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STAT3 Signaling in CD4+ T Cells Is Critical for the Pathogenesis of Chronic Sclerodermatous Graft-Versus-Host Disease in a Murine Model

Vedran Radojcic,* Maria A. Pletneva,*,† Hung-Rong Yen,*,†,‡ Sanja Ivevic,* Angela Panoskaltsis-Mortari,‡ Anita C. Gilliam,§ Charles G. Drake,* Bruce R. Blazar,‡ and Leo Luznik*

Donor CD4+ T cells are thought to be essential for inducing delayed host tissue injury in chronic graft-versus-host disease (GVHD). However, the relative contributions of distinct effector CD4+ T cell subpopulations and the molecular pathways influencing their generation are not known. We investigated the role of the STAT3 pathway in a murine model of chronic sclerodermatous GVHD. This pathway integrates multiple signaling events during the differentiation of naive CD4+ T cells and impacts their homeostasis. We report that chimeras receiving an allograft containing STAT3-ablated donor CD4+ T cells do not develop classic clinical and pathological manifestations of alloimmune tissue injury. Analysis of chimeras showed that abrogation of STAT3 signaling reduced the in vivo expansion of donor-derived CD4+ T cells and their accumulation in GVHD target tissues without abolishing antithost alloreactivity. STAT3 ablation did not significantly affect Th1 differentiation while enhancing CD4+CD25+Foxp3+ T cell reconstitution through thymus-dependent and -independent pathways. Transient depletion of CD25+ T cells in chimeras receiving STAT3-deficient T cells resulted in delayed development of alloimmune gut and liver injury. This delayed de novo GVHD was associated with the emergence of donor hematopoietic stem cell-derived Th1 and Th17 cells. These results suggest that STAT3 signaling in graft CD4+ T cells links the alloimmune tissue injury of donor graft T cells and the emergence of donor hematopoietic stem cell-derived pathogenic effector cells and that both populations contribute, albeit in different ways, to the genesis of chronic GVHD after allogeneic bone marrow transplantation in a murine model. The Journal of Immunology, 2010, 184: 764–774.
CD4+ T cell differentiation and function. Our results demonstrate that STAT3 signaling in donor CD4+ T cells influences the development of chronic sclerodermatous GVHD by enabling sustained alloreactivity and negatively affecting the reconstitution of regulatory T cells (Tregs).

Materials and Methods

Animals

BALB/c-CD45.1 (H-2d, CD45.1+, CD90.2+), B10.D2-Thy1.2 (H-2d, CD45.2+, CD90.2+), B10.D2-Thy1.1 (wild-type [WT]; H-2d, CD45.2+, CD90.1+), and B10.D2-Thy1.1-CD4-Cre STAT3flox/flox [STAT3KO; CD45.2+, CD90.1+] (13) were propagated in the animal facility at the Johns Hopkins University Cancer Research Building I. Thymectomized BALB/c (H-2d, CD45.2+, CD90.2+) mice were obtained from the National Cancer Institute (Frederick, MD). All animals were 8- to 12-wk-old at the time of experimentation. All protocols were approved by the Animal Care and Use Committee of The Johns Hopkins University.

Hematopoietic cell transplantation procedures

A single lethal irradiation dose of 775 cGy was administered using a [137Cs] irradiator. Animals were reconstituted with 107 T cell depleted (TCD) B10.D2-Thy1.2 bone marrow (BM) cells alone or they received TCD BM supplemented with a graft-versus-host (GVH) inoculum consisting of 1.8 × 10^6 WT CD90.1+CD4+ and 0.9 × 10^6 WT CD90.1+CD8+ T cells (WT inoculum) or STAT3KO 1.8 × 10^6 CD90.1+CD4+ and 0.9 × 10^6 WT CD90.1+CD8+ T cells (STAT3KO inoculum). The T cell dose reflects T cells found in 1.2 × 10^7 B10.D2 donor splenocytes, a dose that reproducibly induces GVHD. T cell depletion was performed as previously described (18). Purified populations of donor T cells were obtained using T cell isolation kits (Dynabeads, Invitrogen, Carlsbad, CA). When necessary, cell sorting was done using a FACS Aria cell sorter (BD Biosciences, San Jose, CA). In designated experiments, T cells were labeled with CFSE prior to injection into irradiated recipients, as previously described (18). The purity and viability of T cell isolates in all experiments exceeded 95%. Cells were injected in the lateral tail vein.

For in vivo CD25 depletion, chimeras were injected i.p. with 0.5 mg anti-CD25 (PC61) or isotype control Ab (IgG1; both BioXcell, West Lebanon.

FIGURE 1. STAT3 signaling in CD4+ T cells is critical for the development of chronic GVHD in B10.D2→BALB/c model. On day 0, cohorts of BALB/c-CD45.1 recipients were lethally irradiated and reconstituted with TCD CD90.2+ BM only (○), TCD BM with 1.8 × 10^6 WT CD90.1+CD4+ and 0.9 × 10^6 WT CD90.1+CD8+ T cells (WT GVH inoculum; ♦), or 1.8 × 10^6 STAT3KO CD90.1+CD4+ and 0.9 × 10^6 WT CD90.1+CD8+ T cells (STAT3KO GVH inoculum; □) from B10.D2 donors. All mice were monitored for clinical signs of GVHD using systemic or skin-specific GVHD scoring systems. A, Mean systemic clinical GVHD scores. p < 0.001, ♦ versus ○ and ♦ versus □. B, Mean cutaneous clinical GVHD scores. p < 0.001, ♦ versus ○ and ♦ versus □. C, Representative photographs taken on day 60 after alloBMT show surviving chimeras that received TCD BM only, WT, or STAT3KO GVH inocula. D, i, Representative photomicrographs of histopathological changes in the skin of chimeras depicted in C (H&E stain, original magnification ×200). Note the presence of diffuse epidermal thickening and increased dermal cellularity due to lymphoid infiltration in the skin of WT CD4+ recipients compared with normal-appearing skin of STAT3KO GVH inoculum recipients. ii, GVHD skin scores on day 60 after alloBMT; n = 5 chimeras per group. E and F, Skin was harvested from chimeras, and single-cell suspensions were prepared for flow cytometric analysis of infiltrating inflammatory Gr-1+CD11b+ monocytes (E and F) and the production of IL-6 (F) (♦, WT; □, STAT3KO). G, Gene expression levels of proinflammatory cytokines, chemokines, and their receptors and transcription factors in the skin were determined by real-time quantitative RT-PCR. Obtained values were normalized to 18s rRNA expression. *p < 0.05. Data are expressed as mean ± SD.
Flow cytometry analysis

At designated time points, animals were sacrificed, and organs of interest (spleens, mesenteric lymph nodes [MLNs], livers, skin, and thymuses) were collected. Single-cell suspensions of spleen and MLNs were prepared by passing the organs through a cell strainer. Minced livers were digested for 30 min in complete medium supplemented with Liberase and DNase (both purchased from Roche Applied Science, Indianapolis, IN), and leukocytes were isolated by density gradient centrifugation on Accuprep medium (Accurate Chemical, Oslo, Norway). Minced depilated back skin was incubated for 90 min in complete medium supplemented with Liberase, DNase, and hyaluronidase (Worthington, Lakewood, NJ). For thymic analysis, animals were exsanguinated, thymuses were harvested, and single-cell suspensions were prepared by passing the organs through a cell strainer. Abs against mouse CD4, CD8α, CD11b, CD11c, CD4, CD45, CD45.1, CD45.2, CD62L, CD69, CD90.1, CD90.2, CD103, Gr-1, Annexin V, Foxp3, IL-6 and -17, and IFN-γ and the corresponding isotype controls were obtained from Biolegend (San Diego, CA), BD Biosciences, eBioscience (San Diego, CA), or Invitrogen. For detection of cytokine production, cells were briefly restimulated with PMA and ionomycin, in the presence of monensin (GolgiStop, BD Biosciences), prior to staining for flow cytometric analysis. Annexin V staining was performed according to the manufacturer’s instructions (BD Biosciences) Analysis was performed using an LSR II or FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FACSDiva (BD Biosciences) and FlowJo (Tree Star, Ashland, OR) software.

GVHD analysis

To quantify clinical GVHD, we used a scoring system that sums changes in weight loss, posture, activity, fur texture, and skin integrity (20, 21). Assessment of cutaneous GVHD was done using a separate scoring system (22). GVHD was also evaluated by histopathological analysis of ear skin, liver, colon, and thymus. All samples were prepared for routine histological analysis, stained with H&E, and slides were read by experienced pathologists (skin by A.C.G.; liver, colon, and thymus by A.P-M), in a blinded fashion, using established scoring systems (22, 23). Microphotographs were acquired using a Leica DM IRB microscope, mounted on an Olympus BX51 microscope (Olympus, Melville, NY) with an RT Spot camera (Diagnostic Instruments, Sterling Heights, MI) or an Olympus BX51 microscope (Olympus, Melville, NY) with Spot advanced software, at a magnification of ≥200.

Quantitative analysis of gene expression

Total RNA from snap-frozen tissues and sorted T cells was extracted using an RNeasy mini kit (Qiagen, Valencia, CA) and reverse transcribed using a qScript cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg, MD). cDNA was subjected to PCR amplification using primers for 18s rRNA, IFN-γ, TNF-α, CCL2, CCL5, CCL20, CCR5, IL-6 and -17, T-bet, GATA3, and Foxp3 (24–28) and SYBR Premix Ex Taq (Takara Bio, Madison, WI) in a MyIq light cycler (Bio-Rad, Hercules, CA).

Statistical analysis

Values are presented as the mean ± SEM or mean ± SD, as appropriate. Statistical differences were calculated using one-way ANOVA, the Student t test, or the log-rank test, as appropriate. A value of p < 0.05 was considered statistically significant.

Results

Loss of STAT3 in graft-derived donor CD4+ T cells prevents chronic GVHD development in a B10.D2→BALB/c model.

To assess the role of STAT3 signaling in donor T cells (GVH inocula) during chronic sclerodermatous GVHD development, we used CD4+ T cells from donors in which conditional STAT3 deletion (Cre-Lox system) occurs solely in T cells without altering their number or phenotype (13, 14, 17). Cohorts of chimeras were constructed after conditioning of BALB/c-CD45.1 recipients and transplantation of TCD BM alone or TCD BM supplemented with GVH inocula. Following alloBMT, chimeras were monitored for clinical signs of GVHD using two previously established scoring systems that independently assess acute (20) and chronic GVHD (22). Although recipients of WT inocula developed clinical GVHD, no signs of acute or chronic GVHD were observed in chimeras injected with STAT3KO inocula (Fig. 1A–C; p < 0.001, WT versus STAT3KO). When TCD splenocytes were added to the GVH inocula, the same findings were observed as described above for studies with WT versus STAT3KO T cells without additional TCD splenocytes (data not shown). This finding excluded the possibility that the lack of some other cellular component was responsible for the absence of GVHD in chimeras receiving STAT3KO inocula. Routine histological sections of the ears of mice that received WT inocula revealed increased skin thickness and infiltration with mononuclear cells. In contrast, no histological changes were noted in the chimeras that received STAT3KO/CD4+ T cells (Fig. 1D).

During the course of chronic sclerodermatous GVHD, inflammatory leukocytes invade the skin and produce a range of inflammatory cytokines that affect tissue homeostasis and drive wound healing. Pro-inflammatory cytokines promote fibrosis, neovascularization, and angiogenesis, while anti-inflammatory cytokines have the opposite effect. Understanding the molecular mechanisms that regulate these responses is crucial for developing effective therapeutics to treat this debilitating disease.

**FIGURE 2.** STAT3 ablation abrogates GVHD without impairing the achievement of complete donor chimerism. Lethally irradiated BALB/c-CD45.1 recipients were reconstituted with TCD BM plus WT or STAT3KO GVH inocula. To monitor the fate of graft-derived T cells, we used TCD BM from B10.D2-Thy1.2 mice, whereas all T cells were from donors on a B10.D2-Thy1.1 background. A, Serial analysis of absolute numbers of graft-derived CD90.1+ T cells in spleens and livers of chimeras that received WT or STAT3KO inocula. Data are representative of four independent experiments with a total >12 animals per group and time point. Results are presented as mean ± SEM; *p < 0.05. B, Analysis of CD90.1+CD4+ T cells in the skin on days 14 and 42 after alloBMT. C, Changes in donor chimerism were serially monitored in spleen and liver of chimeras that received TCD BM (●), TCD BM plus WT (●), or STAT3KO (○) GVH inocula based on the differential expression of CD45 on donor B10.D2 (CD45.2+) and host (CD45.1+) T cells. There was no difference in the chimerism levels between WT and STAT3KO chimeras at all time points evaluated.
mediators (16). Flow cytometric analysis of single-skin cell preparations revealed that animals receiving STAT3KO CD4+ T cells had reduced inflammatory Gr-1+CD11b+ monocyte infiltration and decreased IL-6 production in comparison with chimeras receiving WT CD4+ T cells (Fig. 1E, 1F). These observations were corroborated by gene expression analysis of inflammatory markers known to be highly expressed in the skin of chimeras with sclerodermatous GVHD (Fig. 1G) (16, 29). Taken together, these findings suggest that STAT3 ablation in graft-derived donor CD4+ T cells disables the inflammatory response that is crucial to the development of chronic sclerodermatous GVHD in this CD4+ T cell-dependent model.

**STAT3 abrogation in donor CD4+ T cells limits their expansion and accumulation in GVHD target tissues but does not impede their GVH reactivity**

We next focused on exploring how abrogation of STAT3 signaling in CD4+ T cells influences their in vivo fate and function after alloBMT. To assess cell fate, we monitored CD4+ and CD8+ T cell expansion and accumulation in secondary lymphoid (spleen) and epithelial (liver and skin) tissues. In conducting these analyses, we exploited the differential expression of CD90 allele on donor graft-derived T cells (CD90.1) and on T cells developing in vivo from transplanted donor HSCs (CD90.2). We found that STAT3 ablation resulted in a 20-fold lower accumulation of STAT3KO CD4+ T cells in the spleen and liver on day 5 after alloBMT (Fig. 2A; p < 0.01). On day 14, the difference was still marked, but of smaller magnitude (5-fold; Fig. 2A; p < 0.05). A similar pattern of CD90.1+CD4+ T cell accumulation was observed in the skin (Fig. 2B). The lower number of STAT3KO CD4+ T cells observed at day 14 was maintained in the spleens and livers of chimeras over time. The expansion of CD90.1+CD8+ T cells only differed in the spleen on day 14 (Fig. 2A), a finding consistent with the dominant role of CD4+ T cells in this model. Next, we compared the GVH reactivity of STAT3KO and WT GVH inocula. Despite lower expansion and accumulation of graft-derived T cells in spleen and epithelial tissues, recipients of STAT3KO GVH inocula rapidly converted to full donor chimerism, with kinetics that were indistinguishable from those observed in WT GVH inoculum recipients (Fig. 2C and data not shown; p = NS for WT versus STAT3KO for all time points assessed).

**STAT3 signaling influences in vivo proliferation and homeostasis of donor-derived CD4+ T cells**

There are three potential explanations for the observed effect of STAT3 ablation on the in vivo fate of donor T cells: STAT3KO T cell proliferation is reduced, alloantigen recognition is altered, or their in vivo survival is impaired. To examine the effect of STAT3 signaling on in vivo proliferation, graft-derived WT and STAT3KO CD4+ T cells were labeled with CFSE and assayed for dye dilution. At day 5 after alloBMT, the overall percentage of proliferated splenic CFSElow STAT3KO CD4+ T cells was markedly lower than that of WT CD4+ T cells (p < 0.001; Fig. 3A). By day 14, the CFSE dilution of STAT3KO CD4+ T cells was complete and indistinguishable from that of WT CD4+ T cells (data not shown).

To determine whether STAT3 signaling influences in vivo alloantigen-induced T cell activation, we examined changes in the expression of characteristic activation markers. We found no difference in the expression of CD25, CD69, CD11a, CD18, and CD29 on splenic and hepatic CD4+ T cells between the two sets of chimeras on day 14 after alloBMT (Fig. 3B and data not shown). At this time point, WT and STAT3KO CD4+ T cells in the spleen and liver were predominantly of an effector memory phenotype (CD44high CD62Llow−; Fig. 3C; p = NS).

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** STAT3 ablation in CD4+ T cells influences their in vivo proliferation and expansion. BALB/c-CD45.1 recipients were lethally irradiated and reconstituted with WT or STAT3KO GVH inocula containing CFSE-labeled graft-derived CD90.1+ T cells. A. Representative histogram and CFSE dilution profile of graft-derived spleen-infiltrating CD4+ T cells on day 5 after adoptive transfer. Data are representative of five independent experiments, each performed in triplicate (p = 0.001). Expression of CD25/CD69 (B) and CD44/CD62L (C) on graft-derived CFSE+CD4+ T cells retrieved from WT and STAT3KO chimeras on day 14 after alloBMT; p = NS, for all valid comparisons. D. In vivo analysis of apoptosis of WT and STAT3KO CD4+ T cells. Splenic and hepatic graft-derived CD4+ T cells were harvested and stained for Annexin V on day 5 after alloBMT. Representative data from one of three independent experiments are shown.

We next characterized the effect of STAT3 ablation on CD4+ T cell survival. Given that IL-6 expression increases in the posttransplant inflammatory milieu and that IL-6–induced STAT3 activation is indispensable for the prevention of apoptosis (29, 30), we expected that the in vivo survival of STAT3KO T cells would be impaired. Surprisingly, we found a comparable fraction of Annexin V+STAT3KO and WT CD4+ T cells in the spleen and liver of...
chimeras on day 5 posttransplant (Fig. 3D; p = NS). A control in vitro experiment confirmed that IL-6 could prevent apoptosis of optimally stimulated WT, but not STAT3KO, T cells (data not shown). One possible explanation for our in vivo finding is that the postransplant inflammatory milieu may provide other survival factors for STAT3KO/CD4+ T cells. TGF-β mediates antiapoptotic effects via multiple mechanisms, enhances survival of TCR-stimulated CD4+ T cells, and is abundant postransplant in this model (16, 31, 32). We found that TGF-β, alone or in combination with IL-6, prevents apoptosis and rescues stimulated STAT3KO/CD4+ T cells from death in vitro (Supplemental Fig. 1). Thus, multiple signaling pathways influence the survival and apoptosis of STAT3KO/CD4+ T cells after transplantation.

**STAT3 abrogation does not restrain Th1 cells while abrogating the emergence of Th17 cells in allogenic chimeras**

We sought to further determine the impact of STAT3 signaling on donor T cell effector differentiation and cytokine secretion after alloBMT. The percentages of IFN-γ–secreting STAT3KO and WT CD4+ T cells were similar in the skin of chimeras at day 14 after alloBMT (35% versus 33%; Fig. 4A), just prior to the development of cutaneous GVHD. We also found similar percentages of IFN-γ–secreting STAT3KO and WT CD4+ T cells in the spleens and livers of chimeras at all time points analyzed (Supplemental Fig. 2A). Consistent with the reduction in the number of STAT3KO T cells depicted in Fig. 2B, the absolute number of IFN-γ–secreting CD4+ T cells was reduced on day 14 in the spleen and liver (Fig. 4B; *p < 0.01). This reduction in the absolute number of Th1 cells in the spleen and liver was also noted at later time points (Fig. 4B; *p < 0.01).

We were unable to detect any IL-17–secreting cells in the skin of chimeras that received WT or STAT3KO T cells at any time point examined, a result consistent with unpublished microarray data in this model (A.C. Gilliam, unpublished data). We were also unable to detect any significant IL-17 production by graft-derived WT CD4+ T cells in the spleens of chimeras at all time points analyzed (data not shown). However, serial monitoring revealed that the percentage and absolute number of Th17 cells in the livers of chimeras receiving WT T cells, but not STAT3KO T cells, increased over time after alloBMT (Fig. 4C, 4D; Supplemental Fig. 2B). In addition, a substantial percentage of graft-derived WT CD4+ T cells coexpressed IL-17 and IFN-γ. These data indicate that donor graft-derived Th17 cells, as well as IL-17-IFN-γ+CD4+ T cells, are generated in chimeras that develop chronic GVHD but that their emergence is delayed and organ dependent.

**Abrogation of STAT3 signaling promotes Treg development**

In addition to the Th1 and Th17 subsets, CD4+ T cells can differentiate into a distinct subset characterized by the expression of the forkhead box transcription factor FoxP3, considered to represent Tregs (33). Several groups reported marked conversion of STAT3KO T cells into CD4+Foxp3+ Tregs upon in vitro stimulation in the presence of IL-6 and TGF-β (17, 26, 34). TGF-β and IL-6 are present in abundance in allogeneic chimeras (29, 31). We hypothesized that this postransplant milieu skews the fate of naive graft-derived STAT3KO T cells toward a regulatory phenotype. To test this hypothesis, we sorted splenic- and liver-infiltrating graft-derived CD4+ T cells and analyzed the mRNA expression of lineage-specific transcription factors, representative cytokines, chemokines, and their receptors. We found that increased Foxp3 mRNA expression is a predominant characteristic of STAT3KO versus WT CD4+ T cells, especially in the liver (Fig. 5A). Determining whether increased expression of Foxp3 and GATA3 mRNA detected in STAT3KO CD4+ T cells retrieved from spleen indicates their differentiation toward Th2 and Foxp3+ cells or whether these cells represent previously described Tregs known to express both canonical transcription factors (35) will require further studies. Consistent with the reduced expression of IFN-γ protein as assayed by intracellular cytokine staining, STAT3KO/CD4+ T cells also exhibited decreased expression of IFN-γ (Fig. 5A). The expression of mRNA encoding proinflammatory chemokines CCL2, CCL5, and CCL20 and the CCR5 receptor was also reduced in STAT3-deficient CD4+ T cells compared with that in WT CD4+ T cells.

Next, we compared the percentage of Foxp3+ cells among STAT3KO and WT CD4+ T cells retrieved from the spleens and livers of chimeras at multiple time points after alloBMT. The proportion of CD4+Foxp3+ T cells among all GVH inoculum-derived CD4+ T cells, although similar in both sets of chimeras at day 14, was substantially increased starting on day 28 in the group that received STAT3KO/CD4+ T cells, although similar in both sets of chimeras at day 14, was substantially increased starting on day 28 in the group that received STAT3KO/CD4+ T cells (Fig. 5B; *p < 0.05 for all time points after day 14). This prompted us to assess whether the increased percentage of STAT3KO/CD4+Foxp3+ T cells represents expansion of naturally occurring, thymus-derived CD4+CD25+ Tregs or adaptive CD4+CD25+ Tregs that are induced from CD25+ precursors in peripheral organs (induced Tregs [iTregs]). To address this, we constructed GVH inocula using CD4+CD25− T cells that were sorted from WT or STAT3KO CD4+ T cells to a purity exceeding 98%. The CD4+CD25− fraction was then combined with donor

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**FIGURE 4.** Abrogation of STAT3 signaling prevents late emergence of Th17 cells while minimally affecting the Th1 response. Lethally irradiated BALB/c–CD45.1 recipients were reconstituted with TCD BM or TCD BM with WT or STAT3KO GVH inocula. Skin and splenic and liver mononuclear cells were serially retrieved and analyzed for cytokine production by flow cytometry. A, Percentage of IFN-γ–producing graft-derived CD90.1+CD4+ T cells in skin of chimeras (day 14 after alloBMT). B, Production of IFN-γ in graft-derived CD4+T cells after alloBMT. C, Representative contour plots showing IFN-γ and IL-17 production in graft-derived CD4+ T cells harvested from spleens and livers of WT and STAT3KO chimeras 42 d after alloBMT. D, Serial analysis of IL-17 production in graft-derived CD4+ T cells in the liver points to their emergence later during GVHD evolution. *p < 0.05 for WT versus STAT3KO GVH inoculum recipients. Data are representative of at least three experiments with >15 mice per group.
We examined the role of STAT3 signaling in graft-derived CD4+ T cells on the overall quantitative reconstitution of the peripheral CD4+Foxp3+ Treg pool posttransplant. Cohorts of chimeras that received WT or STAT3KO GVH inocula were serially analyzed, and the absolute numbers of CD4+Foxp3+ T cells were quantified. As shown in Fig. 6Ai, the absolute numbers of CD4+Foxp3+ T cells were higher in animals that received STAT3KO inocula than in recipients of WT GVH inocula from day 28 onward; this was especially evident on day 60 (Fig. 6Ai).

The peripheral Foxp3+ Treg pool can be restored posttransplant by residual host Tregs, homeostatically expanded graft Tregs, and new Tregs generated from donor HSC-derived progenitors by thymopoiesis. To evaluate these possibilities, we monitored the kinetics of Treg reconstitution and the relative contribution of each pathway in cohorts of chimeras that received WT or STAT3KO GVH inocula. TCD BM recipients served as a control. The radio-resistant host CD4+Foxp3+ T cells were not detectable beyond day 7 posttransplant in either type of chimera receiving mature donor T cells with the graft (data not shown). When analyzed separately, the absolute number of graft-derived WT CD4+ Foxp3+ T cells rapidly increased in comparison with STAT3KO CD4+Foxp3+ Tregs (Fig. 6Aii; p < 0.05 for days 14 and 28). This early expansion of WT CD4+Foxp3+ Tregs is consistent with the overall proliferative advantage of T cells with intact STAT3 signaling (Figs. 2, 3). From day 28 onward, the absolute numbers of graft-derived WT CD4+Foxp3+ T cells in both groups declined; by day 60, these numbers were significantly reduced in both sets of chimeras. Interestingly, in chimeras that received STAT3KO GVH inocula, thymus-derived CD490.2+CD4Foxp3+ T cells rapidly increased after day 28 and represented the majority of Tregs in the peripheral Treg pool by day 60. In contrast, Treg reconstitution by thymopoiesis in chimeras that received WT GVH inocula was minimal (Fig. 6Aiii; p < 0.05 from day 28 onward).

We next investigated whether the apparent failure in central pathway reconstitution of HSC-derived CD4+Foxp3+ Tregs in recipients of WT GVH inocula was due to thymic damage. We observed that the thymuses of chimeras receiving WT CD4+ T cells were severely atrophic, an observation confirmed by histopathologic analysis and decreased overall thymic cellularity (Fig. 6Bi). Flow cytometric analysis also revealed a decrease in the percentage of double-positive CD4+CD8+ thymocytes in WT CD4+ T cell recipients (Fig. 6Bii; 38.6% versus 80.2%; p < 0.001 WT versus STAT3KO). In contrast, the appearance and cellularity of thymuses and the phenotypic characteristics of T cell populations in STAT3KO CD4+ T cell recipients were indistinguishable from those of chimeras that received only TCD BM. Moreover, the corresponding total numbers of naïve T cells and CD90.2+ Tregs were significantly higher in STAT3KO recipients than in WT CD4+ T cell recipients (data not shown). These results were not due to an inability of STAT3KO T cells to infiltrate the host thymus, because comparable numbers of WT and STAT3KO CD90.1+CD4+ T cells were found in thymus of both groups of chimeras (Fig. 6Biii; p = NS).

Taken together, our results suggest that the ablation of STAT3 signaling in graft-derived CD4+ T cells results in robust thymic-
Dependent Treg production. Furthermore, these results provide evidence that, consistent with other models (11, 37), the development of chronic GVHD in this model is also associated with dysregulated Treg reconstitution. However, here, it is primarily due to failed donor HSC-derived, thymus-dependent immunoreconstitution.

**Donor graft-derived Tregs and preserved thymic function contribute to chronic GVHD prevention in chimeras that received STAT3KO GVH inocula**

We next investigated the contribution of graft-derived Tregs and thymic output to chronic GVHD prevention in chimeras receiving STAT3KO GVH inocula. To study the role of graft-derived Tregs, we depleted CD25+ cells in vivo using the anti-CD25 mAb PC61 (38). This strategy was used previously in multiple models to assess Treg contributions (39, 40). Anti-CD25 mAb treatment resulted in profound depletion of CD4+CD25+Foxp3+ T cells in all organs examined (data not shown). Consistent with previous studies, we observed that PC61 administration did not increase the severity of GVHD in animals receiving WT CD4+ T cells (19). In contrast, animals receiving STAT3KO inocula followed by PC61 did not differ from counterparts that received control IgG1 isotype until day 30, when they started to develop systemic GVHD. This condition was characterized by diarrhea, weight loss, hunched posture, and ruffled fur but interestingly not by cutaneous GVHD (Fig. 7A). Meanwhile, control chimeras reconstituted with only donor TCD BM that received PC61 remained healthy throughout the course of the experiment, confirming the role of adoptively transferred T cells in the development of GVHD in this setting. Histopathological examination of tissues obtained from WT inoculum recipients and STAT3KO inoculum recipients treated with PC61 showed signs of extensive intestinal and hepatic GVHD. Epidermal hyperplasia and dermal and subdermal inflammation with destruction of the fatty layer were seen only in mice receiving WT CD4+ T cells (Fig. 7B). Dysregulated inflammatory response caused by CD25 depletion was also reflected in the serum levels of proinflammatory cytokines (Supplemental Fig. 3). Thus, a lack of Tregs early after transplantation does not directly exacerbate clinical GVHD, but their absence is critical for the induction of late de novo GVHD.

To examine the role of thymic output in preventing chronic GVHD, we conducted experiments using thymectomized BALB/c mice. Thymectomized BALB/c recipients of WT GVH inocula developed GVHD with similar penetrance, although their disease was more lethal in comparison with that of irradiated euthymic mice (data not shown). Interestingly, irradiated thymectomized BALB/c recipients of STAT3KO GVH inocula developed GVHD, including characteristic skin manifestations, although with delayed kinetics (Fig. 7C). These findings suggest that STAT3KO CD4+ T cells are capable of inducing tissue injury and that their inability to trigger GVHD is dependent on an intact thymus.

**De novo systemic GVHD induced by CD25+ Treg depletion in recipients of STAT3KO GVH inocula is mediated by donor HSC-derived Th1 and Th17 cells**

We hypothesized that the delayed alloimmune injury in chimeras that received STAT3KO GVH inocula and were treated with PC61 was the result of an exaggerated graft-derived effector T cell response in the absence of Tregs, resulting in the de novo generation of pathogenic HSC-derived T cells. Consistent with our prediction, we found increased IFN-γ production in donor splenic graft-derived CD90.1+ and HSC-derived CD90.2+CD4+ T cells in chimeras that received STAT3KO/CD4+ T cells and were treated with PC61 (Fig. 8A). Because GVHD was mostly focused on the gastrointestinal system, we also examined the effector phenotype of CD4+ T cells in the MLNs. Macroscopically, STAT3KO GVH inoculum recipients that received PC61 remained healthy until day 60 (Supplemental Fig. 3). Thus, a lack of Tregs early after transplantation does not directly exacerbate clinical GVHD, but their absence is critical for the induction of late de novo GVHD.

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**FIGURE 6.** Unperturbed reconstitution of donor HSC-derived Tregs is a dominant feature in chimeras receiving STAT3KO GVH inocula. Irradiated BALB/c-CD45.1 recipients were transplanted with TCD BM or TCD BM plus WT or STAT3KO GVH inocula. A, Longitudinal analysis of total splenic (i), CD90.1+ graft-derived (ii), and CD90.2+ HSC-derived (iii) Foxp3+CD4+ Treg reconstitution. B, Thymic output to chronic GVHD prevention in chimeras receiving STAT3KO GVH inocula. Donor graft-derived Tregs and preserved thymic function contribute to chronic GVHD prevention in chimeras that received STAT3KO GVH inocula.
absolute numbers, although PC61 treatment did not change the overall number of CD90.1+CD4+ T cells (Supplemental Fig. 4). Examination of HSC-derived CD90.2+ cells revealed a significantly increased percentage of CD4+ T cells secreting IFN-\(\gamma\) and IL-17, including those secreting both cytokines (Fig. 8B). The observed increase in double-positive (IFN-\(\gamma\)+IL-17+) CD4+ T cells is reminiscent of the population described in animals with autoimmune disease and those with autoimmune manifestations of chronic GVHD (11). These results strongly suggest that STAT3KO Tregs play a critical role in preventing the dysregulation of alloreactive graft-derived T cells, which can offset the in vivo generation of Th1 and Th17 HSC-derived T cells.

**Discussion**

In this study, we used a CD4+ T cell-dependent, MHC-matched model of alloBMT to examine the relevance of the STAT3 pathway in CD4+ T cells to chronic sclerodermatous GVHD development. Our studies revealed that STAT3 signaling in donor CD4+ T cells influences several key events in the pathogenesis of chronic GVHD. We found that abrogation of STAT3 signaling limits the in vivo proliferation and expansion of alloreactive CD4+ T cells. Decreased accumulation of these and other inflammatory cells in GVHD target tissues was also noted. Although STAT3 did not impact the capacity of naive CD4+ T cells to differentiate into...
Th1 effectors, it did promote CD4+Foxp3+ T cell reconstitution via thymus-dependent and -independent pathways, whereas WT counterparts experienced a profound loss of Tregs.

Our study provides new insight into the role of STAT3 signaling in naive T cell differentiation, which extends beyond its effect on Th17 generation. First, our results reveal that in an alloBMT setting, STAT3 ablation in alloreactive CD4+ T cells limits their in vivo proliferation and expansion in the secondary lymphoid tissues. A high T cell proliferative rate is known to correlate with acute GVHD, and the control of alloreactive T cell proliferation is the mechanism behind the function of pharmacological agents used for GVHD prophylaxis after alloBMT. Although STAT3KO CD4+ T cells appeared to infiltrate the chimeras’ tissues in proportion to their level of expansion, their reduced CCR5, CCL2, CCL5, and CCL20 expression suggests that the ability of these cells to accumulate in GVHD target organs and orchestrate an inflammatory response is diminished. This is consistent with the known importance of IL-6–driven STAT3 signaling in the chemokine regulation of T cell recruitment to inflamed tissues (41). Despite these findings, the use of STAT3KO GVH inocula still enabled conversion to complete donor chimerism, a reliable outcome after alloBMT. The exact role of Th17 cells originating from donor mature T cells and HSC-derived, in vivo-generated Th1- and Th17-cell subsets in the pathogenesis of liver and gut GVHD needs to be examined further.

Finally, our most relevant observation was that STAT3 signaling in donor mature T cells provides a direct link between alloreactive T cell-mediated tissue damage and favorable Treg reconstitution posttransplant. There are several mechanisms behind this observation. Clearly, STAT3 abrogation limits the generation of alloreactive effector cells and damage to the host thymus, thus allowing early reconstitution of HSC-derived Tregs via the central pathway. The best confirmation for this is seen in the development of GVHD in thymectomized recipients of STAT3KO GVH inocula. In addition, the role of STAT3 in thymus-independent Treg reconstitution is supported by the emergence of pathogenic donor HSC-derived T cells in chimeras reconstituted with STAT3KO inocula and treated with anti-CD25 mAb. The absence of STAT3 signaling in donor graft-derived CD4+CD25+ T cells was also associated with increased conversion of CD4+CD25+ precursors to CD4+CD25+ Foxp3+ Tregs in vivo. However, the overall quantitative contribution of iTregs to the regenerated peripheral Treg pool seems to be minor. Ultimately, a lack of GVHD is correlated with a renewal of thymic activity.

The early in vivo fate of mature graft-derived T cells in the current model differs from that reported for the MHC-mismatched

**FIGURE 8.** HSC-derived Th1 and Th17 are mediators of GVHD in chimeras treated with PC61 mAb. Groups of chimeras were constructed and treated with anti-CD25 or isotype control Abs as described in Fig. 7. On day 28 after alloBMT, spleen and MLNs were retrieved and analyzed for production of IFN-γ and IL-17 in graft (CD90.1+)- and HSC (CD90.2+)-derived T cells. A, Representative contour plots show the percentage of IFN-γ- and IL-17-secreting cells within the gated graft- and HSC-derived CD4+ T cells harvested from spleens of designated chimeras. Note the difference in IFN-γ production between anti-CD25 and isotype-treated STAT3KO GVH inoculum recipients. B, Production of IFN-γ and/or IL-17 in donor HSC-derived CD4+ T cells harvested from MLNs. i, Representative contour plots depicting cytokine secretion profile of CD90.2+CD4+ T cells. Profound effects of CD25 deletion are most evident in the emergence of Th1 and Th17 HSC-derived CD4+ T cells; *p < 0.05. Cumulative results of two independent experiments with a total >10 animals/group analyzed are presented.
model used to study chronic GVHD pathogenesis (11). Although the mechanisms behind these disparities are unknown, the differential kinetics of effector and Treg reconstitution noted in our study are especially relevant to the minor histocompatibility Ag-driven response that typically drives GVHD generation in the clinic. In addition, our observations provide insight into several published contradictory findings related to chronic GVHD pathophysiology. For example, thymus-dependent T cells play a role in mediating chronic GVHD in some, but not all, models (5, 9, 10), and in its sclerodermatous form, GVHD can occur after the administration of mature donor-derived, postthymic T cells in the form of donor lymphocyte infusions (43). Based on our findings, mature donor T cells administered with a graft are dominant and sufficient inducers of sclerodermatous GVHD and can induce GVHD in the absence of a thymus. We also found that pathogenic donor HSC-derived T cells can appear late posttransplant and that their emergence is not necessarily preceded by clinical signs of acute GVHD if donor Tregs are depleted early after alloBMT.

Several groups showed that in vitro stimulation of naïve STAT3-CD4+ T cells in the presence of TGF-β and IL-6 results in more Foxp3-expressing CD4+ cells (17, 26, 34). Our results showed that the in vivo fate of CD4+ T cells with targeted deletion of STAT3 recapitulates previous in vitro observations. In addition, we provide unequivocal evidence for the role of the STAT3 pathway in GVHD, whose relevance was previously examined only by ex vivo treatment of donor T cells using small molecule inhibitors (44). However, our findings are contradictory to a report by Pallandre et al. (45), who showed that in vivo STAT3 neutralization results in exacerbation of acute GVHD. The most likely explanation for this contradiction is that in the referenced study, CD4+ T cells were retrieved from donor mice in which STAT3 was ablated using small interfering RNA in HSCs, whereas in our studies, STAT3 was ablated only in T cells. STAT3 abrogation in HSCs activates innate immunity, resulting in much stronger T cell responses, and causes autoimmunity (46, 47). This is not the case when STAT3 is ablated only in CD4+ T cells, because these animals possess a normal phenotype and numbers of Tregs at steady state (17). In that regard, it is important to emphasize that all observations on the effects of abrogating STAT3 signaling need to be considered in the context of the effects on other cell populations and the host cytokine milieu.

In summary, STAT3 signaling in donor graft-derived CD4+ T cells promotes their expansion and trafficking to epithelial organs and, in the setting of alloBMT, hinders their differentiation into Tregs. The resulting increase in Tregs in levels directly outweighs the Th1 effector-dominated alloimmune response and tissue damage, preventing the loss of self-tolerance and the emergence of donor HSC-derived pathogenic Th1 and Th17 cells. Therefore, STAT3 signaling in donor graft CD4+ T cells plays an important role in chronic GVHD pathogenesis; abrogation of this signaling may have a critical effect on chronic GVHD modulation after alloBMT.

Disclosures

The authors have no financial conflicts of interest.

References


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Supplementary Figure 1. TGF-β rescues optimally stimulated STAT3KO CD4+ T-cells from apoptotic death. STAT3KO CD4+ T-cells were cultured in anti-CD3/anti-CD28 pre-coated wells in complete medium supplemented with nothing, TGF-β (5 ng/ml), or both TGF-β and IL-6 (20 ng/ml). After 84 hours, cells were harvested, stained with Annexin V mAb and analyzed by flow cytometry. Numbers on representative histogram plots show percentages of Annexin V positive STAT3KO CD4+ T-cells.

Supplementary Figure 2. STAT3 ablation does not interfere with the commitment of STAT3KO CD4+ T-cells toward a Th1 phenotype while preventing the late emergence of Th17 cells. B10.D2→BALB/c-CD45.1 chimeras were constructed by transplanting TCD BM with 1.8 × 10^6 WT CD4+ and 0.9 × 10^6 WT CD8+ T-cells (♦) or 1.8 × 10^6 STAT3KO CD4+ and 0.9 × 10^6 WT CD8+ T-cells (◇). At designated time-points designated organs were retrieved and the isolated mononuclear fraction was analyzed for production of IFN-γ and IL-17 after a brief in vitro stimulation with PMA and ionomycin in the presence of GolgiStop. Graft-derived CD90.1+ CD4+ and CD8+ T cells were gated and examined for cytokine production and origin. Serial changes in IFN-γ (A) and IL-17 (B) production by graft-derived CD4+ T-cells in spleens and livers of chimeras that received WT and STAT3KO GVH inocula. * indicates p < 0.05.

Supplementary Figure 3. Dysregulated inflammatory response in CD25-depleted STAT3KO chimeras is accompanied by systemic changes in cytokine levels. Serum was collected from chimeras on day 28 after alloBMT and analyzed for presence of IFN-γ, IL-17, TNF-α and KC on a Luminex 100 IS analyzer (Luminex Corporation) using Cytokine Mouse 20-plex Panel kit (Invitrogen), according to the manufacturer’s instructions. * indicates p < 0.05.
Supplementary Figure 4. In vivo CD25 depletion in STAT3\textsuperscript{KO} chimeras leads to selective expansion of graft-derived Th1 cells. STAT3\textsuperscript{KO} chimeras were treated with anti-CD25 or isotype control Abs as described in Figure 7. Animals were sacrificed and spleens, livers and MLNs were harvested on day 28 after alloBMT, just prior to the start of clinical symptoms for flow cytometric analysis. No difference in total CD4\textsuperscript{+} T-cell expansion between anti-CD25 and isotype control treated chimeras was observed. Anti-CD25 treatment allowed for prominent, selective expansion of IFN-\(\gamma\)-secreting graft-derived STAT3\textsuperscript{KO} CD4\textsuperscript{+} T-cells. * indicates \(p < 0.05\).