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Stat6-Dependent and -Independent Pathways for IL-4 Production

Mark H. Kaplan,* Andrea L. Wurster, † Stephen T. Smiley, 2† and Michael J. Grusby 3‡

Stat6 has been shown to have a crucial role in the IL-4-dependent differentiation of Th2 cells. In this report, we explore whether in vitro Th2 differentiation driven by altered costimulatory signals or Ag dose is Stat6 dependent. We find that blocking B7-1 signaling in vitro promotes the differentiation of IL-4-secreting Th2 cells in wild-type but not Stat6-deficient T cell cultures. Additionally, stimulation with peptide Ag doses that normally result in the production of Th2 cells in vitro fails to do so in cultures of Stat6-deficient cells. We also demonstrate that Stat6 is required for the in vitro differentiation of CD8⁺ T cells into IL-4-secreting cytotoxic T cell type 2 cells. However, IL-4 expression is not absolutely dependent on Stat6. We demonstrate that populations of T cells that do not require IL-4 for their development, such as NK T cells, are still competent to secrete IL-4 in the absence of Stat6. These results demonstrate that Stat6 is required for the differentiation program leading to the generation of Th2 and cytotoxic T cell type 2 cells but not for IL-4 expression in cells that do not undergo differentiation in response to IL-4. The Journal of Immunology, 1999, 163: 6536–6540.

Stat6 is a member of the STAT family of proteins, first characterized and cloned as an IL-4-activated factor (1–3). Stat6 is the only STAT protein activated by IL-4 and IL-13 and has been shown to be a crucial signaling molecule for these cytokines (4–7). B lymphocytes from mice deficient in Stat6 lack the ability both to undergo class switching to IgE and modulate expression of IL-4-dependent surface markers and have impaired proliferative responses to IL-4. Importantly, Stat6-deficient T cells stimulated with either IL-4 in vitro (5, 8) or helminthic parasites in vivo (6, 9) do not differentiate into Th2 cells secreting high levels of IL-4, IL-5, and IL-10.

The inability of Stat6-deficient T cells to become Th2 cells is likely due to a requirement for Stat6 in the activation of a differentiation genetic program. While this program is not well understood, it may involve subsequent expression of other transcription factors such as GATA-3 and c-maf, both of which have been shown to activate Th2 cytokine expression (10–12). However, this does not rule out a direct effect of Stat6 on cytokine gene transcription. Indeed, Stat6-responsive elements have been identified in the promoters of both the murine and human IL-4 genes (13, 14).

To determine whether Stat6-deficient T cells are capable of making IL-4, we employed several in vitro methods to generate IL-4-secreting T cells. In this report, we find that elimination of B7-1 signaling in vitro, which normally favors the generation of Th2 cells, fails to do so in Stat6-deficient T cells. Additionally, Ag doses that normally drive Th2 differentiation in vitro also fail to drive Stat6-deficient T cells to become IL-4-secreting Th2 cells. Furthermore, in vitro differentiation of IL-4-secreting CD8⁺ T cells (cytotoxic T cell type 2 (Tc2)) does not occur in the absence of Stat6. However, Stat6-deficient NK T cells are competent to secrete IL-4, albeit at somewhat reduced levels. These results suggest that although Stat6 is required for IL-4 dependent differentiation events in vitro, it is not required for the transcription of IL-4.

Materials and Methods

Mice
Generation of Stat6-deficient mice has been described previously (4). All mice were from the tenth backcross generation to BALB/c. Stat6-deficient mice were mated to DO11.10 TCR transgenic mice to generate Stat6-deficient DO11.10 mice (15).

Anti-IgD treatment
Wild-type or Stat6-deficient mice were injected s.c. with 100 μg each of two mAbs against IgD⁺ (FF1-4D5; Hda/1) as previously described (4, 16). Ten days following injection, splenocytes were stimulated overnight with plate-bound anti-CD3. Seven hours after anti-CD3 stimulation, the cells were analyzed for DX5 expression and IL-4 production by FACS (17). In duplicate wells, culture supernatants were tested by ELISA for IL-4 production after 24 h of anti-CD3 stimulation.

In vitro cultures
Splenocytes from wild-type or Stat6-deficient mice were stimulated in vitro with anti-CD3 and cultured in the presence of 10 μg/ml anti-IFN-γ (R4/6A2) and either 1000 U/ml IL-4 (Genzyme, Cambridge, MA) or 20 μg/ml anti-B7-1 (1G10). IL-2 (20 U/ml; Boehringer Mannheim, Mannheim, Germany) was added after 48 h, and cells were cultured for an additional 4 days. Cells were then washed and restimulated with plate-bound anti-CD3 alone for 24 h. Supernatants were tested for IL-4 and IFN-γ concentrations by ELISA.

Splenocytes from wild-type or Stat6-deficient mice were stimulated in vitro with various doses of peptide in the presence of 10 μg/ml anti-IFN-γ (R4/6A2) and either 1000 U/ml IL-4 (Genzyme, Cambridge, MA) or 20 μg/ml anti-B7-1 (1G10). IL-2 (20 U/ml; Boehringer Mannheim, Mannheim, Germany) was added after 48 h, and cells were cultured for an additional 4 days. Cells were then washed and restimulated with plate-bound anti-CD3 alone for 24 h. Supernatants were tested for IL-4 and IFN-γ concentrations by ELISA.

Splenocytes from wild-type or Stat6-deficient mice were stimulated in vitro with various doses of peptide in the presence of 10 μg/ml anti-IFN-γ. After 1 wk in culture, cells were washed and restimulated with peptide plus irradiated BALB/c splenocytes. Supernatants were collected after 48 h and tested for levels of IL-4.

Tc2 cells were differentiated by enriching for CD8⁺ cells from wild-type and Stat6-deficient spleens. The CD8⁺ cells were purified by positive selection for CD8 by using MiniMacs (Milteny) Biotec, Gladbach, Germany) columns according to the supplier’s instructions. The resulting populations (70–80% CD8⁺) were stimulated with plate-bound anti-CD3 and cultured for 1 wk in culture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked 4 Abbreviations used in this paper: Tc2, cytotoxic T cell type 2; HSA, heat stable Ag.

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Additionally, the Stat6-deficient cells produced high levels of IL-4 upon restimulation, while Stat6-deficient cells cultured with anti-CD3 under identical conditions secrete little IL-4 (4, 5) (Fig. 1). As shown previously, wild-type cells cultured with IL-4 secrete high levels of IL-4 in the presence of anti-IFN-γ and either IL-4 or anti-B7-1. After 1 wk in culture, the cells were washed and restimulated with anti-CD3 for 24 h. IFN-γ (left) and IL-4 (right) levels in culture supernatants were assessed by ELISA in duplicate. Results shown are representative of two separate experiments.

**Results**

**Stat6 is required for Th2 differentiation in vitro**

Th2 cells to a Th2 phenotype is dependent on Stat6. However, several other in vitro protocols have also been described that can drive a population of primary T cells into Th2 cells, and it has not been established whether these are strictly Stat6 dependent as well. Recent reports have suggested that costimulation of primary T cells with B7-2, rather than B7-1, will lead to the development of Th2 cells (18–24). To determine whether this process is dependent on Stat6 activation, we differentiated wild-type and Stat6-deficient splenocytes in the presence or absence of Abs that block B7-1 costimulation. After 1 wk in culture, the cells were restimulated with plate-bound anti-CD3, and supernatants were collected after 48 h for ELISA analysis.

**Stat6 is required for Tc2 differentiation in vitro**

CD8+ T cells populations have also been shown to secrete IL-4 after culture under skewing conditions and have been termed Tc2 cells (28). To determine whether Tc2 differentiation is also dependent on Stat6, we enriched CD8+ cells from wild-type and Stat6-deficient mice and cultured these cells under Tc2-skewing conditions (10 ng/ml IL-4 and 10 μg/ml anti-IFN-γ). The cells were cultured with 10 ng/ml IL-4 (Peprotech, Boston, MA) and 10 μg/ml anti-IFN-γ (PharMingen, San Diego, CA) as previously described. Cultures were supplemented with 100 U/ml IL-2 2 days and 4 days after initial stimulation. After 6 days in culture, CD8+ cells were restimulated with anti-CD3 under Tc2 conditions as above. Resulting populations were >98% CD8+. On day 14, the CD8 cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 h and analyzed for cytokine production by intracellular cytokine staining (17).

NK T cells were prepared from thymocytes of wild-type or Stat6-deficient mice enriched for heat stable Ag (HSA)low cells by treatment with J11d and complement as previously described (16). These cells were restituted with anti-CD3, and supernatants were collected after 48 h for ELISA analysis.

It has previously been shown that IL-4-stimulated differentiation of CD4+ T cells to a Th2 phenotype is dependent on Stat6. However, this method of driving Th2 differentiation is Stat6 dependent because Stat6-deficient cells cultured under these conditions failed to produce detectable IL-4 and IL-5 but did make high levels of IFN-γ (Fig. 1 and data not shown).

**FIGURE 1.** Blockade of B7-1 costimulation is unable to promote Th2 differentiation in Stat6-deficient T cells. Splenocytes from wild-type or Stat6-deficient mice were stimulated with anti-CD3 in the presence of anti-IFN-γ and either IL-4 or anti-B7-1. After 1 wk in culture, the cells were washed and restimulated with anti-CD3 for 24 h. IFN-γ (left) and IL-4 (right) levels in culture supernatants were assessed by ELISA in duplicate. Results shown are representative of two separate experiments.

**FIGURE 2.** Variation of Ag dose is unable to drive Th2 differentiation in Stat6-deficient T cells. Splenocytes from wild-type or Stat6-deficient mice were stimulated with the indicated doses of peptide Ag and APC. Culture supernatants were collected 48 h later and tested for IL-4 levels by ELISA. Fig. 2 (left) demonstrates that cell growth in response to peptide stimulation is identical in both wild-type and Stat6-deficient cultures. After 1 wk in culture, cells were restimulated with peptide Ag and APC. Culture supernatants were collected 48 h later and tested for IL-4 levels by ELISA. Fig. 2 (right) demonstrates that a specific range of peptide concentrations (0.1–100 μg/ml) will drive Th2 differentiation in wild-type cells. However, restimulated Stat6-deficient cells do not make detectable IL-4 regardless of peptide concentration, supporting the notion that Th2 cell generation by this in vitro differentiation protocol is also Stat6 dependent.
restimulated under Tc2 conditions on day 6 and then analyzed for cytokine production after PMA and ionomycin stimulation by intracellular cytokine staining on day 14. As was shown for Th2 differentiation, wild-type cells were composed of mostly IL-4- and IL-5-producing cells (Fig. 3). Interestingly, even after 2 wk in culture under Tc2-skewing conditions a significant number of cells coexpress IL-4 and IFN-γ. The Stat6-deficient cells only produced IFN-γ (Fig. 3). This demonstrates the requirement for Stat6 in Tc2 generation in vitro.

Stat6 is not required for IL-4 production

Because all in vitro methods of deriving both Th2 and Tc2 cells rely on Stat6 activation, we next asked whether any T cells in Stat6-deficient mice could secrete IL-4. CD1-dependent NK T cells were originally identified as being prompt producers of IL-4 following anti-CD3 stimulation (16, 29). To determine whether this population still produces IL-4 in the absence of Stat6, HSA<sub>low</sub> thymocytes were isolated and stimulated with anti-CD3 for 48 h. IL-4 was produced by both wild-type and Stat6-deficient cells, demonstrating that Stat6 is not absolutely required for IL-4 expression and secretion (Fig. 4). However, IL-4 secretion was some-

what lower in Stat6-deficient cultures. Intracellular cytokine staining of these cells indicated that the IL-4 producers express high levels of CD44, intermediate levels of V<sub>β</sub>8, and low levels of CD62L, supporting the notion that the IL-4-producing cells are NK T cells (data not shown). Similar results are also seen when total splenocytes are stimulated with anti-CD3 (data not shown).

Injection of wild-type mice with mAb toward IgD results in polyclonal B cell activation and increased class switching. We and others have previously shown that Stat6-deficient mice still respond to anti-IgD treatment but that class switching to IgE is abolished (4, 5). Because anti-IgD injection has previously been shown to result in IL-4 secretion, we tested whether IL-4 production could be detected from stimulated spleen cells isolated from anti-IgD-injected wild-type and Stat6-deficient mice. Ten days after injection, spleen cells were isolated and stimulated with anti-CD3. After 24 h, supernatants were collected and tested for IL-4 levels by ELISA. Both wild-type and Stat6-deficient cells produced IL-4 (Fig. 5A); again showing that Stat6 is not absolutely required for

FIGURE 3. Stat6 is required for Tc2 differentiation in vitro. Splenocytes from wild-type and Stat6-deficient BALB/c mice enriched for CD8<sup>+</sup> T cells were stimulated with plate-bound anti-CD3 in the presence of IL-4 and anti-IFN-γ. After 6 days in culture, CD8<sup>+</sup> cells were restimulated with anti-CD3 and then analyzed by intracellular cytokine staining for IL-4, IL-5, and IFN-γ on day 14. Results shown are representative of three separate experiments.

FIGURE 4. NK T cells do not require Stat6 to produce IL-4. HSA<sub>low</sub>-enriched thymocytes from wild-type and Stat6-deficient mice were stimulated with anti-CD3, and culture supernatants were collected 48 h later. IL-4 levels were determined by ELISA in duplicate. Results shown are representative of three separate experiments.

FIGURE 5. Anti-IgD-stimulated splenocytes do not require Stat6 to produce IL-4. Wild-type and Stat6-deficient mice were injected s.c. with anti-IgD. After 10 days, splenocytes were isolated and stimulated with anti-CD3. A, Supernatants from 24 h cultures were assessed for IL-4 production by ELISA. The graph represents the average of four different experiments. B, Splenocytes were activated for 7 h and then analyzed for DX5 and IL-4 expression by FACS. Results shown in B are representative of two separate experiments.
IL-4 expression. However, similar to the results with HSA\textsuperscript{low} thymocytes, IL-4 production by Stat6-deficient cells was reduced compared with wild-type cells. Intracellular cytokine staining of the splenocytes indicated that the IL-4-producing cells coexpress the NK cell marker DX5 and CD3, demonstrating that the IL-4 producers in this assay are NK T cells (Fig. 5B and data not shown). Supernatants from wild-type and Stat6-deficient cultures also had minimal levels of IL-5 and IL-10, suggesting that the T cells producing IL-4 in this assay are not Th2 cells but rather NK T cells (data not shown).

**Discussion**

Stat6 has an indispensable role in IL-4 signaling. It has recently been established that IL-4 and nematode-driven Th2 differentiation both in vitro and in vivo are dependent on the activation of Stat6 (4–6, 9). The results in this report demonstrate a central role for Stat6 following other in vitro differentiation conditions that lead to the development of IL-4-producing T cells. However, we also present evidence that Stat6 is not absolutely required for IL-4 production in all cell types in vivo. We demonstrate that IL-4 is produced from HSA\textsuperscript{low} thymocytes in vitro and NK T cells from anti-IgD-treated Stat6-deficient mice.

The dependence of alternative in vitro pathways of Th2 generation on Stat6, and therefore IL-4, may not be surprising. IL-4 has previously been shown to be able to replace absent costimulatory signals (22). TCR transgenic T cells given Th2-skewing doses of Ag also failed to differentiate into Th2 cells when incubated with anti-IL-4 (26). Thus, while the development of Th2 cells can be influenced by many factors, these factors do not circumvent the requirement for Stat6 in Th2 differentiation in vitro. The demonstration that Tc2 differentiation is also controlled by Stat6 provides the first evidence of a role for Stat6 in CD8\textsuperscript{T} cells. It further suggests that the same differentiation program that is activated during CD4\textsuperscript{T} cell differentiation is responsible for the acquisition of an IL-4-secreting phenotype in CD8\textsuperscript{T} cells. Thus, Stat6 appears to be universally required for the acquisition of an IL-4-secreting phenotype in differentiated Th2 and Tc2 cells.

The requirement for Stat6 in IL-4 gene transcription has been controversial. The identification of a Stat6-responsive element in the IL-4 promoter suggested that IL-4 could activate its own promoter in a positive feedback loop (13, 14). However, recent reports showing IL-4 production from IL-4R\textsuperscript{−/−} NK T cells (30) and a lack of effect of IL-4 stimulation on IL-4-producing Th2 cells (31) have argued against a crucial role for Stat6 in IL-4 gene regulation. Our results, as well as other recent reports, support this conclusion because IL-4 production can be detected from Stat6-deficient NK T cells and Stat6-deficient mast cells as well (data not shown) (32, 33). However, we do reproducibly see a reduced level of IL-4 production from Stat6-deficient cells. This may be due to a direct effect of Stat6 on IL-4 transcription, but could also be due to developmental defects in these cells caused by the absence of Stat6 in vivo.

In this report, the IL-4-producing cells identified in the Stat6-deficient mice appear to be composed of NK T cells. As stated earlier, the IL-4-producing cells in anti-IgD-injected mice express the NK marker DX5, and, although DX5 is not an appropriate marker for thymic NK T cells, the IL-4-expressing J11d\textsuperscript{low} thymocytes expressed other markers characteristic of NK T cells (34). In a previous report, we also demonstrated that a large portion of the IL-4-producing cells in anti-IgD-treated mice express DX5 (35). Additionally, the IL-4-producing cells from anti-IgD-treated mice did not produce detectable amounts of IL-5 or IL-10, suggesting that they are not classical Th2 cells. However, T cells from CD1-deficient mice that lack CD1 restricted NK T cells have normal responses to anti-IgD (data not shown) (16, 36). Also, in a recent report it was demonstrated that MHC class II-deficient mice are unable to make IL-4 in response to anti-IgD, which suggests that the IL-4-producing cells are class II-restricted T cells (37). Therefore, it is possible that some of the IL-4-producing NK T cells found after anti-IgD treatment are not CD1 dependent and could even be class II-restricted (34). Alternatively, class II MHC could be necessary for the NK T cell response indirectly. Regardless, we have demonstrated here that NK T cells from Stat6-deficient mice are capable of producing IL-4.

In this report, we have demonstrated that the generation of IL-4-secreting Th2 cells in vitro is critically dependent on Stat6 signaling. There is increasing evidence, though, that alternative Stat6-independent pathways of Th2 cell generation exist in vivo. This is most clearly demonstrated in BCL-6/Stat6-deficient mice (38). In these mice, the loss of the transcription factor, BCL-6, permitted the development of a Th2 inflammatory response in vivo in the absence of Stat6. Interestingly, the generation of BCL-6/Stat6-deficient Th2 cells could not be replicated under in vitro Th2-skewing conditions. This implies that a unique environment exists in vivo that is capable of driving a Th2 response independent of Stat6 signaling. Interestingly, a recent report described a human dendritic cell population that is capable of inducing Th2 differentiation in human T cells in vitro in an IL-4-independent manner (39). It will be interesting to see if an analogous cell population is found in mice and whether its development and/or function is Stat6 dependent.

These studies highlight the distinction between a direct role for Stat6 in gene regulation vs a differentiating signal that leads to gene activation. We have demonstrated that while Stat6 is absolutely required for the differentiation of precursor T cells to effector Th2 and Tc2 populations in vitro, it is not required for IL-4 gene expression per se. The identification of the genes involved in determination of the Th2/Tc2 phenotype will aid in further understanding of how IL-4 gene expression is controlled in different subsets of T cells.

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**References**

STAT6 AND IL-4 PRODUCTION


