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Th1 Cells Regulate Hematopoietic Progenitor Cell Homeostasis by Production of Oncostatin M

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Summary

Regulation of hematopoietic progenitor cell homeostasis is crucial for maintenance of innate immunity and the ability of the body to respond to injury and infection. In this report, we demonstrate that progenitor cell numbers and cycling status in vivo are dramatically increased in mice deficient in Stat6 and decreased in mice deficient in Stat4, targeted mutations which also alter T helper cell polarization. Experiments using mice that have T cell restricted transgenic expression of Stat4 or Stat6 or have been in vivo depleted of T cell subsets demonstrate that $CD4^+$ T cells regulate progenitor cell activity. Injection of the Th1 cytokine Oncostatin M but not other cytokines into Stat4-deficient mice recovers progenitor cell activity to wild-type levels. Thus, T helper cells actively regulate hematopoietic progenitor cell homeostasis.

Introduction

Myeloid cells are integral components of the innate immune system. They are often the first line of defense following infection and injury. Some myeloid cells are also involved in recruiting and activating antigen-specific cells, components of the acquired immune system, at sites of inflammation. T cells can amplify the inflammatory response by additional myeloid recruitment. T helper subsets regulate distinct types of inflammation (reviewed in Abbas et al., 1996; Glimcher and Murphy, 2000). Th1 cells, which predominantly secrete IFN-γ and LTα, regulate cell-mediated immunity, which includes the recruitment and activation of neutrophils and antigen-presenting cells (macrophages and monocytes). By contrast, Th2 cells, which secrete IL-4, -5, -6, -10, and -13, mediate allergic inflammatory disease. Recruitment of myeloid cells to sites of inflammation reduces the number of cells available for innate immune system responses. These cells must then be replaced by the induction of differentiation in the myeloid stem and progenitor cell populations in bone marrow, spleen, and other hematopoietic organs. The maintenance of homeostatic levels of myeloid progenitors, as well as mature myeloid cells, requires ongoing regulation of progenitor cell populations. Many cytokines, including some that are secreted by Th1 and Th2 cells, have been shown to have positive and negative regulatory effects on myeloid progenitor cells (Bacon et al., 1983; Greenberger et al., 1984; Broxmeyer et al., 1988; Bonomo et al., 1990; Sonoda et al., 1990; Eng et al., 1995; Lai et al., 1996). Despite this, there has been little evidence that Th cells can directly regulate myeloid progenitor cell activity in vivo.

The commitment to a T helper cell differentiated state is determined by members of the signal transducer and activator of transcription (STAT) family of proteins. Stat4 is expressed primarily in lymphoid and myeloid cells and in mice is activated by IL-12 and the related cytokine IL-23 (Yamamoto et al., 1994; Zhong et al., 1994; Bacon et al., 1995; Jacobson et al., 1995; Oppmann et al., 2000). Stat4-deficient mice lack IL-12-stimulated responses, including the induction of IFN-γ, and have impaired development of Th1 cells (Kaplan et al., 1996b; Thierfelder et al., 1996). In contrast, Stat6 is ubiquitously expressed and is primarily activated by IL-4 and IL-13 (Hou et al., 1994; Lin et al., 1995; Quelle et al., 1995). In Stat6-deficient mice, the ability of T cells to differentiate into normal Th2 cells is greatly decreased (Kaplan et al., 1996a; Shimoda et al., 1996; Takeda et al., 1996). The lack of Th cell subsets in vivo has allowed these mice to be used as models for examining the roles of Th1 and Th2 cells in diseases and other immunological responses in vivo (Kaplan et al., 1998a, 1998b; Kuperman et al., 1998; Tarleton et al., 2000).

In this report, we demonstrate that deficiencies in Stat4 and Stat6 also dramatically affect myeloid progenitor cell numbers and their cycling activity. In the absence of Stat4, progenitor cell numbers and cycling status are decreased. Conversely, in the absence of Stat6, progenitor cell numbers and cycling status are increased. Expression of Stat4 restricted to T cells of Stat4-deficient mice, or of a constitutively active mutant of Stat6 restricted to T cells of Stat6-deficient mice, returns the progenitor activity to normal. Depletion of $CD4^+$ cells in Stat6-deficient mice reduces progenitor cell activity to normal or below normal levels while having no effect on Stat4-deficient mice. The phenotype in Stat4-deficient mice correlates with decreased secretion of Oncostatin M (OSM) in Stat4-deficient Th1 cells, and in vivo injection of OSM into Stat4-deficient mice results in normal levels of hematopoietic progenitor cell numbers and levels of cycling. Together, this data suggests that T helper subsets actively regulate myeloid progenitor cell homeostasis.

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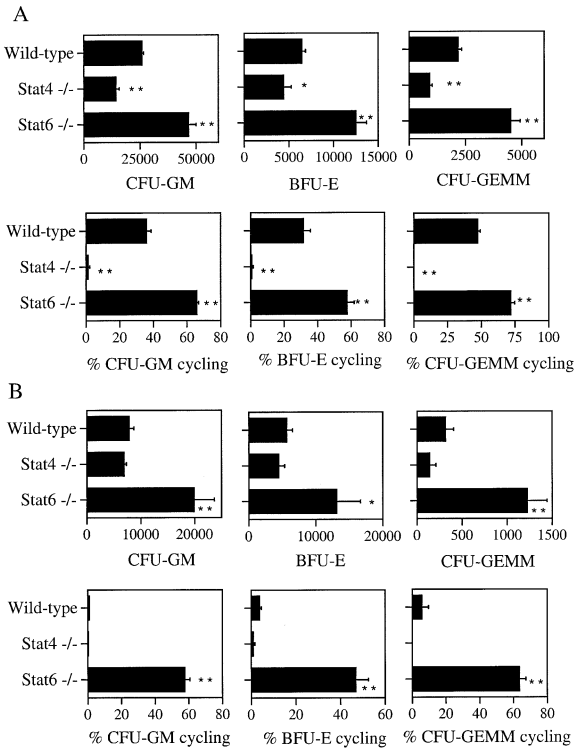

Figure 1. Altered Homeostasis of Hematopoietic Progenitor Cells in Stat4- and Stat6-Deficient Mice

Bone marrow (A) or spleen (B) cells were analyzed for hematopoietic progenitor cell numbers (top) and cycling status (bottom) from mice of the indicated genotype. Results are the average ± SEM of eleven Balb/c, seven Stat4-deficient, or seven Stat6-deficient mice, all individually assessed from a total of two separate experiments. Asterisks indicate significant difference from wild-type mice: **, $p < 0.0025$; *, $p < 0.03$.

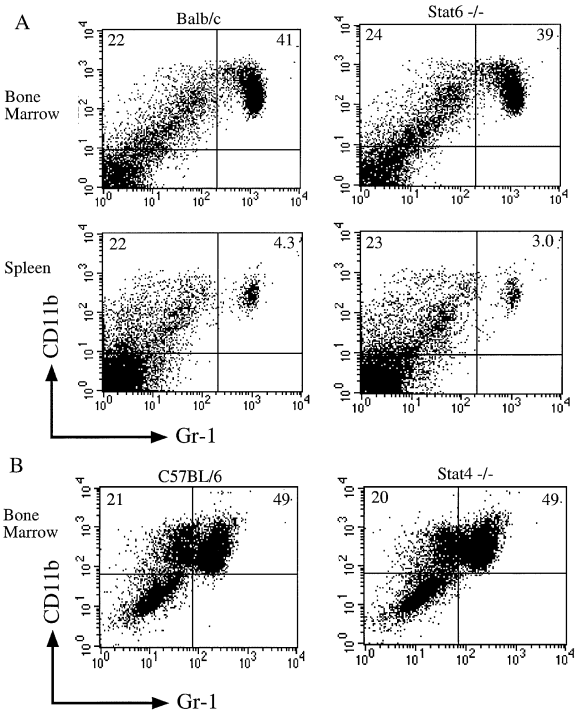

Figure 2. Normal Levels of Splenic and Bone Marrow Myeloid Cells in Stat4- and Stat6-Deficient Mice

(A) Balb/c wild-type and Stat6-deficient spleen and bone marrow were analyzed by flow cytometry for the levels of $CD11b^+$ and $Gr\text{-}1^+$ cells. Numbers indicate the percentages of cells in each quadrant and are representative of triplicate analysis.

(B) C57BL/6 wild-type and Stat4-deficient bone marrow were analyzed by flow cytometry for the levels of $CD11b^+$ and $Gr\text{-}1^+$ cells as above.

Results

Altered Hematopoietic Progenitor Cell Homeostasis in Stat4- and Stat6-Deficient Mice

Since Stat4- and Stat6-deficient mice have dramatic alterations in their in vivo T helper subset profile, we wanted to determine whether these changes had any affects on the pool of hematopoietic progenitor cells. To test this, we performed ex vivo hematopoietic progenitor cell colony-forming assays on bone marrow and spleen cells from wild-type Balb/c and Stat4- or Stat6-deficient mice on the Balb/c genetic background. Total cells from bone marrow and spleen are plated in the presence of a cytokine cocktail that stimulates optimal colony formation from progenitor cells present in the organs. This allows assessment of the absolute number of subtypes of progenitor cells in vivo by differentiating the progenitor cells to colonies in vitro. Pulse exposure of cells with high specific activity ^{3}H-thymidine prior to plating allows assessment of the proportion of progenitor cells proliferating in vivo. Figure 1A demonstrates that in the absence of Stat4, absolute numbers of granulocyte-macrophage (CFU-GM), erythroid (BFU-E), and multipotential (CFU-GEMM) progenitor cells present in the bone marrow are significantly decreased compared to control mice. The cycling status of Stat4-deficient progenitor cells is also significantly decreased compared to wild-type controls such that these cells are in a slow or noncycling state (Figure 1A). In contrast, absolute progenitor cell numbers from Stat6-deficient bone marrow are increased in number and have greatly increased percentages of cycling cells (Figure 1A). In the spleen, the differences between wild-type and Stat4-deficient cells are reduced, though there is still a trend toward reduced numbers and cycling (Figure 1B). Stat6-deficient cells, however, show significant increases in absolute progenitor cell numbers and striking increases in the percentages of cells that are cycling in spleen (Figure 1). These studies demonstrate that the absence of Stat4 or Stat6 in vivo alters the homeostasis of hematopoietic progenitor cells in bone marrow and spleen.

To determine if the alterations in hematopoietic progenitor cell activity results in altered levels of mature cells in these organs, we analyzed the levels of $Gr\text{-}1^+$ and $CD11b^+$ cells by flow cytometry. Figure 2A demonstrates that similar levels of $CD11b^+$ and $CD11b^+/Gr\text{-}1^+$ cells are observed in Balb/c wild-type and Stat6-deficient bone marrow and spleen populations. There are also no significant differences observed between C57BL/6 wild-type and Stat4-deficient bone marrow cells (Figure 2B). These results suggest that despite the altered homeostasis of progenitor cells in the spleen and bone marrow

of Stat4- and Stat6-deficient mice, the homeostasis of mature myeloid cells occurs normally in the absence of Stat4 and Stat6.

Altered Hematopoietic Progenitor Cell Activity in IL-4-, IL-12-, and IL-13-Deficient Mice

We next wanted to determine the mechanism of Stat4- and Stat6-dependent hematopoietic progenitor cell regulation. We first determined if mice lacking in the cytokines that activate Stat4 and Stat6 have similar phenotypes in the progenitor cell populations. IL-12 has been shown to be a myeloid progenitor cell growth factor, though most of its positive effects on growth are negated by the simultaneous induction of IFN-γ secretion that is a negative regulator of progenitor growth (Jacobsen et al., 1993; Eng et al., 1995). Similarly, IL-4 and IL-13 have been shown to be positive factors (Broxmeyer et al., 1988; Sonoda et al., 1990), a phenotype that apparently conflicts with that observed in the Stat6-deficient mice. To test this, we performed assays of progenitor cell numbers and cycling status on mice deficient in IL-12 p40, IL-4, or IL-13. The phenotype of IL-12 p40-deficient mice was remarkably similar to that seen in Stat4-deficient mice (Figure 3A). Numbers and cycling of progenitors in the bone marrow were significantly reduced from control values. This result suggests that the phenotype observed in Stat4-deficient mice correlates with the biological function of IL-12 p40, which may include IL-12 and IL-23 activity, and not novel Stat4-activating cytokines.

The phenotype of progenitor cells in the IL-4-deficient mice, however, was completely dissimilar to the observed phenotype of progenitor cells in the Stat6-deficient mice. In bone marrow, progenitor numbers and cycling were significantly decreased compared to control mice (Figure 3A), agreeing with previously published reports. To determine if IL-13 has an effect on hematopoietic progenitor cells in vivo, we next analyzed IL-13-deficient mice. Importantly, because of the linkage of the IL-4 and IL-13 genes, IL-13-deficient mice also have decreased endogenous IL-4 production, leading to a profound deficiency in Th2 activity (McKenzie et al., 1998b; Guo et al., 2001). Because the IL-13-deficient mice are on a mixed C57BL/6 $\times$ 129 genetic background, we examined progenitor activity in C57BL/6 and 129/Sv mice as controls. IL-13-deficient mice had significantly increased progenitor numbers and cycling in the bone marrow (Figure 3B) and spleen (Figure 3C) compared to either wild-type background strain. Thus, an in vivo environment that lacks IL-13, has reduced IL-4, and might have an increased Th1 environment produces a hematopoietic progenitor cell phenotype that is similar to the Stat6-deficient phenotype.

T Helper Cells Regulate Hematopoietic Progenitor Cell Activity

We next wanted to test whether it was possible for IL-4, IL-13, and IL-12 to be exerting Stat4- and Stat6-dependent functions in progenitor populations. While Stat6 is ubiquitously expressed (Hou et al., 1994; Quelle et al., 1995), constitutive Stat4 expression is restricted to lymphoid tissues and can be induced in myeloid cells (Yamamoto et al., 1994; Zhong et al., 1994; Frucht et al., 2000). The Stat4 mRNA has also been observed in bone marrow and myeloid cell lines (Yamamoto et al., 1994). We performed immunoblot analysis of lymph node and bone marrow cell extracts for the expression of Stat4 and Stat6 (Figure 4A). As expected, Stat6 is expressed at equivalent levels in both tissues. However, while expression of Stat4 was detected in lymph node extracts, it could not be detected in bone marrow extracts (Figure 4A). We also saw no expression of Stat4 in bone marrow cells enriched for the lineage-negative, ckit$^+$ population (data not shown). Thus, while IL-12 may have some direct effects on colony-forming cells, the lack of detected expression of Stat4 suggests that these functions must be independent of Stat4. These results suggest that Stat4 has indirect effects on the hematopoietic progenitor cell populations, possibly through the ability to regulate T helper (Th) cell differentiation.

The reciprocal phenotypes of progenitor cell activity in Stat4- and Stat6-deficient mice are reminiscent of the alterations in T helper cell subset development. It is

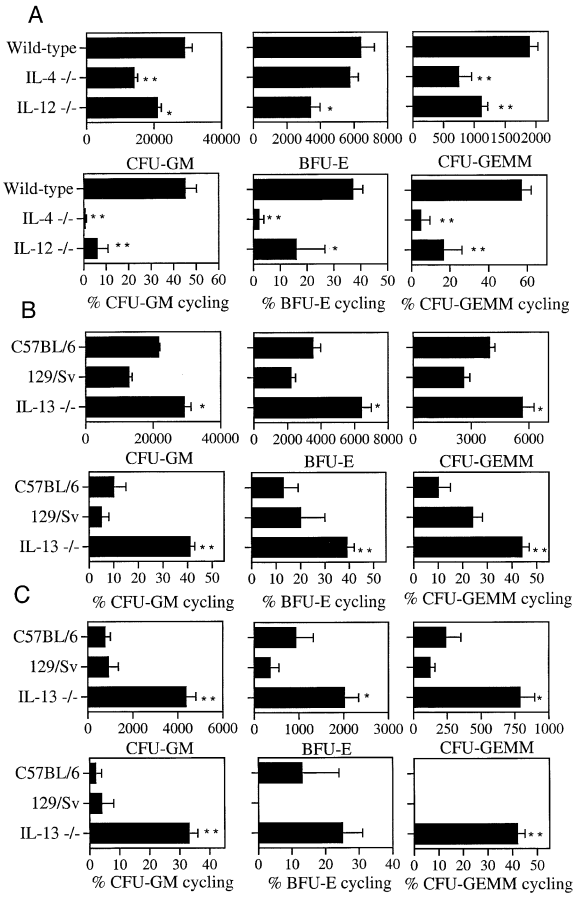

Figure 3. Altered Homeostasis of Hematopoietic Progenitor Cells in IL-4-, IL-12-, and IL-13-Deficient Mice

Bone marrow cells (A and B) and spleen cells (C) were analyzed for hematopoietic progenitor cell numbers (top) and cycling status (bottom). Results are the average $\pm$ SEM of three individually assessed C57BL/6, 129/Sv, Balb/c, IL-4-deficient, or IL-12-deficient mice and five individually assessed IL-13-deficient mice. Asterisks indicate significant difference from wild-type mice in (A) and from C57BL/6 mice in (B) and (C): **, $p < 0.005$; *, $p < 0.05$.

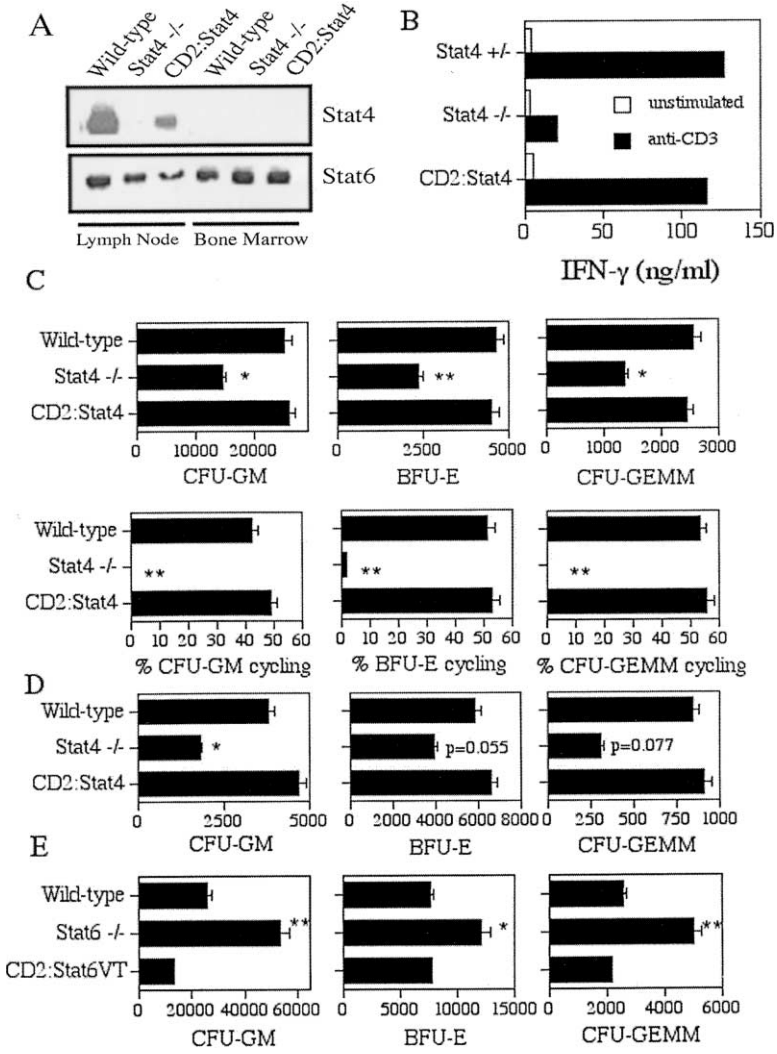

Figure 4. T Cell Expression of Stat4 or Stat6 Recovers Normal Hematopoietic Progenitor Cell Homeostasis on the Stat4- or Stat6-Deficient Background

(A) Total cell extracts (100 μg) from wild-type, Stat4-deficient, or CD2:Stat4 lymph node and bone marrow were analyzed by immunoblot for Stat4 and Stat6 expression.

(B) $CD4^+$ cells from $Stat4^{+/-}$, Stat4-deficient, or CD2:Stat4 were cultured under Th1 promoting conditions for 6 days. Cells were washed and restimulated with anti-CD3. Twenty-four hour supernatants were analyzed for IFN-γ levels by ELISA.

(C and D) Bone marrow (C) or spleen (D) cells were analyzed for hematopoietic progenitor cell numbers (top) and cycling status (bottom). Results are the average ± SEM of three individually assessed C57BL/6, Stat4-deficient, or CD2:Stat4 mice.

(E) Bone marrow cells were analyzed for hematopoietic progenitor cell numbers in mice with the wild-type, Stat6-deficient mice, or CD2:Stat6VT genotype. Asterisks indicate significant difference from wild-type mice: **, $p < 0.005$; *, $p < 0.05$.

possible that reciprocal Th subsets are regulating hematopoietic progenitor cell homeostasis. To directly test whether T cell-restricted expression of Stat4 was sufficient to recover normal progenitor cell activity, we generated transgenic mice that express Stat4 under control of the CD2 locus control region. These transgenic mice were backcrossed to C57BL/6 mice for two generations and to $Stat4^{-/-}$ C57BL/6 for two generations to generate Stat4-deficient transgene-positive mice (CD2:Stat4). Figure 4A demonstrates the expression of transgenic Stat4 in lymph node cells of transgene-positive mice at levels somewhat less than that seen in wild-type mice. We observed no expression of the transgenic Stat4 in bone marrow supporting the specificity of expression of the transgene. With purified cells and other tissues, we primarily see expression of the transgene in T cells, with low expression in B cells and no expression detected in nonlymphoid cells. Since the transgenic mice were on the C57BL/6 background, we performed the analysis of these mice with wild-type and Stat4-deficient mice on the C57BL/6 background. To demonstrate that the transgenic Stat4 is capable of recovering endogenous Stat4 function, we differentiated purified $CD4^+$ T cells from wild-type, Stat4-deficient, and CD2:Stat4 mice. After 6 days in culture, cells were washed and left unstimulated or stimulated with anti-CD3. After 24 hr, supernatants were recovered and tested for levels of IFN-γ secretion. As reported, IFN-γ secretion is dramatically reduced in the Stat4-deficient cultures (Figure 4B). Importantly, transgenic expression of Stat4 recovers the ability of CD2:Stat4 cells to become IFN-γ-secreting Th1 cells (Figure 4B). We then repeated the analysis of hematopoietic progenitor cell characteristics in wild-type, Stat4-deficient, and CD2:Stat4 mice, as performed in Figure 1. As seen previously, Stat4 deficiency leads to decreases in bone marrow and spleen progenitor numbers even more dramatically than seen from animals on the Balb/c genetic background (Figures 4C and 4D compared to Figure 1). Cycling of progenitors in the bone marrow was also dramatically reduced (Figure 4C). Importantly, T cell-specific expression of Stat4 in the CD2:Stat4 mice was sufficient to restore all parameters to wild-type levels (Figures 4C and 4D).

To test the converse, whether T cell-specific expression of Stat6 can restore increased progenitor activity to normal, we generated mice that express a constitutively active form of Stat6 (termed Stat6VT) under control of the CD2 LCR (Daniel et al., 2000). These mice will be

described in detail elsewhere. Transgene-positive mice were mated to Stat6-deficient mice to generate Stat6VT-expressing, Stat6-deficient mice (CD2:Stat6VT). While Stat6-deficient mice have increased numbers of hematopoietic progenitors, expression of Stat6VT in the T cell compartment is sufficient to reduce progenitor numbers to normal or below normal (Figure 4E). Thus, the expression of Stat4 or Stat6 in T cells regulates colony-forming cell activity in vivo.

To continue to characterize the role of T helper cells in regulation of hematopoietic progenitor cells, we hypothesized that depletion of $CD4^+$ cells in vivo would alter the progenitor cell phenotypes observed in the STAT-deficient mice. In vivo depletion of $CD4^+$ cells from Stat6-deficient mice should lead to a decrease or normalization of hematopoietic progenitor cell activity following the elimination of the Stat4-dependent population responsible for the phenotype. To test this, Stat6-deficient mice were injected intraperitoneally with PBS or 200 μg anti-CD4 or anti-CD8 every 3 days for 9 days. As a control, wild-type Balb/c mice were injected at the same times with PBS. Twenty-four hours after the last injection, mice were sacrificed for analysis as in Figure 1. FACS analysis demonstrated no significant differences in the splenic CD4 and CD8 populations of wild-type and Stat6-deficient mice. Percentages of splenic $CD4^+$ cells in anti-CD4-treated Stat6-deficient mice were greater than 97% depleted, though importantly the percentage of Lin-Sca-1^+c-kit^+ cells in the bone marrow was not affected (data not shown). Similarly, anti-CD8-treated Stat6-deficient mice showed 98% depletion of splenic $CD8^+$ cells. These T cell-depleted mice were then used for hematopoietic progenitor cell analysis as described above. In the bone marrow, $CD4^+$ cell depletion decreased progenitor cell numbers to levels indistinguishable from wild-type cells (Figure 5A, top). Treatment with anti-CD8 did not have as dramatic an effect, and only CFU-GM were significantly below numbers of colonies in PBS-treated Stat6-deficient mice. Cycling in the bone marrow was reduced below wild-type levels in anti-CD4-treated Stat6-deficient mice and was unaffected by treatment with anti-CD8 (Figure 5A, bottom). In the spleen, the results were even more dramatic. Anti-CD4 treatment of Stat6-deficient mice reduced hematopoietic progenitor numbers to wild-type or below wild-type levels (Figure 5B, top). Treatment with anti-CD8 increased progenitor numbers above that seen in PBS-treated Stat6-deficient mice. Strikingly, anti-CD4 treatment of Stat6-deficient mice completely abolished the levels of cycling cells, while anti-CD8 treatment had no effect (Figure 5B, bottom). To demonstrate that $CD4^+$ cell depletion was only affecting a positive and not a negative factor, we also performed CD4 depletions on wild-type and Stat4-deficient mice. As in the Stat6-deficient mice, CD4 depletion in wild-type mice also reduced both progenitor numbers and cycling in the bone marrow (Figure 5C). In contrast, CD4 depletion of Stat4-deficient mice had only minimal and statistically insignificant effects on progenitor numbers or cycling in the bone marrow (Figure 5C). Together these data suggest that Th cells positively regulate hematopoietic progenitor cell activity and alterations in the Th subset profile within mice can dramatically affect progenitor numbers and cycling status in the spleen and bone marrow.

Oncostatin M Stimulates Progenitor Activity

If T helper subsets are regulating hematopoietic progenitor cell activity, it seems likely that this is due to the Th1-specific secretion of a cytokine. To determine if Stat4 was required for the expression of a cytokine required for normal colony-forming cell activity, we performed ribonuclease protection analysis of cytokine expression in wild-type and Stat4-deficient Th1 cultures. IL-3 and GM-CSF have been described as being secreted by both Th1 and Th2 subsets (Mosmann et al., 1986). Expression of both of these cytokines was observed in wild-type Th1 cells, and expression was not affected by the absence of Stat4 (Figure 6A). We also observed expression of M-CSF and IL-6 in wild-type Th1 cells. Expression of M-CSF was reduced in the absence of Stat4 (Figure 6A). However, it is unlikely that M-CSF is responsible for the phenotype, since it does not have effects on all the colony-forming cells studied and since expression was also observed in Th2 cells (data not shown). Other cytokines which are known to have an affect on many types of colony-forming cells, including IL-11, LIF, G-CSF, SCF/SLF, and Flt3L, were not expressed in Th1 or Th2 cells (Figure 6A and data not shown). Another cytokine, OSM, is known to affect hematopoietic progenitor cell activity and has recently been described as preferentially expressed in human Th1 cells (Rogge et al., 2000). To determine if OSM was also expressed in murine Th1 cells and if that expression was Stat4 dependent, we differentiated $CD4^+$ T cells under Th1- or Th2-promoting conditions. After 6 days in culture, cells were washed and restimulated with anti-CD3. Supernatants were collected after 72 hr and tested by ELISA for the level of OSM. OSM could be detected in wild-type Th1 cells. The levels of OSM detected in supernatants of Stat4-deficient cultures were greatly reduced compared to wild-type cells (Figure 6B). Minimal OSM secretion was detected in wild-type Th2 cultures though considerably higher levels were detected in Stat6-deficient Th2 cultures (Figure 6B). Stimulation of primary $CD4^+$ cells with anti-CD3 produced slightly lower levels of OSM from Stat4-deficient cells than from wild-type cells at several time points (Figure 6C). To determine if regulation of OSM in Th subsets was transcriptional, we differentiated $CD4^+$ Th subsets as above and isolated total RNA after 24 hr of stimulation. OSM expression was inducible by anti-CD3 in Th1 but not Th2 cultures (Figure 6D), supporting the ELISA data and agreeing with Rogge et al. (2000). In contrast to that report, however, we did not observe IL-12 (data not shown) or IL-12 + IL-18 inducible OSM expression in murine wild-type Th1 cells (Figure 6D). Stat6-deficient Th2 cultures had increased expression of OSM compared to wild-type Th2 cells. In contrast to the ELISA results, however, OSM was still inducible by anti-CD3 in Stat4-deficient Th1 cultures. These results suggest that OSM production may be controlled both transcriptionally, as observed between Th1 and Th2 cells, and by Stat4-regulated processes that may enhance translation or secretion. Thus, OSM was considered a candidate cytokine that might be responsible for the reduced hematopoietic progenitor cell activity in Stat4-deficient mice.

To test whether replacement of OSM would correct the phenotype of Stat4-deficient mice, we injected OSM

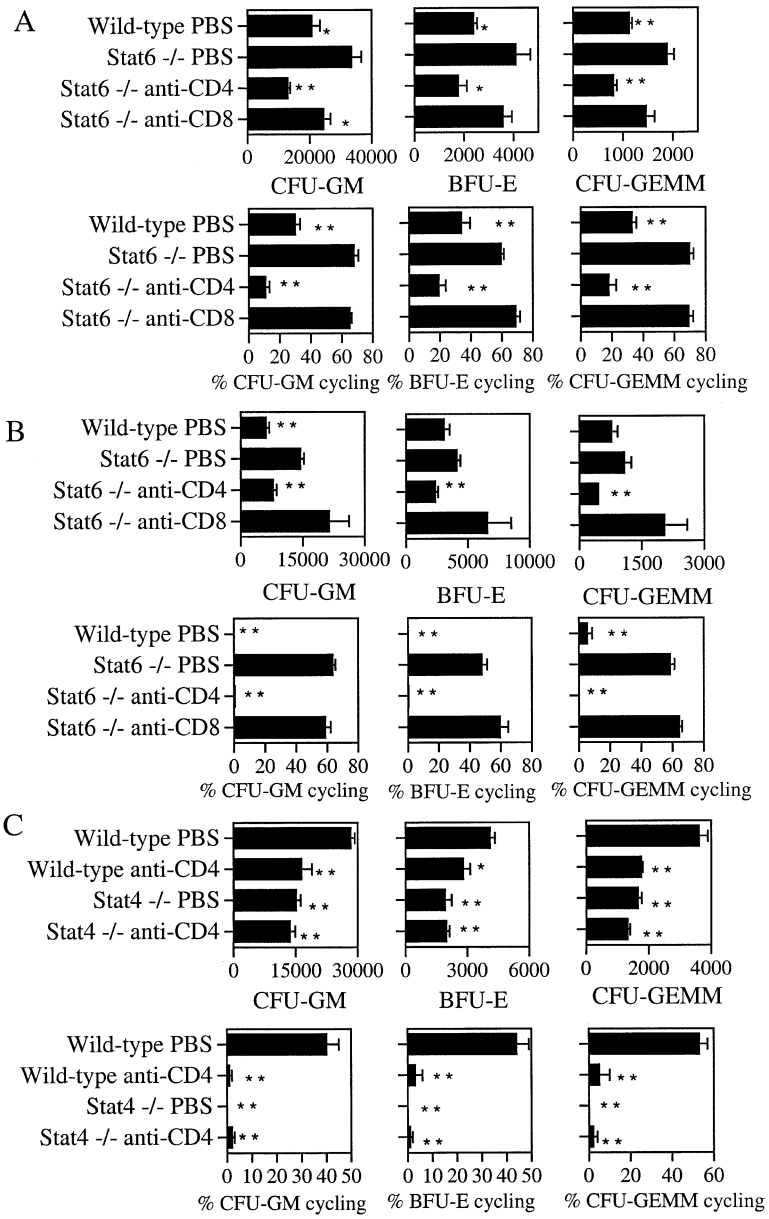

Figure 5. Depletion of CD4$^+$ Cells In Vivo Alters Hematopoietic Progenitor Cell Homeostasis in Wild-Type and Stat6-Deficient but Not Stat4-Deficient Mice

(A and B) Bone marrow (A) or spleen (B) cells were analyzed for hematopoietic progenitor cell numbers (top) and cycling status (bottom). Results are the average ± SEM of six individually assessed Balb/c, Stat6-deficient, anti-CD4-treated Stat6-deficient, or anti-CD8-treated Stat6-deficient mice from a total of two separate experiments.

(C) Bone marrow cells were analyzed for hematopoietic progenitor cell numbers (top) and cycling status (bottom) of Balb/c or Stat4-deficient mice injected with PBS or anti-CD4 as indicated. Results are the average ± SEM of 3–4 individually assessed mice per group. Asterisks indicate significant difference from Stat6-deficient mice ([A] and [B]) or wild-type mice (C): **, $p < 0.005$; *, $p < 0.05$.

or PBS as a control into wild-type and Stat4-deficient mice. As an additional control for the specificity of OSM function, we also injected IL-6, which belongs to the same cytokine family as OSM and has been shown to be involved in the regulation of hematopoietic colony-forming cells (Bernad et al., 1994). IL-6R and OSMR also share the gp130 component of their receptors (Kamiya et al., 1999; Tanaka et al., 1999). Cytokines were injected twice a day for 2 days, and mice were sacrificed for analysis of bone marrow progenitor activity 12 hr after the final injection. Analysis demonstrated that Stat4-deficient cells had decreased hematopoietic progenitor numbers and cycling (Figure 6E), as seen previously (Figures 1 and 4). Injection of IL-6 had no significant effect on either progenitor numbers or cycling status in wild-type or Stat4-deficient mice (Figure 6E) in this assay. OSM injection resulted in modest though insignificant increases in wild-type progenitor numbers. Strikingly, OSM increased Stat4-deficient progenitor numbers to levels that were indistinguishable from wild-type levels (Figure 6E). OSM also increased the cycling status of wild-type and Stat4-deficient progenitor cells to levels that were significantly increased over PBS-injected wild-type mice (Figure 6E). Thus, these data suggest that one potential regulatory mechanism of hematopoietic progenitor cell homeostasis by T helper cells is through Th1-specific secretion of OSM.

Discussion

These results describe a new role for T helper cells: regulating hematopoietic progenitor cell homeostasis. Stat4-deficient mice, which have impaired development of Th1 cells, have diminished levels of hematopoietic cell numbers and cycling. In contrast, Stat6-deficient mice have increased progenitor numbers and cycling,

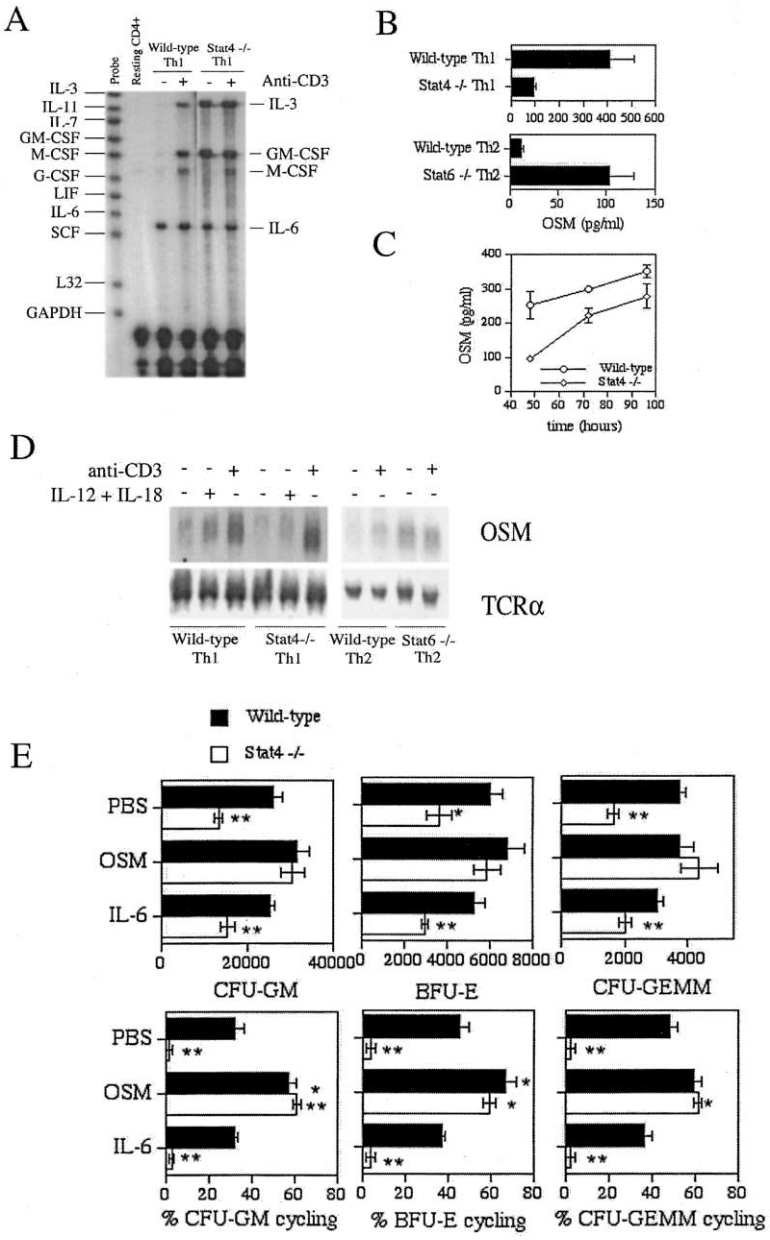

Figure 6. Oncostatin M Administration In Vivo Rescues the Activity of Stat4-Deficient Hematopoietic Progenitor Cells

(A) Wild-type or Stat4-deficient CD4$^+$ T cells were incubated for 6 days under Th1 culture conditions and left unstimulated or stimulated with 2 μg/ml plate bound anti-CD3 for 24 hr. RNA was isolated and used for ribonuclease protection analysis. The identity of the probe bands and protected bands is indicated.

(B) Th1 or Th2 cultures of the indicated genotypes were stimulated for 72 hr with plate-bound anti-CD3. Supernatants were harvested for analysis using an OSM-specific ELISA.

(C) Primary CD4$^+$ T cells were stimulated with anti-CD3. At the indicated time points, supernatants were removed and analyzed for OSM levels by ELISA.

(D) RNA was isolated from Th1 or Th2 cultures of the indicated genotype left unstimulated or stimulated with 2 μg/ml anti-CD3 or 2 ng/ml IL-12 + 25 ng/ml IL-18 as indicated for 24 hr. Following transfer to a nylon membrane, RNA was hybridized with labeled Oncostatin M cDNA. Membranes were stripped and reprobed for TCRα expression as a control.

(E) Wild-type or Stat4-deficient mice were injected with PBS, IL-6, or OSM (2 μg) twice a day for 2 days. Twelve hours after the last injection, bone marrow cells were harvested for analysis of colony-forming cell numbers (top) and colony-forming cell cycling status (bottom) as described above. Results are the average of three wild-type and four Stat4-deficient individually assessed mice in each group and are representative of two separate experiments. Asterisks indicate significant difference from wild-type mice: **, $p < 0.005$; *, $p < 0.05$.

with increased Th1 activity and impaired Th2 activity in vivo. We hypothesized that T helper cells may be important for regulating hematopoietic progenitor cell activity in vivo. To test this, we used mice that had T cell-specific transgenic expression of Stat4 on a Stat4-deficient background or a constitutively active Stat6 on a Stat6-deficient background. These mice had normal levels of progenitor activity. Furthermore, in vivo depletion of CD4$^+$ cells but not CD8$^+$ cells reduced the level of progenitor activity in Stat6-deficient mice to normal or below normal levels. In an examination of cytokines that might be responsible for this phenotype, we observed Oncostatin M secretion reduced in Stat4-deficient Th1 cultures. Moreover, the diminished in vivo Th1 responses of Stat4-deficient mice make it likely that the levels of OSM in vivo would be lower and comparable to Th2 levels. Injection of OSM in vivo recovered wild-type levels of progenitor numbers and cycling in Stat4-deficient mice. These data are consistent with a hypothesis wherein T helper cells are actively involved in regulating hematopoietic progenitor cell homeostasis.

There are several lines of evidence supporting our hypothesis that the role of Stat4 and Stat6 in regulation of hematopoietic progenitor cells is indirect, affecting Th cell polarization. First, gene-deficient mice lacking in cytokines involved in T helper differentiation had similar defects. IL-12 p40-deficient mice had a similar phenotype to Stat4-deficient mice. IL-13-deficient mice, which also have reduced IL-4 production and presumably an increased Th1 environment, had a phenotype similar to the Stat6-deficient mice. Second, we found no expression of Stat4 in primary bone marrow or purified lineage-negative, c-kit$^+$ cells (Figure 4). If these cells do not express detectable Stat4, it is unlikely that they are di-

rectly affected by Stat4 deficiency. Third, T cell-specific transgenic expression of Stat4 or Stat6 recovers progenitor cell function. The transgene that directs expression of Stat4 and Stat6 is based on the CD2 locus control region (Zhumabekov et al., 1995). In addition to T cells, CD2 can be expressed at low levels on B cells and NK cells but has not been detected on nonlymphoid cells. We also did not detect expression of the transgenic Stat4 in bone marrow cells or cells of nonlymphoid tissues (Figure 4 and data not shown). Fourth, in vivo depletion of $CD4^+$ cells eliminates the hyperactivity of hematopoietic progenitor cells in Stat6-deficient mice and reduces progenitors in wild-type mice (Figure 5). Finally, the injection of a Th1 cytokine into Stat4-deficient mice increases hematopoietic progenitor cell activity to wild-type levels (Figure 6). Together, these data support a model in which Th1 cells regulate hematopoietic progenitor cell numbers and cycling status.

It is likely that progenitor cell activity is regulated by many cell types other than Th cells. In addition to the positive function of Th1 cells on progenitor cells we have described, it is possible that Th2 cells might also exert a negative effect on hematopoietic progenitor cell activity. However, we think a regulatory role for Th2 cells is unlikely. First, OSM was able to recover progenitor cell activity in Stat4-deficient mice in vivo in the absence of manipulation of the existing in vivo Th2 populations. Additionally, IFN-γ-deficient mice, which also develop strong Th2 responses, have increased numbers of colony-forming cells (Murray et al., 1998), suggesting that an active Th2 population by itself does not result in decreased progenitor cell activity. In vivo depletion of $CD4^+$ cells in Stat4-deficient mice did not increase progenitor numbers or cycling, suggesting that there is not a Th suppressor factor in these mice. Furthermore, Th2 cytokines are not known to have functions that directly suppress progenitor activity. IL-4 is a growth factor, as demonstrated both in gene-deficient mice (Figure 3) and in vivo injections (data not shown). While IL-13-deficient mice have increased progenitor numbers, IL-13 had positive effects when injected in vivo and did not have any effects on progenitor colony formation in in vitro assays of four different strains of mice (data not shown). Thus, a factor in Th1 cells is responsible for the maintenance of hematopoietic progenitor cell homeostasis.

The differences in the progenitor cell phenotypes between the IL-4- and Stat6-deficient mice can be explained in several ways. IL-4 clearly functions as a growth factor for hematopoietic progenitors both in vitro (Broxmeyer et al., 1988) and in vivo (Figure 3 and data not shown). Some IL-4-induced growth affects, as well as protection from apoptosis, may signal through molecules distinct from Stat6, including IRS proteins (Nelms et al., 1999). In IL-4-deficient mice, any IL-4-activated pathways would be absent, producing the phenotype observed in vivo. In contrast, the Stat6-deficient mice lack particular IL-4-stimulated functions, including Th2 generation. The positive and Stat6-independent effects of IL-4 on the progenitor populations may still be activated in Stat6-deficient mice, wherein increased Th1 activity would also increase progenitor activity. Gene targeting of IL-13 also decreases IL-4 levels (McKenzie et al., 1998b; Guo et al., 2001), and this composite defect appears to result in a progenitor phenotype more similar to Stat6-deficient mice. Thus, hematopoietic progenitor cell function is an interesting example of a response where cytokine deficiency and STAT deficiency yield opposite phenotypes.

Murine oncostatin M was first described as an IL-3-inducible gene (Yoshimura et al., 1996). It has since been shown to have pleiotropic functions on many cell types, including hematopoietic cells (Malik et al., 1995; Clegg et al., 1996; Kamiya et al., 1999; Kinoshita et al., 1999; Wallace et al., 1999; Boileau et al., 2000). OSM has been specifically linked to the myeloproliferative disorder associated with TEL/JAK2-transduced bone marrow (Schwaller et al., 2000). Th1 cells may not be the only source of in vivo OSM production, since activated T cells and macrophages also secrete OSM (Wallace et al., 1999). While OSM injection was sufficient to increase levels of hematopoietic progenitor cell numbers and cycling to wild-type levels, it does not exclude the role of other cytokines in this process and may be acting indirectly, since OSM had little effect by itself in vitro in the absence of other growth factors. Importantly, OSM appears to be the only candidate cytokine with an expression pattern that correlates with T helper cell regulation of hematopoietic progenitor cells.

We have demonstrated that OSM is one potential component of T cell-hematopoietic progenitor cell communication that increases progenitor cell activity. It may be that stimulation of Th1 cells at sites of inflammation leads to OSM secretion. Since so many cytokines have effects on progenitor cell activity in vivo, and those cytokines can be secreted by many cells, including T cells, myeloid cells, stromal cells, and endothelial cells, an understanding of how the milieu regulates progenitor activity is not yet apparent. It is also intriguing that mature $CD11b^+$ or $Gr\text{-}1^+$ populations are not dramatically altered by the changes in the progenitor compartment of either Stat4- or Stat6-deficient mice. It is likely that the progenitor and mature cell "compartments" have separate homeostatic controls. However, we hypothesize that under immunological stress, when granulocytes and macrophages are recruited to sites of inflammation, the Stat4-deficient mice would be less capable of repopulating mature myeloid cells in various organs. Indeed, we have observed that 1 week after immunization Stat4-deficient mice have only 50% of the wild-type level of mature granulocytes present in the bone marrow (data not shown). Thus, this provides another level of control that Th1 and Th2 cells can respectively perpetuate and inhibit inflammatory responses. The role of Th cells in regulating this process will be the focus of ongoing investigation.

This report describes a regulatory role for T helper cells in hematopoietic progenitor cell homeostasis. We propose that this regulation is critical for the innate immune system to maintain its ability to effectively respond to infection and initiate wound repair.

Experimental Procedures

Mice

Generation of Stat4- and Stat6-deficient mice has been described (Kaplan et al., 1996a, 1996b), and mice were backcrossed ten generations to the Balb/c or eight generations to the C57BL/6 genetic background. The transgenic vector was generated by cloning the

human Stat4 cDNA or the Stat6VT cDNA into the CD2 locus control region (Zhumabekov et al., 1995). Stat4 and Stat6VT transgenic mice were generated by the IU transgenic facility (on a pure C3H genetic background) and backcrossed two generations to C57BL/6 mice and two generations to Stat4$^{-/-}$ C57BL/6 mice or three generations to the Balb/c Stat6$^{-/-}$ mice, respectively. IL-4-deficient mice on the Balb/c genetic background were purchased from Jackson Laboratories (Bar Harbor, Maine). IL-12 p40-deficient mice were kindly provided by Bruce Blazar. Generation of IL-13-deficient mice on a mixed C57BL/6 $\times$ 129 background has been previously described (McKenzie et al., 1998a, 1998b). Control (wild-type) mice were purchased from Jackson laboratories or Harlan Bioproducts (Indianapolis, IN).

Assay of Hematopoietic Progenitor Numbers and Cycling

Total bone marrow cells were plated at 5×10^4/ml, and total splenocytes were plated at 5×10^5/ml in 1% methylcellulose culture medium containing growth factors (30% v/v fetal bovine serum [Hyclone, Logan, UT], 1 U/ml human erythropoietin [Amgen Biologicals, Thousand Oaks, CA], 50 ng/ml murine steel factor [Immunex Corp., Seattle, WA], 5% v/v pokeweed mitogen mouse spleen cell-conditioned medium, and 0.1 mM hemin [Eastman Kodak Co.]). Colonies derived from granulocyte-macrophage (CFU-GM), erythroid (BFU-E), and multipotential (CFU-GEMM) progenitor cells were scored after 7 days of incubation in a humidified environment at 5% CO_2 and lowered (5%) O_2 as previously described (Cooper and Broxmeyer, 1991; Kim et al., 1999). CFU and BFU are expressed as the total number of colonies per femur or spleen.

Absolute numbers of progenitors per organ were calculated based on the number of viable, total nucleated cells per femur or spleen and of the number of colonies scored per number of cells plated. The percentage of progenitors in S phase was estimated by the high-specific-activity ^{3}H-thymidine-kill technique that eliminates cells in cycle from dividing in culture to form colonies (Cooper and Broxmeyer, 1991; Kim et al., 1999). Average control colony numbers, on which the percentage of progenitors in S phase were based, ranged from 30–150, 7–25, and 4–20, respectively, for CFU-GM, BFU-E, and CFU-GEMM.

Statistics were performed using a Student's t test. P values are indicated in figure legends.

In Vivo Depletion of CD4 and CD8 Cells

Hybridomas secreting anti-CD4 (GK1.5) and anti-CD8 (2.43) were purchased from ATCC (Manassas, VA). Monoclonals were purified from culture supernatants with protein-G agarose and dialyzed against PBS. Mice were injected with 200 μg antibody or PBS as indicated every 3 days for 9 days (four doses of mAb). Ten days after the first injection, mice were sacrificed for analysis. Spleens were monitored for depletion of cells by flow cytometry using FITC- and PE-labeled antibodies that do not compete for the binding sites of GK1.5 and 2.43 (BD Pharmingen, San Diego, CA). Analysis of the bone marrow demonstrated that depletion of CD4$^+$ cells did not affect the percentages of Lin-Sca-1$^+$c-kit$^+$ cells in the bone marrow. To further demonstrate that CD4$^+$ cell depletion did not directly affect progenitor cells, it was also determined that in vitro depletion of bone marrow cells with anti-CD4 + complement did not affect the development of colonies in the assay.

Analysis of CD2:Stat4 and CD2:Stat6VT Transgenic Mice

Mice were typed for the presence of the transgene either by Southern using the Stat4 or Stat6 cDNA as a probe or by PCR. The presence of wild-type and Stat4- or Stat6-targeted alleles were typed by PCR. Western analysis was performed by using 100 μg of total cellular extract and anti-Stat4 polyclonal specific for the C-terminal portion of Stat4 (Santa Cruz, Santa Cruz, CA). As a control, immunoblots were reprobed with anti-Stat6 (Santa Cruz). Differentiation and analysis of Th1 cultures was performed as described (Zhang et al., 2000). Complete characterization of the Stat4 transgenic and Stat6 transgenic mice will be described elsewhere.

Analysis of Cytokine Gene Expression and Production

Th1 cultures were generated as described (Zhang et al., 2000). Cultures were left unstimulated or stimulated with plate-bound anti-CD3 as noted. RNA was isolated 24 hr following stimulation and used for an RNase protection assay using the probe template mCK-4 (BD Pharmingen). OSM levels in supernatants were determined by ELISA using specific polyclonal antibodies (R&D Systems, Minneapolis, MN) and recombinant OSM as the standard. Oncostatin M expression was detected by Northern analysis as described (Zhang et al., 2000).

In Vivo Cytokine Injections

Oncostatin M (R&D Systems), IL-6 (Peprotech, Rocky Hill, NJ), or PBS was injected intraperitoneally twice daily (2 μg/dose) for 2 days. Mice were sacrificed 12 hr after the final injection, and colony-forming cell numbers and cycling status were analyzed as above.

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