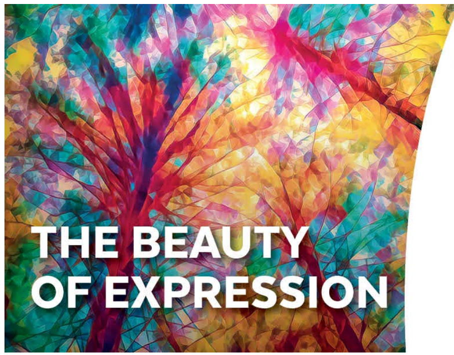

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Defining Th1 and Th2 Immune Responses in a Reciprocal Cytokine Environment In Vivo[1]

Tanuja Chitnis,* Alan D. Salama,† Michael J. Grusby,‡ Mohamed H. Sayegh,† and Samia J. Khoury[2]*

The ability of committed Th1 and Th2 cells to function in altered cytokine environments is a central issue in autoimmune and immune-mediated diseases. Therefore, it is of interest to study the ability of Th1 or Th2 cells to expand and produce cytokine reciprocal environments in vivo. Using STAT4- and STAT6-deficient mice, we studied the expansion and cytokine production of Ag-specific Th1 or Th2 cells after transfer into Th1, Th2, or wild-type recipients. Our data show that these Th1 or Th2 cells proliferated and clonally expanded normally, regardless of the in vivo cytokine environment. These data have implications for the treatment of immune-mediated diseases by immunomodulatory agents that alter the cytokine milieu in vivo. *The Journal of Immunology,* **2004, 172: 4260–4265.**

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The ability of committed Th1 and Th2 cells to function in altered cytokine environments is a central issue in autoimmune and immune-mediated diseases (1, 2). In several immune-mediated diseases (autoimmune, allergic, infectious), skewing of Th phenotype to either Th1 or Th2 is associated with pathogenicity or protection from disease. In addition, novel immunotherapies in experimental or clinical development are designed to alter the cytokine environment, and suppress activation and/or function of the pathogenic Th1/Th2 cells (3–5). Therefore, it is of interest to study the ability of Th1 or Th2 cells to function in altered environments in vivo. Indeed, there are no published data on the behavior and/or function of Th1 or Th2 cells in a reciprocal environment in vivo.

Intracellular signaling mechanisms provide the link between the binding of the cytokine with its receptor and the effect of the cytokine on cellular function. The Janus kinase and STAT (Jak/STAT)[3] family of transducer/transcription-activating factors play a critical role in the signaling of many cytokine receptors. There are four main Jak molecules and six major STAT proteins, of which typically one or two of each are associated with a particular cytokine receptor. The IL-4R is associated with Jak 1–3 and the STAT6 pathway (6, 7). The IL-12R is associated with Jak 2 and STAT3 and 4 (8), whereas the IFN-γ receptor is associated with Jak 1, 2 and STAT1α. STAT4-deficient (STAT4 knockout (STAT4KO)) mice are unable to process IL-12R-induced signals, and have a predominantly Th2 phenotype (8, 9). In contrast, STAT6-deficient (STAT6KO) mice are unable to process IL-4R-related signals, and therefore, have a Th1 phenotype (6, 7)

In this study, we used an adoptive transfer model system of TCR transgenic cells of defined Th1/Th2 phenotype to investigate the effect of the in vivo environment on the ability of Th1 and Th2 cells to proliferate and differentiate. KJ126 TCR transgenic (specific for OVA) mice (also called DO11 mice) were backcrossed onto either STAT4KO or STAT6KO backgrounds, yielding either STAT4KO-DO11 or STAT6KO-DO11 TCR transgenic mice, which have a predominantly Th2 or Th1 phenotype, respectively. STAT4KO-DO11 cells or STAT6KO-DO11 splenocytes were transferred into either STAT4KO (Th2) or STAT6KO (Th1) or wild-type (Th0) recipients. Our data show that Th1 or Th2 cells proliferated and clonally expanded normally, regardless of the in vivo cytokine environment. These data have implications for the treatment of immune-mediated diseases by immunomodulatory agents that alter the cytokine milieu in vivo.

Materials and Methods

Mice

STAT4KODO11-BALB/c, STAT6KODO11-BALB/c, STAT4KO/BALB/c, and STAT6KO/BALB/c mice were provided by M. Grusby (Harvard University, Boston, MA). Females 6–9 wk of age were used for these experiments.

Passive transfer of KJ126$^+$/CD4$^+$ cells

Spleens from nonimmunized STAT4KO-DO11/BALB/c or STAT6KO-DO11/BALB/c mice were harvested, and a single cell suspension was prepared. The cells were counted and resuspended in PBS. The cells were then double stained with anti-KJ126 and anti-CD4 Abs, as described below, and the frequency of KJ126$^+$CD4$^+$ double-positive cells was calculated. A total of 10^7 double-positive lymphocytes in 500 μl of PBS was transferred by i.p. injection to STAT4KO/BALB/c or STAT6KO/BALB/c or wild-type BALB/c mice. On day 0, the recipients were immunized with OVA peptide 323–339, as described below.

Immunization with OVA peptide

STAT4KO/BALB/c, STAT6KO/BALB/c mice, and wild-type BALB/c mice were immunized on day 0 with 1000 μg of OVA from hen egg white (OVA) (10) (Sigma-Aldrich, St. Louis, MO) in 0.025 ml of PBS and 0.025 ml of CFA containing 0.4 mg of *Mycobacterium tuberculosis* (H37Ra; Difco, Detroit, MI) s.c. in the flank and in one footpad.

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[3] Abbreviations used in this paper: Jak, Janus kinase; KO, knockout; LN, lymph node.

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FACS analysis of donor KJ126$^+$/CD4$^+$ cells

Splenocytes from either STAT4KO-DO11/BALB/c or STAT6KO-DO11/BALB/c donors were washed and resuspended in PBS to a concentration of 10^7 cells/ml. The cells were incubated on ice with 5 μg/10^6 cells of rat anti-mouse CD4 PE-conjugated Ab (Caltag Laboratories, Burlingame, CA) and mouse anti-mouse TCR clonotype (KJ126) Trichrome-conjugated Ab for 20 min (Caltag Laboratories). Cells were then washed and analyzed by flow cytometry on a FACScan (BD Immunocytometry Systems, San Jose, CA). The percentage of double-positive cells per sample group was calculated (Fig. 1).

FACS analysis of recipient KJ126$^+$/CD4$^+$ cells

Draining lymph nodes (LN) from recipient STAT4KO, STAT6KO, or wild-type BALB/c mice were harvested, and a cell suspension was prepared. The cells were incubated in DMEM culture medium with 50 μg/ml OVA peptide 323–339 for 48 h. The cells were then washed and resuspended in PBS. Using the standard protocol for intracellular staining, cells were triple stained with anti-CD4 Ab, anti-TCR clonotype (KJ126) (Caltag Laboratories), and anti-IFN-γ or anti-IL-10 or anti-IL-4 Abs (BD PharMingen, San Diego, CA). Samples were analyzed by flow cytometry on FACScan (BD Immunocytometry Systems). The frequency of KJ126$^+$CD4$^+$ cells and the frequency of cytokine-producing KJ126$^+$CD4$^+$ cells were calculated.

Cell culture

The recipient mice were harvested on day 5, draining inguinal and popliteal LN were collected, and a single cell suspension was prepared. For proliferation and cytokine measurement, cells were cultured in 96-well plates (Costar, Cambridge, MA). Cells were analyzed via FACS for precusor frequency of KJ126$^+$/CD4$^+$ double positive. Media used for proliferation and cytokine assays consisted of DMEM medium (BioWhittaker, Walkersville, MD) containing 75 mM/ml L-glutamine, 100 U/ml penicillin and streptomycin, 1 ml/100 ml medium of a 100× concentrated nonessential amino acid solution, 0.1 mM HEPES/ml, 1 mM/ml sodium pyruvate (all BioWhittaker), and 0.05 mM/ml 2-ME (Sigma-Aldrich). Cells were incubated at 37°C in humidified air containing 7% CO_2.

Proliferation assay

Cells were cultured at a concentration of 2×10^6 cells/ml and 200 μl/well with various Ag concentrations. After 48 h of culture, 1 μCi of [^{3}H]thymidine (NEN, Boston, MA) was added in 10 μl of medium to each well for another 16 h. Cells were harvested on filter mats, dried, and counted.

Cytokine ELISA

For cytokine assay, cells were cultured at a concentration of 4×10^6 cells/ml in 200 μl of medium at various Ag concentrations. Supernatants were collected after 48 h of culture. Quantitative ELISAs for IL-10 and IFN-γ were performed using paired Abs and recombinant cytokines from BD PharMingen, according to manufacturer's recommendations.

Results

Expansion of Ag-specific Th1 or Th2 cells is independent of the cytokine environment in vivo

To investigate the survival and expansion of Th1 cells in different cytokine environments, we used splenocytes from STAT6KO mice backcrossed onto KJ126 TCR transgenic mice (DO11/BALB/c mice). A total of 10^7 STAT6KO-KJ126$^+$CD4$^+$ double-positive cells (Fig. 1) was transferred into recipient wild-type BALB/c or STAT4KO or STAT6KO mice. Recipients were immunized with OVA peptide, and draining LN were harvested after 5 days. Cells were incubated with 50 μg/ml OVA peptide for 48 h, and using flow cytometry the number of KJ126$^+$CD4$^+$ cells was calculated for each recipient group. Fig. 2 shows the percentage of STAT6KO-KJ126$^+$CD4$^+$ cells in each recipient, and demonstrates that there were no significant differences in the cells' ability to expand in wild-type mice vs a Th1- or Th2-biased environment. Similarly, no differences were noted in the expansion of STAT4KO-KJ126$^+$CD4$^+$ cells transferred into similar recipients (Fig. 2). As a negative control, in the absence of transfer of KJ126$^+$CD4$^+$ cells, there was no expansion of KJ126$^+$CD4$^+$ OVA-reactive cells 5 days after immunization with OVA peptide.

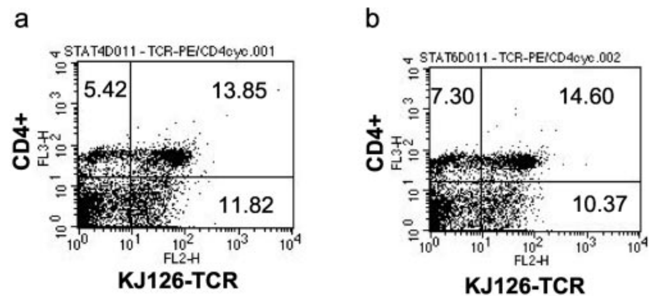

FIGURE 1. Representative FACS profile of donor KJ126$^+$CD4$^+$ cells from STAT4KO-DO11 and STAT6KO-DO11 mice. Spleens from naive STAT4KO-DO11 or STAT6KO-DO11 mice were harvested, and a cell suspension was prepared. Cells were then double stained with fluorescently labeled anti-KJ126 TCR and anti-CD4 Abs. Using flow cytometric (FACS) analysis, the frequency of KJ126$^+$CD4$^+$ double-positive cells from STAT4KO-DO11 (*a*) and STAT6KO-DO11 (*b*) was calculated. A total of 10^7 double-positive lymphocytes in 500 μl of PBS was subsequently transferred by i.p. injection to STAT4KO/BALB/c or STAT6KO/BALB/c or wild-type BALB/c mice.

Proliferative responses to Ag using [^{3}H]thymidine uptake reflect the frequency and responsiveness of Ag-specific cells harvested from the recipients. Using the same experimental design as described above, draining LN cells from immunized recipients were harvested on day 5 and exposed to OVA peptide in vitro. Proliferation of cells to OVA peptide was measured and adjusted to the number of KJ126$^+$CD4$^+$ cells recovered. Consistent with the expansion data outlined above and in Fig. 2, there were no significant differences in proliferation of STAT4 or STAT6 cells harvested from wild-type, Th1-, or Th2-biased environments (Fig. 3).

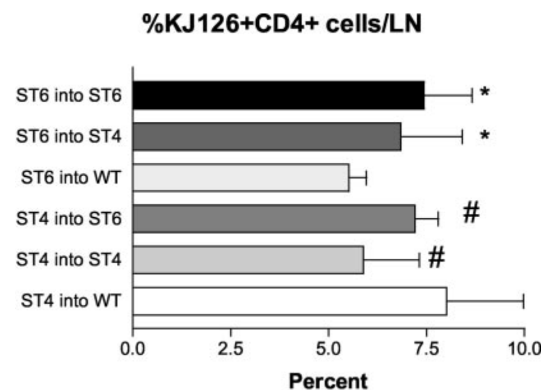

FIGURE 2. Proportion of STAT4KO or STAT6KO KJ126$^+$CD4$^+$ cells is similar in either Th0, Th1, or Th2 environments. A total of 10^7 KJ126$^+$CD4$^+$ cells harvested from either STAT4KO-DO11 or STAT6KO-DO11 mice was transferred into either wild-type BALB/c, STAT4KO, or STAT6KO hosts. Recipients were immunized with OVA in the footpad, and draining LN were harvested on day 5, and stimulated in vitro for 48 h with 50 μg/ml OVA peptide. Cells were then stained with fluorescently conjugated anti-CD4 Ab and anti-KJ126 Abs. The percentage of KJ126$^+$CD4$^+$ cells/LN in the different donor-recipient groups was calculated. Using unpaired t test, the proportion of either STAT4KO or STAT6KO-KJ126$^+$CD4$^+$ cells harvested from STAT4KO or STAT6KO mice was not significantly different from the proportion found in wild-type BALB/c hosts (p = NS; *, STAT6KO-DO11 donor; #, STAT4KO-DO11 donor).

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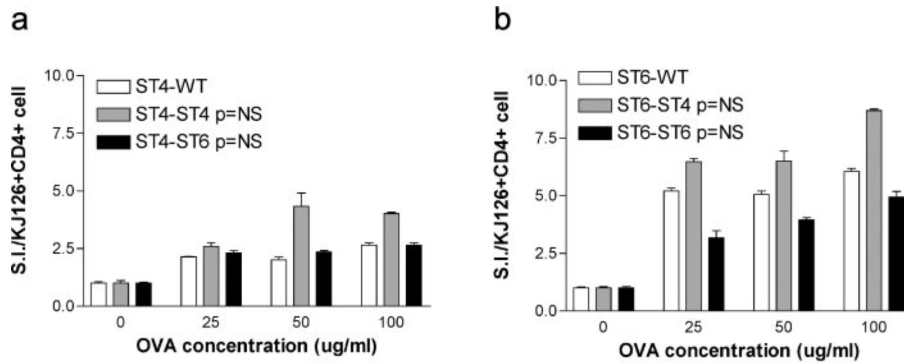

FIGURE 3. Ex vivo proliferative response to OVA by either STAT4KO or STAT6KO KJ126$^+$CD4$^+$ cells harvested from either Th0, Th1, or Th2 environments. A total of 2×10^5 cells/well from LN suspensions harvested from either wild-type, STAT4KO, or STAT6KO recipient mice, 5 days after transfer of either STAT4KO-DO11 (*a*) or STAT6KO-DO11 (*b*) donor splenocytes, was cultured with increasing concentrations of OVA peptide in vitro. The figure shows stimulation indices after 48 h of culture and addition of [^{3}H]thymidine. Using the percentage of KJ126$^+$CD4$^+$ cells/LN calculated in Fig. 2, the total number of KJ126$^+$CD4$^+$ cells/well was calculated. Results are expressed as stimulation index (S.I.)/KJ126$^+$CD4$^+$ cells. The paired *t* test revealed no significant difference between transfer of either STAT4KO-DO11 or STAT6KO-DO11 cells into Th0 environments compared with Th1 or Th2 environments (p = NS).

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Cytokine profile of committed Th1 or Th2 cells is not altered in Th0, Th1, or Th2 environments

Th1 cytokines can suppress Th2 cytokine production and vice versa. We asked, whether Ag-specific cytokine production was altered by in vivo exposure of Ag-specific Th1 or Th2 cells to Th1, Th2, or Th0 cytokine environments. Using the same experimental design as above, STAT4KO or STAT6KO-KJ126$^+$CD4$^+$ cells were transferred into recipient wild-type BALB/c, STAT4KO, or STAT6KO mice. Recipients were immunized with OVA peptide, and draining LN were harvested after 5 days. Using flow cytometry, the number of KJ126$^+$CD4$^+$ cells recovered was calculated for each recipient group. Lymphocytes were cultured with OVA

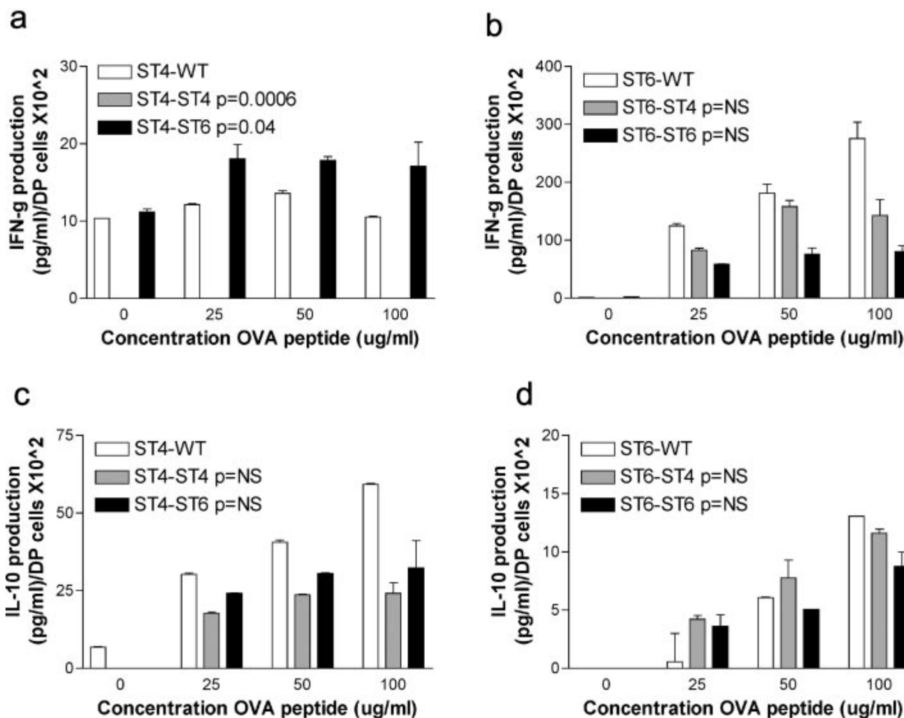

FIGURE 4. Ex vivo Ag-specific cytokine production by either STAT4KO or STAT6KO KJ126$^+$CD4$^+$ cells harvested from either Th0, Th1, or Th2 environments. A total of 4×10^5 cells/well from LN suspensions harvested from either wild-type, STAT4KO, or STAT6KO recipient mice, 5 days after transfer of either STAT4KO-DO11 or STAT6KO-DO11 donor splenocytes, was cultured with increasing concentrations of OVA peptide in vitro. After 48 h, supernatants were harvested for analysis of cytokine production by ELISA. Results are expressed as cytokine production per KJ126$^+$CD4$^+$ cell. IFN-γ production by double-positive cells from STAT4KO-DO11 mice (*a*) and STAT6-DO11 mice (*b*) and IL-10 production by STAT4KO-DO11 mice (*c*) and STAT6KO-DO11 mice (*d*) were analyzed. Production of IFN-γ was low in LN supernatants from STAT4KO-DO11 after transfer into STAT4KO mice, but there was significant background production of IFN-γ in cultures from STAT4KO-DO11 into wild type ($p = 0.0006$ by paired Student's *t* test) and an even higher IFN-γ production in cultures from STAT4KO-DO11 into STAT6KO mice ($p = 0.04$ compared with transfer into wild-type mice). There was no significant difference in IFN-γ production after transfer of STAT6KO-DO11 cells into either STAT4KO (Th2) or STAT6KO (Th1) recipients compared with wild-type recipients.

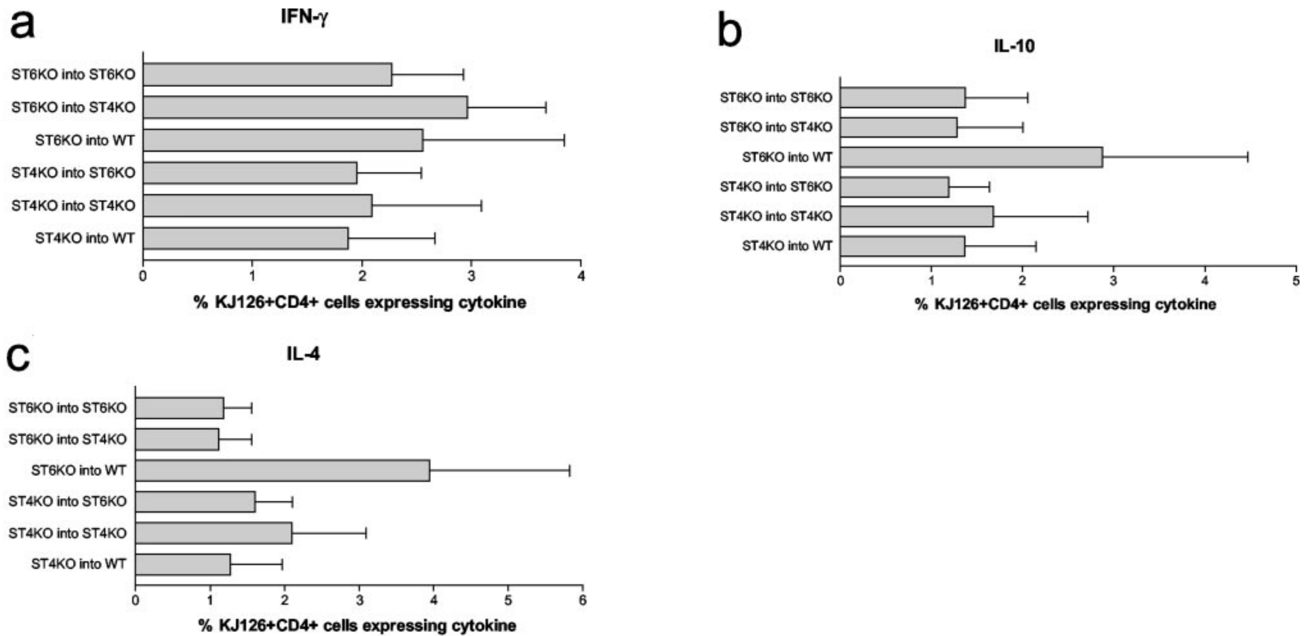

FIGURE 5. Intracellular cytokine production by KJ126$^+$CD4$^+$ cells transferred to wild-type, STAT4KO, or STAT6KO mice in vivo. LN suspensions harvested from either wild-type, STAT4KO, or STAT6KO mice given either STAT4KO-DO11 or STAT6KO-DO11 donor splenocytes were plated in 96-well plates and stimulated with 50 μg/ml OVA for 48 h. Cells were harvested and, using triple-color intracellular fluorescence staining, were labeled with either anti-IFN-γ (*a*), IL-10 (*b*), or IL-4 (*c*) Abs, and all samples were labeled with anti-CD4 and anti-KJ126 Abs. Fluorescence labeling was analyzed using flow cytometry, and percentage of cytokine-expressing KJ126$^+$CD4$^+$ cells is presented. Using unpaired *t* test, cytokine production from either STAT4KO or STAT6KO-KJ126$^+$CD4$^+$ cells harvested from STAT4KO or STAT6KO was not significantly different from that found in wild-type BALB/c hosts (p = NS).

peptide in vitro, and cytokine production was examined using ELISA. Results were adjusted to the number of CD4$^+$KJ126$^+$ cells recovered.

Production of IFN-γ and IL-10 by STAT6KO-KJ126$^+$CD4$^+$ cells was similar in Th0, Th1, and Th2 environments (Fig. 4, *b* and *d*). STAT4KO-KJ126$^+$CD4$^+$ cells harvested from Th0, Th1, and Th2 recipients produced similar amounts of IL-10 (Fig. 4*c*); however, production of IFN-γ by STAT4KO-KJ126$^+$CD4$^+$ was lower after transfer into STAT4KO (Th2) recipients, and slightly higher after transfer into STAT6KO (Th1) recipients (Fig. 4*a*). This difference was attributed to production of IFN-γ by the recipient's LN. A similar background production of IFN-γ was seen in the cultures of STAT6KO into wild type, STAT4KO, and STAT6KO, but is less apparent in the figure because of the much higher IFN-γ production of STAT6KO cells (the *y*-axis is 10-fold higher). Moreover, IFN-γ production is seen without Ag restimulation, suggesting that this is nonspecific production by recipient LN cells.

To evaluate cytokine production specifically by Ag-stimulated KJ126$^+$CD4$^+$ donor cells harvested from recipient LN, we used flow cytometry to analyze intracellular cytokine production. LN from donor-recipient groups were harvested and stimulated with 50 μg/ml OVA peptide, and production of IFN-γ, IL-10, and IL-4 was evaluated (Fig. 5). IL-4 was not measured using the ELISA, because it is typically consumed quickly in supernatants, and is therefore undetectable using this method. Although there was variation between experiments, cytokine production by either STAT4KO-KJ126$^+$CD4$^+$ or STAT6KO-KJ126$^+$CD4$^+$ cells was not significantly different after exposure to either Th1 or Th2 compared with Th0 environment.

Discussion

The current paradigm for Th subset differentiation indicates that differentiated T cells remain committed to either a Th1 or Th2 lineage. However, previous studies have reported reversal of Th1 or Th2 lineage commitment after in vitro restimulation with the appropriate cytokines (11). In contrast, in long-term Th1 and Th2 populations arising originally from repeated stimulation with IL-12 or IL-4 in vitro, Th commitment was not reversible (11). However, there are little data on the effects of cytokine environment on committed Th1 or Th2 cells in vivo.

Many immune-mediated diseases are thought to be based on the Th1/Th2 paradigm, and thus, T cell skewing to either lineage may be associated with either pathogenicity or protection from disease. In addition, novel immunotherapies in experimental or clinical development are designed to alter the cytokine environment, and suppress activation and/or function of the pathogenic Th1/Th2 cells (3–5). Furthermore, there is always the risk that switching an immune response from one phenotype to another may ameliorate one disease, but either precipitate or worsen another disease. For example, administration of an altered peptide ligand of myelin basic protein to multiple sclerosis patients reduced the frequency of new gadolinium-enhancing magnetic resonance imaging lesions; however, it induced a hypersensitivity response that was associated with Th2 response to the altered peptide ligand (12). T-bet-deficient mice, which have an impaired Th1 response, develop an asthma-like syndrome that is associated with the infiltration of Th2 cells in the airway (13). Thus, understanding the ability of the environment to influence the phenotype of committed Th1 or Th2 cells in vivo has significant implications for understanding the pathogenesis of immune-mediated diseases as well as for immunotherapies.

We used STAT4KO mice that lack IL-12R-mediated Th1 differentiation and therefore have a Th2 phenotype; and STAT6KO mice that cannot respond to IL-4R-mediated Th2 differentiation and therefore have a Th1 phenotype. Mice of either lineage were

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backcrossed to OVA-TCR transgenic mice (DO11), thereby generating STAT4KO-DO11 or STAT6KO-DO11 mice. T cells from these mice were transferred into STAT6KO (Th1), STAT4KO (Th2), or wild-type recipient mice to assess their ability to expand and produce cytokines in vivo. Our results establish, using this system, that STAT6KO-DO11 and STAT4KO-DO11 cells exhibited similar expansion and cytokine production patterns in wild-type, Th1, and Th2 environments in vivo. These results support the view that committed Th1 or Th2 Ag-specific cells are not influenced by the cytokine milieu, at least for the short-term.

Interestingly, survival and expansion of both STAT6KO (Th1) or STAT4KO (Th2) cells were the same in Th0, Th1, and Th2 environments, suggesting that signals for cell survival and expansion are independent of Th environment. Prior studies have shown that in vitro, in the presence of Th2-polarizing conditions, STAT6KO cells cease dividing after 3 days and display a phenotype of incomplete activation (14). The same study shows that under Th1-polarizing conditions, STAT4KO cells are incompletely activated. The results obtained using our in vivo system suggest that other growth factors such as TGF-β may promote expansion of T cells unable to respond to polarizing Th1 or Th2 cytokines (15–17).

We have demonstrated that STAT4KO-DO11 cells exhibit similar cytokine profiles despite being placed in a Th0, Th1, or Th2 environment in vivo. Prior studies have shown that when Th2 cells isolated from asthmatic patients were restimulated in the presence of IL-12, this resulted in a shift toward a Th0/Th1 phenotype with fully restored IL-12Rβ2 chain expression IL-12-inducible STAT4 activation (18). In our system, we would not expect to see restoration of STAT4 signaling after Th1 deviation in STAT4KO mice. However, our results confirm that in vivo, non-STAT4-dependent mechanisms are insufficient to cause a Th1 deviation or to suppress Th2 cytokine production in STAT4KO cells.

STAT4 and STAT6 double-KO mice surprisingly develop a Th1 phenotype in vitro (9), suggesting that a STAT4-independent pathway of Th1 differentiation exists. IL-18 has been recently described as an IFN-γ-inducing factor (19, 20). Although both IL-12 and IL-18 enhance the production of IFN-γ by Th1 cells, each may do so using different intracellular signaling pathways (8, 21). IL-12 is dependent on STAT4 for IFN-γ production; however, IL-18 binding results in direct activation of the downstream AP-1 site required for IFN-γ production (22). In addition, IL-12 may affect IL-18-dependent IFN-γ production (23–26), and IL-18 has been shown to enhance expression of the IL-12Rβ2 subunit (25), demonstrating interdependence of these two Th1-inducing cytokines.

Although STAT6 may be an important determinant of the final frequency of Th2 cells (27), it may not be necessary for Th2 cell development, because Th2 cells have been shown to develop in STAT6KO mice (27–30) as well as IL-4-deficient and IL-4R-deficient mice (31–33). The transcription factor GATA-3 (34) is present in IL-4-producing STAT6KO cells (30), and may be the primary factor responsible for Th2 development. Once induced, GATA-3 can augment its own expression, independent of STAT6 (30), and can down-regulate IFN-γ production by developing Th1 cells (35). In our model, we would not expect deviation of STAT6KO cells to a Th2 phenotype. However, we expected an inhibition of proliferation or limited expansion of the Ag-specific cells transferred to STAT4KO (Th2) recipients. Our data showed no such limitation in proliferation or cytokine production.

In summary, we conclude that the cytokine environment in vivo does not affect the ability of polarized Th1 and Th2 cells to divide and secrete cytokines. In addition, absence of STAT4 or STAT6 signaling is sufficient to ensure maintenance of the Th2 or Th1 phenotypes, respectively, despite polarizing conditions. With the caveats that our studies are conducted in TCR transgenic animals, these data have implications for understanding the pathogenesis of immune-mediated diseases as well as development of novel safe immunotherapeutic strategies in humans.

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