAT3 phosphorylation in CD28 costimulated lymphocytes (Calbiochem).

Several approaches were used to directly block STAT3 activation. AG490 (Tyrosophin B42) and the STAT3 cell-permeable inhibitor peptide PyVLKKT-mts were purchased from Calbiochem. STA-21, a selective inhibitor of STAT3 dimerization and DNA binding activity was purchased from BIOMOL.

**Purification of CD4+CD25+ T cells**

CD4+CD25+ T lymphocytes were purified from human peripheral blood lymphocytes or from C57BL/6 splenocytes using a negative magnetic selection (Treg kits were purchased from Miltenyi Biotec) according to manufacturer’s recommendations. For Foxp3+ T cell purity ranged from 96 to 99%.

**Flow cytometry**

We prepared single-cell suspensions by mechanic dispersion of spleens or thymus in rodent experiments. Cells were washed and suspended in PBS plus 2% FCS. In mouse experiments, cells were incubated with anti-FcγRII/III-specific Ab (2.4G2; BD Pharningen) to block nonspecific binding. Cells were then stained with the appropriate combination of fluorescein-chromophore-coupled Abs and analyzed on a CyAn LX flow cytometer (DakoCytomation,) using Summit (DakoCytomation) software.

**Real-time quantitative PCR for Foxp3 or TGF-β1 mRNA**

Total RNA were extracted using Kit RNeasy mini kit (Qiagen) and reverse transcribed using random hexamers and M-MLV reverse transcriptase (In-vitrogen Life Technologies). Duplicate samples were subjected to quantitative real-time PCR (QRT-PCR). Murine Foxp3 or mRNA were quantified as previously described (20). Data were expressed as normalized Foxp3 mRNA expression, which was obtained by dividing the relative amount of Foxp3 mRNA for each sample by the relative amount of GAPDH mRNA of the same sample. Primer pairs and fluorescent probes murine TGF-β1 were as follow (sense, anti-sense, and probe, respectively): 5’ACCACT GCCAACATCTGCATG3’, 5’CGGTTGTGTTGTTGTAAG3’, and 5’-FAM-CCGTGGAATTACACGGGATCAGCC-TAMRA-3’. These primer pairs and fluorescent probes murine TGF-β1 were as follow (sense, anti-sense, and probe, respectively): 5’ACCACT GCCAACATCTGCATG3’, 5’CGGTTGTGTTGTTGTAAG3’, and 5’-FAM-CCGTGGAATTACACGGGATCAGCC-TAMRA-3’. Small interfering RNA (siRNA) synthesis, transfection and plasmid constructs

The siRNA was designed to target the sequences 5’-GAGTCACATGC ACAGTGG-3’ of mouse STAT3. The sense (5’-AAAGGAGGACTCAC AGTGGTG-3’) and anti-sense (5’-AAACCAACTGGTGACCATG TGCACC-3’) sequences were annealed and cloned into the BbsI site of the 3’ LTR of pFIV-H1/6 vector according to manufacturer’s instructions (System Biosciences). Scrambled siRNA were included as control (5’- AAAGGAGGAGCAGTGGTGCG-3’ and 5’-AAACCAACTGGTGACCATG CTGCACC-3’). Recombinant viruses were produced by transient transfection into 293T cells by pFIV-Pack Lentiviral packaging kit (System Biosciences). Infectious lentiviruses were collected 36–48 h. Bone marrow cells from C57BL/6 mice were depleted in CD4+ T cell lymphocytes (MACS selection; Miltenyi Biotec) and infected with lentiviruses containing STAT3 siRNA or a control siRNA. After 24 h of culture, the cells were washed with a complete medium and infected in lethally irradiated C57BL/6 mice (2x10^6 bone marrow cells per mice). Because GFP is encoded in the lentivirus transduction vector, GFP was used as a marker to track siRNA expressing lymphocytes upon hemopoietic reconstitution. After infection with lentiviruses, 37 ± 4% of total bone marrow cells and 70 ± 9% of CD34+ hemopoietic progenitors were GFP+. After hemopoietic reconstitution, 71 ± 14% of differentiated CD4+ T cells expressed GFP. For Treg knockdown experiments, CD4+CD25+ T lymphocytes were purified from C57BL/6 mice cultured in RPMI 1640 medium supplemented with 10% FBS at 37°C. 5% CO2. They were stimulated for 3 days with recombinant human IL-2 (100 IU/ml) and anti-CD3/CD28 T cell expander beads (bead-to-cell ratio of 1:1; Dynal Biotech). Transduction of T cells were performed on 48-well plates with the addition of 5 μg/ml Polybrene (Sigma-Aldrich). The cells were exposed to lentiviral concentrations at a concentration of 5,10^5 cells/ml. After 8 h of transduction, the cells were washed with PBS and further incubated in fresh tissue culture medium and 20 IU IL-2/ml for at least two more days. A total of 60–68% of Treg cells were GFP-positive at the end of the culture.

Murine STAT3C in pBABE vectors were provided by Dr. J. Bromberg and were previously described (38).
Mixed allogeneic lymphocyte reactions

CD4+CD25+ T lymphocytes from C57BL/6 (H-2b) mice were used as responder cells. A total of 10^5 responder CD4+CD25+ T lymphocytes were cultured with 10^5 APC cells from BALB/c (H-2d) mice during 5 days. In some conditions, 5.10^5 CD4+CD25+ T lymphocytes from control or STAT3-deficient mice were added to the culture. Cultures were incubated in 96-well round-bottom plates (BD Biosciences) in total volume of 200 μl. Cells were cultured in RPMI1640 (Invitrogen Life Technologies) supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin (all Invitrogen Life Technologies), and 10% heat inactivated endotoxin free FCS (PAN Biotech GmbH). Proliferation was assessed in vitro using a BrdU proliferation kit (Delta Proliferation kit; PerkinElmer®). Incorporated BrdU is detected using europium-labeled Ab. The fluorescence measured with Delfia technology is proportional to the DNA synthesis.

Cytokine detection and quantification (human and mouse IFN-γ)

After lymphocyte activation, supernatants were harvested, stored at −80°C and assayed either directly or after 2–10 times dilution using commercial ELISA kits (IFN-γ Eilpair kit; Diaclone). The sensitivity of the mouse IFN-γ kit was >9.4 pg/ml (Mouse IFN-γ Quantikine Six Pack; R&D Systems).

In vivo conversion of peripheral CD4+CD25− T cells to CD4+CD25+ Foxp3+ T cells experiments.

Five million freshly isolated CD4+CD25 Foxp3− T cells from CD45.2+ C57BL/6 mice were sorted and infused i.v. into lethally irradiated C57BL/6 mice. Spleenocytes were harvested 4–6 wk after adoptive transfer. The CD45.2 isotype was used to track cells in syngeneic recipients. Converted Treg were assessed at single cell level using FACS analysis. Percentages of CD45.2+Foxp3+ T cells were reported among CD45.2+ splenocytes.

Bone marrow transplantation experiments

We used a full MHC-mismatched bone marrow transplantation model in the C57BL/6 (H-2b) strain combination. BALB/c mice were used for engraftment assessment using specific anti-H-2 Abs (BD Biosciences) and flow cytometry analysis as previously described (40).

Tumor model

T cell leukemia/lymphoma cell line EL4 was purchased from the American Type Culture Collection and were cultured in RPMI1640 (Invitrogen Life Technologies) supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin (all Invitrogen Life Technologies), and 10% heat inactivated endotoxin free FCS (PAN Biotech GmbH). A new vial of working bank of EL4 cells were thawed for the development of clinical signs of GvHD according to the scoring system initially proposed by Ferrara’s group (39). Fresh blood cells were used for engraftment assessment using specific anti-H-2 Abs (BD Biosciences) and flow cytometry analysis as previously described (40).

Statistical analysis

Results are expressed as the mean ± SEM. Group comparisons were performed using Student’s t test. Differences were considered significant at p < 0.05.

Results

CD28 costimulation induces STAT3 phosphorylation in CD4+CD25− T lymphocytes

The role of STAT3 in inflammatory cytokine production and gut-associated autoimmune syndrome prompted us to identify the precise role of STAT3 in CD28-mediated FOXP3 expression (41–43). Because foxp3 transcription in CD4+CD25− T cells is mediated by CD28 triggering, we decided to assess whether CD28 costimulation leads to STAT3 activation in CD4+CD25− T cells (17). It was previously demonstrated in a rodent model, that CD28-mediated Lck tyrosine kinase activation is mandatory for FOXP3 induction in developing thymocytes (20). Src family tyrosine kinase Lck is essential for TCR signaling. CD4 recruits Lck to the T cell-APC interface, whereas CD28 sustains Lck activation (44). According to these observations, we investigated the ability of CD28 and Lck to activate STAT3. First, human CD4+CD25+ T cells were stimulated with anti-CD3/CD28 microbeads for 2–4 h before determining the phosphorylation status of STAT3 on Tyr705 in CD4+CD25− T cells, by Western blotting analysis. In some conditions, the pharmacological Src family kinase inhibitor PP2 was used to block Lck activity (45). CD4+CD25− T cells were incubated with 10 nM of PP2 before anti-CD3/CD28 (Fig. 1A) stimulation. AG490, known to inhibit the constitutive activation of STAT3 (46), was used as a control for STAT3 phosphorylation inhibition. As shown in Fig. 1, STAT3 was constitutively expressed in human CD4+CD25− T cells. Resting CD4+CD25− T lymphocytes did not exhibit STAT3 phosphorylation on Tyr705. In contrast, CD28 signaling in human CD4+CD25− T lymphocytes was associated with STAT3 phosphorylation (Fig. 1A). Pharmacological inhibition of the Src family kinase prevented the CD28-induced STAT3 phosphorylation in CD4+CD25− T cells (Fig. 1A). These results showed that CD28 signaling in human CD4+CD25− T cells leads to STAT3 phosphorylation. Then, we reproduced these experiments in murine CD4+CD25− T cells. In rodents, conversion of naive peripheral CD4+CD25− T cells into suppressor CD4+CD25+ FOXP3+ can be achieved through costimulation with TCR/CD28 and TGF-β1 (13). Purified CD4+CD25− T cells from spleens of normal C57BL/6 mice were cultured with anti-CD3 and anti-CD28 microbeads plus TGF-β1 in the presence or in the absence of PP2 (10 nM), during 2–4 h. STAT3 was phosphorylated in response to CD3/CD28 activation of CD4+CD25− T cells. PP2 treatment decreased murine STAT3 phosphorylation in this setting confirming a potential interaction between Src family tyrosine kinase Lck activation and STAT3 phosphorylation in the signaling pathway driven by CD28 in CD4+CD25− T lymphocytes (Fig. 1B).

To confirm a specific role of STAT3 in the molecular signaling initiated by CD28, several strategies were used to interrupt STAT3 activation. In one approach, CD4+CD25− T cells were exposed to the protein kinase inhibitor AG490. In another approach, a peptide based STAT3 inhibitor was used to target the STAT3 SH2 domain to block STAT3 functions in vitro. We used a STAT3-derived phospho-hexapeptide corresponding to the region encompassing the Tyr705 residue interacting with the SH2 domain of another STAT3 monomer (47, 48). Similarly, the small molecule (STA-21) that binds to the STAT3 SH2 domain has been used to directly block STAT3 dimerization and activity (49). STAT3 promotes TGF-β1, IL-10, and IL-4 transcription in CD4+ T cells (37, 50). Moreover, STAT3 can bind directly to the promoter of the genes encoding TGF-β1 as well as IL-10 (37). To substantiate a cell autonomous role of STAT3 in mediating CD28 signaling, we assessed the transcription of these cytokines during in vitro Treg conversion of CD4+CD25− T cells. Costimulation with TCR/CD28 and TGF-β1 of rodent CD4+CD25− T cells increased IL-4, IL-10, and TGF-β1 transcription. The transcription of these cytokines was decreased by pharmacological strategies interfering with Src kinase family or STAT3 (Fig. 1C). Overall, these experiments show a biological activity of STAT3 during activation of naive peripheral CD4+CD25− T cells through costimulation with TCR/CD28 and TGF-β1.
protein expression in CD4+ T lymphocytes was performed. AG490 was used as a control of STAT3 phosphorylation inhibition. One representative of three experiments is depicted. Similar experiments were performed using murine CD4+ T lymphocytes exposed to 10 nM PP2 or DMSO and activated using anti-CD3/CD28 microbeads for 2 to 4 h (A). Equivalent amounts of protein from cell lysates were separated on SDS-PAGE. Western blotting analysis with specific anti-STAT3 or anti-phospho-STAT3 antibodies was performed. AG490 was used as a control of STAT3 phosphorylation inhibition. One representative of three experiments is depicted. Similar experiments were performed using murine CD4+ T lymphocytes exposed to 10 nM PP2 or DMSO and activated using anti-CD3/CD28 microbeads plus TGF-β1 (B). IL-4, IL-10, and Foxp3 mRNA transcription levels in murine CD4+ T lymphocytes activated with anti-CD3/CD28 and TGF-β1 were determined by QRT-PCR. A representative experiment of five is shown (C). *p < 0.05 compared with CD4+ CD25+ T lymphocytes activated with anti-CD3/CD28 and TGF-β1.

**FIGURE 1.** The Src tyrosine kinase family inhibitor PP2 inhibits STAT3 phosphorylation induced by CD28 costimulation. Five millions of human CD4+CD25+ T lymphocytes per condition were exposed to 10 nM PP2 or DMSO and activated using anti-CD3/CD28 microbeads for 2 to 4 h (A). Equivalent amounts of protein from cell lysates were separated on SDS-PAGE. Western blotting analysis with specific anti-STAT3 or anti-phospho-STAT3 antibodies was performed. AG490 was used as a control of STAT3 phosphorylation inhibition. One representative of three experiments is depicted. Similar experiments were performed using murine CD4+ T lymphocytes exposed to 10 nM PP2 or DMSO and activated using anti-CD3/CD28 microbeads plus TGF-β1 (B). IL-4, IL-10, and Foxp3 mRNA transcription levels in murine CD4+ T lymphocytes activated with anti-CD3/CD28 and TGF-β1 were determined by QRT-PCR. A representative experiment of five is shown (C). *p < 0.05 compared with CD4+ CD25+ T lymphocytes activated with anti-CD3/CD28 and TGF-β1.

STAT3 activation is critical for FOXP3 expression and Treg conversion

Lck-binding activity of CD28 was determined as an early step of the molecular signaling events leading to foxp3 transcription and Treg cell differentiation (20). Because our previous experiments suggested a direct interaction between CD28 and STAT3 phosphorylation, we further examined whether STAT3 is involved in FOXP3 expression and Treg functions. CD4+CD25− T cells (2 × 10^6) purified from C57BL/6 splenocytes, were treated with AG490, STA-21, or with a STAT3-derived phospho-hexapeptide corresponding to the region encompassing the Tyr705 residue. Afterward, these lymphocytes were exposed to anti-CD3/CD28 microbeads and TGF-β1 (1 ng/ml) for 72 h. Absolute number of surviving cells was determined by trypan blue exclusion assay. 3.4 ± 0.7 × 10^6, 1.9 ± 0.2 × 10^6, 2.28 ± 0.3 × 10^6 viable lymphocytes were recovered from CD4+CD25− T cells initially stimulated with CD3/CD28, CD3/CD28 and TGF-β1, CD3/CD28, TGF-β1, and STAT3 inhibitor. Both absolute number and viability of the recovered-cells were not influenced by STAT3 inhibitory peptide treatment (data not shown). As previously described in these conditions, CD28 costimulation induced FOXP3 (Fig. 2A, left panel) and CD62L proteins (Fig. 2A, right panel) in CD4+CD25− T cells (13). We observed a decreased FOXP3 protein expression in CD4+CD25− T lymphocytes treated with a STAT3-derived phospho-hexapeptide and activated by CD3/CD28. STAT3 blockade decreased CD62L expression while it did not affect the expression of the activation marker CD25 (data not shown). FOXP3 expression according to STAT3 modulation was also tested in RT-PCR assays. STAT3 inhibition decreased foxp3 transcription driven by CD3/CD28 activation (Fig. 2B). Similar results were obtained using AG490 or STA-21 to prevent STAT3 phosphorylation in CD4+ T lymphocytes (data not shown). Next, we defined the functional characteristics of CD4+CD25− T cells recovered from CD4+CD25− T cells initially stimulated with CD3/CD28 beads, TGF-β1 with or without STAT3 inhibitors. STAT3 neutralization during naive peripheral CD4+CD25− T cell conversion into Treg through costimulation with TCR/CD28 and TGF-β1, restore the ability of the converted lymphocytes to produce IL-2 and IFN-γ (Fig. 2C). Of note, STAT3 inhibition decreased IL-10 (Fig. 2C) and TGF-β1 (Fig. 1C) synthesis following TCR/CD28 and TGF-β1 costimulation.

Previous experiments were reproduced and CD4+CD25− T cells recovered three days following initial in vitro stimulation were cell-sorted and analyzed for their ability to suppress conventional lymphocyte proliferation. Bone marrow-derived dendritic cells propagated from BALB/c mice and activated by LPS were used as APC. C57BL/6 lymphocytes were used as responding cells. Naturally CD4+CD25− Treg freshly purified from C57BL/6 splenocytes were used as control. As expected, converted CD4+CD25− T cells derived from CD4+CD25− T cells initially stimulated with CD3/CD28 beads and TGF-β1 inhibited the proliferation of conventional T lymphocytes (Fig. 2D). Initial treatment with STAT3 inhibitory peptide (Fig. 2D) or AG490 (data not shown) abolished the suppressive functions of the converted CD4+CD25− T cells in mixed lymphocyte reactions. The precise role of STAT3 in FOXP3 expression was further substantiated using a constitutive active STAT3 protein (STAT3-C). CD4+ T cells were stimulated in vitro with 2.5 μg/ml ConA plus 20 IU/ml IL-2 for 2 days before transduction. STAT3-C or control pBABE were introduced into ConA activated CD4+ T cells. After infection, cells were cultured for 1 day in 20 IU IL-2, followed by two more days with puromycin (1 μg/ml) and used in functional experiments. To directly test whether STAT3 activity would induce FOXP3 expression, STAT3-C or control pBABE containing lymphocytes were activated with anti-CD3 ± TGF-β1 during 72 h and
FIGURE 2. STAT3 inhibition decreases Foxp3 expression and suppressive functions in CD28-converted Treg. CD4⁺CD25⁻ T lymphocytes (2 × 10⁶) were treated with AG490 (1 μM) or with a STAT3-derived phospho-hexapeptide corresponding to the region encompassing the Tyr⁷₀₅ residue interacting with the SH2 domain and neutralizing STAT3 dimerization. Afterward, these lymphocytes were exposed to anti-CD3/CD28 microbeads and TGF-β1 (1 ng/ml) for 72 h. At day 3, Foxp3 expression was determined by FACS analysis (A) or QRT-PCR (B). Flow cytometry detection of human Foxp3 and CD62L is shown in gated CD4⁺T lymphocytes (A). Foxp3 mRNA levels of Treg or converted CD4⁺CD25⁻ T lymphocytes were reported to CD4⁺CD25⁺ Foxp3 mRNA level (B). IL-10, IL-2, and IFN-γ production of CD4⁺ T cells activated with anti-CD3/CD28 microbeads and TGF-β1 (1 ng/ml), was assessed by ELISA in culture supernatants. Several STAT3 neutralization strategies were used to study STAT3 influence on cytokine production. One representative experiment of three is shown (C). Next, converted-CD4⁺CD25⁻ T lymphocytes were magnetically cell-sorted and analyzed for their ability to suppress conventional lymphocyte proliferation. Bone marrow-derived dendritic cells propagated from BALB/c mice and activated by LPS were used as APC. C57BL/6 lymphocytes were used as responding cells. Naturally CD4⁺CD25⁺ Treg freshly purified from C57BL/6 splenocytes were used as control. A total of 10⁵ responder lymphocytes were cultured with 10⁴ allogenic APC during 5 days. In some conditions, 5 × 10⁴ converted-CD4⁺CD25⁻ T lymphocytes were added to the culture (D). Proliferation was determined in BrdU incorporation assays. One representative experiment of three is depicted. Data are means ± SD from triplicate assays. * p < 0.05 compared with CD4⁺CD25⁻ T lymphocytes activated with anti-CD3/CD28 and TGF-β1. In the next set of experiments, STAT3-C or control pBABE (p-B) were introduced into ConA activated CD4⁺CD25⁻ T lymphocytes. Then, these lymphocytes were activated during 72 h by IL-2, anti-CD3 ± TGF-β1 (10 ng/ml). Foxp3 expression was assessed by flow cytometry. One representative experiment of three is shown (E).
FOXP3 expression assessed by flow cytometry. STAT3-C promoted FOXP3 expression in CD3 ± TGF-β1-activated T cells, whereas FOXP3 was not detected in ctrl-pBABEL T cells exposed to the same stimulation (Fig. 2E). Thus, in the absence of CD28 signaling, the single activation of STAT3 allows FOXP3 expression in CD4⁺ T cells activated by CD3 ± TGF-β1. Therefore, along with previous experiments, these findings suggest that STAT3 activation is a molecular event required upon CD28 co-stimulation to promote foxp3 transcription, and Treg suppressive function acquisition.

**STAT3 contributes to the maintenance of natural Treg phenotype and functions**

To better extend these results, a next set of experiments was designed to assess the role of STAT3 in naturally differentiated Treg. Our preliminary experiments showed that STAT3 is constitutively phosphorylated on Tyr705 in Treg (data not shown). Thus, we decided to inhibit STAT3 expression in naturally differentiated Treg. CD4⁺CD25⁺ T lymphocytes were purified from C57BL/6 splenocytes and treated with a lentivirus encoding a specifically designed siRNA for STAT3. A non specific siRNA was used as a control. These cells were then washed and maintained 48 h in culture with 100 UI interleukine 2 (1 × 10⁶ cells/condition). Then these lymphocytes were analyzed for their Treg phenotype and their ability to suppress conventional lymphocyte proliferation. Because GFP is encoded in the lentivirus transduction vector, GFP was used as a marker to track siRNA expressing lymphocytes and characterize the influence of STAT3 neutralization on Treg molecules. We first observed that STAT3 inhibition did not influence the absolute number of cells recovered in these experiments. A total of 0.62 ± 0.15 × 10⁶ and 0.65 ± 0.2 × 10⁶ Treg exposed to control or STAT3 siRNA were recovered, compared with 0.72 ± 0.22 × 10⁶ of nontransfected Treg, suggesting that STAT3 knockdown did not influence Treg viability. GFP⁺ lymphocytes displayed a decreased Foxp3, CD62L, and LAG3 expression (Fig. 3A), suggesting an interaction between STAT 3 and Treg related-molecule expression. In contrast, CD25 and CD45RA expressions were not modified by STAT3 neutralization (Fig. 3A). Then, these CD4⁺CD25⁺ T lymphocytes were assessed for their ability to suppress allophenic T cell proliferation as described above. STAT3 inhibition abrogated Treg suppressive functions (Fig. 3B). These results suggest that STAT3 signaling is involved in Foxp3 expression and Treg suppressive function maintenance and prompted us to assess the precise role of STAT3 on established in vivo models, in which Treg control inflammatory disease onset.

**STAT3 is involved in Treg differentiation in vivo**

In the first step, we decided to analyze the influence of STAT3 on in vivo Treg generation and functions. We used lentivirus-mediated gene transfer to deliver a specifically designed siRNA for mouse STAT3 into mouse bone marrow cells. Then, lethally irradiated wild-type C57BL/6 mice were reconstituted with 2 × 10⁶ bone marrow cells transfected with STAT3 or control siRNA. Presence of gene-modified cells was monitored by GFP fluorescence. Splenocytes or thymocytes were recovered three to five weeks after reconstitution. STAT3 expression was decreased in GFP⁺ T lymphocytes recovered from STAT3 siRNA reconstituted mice (Fig. 4A). Both the absolute numbers and the percentages of CD4⁺ or CD4⁺CD25⁺ T cells in spleen did not significantly differ according to STAT3 expression (1.15 ± 0.3 × 10⁶ CD4⁺ T cells for control mice vs 1.11 ± 0.25 × 10⁶ CD4⁺ T cells for STAT3 knockdown mice; 1.03 ± 0.45 × 10⁶ CD4⁺CD25⁺ T cells for control mice vs 1.01 ± 0.48 × 10⁶ for STAT3 knockdown mice; n = 21). In contrast, STAT3 knockdown decreased FOXP3 expression in CD4⁺CD25⁺ T cells (Fig. 4B), whereas it did not affect CD25 or CTLA-4 expression on CD4⁺ lymphocytes (data not shown). As assessed by flow cytometry, 85 ± 10% of CD4⁺CD25⁺ T cells expressed FOXP3 in control mice compared with 35 ± 12% of CD4⁺CD25⁺ T cells in the STAT3 knockdown group (p < 0.001). The same results were observed in the thymus (1.06 ± 0.36 × 10⁶ CD4⁺CD25⁺FOXP3⁺ thymocytes in control mice vs 2.18 ± 0.19 × 10⁶ CD4⁺CD25⁺FOXP3⁺ cells in STAT3 knockdown mice, p < 0.05; n = 12). Of note, CD4⁺CD25⁺GFP⁺ T lymphocytes from both control and STAT3-depleted groups expressed the same level of FOXP3 (Fig. 4B). To confirm the decreased expression of FOXP3 in STAT3 knockdown mice, CD4⁺CD25⁺ T cells were magnetically cell sorted and Foxp3 mRNA analyzed by real-time RT-PCR. As shown in Fig. 4C, Foxp3 mRNA expression was significantly decreased by STAT3 knockdown in CD4⁺CD25⁺ T cells, whereas STAT3 inhibition

**FIGURE 3.** STAT3 inhibition reverses the suppressive functions of naturally differentiated Treg. CD4⁺CD25⁺ T lymphocytes were purified from C57BL/6 splenocytes and treated with a lentivirus encoding a specifically designed siRNA for STAT3. A non specific siRNA was used as a control. These cells were then washed and maintained 48 h in culture with 100 UI IL-2 (1 × 10⁶ cells/condition). Then these lymphocytes were analyzed for their Treg marker expression (A). The black line depicted the isotypic control. Then, 10⁵ responder CD4⁺CD25⁻ T lymphocytes were cultured with 10⁵ APC cells from BALB/c (H-2b) mice during 5 days. In some conditions, 5 × 10⁵ CD4⁺CD25⁺ T lymphocytes treated with control or STAT3 siRNA were added to the culture (B). One representative experiment of three is depicted.
The proliferation of conventional T lymphocytes (Fig. 5) knockdown abolished the suppressive functions of CD4+ CD25+ T cells. Three to five weeks following reconstitution splenocytes were harvested and analyzed for CD4+ CD25+ and Foxp3 expression. Gated GFP+ splenocytes are shown. A, STAT3 knockdown was monitored in CD4+ GFP+ T lymphocytes recovered from reconstituted mice. Splenocytes of reconstituted mice were harvested and stained with anti-CD4, intracellular anti-STAT3, and anti-GFP. B, Three to five weeks following reconstitution, Foxp3 expression was determined at the protein level. Flow cytometric detection of Foxp3 is shown in gated CD4+ CD25+ T lymphocytes. The black line depicted the isotypic control. C, RNA was extracted from purified CD4+CD25+ or CD4+CD25− T cells and analyzed by QRT-PCR using specific primers for Foxp3. Foxp3 RNA relative level was obtained by dividing the relative amount of Foxp3 mRNA for each sample by the relative amount of GAPDH mRNA of the same sample (D). QRT-PCR analysis was also performed to determine TGF-β1 expression in CD4+ C25+ or CD4+ C25− lymphocytes purified from control or STAT3 knockdown mice.

did not affect Foxp3 mRNA levels in CD4+CD25− T cells (Fig. 4C). Then, the production of the immunosuppressive cytokine TGF-β1, involved in Treg functions, was examined by RT-PCR in CD4+CD25− or CD4+CD25+ T lymphocytes derived from STAT3 knockdown and control mice. TGF-β1 mRNA level was decreased by STAT3 ablation in CD4+CD25+ T cells (Fig. 4D). These results suggest a role of STAT3 in vivo in Treg generation.

Next, we defined the functional characteristics of splenic CD4+ T lymphocytes recovered from STAT3 or control siRNA reconstituted mice. Bone marrow-derived dendritic cells propagated from BALB/c mice and activated by LPS were used as APC. C57BL/6 lymphocytes were used as responding cells. As expected, CD4+CD25− T cells from control mice but not CD4+CD25+ T cells, to induce Foxp3 mRNA levels in CD4+CD25− T lymphocytes peripheral conversion (14). Liang S et al. demonstrated that CD4+CD25− T lymphocytes peripheral conversion depends on CD28 signaling. Therefore, we addressed the question as to whether Treg peripheral conversion in vivo would involve STAT3 signaling. We took advantage of an adoptive transfer model where 5 × 10^7 freshly isolated CD4+CD25− Foxp3+ T cells from STAT3 knockdown or control DC45.2+ mice were sorted and infused i.v. into sublethally irradiated CD45.1+ syngeneic wild-type mice. Splenocytes were harvested 4–6 wk after infusion. Donor CD45.2+CD4+ T lymphocytes were gated and analyzed for the presence of CD4+CD25−Foxp3+ T cells. As depicted in the Figs. 6 and 8, 8.08 ± 2.5% of CD45.2+CD4+CD25− T cells converted into CD45.2+CD4+Foxp3+ T cells in the control group compared with 3.6 ± 0.8% when STAT3 was knocked down (p < 0.05). Taken together, these experiments showed that STAT3 is a critical component of naturally occurring Treg differentiation as well as Treg peripheral conversion.

**STAT3 ablation promotes lethal acute GvHD**

Donor-derived CD4+CD25+ Treg can suppress lethal acute GvHD in murine models of allogeneic bone marrow transplantation (21–23). Acute GvHD can be induced by adoptive transfer of C57BL/6 (H-2b) CD4+CD25− T cells into lethally irradiated BALB/c (H-2d) mice. To determine the functional role of STAT3 in CD4+ T cells, we compared the capacity of CD4+ T lymphocytes depleted or not in CD4+CD25+ T cells, to induce acute GvHD in lethally irradiated BALB/c reconstituted with 1 × 10^7 bone marrow cells from C57BL/6 mice. Engraftment was monitored at day 10 by assessing H2b expression on recipient PBMC. Engraftment was achieved in all mice without any signif-
and significant differences between experimental groups (data not shown). The adoptive transfer of $5 \times 10^5$ total control CD4+ T cells led to a moderate GvHD with no death occurring within the first 35 days of follow up (Fig. 7, A–C). In contrast, the same number of total CD4+ T lymphocytes derived from STAT3-deficient mice induced an acute lethal GvHD as all mice died within 35 days (Fig. 7, A–C). These results indicate that STAT3 ablation in CD4+ T lymphocytes favors acute GvHD. These experiments might suggest that STAT3 depletion abrogated the suppressive activities included in a CD4+ T cell lymphocyte subpopulation or improved CD4+ T lymphocyte effector’s functions. To decipher which mechanism is involved in this process, we assess the capacity of CD4+CD25+ T cells to control conventional T lymphocyte activation in vivo, according to STAT3 expression. A total of $5 \times 10^5$ CD4+CD25+ T cells from siRNA control C57BL/6 mice were injected into lethally irradiated BALB/c (H-2b) mice to induce an acute GvHD. To define the immunosuppressive functions of Treg, $5 \times 10^5$ CD4+CD25+ T cells from control or STAT3-deficient mice were coinjected, resulting in a 1:1 effector/regulatory T cell ratio. The addition of control CD4+CD25+ T cells led to the inhibition of acute GvHD induced by conventional CD4+CD25+ T cells (Fig. 7D). In contrast, STAT3-deficient CD4+CD25+ T cells were unable to suppress acute GvHD induced by conventional CD4+CD25+ T cells and all the mice died within 30 days. In addition, $5 \times 10^5$ CD4+CD25+ T cells from STAT3 knockdown mice were injected into lethally irradiated BALB/c (H-2b) mice.

Control or STAT3 knockdown CD4+CD25+ T cells did not significantly differ in their ability to trigger an acute GvHD (Fig. 7D), confirming that STAT3 modulates suppressive rather than effector’s CD4+ T lymphocyte functions. Moreover, in these experiments, sera were collected on day 10 after BALB/c transplantation. IFN-γ levels in the serum of nontreated C57BL/6 mice or BALB/c mice were $57 \pm 5$ pg/ml. BALB/c mice adoptively transferred with $5 \times 10^5$ CD4+CD25+ T cells exhibited increased IFN-γ levels in the serum compared with recipients receiving C57BL/6 bone marrow alone ($120 \pm 5$ pg/ml). Animals receiving Treg cells had a significantly lower IFN-γ serum levels ($56 \pm 11$ pg/ml) as compared with STAT3-deficient CD4+CD25+ T cells ($137 \pm 8$ pg/ml). These results support the conclusion that CD4+CD25+ T cells differentiated in the absence of STAT3 are devoid of Treg functions in vivo.

**STAT3 ablation in CD4+ T lymphocytes elicits an efficient antitumor immunity**

Treg expansion has been observed during cancer progression in rodent models as well as in human settings (24–29). Kortylewski
et al. (36) showed that STAT3 inhibition in the whole immune system elicits an efficient antitumor immunity. Lastly, due to the known role of Treg in tumor immune-surveillance, the next set of experiments was designed to address the role of STAT3 ablation in CD4+ T lymphocytes during anti-tumor immune responses. C57BL/6 mice were nonlethally irradiated (6 Gy) and used as recipients. Twenty four hours after irradiation, these mice were subcutaneously challenged with the host-type T cell leukemia/lymphoma EL4 (10^6 cells). At the same time, wild-type C57BL/6 splenocytes previously depleted in CD4+ T lymphocytes were reconstituted with control or STAT3-deficient CD4+ T cells and injected i.v. into EL4-bearing mice. Each animal received 2 × 10^6 control or STAT3-deficient CD4+ T lymphocytes and 10^5 wild-type C57BL/6 splenocytes previously depleted from CD4+ T cells. As control, additional animals were only injected with PBS. EL4 rapidly appeared in irradiated mice in the PBS group (Fig. 8). Splenocytes containing control CD4+ T lymphocytes delayed EL4 growth kinetic but tumors were observed in all mice. In contrast, STAT3 ablation in CD4+ T lymphocytes significantly hampered EL4 tumor growth. Although all mice injected with control CD4+ lymphocytes developed measurable tumors, 80% of the mice treated with STAT3-deficient CD4+ T lymphocytes were tumor free at the end of the experiments (Fig. 8; p < 0.05). These results further indicate that STAT3 inhibition in CD4+ T lymphocytes is sufficient to elicit an effective antitumor immunity. These observations underline the potential importance of finding mediators involved in FOXP3 expression and pointed to a critical role of STAT3 in CD4+ lymphocytes to regulate immune responses targeting cancer cells.

**Discussion**

FOXP3 is a transcription factor dedicated to the genetic programming of CD4+CD25+ Treg differentiation and functions in mice (8–10). Patients bearing foxp3 mutations develop symptoms of immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), and do not exhibit peripheral Treg cells (3, 51–53). These observations indicate that foxp3 is a gene involved in Treg development and homeostasis which are essential for maintaining immune tolerance. Besides naturally occurring Treg, a subset of human CD4+ T lymphocytes up-regulates FOXP3 upon in vitro activation (18, 54). The functional consequences of this transient FOXP3 expression in humans have been controversial. CD4+CD25+FOX3+ T lymphocytes are hyporesponsive to restimulation and display poor proliferation and IFN-γ production capacities (55). In mice, Treg can be converted from peripheral CD4+CD25+ T cells through costimulation of TCR and TGF-β1 signaling (13). Although the relevance of the transcription factor FOXP3 is well documented in Treg generation and functions, the molecular pathways responsible for CD28-mediated Treg differentiation and peripheral conversion are still unclear. In this study, we propose that STAT3 directly drives foxp3 transcription and Treg differentiation. We found that STAT3 is involved in the molecular pathway governing FOXP3 expression and initiated by CD28 triggering.
In vivo, STAT3 knockdown in bone marrow cells of C57BL/6 mice decreased FOXP3 expression in both thymic and splenic (Fig. 4) CD4+CD25+ T cells following hematopoietic reconstitution. In this model, STAT3 deficiency did not significantly influence absolute numbers and percentages of total CD4+ and CD4+CD25+ T cells, suggesting a direct effect on foxp3 transcription. Another possible explanation might be that STAT3 ablation, rather than inhibiting FOXP3+ lymphocyte generation, leads to an enhanced activation of effector’s helper T lymphocytes. However, several lines of evidence support a direct molecular interaction between STAT3 and FOXP3 expression. Ex vivo BrdU experiments failed to identify any proliferation among CD4+CD25+ T cells (data not shown). STAT3 modulation did not either affect CTLA-4 or CD25 expression (data not shown). Moreover, in vitro, STAT3 knockdown in normally differentiated Treg reverse their suppressive functions on naïve CD4+CD25− T lymphocyte proliferation. In vivo, both STAT3-deficient or control CD4+CD25− T cells induced lethal aGVHD without any significant difference, whereas STAT3 ablation abrogated the suppressive functions of CD4+CD25− T lymphocytes (Fig. 7D). These results suggest that STAT3 activation contribute to CD4+ T lymphocyte polarization toward tolerance following CD28 costimulation.

Walker et al. (17) reported that CD28 costimulation in human CD4+CD25− T cells induces FOXP3 expression. However, discrepancies were observed between human and mouse Treg conversion models. In the mouse setting, CD28 costimulation alone does not induce FOXP3. Several authors have clearly established a role for TGF-β1 in association with CD3/CD28 to induce FOXP3 expression (13). We decided to focus our work in the rodent model to address the role of STAT3 in Treg maintenance. GvHD, and cancer. Nevertheless, STAT3 neutralization in human CD4+CD25− T cells decreases the transient expression of FOXP3 induced by CD3 and CD28 costimulation (data not shown), suggesting that the role of STAT3 might be extended in the human setting.

These experiments complete recent findings which contribute to elucidate the molecular events leading to foxp3 transcription. Low-dose IL-2 treatment results in CD4+CD25+FOXP3+ Treg expansion in peripheral blood of cancer patients (56–57). These authors showed that IL-2 exposition enhanced FOXP3 in CD4+CD25+ but not in CD4−CD25− T cells (57). However, Fontenot et al. (58) demonstrated that IL-2 is dispensable for Treg differentiation. Therefore, it appeared that Treg differentiation relies on CD28 signaling, whereas IL-2 seems to be required for maintaining Treg homeostasis and suppressive functions (58, 59). Zorn E et al. (57) identified a STAT3-binding domain located in the first exon of foxp3. Luciferase reporter gene assays in 293 cells confirmed the ability of STAT3 to interact with the proximal region of the foxp3 gene.

Recent findings provided by Kasprzycka et al. (60) contribute to identify a direct molecular association between STAT3 activation and FOXP3 expression. These authors studied T cell lymphomas that express anaplastic lymphoma kinase (ALK). ALK mediates its oncogenic functions through STAT3 activation. ALK+ T cell lymphomas display Treg phenotype and express FOXP3. STAT3 depletion in these lymphomas decreased ALK-mediated foxp3 transcription (60). Our experiments provide compelling evidence indicating that STAT3 governs CD28-driven foxp3 transcription in vitro and in vivo in conventional CD4+CD25− T cells. The fact that IL-2 mediates STAT3 phosphorylation and fails to enhance FOXP3 level in CD4+CD25− T cells, suggests that another factor induced by CD28 and licensing STAT3 mediated-Foxp3 transcription remains to be identified (57). However, our experiments using siRNA point to a nonredundant role of STAT3 in Foxp3 expression because its specific inhibition directly interferes with Treg generation and maintenance.

STAT3 influence on the suppressive functions of CD4+CD25+ T cells was further challenged in vivo. CD4+CD25+ T cells differentiated in the absence of STAT3 cannot prevent GvHD, suggesting that in vivo, STAT3 activation in CD4+ T lymphocytes is a critical event to control inflammatory diseases. These data corroborate previous observations indicating that long-term STAT3 ablation is associated with inflammatory diseases such as Crohn disease-like pathology (41–43). Moreover, recent findings showed that STAT3 activation is associated to T cell-tolerance induced by repetitive Ag stimulation (61). Indeed, the precise role of STAT3 in the onset of T cell dependent inflammatory or autoimmune diseases deserves further studies.

Our results also show a role for STAT3 in Treg suppressive function maintenance (Figs. 3B and 7D), which offers an opportunity to develop targeted therapies to reverse an established tolerance. Foxp3 suppressive functions do not act through a binary mechanism including an on-and-off switch to regulate Treg biology. Wan et al. (62) reported that a partial inhibition of Foxp3 is sufficient to subvert Treg functions. In line with this observation, our experiments using siRNA strategies showed that a decreased STAT3 expression in natural Treg, leading to a partial down-regulation of Foxp3 expression, was significantly correlated to a lack of suppressive functions. These results highlight the central role of STAT3 in Treg functions and suggest that STAT3 neutralization will be an effective strategy to get rid Treg suppressive functions in therapy.

STAT3 has been described as an oncogene involved in tumor progression (38, 63). STAT3 inhibition in tumor cells, using siRNA strategies, decreases invasion, angiogenesis, and reduces tumor spreading (63). In contrast, STAT3 expression in immune cells is associated with cancer tolerance. Kortylewski et al. (36) have shown that STAT3 expression disruption in tumor-bearing mice restored an efficient anti-tumoral immune response. STAT3 inhibition was associated to an enhanced CD8, CD4, and NK cell infiltration in tumors. Moreover, Treg number was decreased in STAT3-deficient mice. It was suggested that STAT3 expression in
tumor environment promotes Treg expansion. Ghiringhelli et al. (30) showed that a subset of immature dendritic cells, exhibiting a myeloid phenotype, selectively induced Treg expansion in a TGF-β1-dependent manner. Because increased STAT3 activity in DC impairs the maturation process and leads to IL-10 production, it was reasonable to consider dendritic cells as the main mediators of STAT3 immunosuppressive functions in STAT3 deficiency rodent models (64). Our results show that STAT3 activation directly acts on CD4+ T lymphocytes to promote Foxp3. This data are in line with the decreased number of Treg observed by Kortylewski et al. (36) following STAT3 ablation in hemopoietic cells. Based on an adoptive transfer model to reconstitute a syngenic immune system in C57BL/6 mice including control or STAT3-deficient CD4+ T cells, we showed that STAT3 inhibition specifically targeting CD4+ T cells avoids tumor engraftment in 80% of the mice (Fig. 8). Taken together, these data confirm the importance of STAT3 in Treg-mediated cancer immune tolerance and prompt the development of therapeutic strategies modulating STAT3 expression in CD4+ T cell lymphocytes.

In conclusion, we extended the description of the molecular pathway leading to FOXP3 expression. STAT3 phosphorylation is mandatory for FOXP3 expression upon CD4+ T cell activation. Taken together, these data indicate that CD28 driven STAT3 activation leads to a signaling specifically involved in regulatory molecule expression. STAT3 activation may be targeted by pharmacological agents or siRNA strategies to promote efficient adaptive immune responses. Such strategies might be of particular importance in vivo to restore anti-tumoral immune responses. In vitro, STAT3 inhibition might be included in T cell based cellular therapy protocols.

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References


