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Regulation of Th2 Cytokine Expression in NKT Cells: Unconventional Use of Stat6, GATA-3, and NFAT2

Zheng-Yu Wang,* Saritha Kusam,* Veerendra Munugalavadla,† Reuben Kapur,† Randy R. Brutkiewicz,* and Alexander L. Dent2*

NKT cells are unique in that they can produce high levels of both Th1 and Th2 cytokines, yet little is known about how NKT cells control the transcription of Th2 cytokines. The expression of IL-4 by NKT cells is independent of the Th2-associated transcription factor Stat6. We have found that Stat6 is critical for the expression of IL-5, IL-10, and IL-13 by NKT cells. However, the Th2 cell-associated transcription factor GATA-3, normally induced by Stat6 activation, is expressed at low levels in NKT cells. CD4+ NKT cells are highly enriched for Th2 cytokine expression compared with CD4+ NKT cells, and we searched for transcription factors that are up-regulated in CD4+ NKT cells that could control Th2 cytokine expression. We found that the NFAT family member NFAT2 is selectively increased in CD4+ NKT cells. We tested the roles of NFAT2 and also GATA-3 in Th2 cytokine expression by retrovirus-mediated gene transduction into NKT cells and nonpolarized conventional T cells. Expression of NFAT2 increased the expression of IL-4 in both NKT cells and conventional T cells, and NFAT2 activated IL-10 in conventional T cells but not in NKT cells. GATA-3 strongly activated IL-4, IL-5, and IL-13 expression in conventional T cells but had comparatively weak effects on these cytokines in NKT cells. Thus, NFAT2, GATA-3, and Stat6 have surprisingly different roles in NKT cells than in conventional T cells. We propose that one mechanism by which CD4+ NKT cells express IL-4 independent of Stat6 is via increased NFAT2 activity. The Journal of Immunology, 2006, 176: 880–888.

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that individual NKT cells can express both IL-4 and IFN-γ. Interestingly, the vast majority of NKT cells appear to express IL-4 and IFN-γ mRNA constitutively, and therefore part of the regulation of Th1 and Th2 cytokine expression by NKT cells may be regulated at the posttranscriptional stage (29). However, this mode of cytokine regulation by NKT cells is not well understood.

The activation of specific transcription factors in T cells during an immune response is critical for the development of polarized Th1 and Th2 cells (reviewed in Ref. 24). The transcription factor T-bet is critical for the development of Th1 cells, and interestingly, mature NKT cells cannot be formed in the absence of T-bet (30). The transcription factor Stat6, which is activated by IL-4 signaling, is vital for the development of a normal Th2 response. Yet NKT cells apparently do not require Stat6 for IL-4 production (29, 31). Mast cells also produce IL-4 independently of Stat6 (32), further showing that the same cytokine can be regulated differently within the hemopoietic system. Mast cells also express different isoforms of the transcription factor NFAT, which also is involved in IL-4 transcription (33). Besides NFAT, GATA-3 is a key transcription factor that controls Th2 cytokine production in conventional T cells (24). Although little is known about the role of GATA-3 in Th2 cytokine expression by NKT cells, increased GATA-3 mRNA correlated with increased IL-4 expression by NKT cells in one study (34). In this study, we analyze in more detail the roles of Stat6, NFAT2, and GATA-3 in Th2 cytokine production by NKT cells.

Materials and Methods

Mice

Animals were maintained under specific pathogen-free conditions in animal facilities certified by the American Association of Laboratory Animal Care; 6- to 10 wk-old wild-type (WT) mice and Stat6−/− mice on a mixed C57BL/6H129 background were used to isolate NKT cells. C57BL/6J mice (The Jackson Laboratory) also were used to obtain NKT cells. Primary Th1 and Th2 cells were isolated as described previously (36).

Cell isolation and culture

Primary NKT cells were obtained by sorting NK1.1+TCRβ+ cells from liver mononuclear cells as described previously (35). Freshly isolated NKT cells apparently do not require Stat6 for IL-4 production (29, 31). Mast cells also produce IL-4 independently of Stat6 (32), further showing that the same cytokine can be regulated differently within the hemopoietic system. Apart from the expression of different isoforms of the transcription factor NFAT, which also is involved in IL-4 transcription (33). Besides NFAT, GATA-3 is a key transcription factor that controls Th2 cytokine production in conventional T cells (24). Although little is known about the role of GATA-3 in Th2 cytokine expression by NKT cells, increased GATA-3 mRNA correlated with increased IL-4 expression by NKT cells in one study (34). In this study, we analyze in more detail the roles of Stat6, NFAT2, and GATA-3 in Th2 cytokine production by NKT cells.

ELISA

Cells were stimulated for 24 h with plate-bound anti-CD3 at 10 μg/ml at 1 × 10^6 cells per ml, and supernatants were collected and analyzed by ELISA. All ELISA reagents were obtained from BD Pharmingen.

Western blots

Whole-cell lysates and nuclear extracts were analyzed by Western blot using standard conditions. Blots were probed with anti-GATA-3 mAb (clone HG3-31), anti-NFAT1 (4G6-5G), or anti-NFAT2 (7A6), all from Santa Cruz Biotechnology. GATA-3 blots were developed with ECL reagents (Amersham Biosciences). NFAT1 and NFAT2 blots were developed with SuperSignal West Femto maximum sensitivity substrate (Pierce). After anti-GATA-3 or anti-NFAT1/2 probing, blots were probed with Ab to β-actin (Sigma-Aldrich) to demonstrate equivalent protein loading.

Transcription array analysis

Nuclear extracts from CD4+ and CD4+cells stimulated with 20 ng/ml PMA and 0.3 nmol iodonium for 6 h were tested in the assay. Thirty micrograms of each nuclear extract was used for each analysis. The TransSignal arrays were performed according to the manufacturer’s protocols (Panomics).

Gel shift assay

Nuclear extracts were prepared, and gel shift assays for NFATs were performed as described previously (37). Binding reactions were performed with labeled probe and 10 μg of nuclear extract from CD4+ and CD4+ NKT and Th2 cells stimulated with 20 ng/ml PMA and 0.3 μM iodonium for 6 h. For NFAT binding, a double-stranded oligonucleotide from the murine IL-4 promoter (−88 to −60) was used: 5’-CTGGTGGATATA AATTTTCATAGTAAA-3’ (top strand shown only) (38). The super-shift Ab for NFAT2 (7A6) was obtained from Santa Cruz Biotechnology.

RT-PCR

RNA isolation, cDNA synthesis, and RT-PCR were performed by standard procedures. Total RNA was isolated from CD4+ and CD4+ NKT cells stimulated with or without anti-CD3 Ab at 10 μg/ml for 3 h. The following primers were used: NFAT2, 5’-GAGGCGGAAACCTTTGCG-3’ and 5’-CAGAGGCACAAGTATCCCG-3’; NFAT2β, 5’-GGAGTCTGACCT CGATTCTCCT-3’ and 5’-CAGAGGCACAAGTATCCCG-3’; and NFAT1, 5’-GATGGGATGTCCTGATCCGA-3’ and 5’-TATCGAAGAAGACCGATCGCC-3’. Primers for β-tubulin were published previously (39). PCR reactions were run under conditions where amplification was linear, generally 28–30 cycles.

Retroviral infection

Amphotropic retrovirus were prepared with the Phoenix-GP system obtained from G. Nolan (Stanford University, Stanford, CA) and used as described (40, 41). The control human CD4 and the GATA-3-human CD4 retroviral vectors were obtained from M. Kaplan (Indiana University School of Medicine) (42). The NFAT2-expressing retrovirus were constructed by inserting blunt-ended NFAT2 cDNA from pSH160C (43) into the retroviral vector pMIEG3 (37) via the blunt-ended XhoI site. Sorted NKT cells were activated with anti-CD3 Abs as described above, then spin-infected with retroviral supernatant on day 5 of growth, put at 32°C overnight, and moved to 37°C for 24 h. Control CD4 and CD4-GATA-3 virus-infected cells were then sorted for either human CD4 expression using PE-labeled anti-CD4 Ab (BD Pharmingen). Cells infected with pMIEG3 alone or pMIEG3-NFAT2 were sorted for GFP expression. Following sorting, cells were put into wells containing immobilized anti-CD3 Abs (10 μg/ml) and stimulated overnight at 37°C. Supernatants were then collected for ELISA analysis. Nonpolarized conventional T cells were obtained by activating lymph node T cells with Con A at 2 μg/ml before infection without the addition of any cytokine except IL-2. Retrovirus infection of nonpolarized conventional T cells followed a similar protocol, except that the cells were infected at day 3 following activation. In general, NKT cells were infected at a much lower rate than conventional T cells by the retroviruses: 2–8% for the NKT cells vs 30–40% for conventional T cells. In pilot experiments, conventional T cells expressing control CD4 or control GFP retrovirus behaved similarly; due to limitations in numbers of NKT cells, only the GFP control retrovirus was used as a control for the GATA-3 and NFAT2 retroviruses.

Results

Stat6 regulates IL-5, IL-10, and IL-13 but not IL-4 expression by NKT cells

Previous published data on the role of Stat6 in NKT cells suggested that IL-4 expression by NKT cells was independent of Stat6 activity (31). To investigate in more detail how Th2 cytokine expression was regulated in NKT cells, we analyzed the role of Stat6 in the expression of other Th2 cytokines (Fig. 1). We found that, consistent with previously published data, Stat6-deficient NKT cells produced similar levels of IL-4 as WT NKT cells. Strikingly, other Th2 cytokines such as IL-5, IL-10, and IL-13, were strongly dependent on Stat6 function in NKT cells. Not surprisingly, IFN-γ expression by NKT cells was unaffected by the loss of Stat6. These data indicate that there are different levels of regulation of Th2 cytokine expression in NKT cells, and that, although IL-4 expression is independent of Stat6 activity, other Th2 cytokines are Stat6 dependent.
Different layers of regulation of Th2 cytokine expression by NKT cells

Previously published data with both human and mouse NKT cells showed that CD4 expression correlates with IL-4 expression, although both CD4$^+$ and CD4$^-$ NKT cells produce high levels of IFN-γ (25–28). Consistent with the findings of Gumperz et al. (26) on human NKT cells, we found that 40–50% of mouse NKT cells were CD4$^+$ (data not shown). We first investigated whether CD4 expression on mouse NKT cells correlates with expression of Th2 cytokines besides IL-4. We found that, with WT CD4$^+$ NKT cells, IL-4, IL-5, and IL-13 are expressed at greatly increased levels, compared with CD4$^-$ NKT cells (Fig. 2A). However, both types of NKT cells produced similar levels of IL-10 and IFN-γ. These data indicate several levels of regulation of Th2 cytokines by NKT cells. First, IL-4 is expressed along with CD4 but independent of Stat6. Second, IL-5 and IL-13 are expressed along with CD4 but are dependent on Stat6. Finally, IL-10 is expressed independently of CD4 but dependent on Stat6. We next asked whether Stat6 affected the development of CD4$^+$ NKT cells or the development of Th2 cytokine expression by CD4$^+$ NKT cells. We found that CD4$^+$ NKT cells from Stat6-deficient mice were found at percentages comparable to that of WT (data not shown). Surprisingly, we observed that, like WT CD4$^+$ NKT cells, CD4$^+$ NKT cells from Stat6-deficient mice are enriched for the expression of IL-4, IL-5, and IL-13 (Fig. 2B). However, if we compare the ratio of ng/ml IL-4 to ng/ml IL-5 secreted by CD4$^+$ NKT cells from WT and Stat6-deficient mice, we find that Stat6-deficient NKT cells express relatively less IL-5, compared with IL-4, than WT NKT cells.
CD4⁺ production by these cells, the overall low levels of GATA-3 in GATA-3 accounted for the increased Th2 cytokine expression in We next wondered whether other factors besides Stat6 and cells that, before CD3 stimulation, CD4⁺ cytokines in Stat6-deficient NKT cells correlated with changes in GATA-3 expression. We used Western blots of whole-cell lysates to assess GATA-3 protein levels in NKT cells from WT and Stat6-deficient mice, as well as conventional Th1 and Th2 cells and T cells from Stat6-deficient mice cultured under Th2 conditions. GATA-3 was expressed at very low levels in NKT cells, compared with conventional Th2 cells, and there was little difference in GATA-3 expression between resting or stimulated WT and Stat6-deficient NKT cells (Fig. 3A). As expected, GATA-3 expression was low in T cells from Stat6-deficient mice cultured under Th2 conditions. These data indicate that, in NKT cells, unlike conventional T cells, Stat6 does not up-regulate GATA-3 expression. We next tested whether GATA-3 is up-regulated specifically in CD4⁺ NKT cells, compared with CD4⁺ NKT cells (Fig. 3B). We found that, before CD3 stimulation, CD4⁺ NKT cells had more GATA-3 than CD4⁻ NKT cells, although the levels were still low. Following CD3 stimulation, GATA-3 levels were comparable between CD4⁺ and CD4⁻ NKT cells. Although the increased GATA-3 in CD4⁺ NKT cells may account for the increase in Th2 cytokine production by these cells, the overall low levels of GATA-3 in CD4⁻ NKT cells suggests that these cells regulate Th2 cytokine transcription by a very different mechanism than conventional Th2 cells.

Identification of NFAT2 as a factor up-regulated in CD4⁺ NKT cells

We next wondered whether other factors besides Stat6 and GATA-3 accounted for the increased Th2 cytokine expression in CD4⁺ NKT cells. We therefore used a TranSignal protein/DNA array kit to search for transcription factors up-regulated specifically in CD4⁺ NKT cells, compared with CD4⁺ NKT cells. We used a TranSignal protein/DNA array kit to search for transcription factors up-regulated specifically in CD4⁺ NKT cells, compared with CD4⁺ NKT cells. We used Western blots of whole-cell lysates to assess GATA-3 protein levels in NKT cells from WT and Stat6-deficient mice, as well as conventional Th1 and Th2 cells and T cells from Stat6-deficient mice cultured under Th2 conditions. GATA-3 was expressed at very low levels in NKT cells, compared with conventional Th2 cells, and there was little difference in GATA-3 expression between resting or stimulated WT and Stat6-deficient NKT cells (Fig. 3A). As expected, GATA-3 expression was low in T cells from Stat6-deficient mice cultured under Th2 conditions. These data indicate that, in NKT cells, unlike conventional T cells, Stat6 does not up-regulate GATA-3 expression. We next tested whether GATA-3 is up-regulated specifically in CD4⁺ NKT cells, compared with CD4⁺ NKT cells (Fig. 3B). We found that, before CD3 stimulation, CD4⁺ NKT cells had more GATA-3 than CD4⁻ NKT cells, although the levels were still low. Following CD3 stimulation, GATA-3 levels were comparable between CD4⁺ and CD4⁻ NKT cells. Although the increased GATA-3 in CD4⁺ NKT cells may account for the increase in Th2 cytokine production by these cells, the overall low levels of GATA-3 in CD4⁻ NKT cells suggests that these cells regulate Th2 cytokine transcription by a very different mechanism than conventional Th2 cells.

**Identification of NFAT2 as a factor up-regulated in CD4⁺ NKT cells**

We next wondered whether other factors besides Stat6 and GATA-3 accounted for the increased Th2 cytokine expression in CD4⁺ NKT cells. Gel shifts with nuclear extracts from CD4⁺ NKT cells and CD4⁻ NKT cells showed increased total NFAT DNA binding (data not shown), consistent with the TranSignal array results. Because NFAT can be composed of one of five different family members (44), we wanted to determine which NFAT family member is up-regulated in CD4⁺ NKT cells. The regulation of IL-4 by NFAT is complex, and of the three NFAT family members expressed in T cells, NFAT1 (also known as NFATp or NFATc2), NFAT2, (also known as NFATc or NFATc1), and NFAT4 (also known as NFATc3), only NFAT2 can clearly activate IL-4 expression (24, 45). Mice deficient in NFAT1 and NFAT4 express more IL-4 than normal mice, suggesting that these proteins repress IL-4 (24). In contrast, NFAT2-deficient mice are defective in expressing normal levels of IL-4 (24). Moreover, other studies have suggested a positive role for NFAT2 in IL-4 transcription (38). We therefore initially examined the expression of NFAT1 and NFAT2 proteins in nuclear extracts from CD4⁺ NKT cells and CD4⁻ NKT cells (Fig. 5A). We found that NFAT2 was significantly up-regulated in nuclear extracts from PMA plus ionomycin (P/I)-stimulated CD4⁺ NKT cells, compared with nuclear extracts from P/I-stimulated CD4⁻ NKT cells. At the same time, little difference was seen in the expression of NFAT1 in these nuclear extracts. We next examined NFAT2 expression in total cell extracts and found that NFAT2 protein expression was up-regulated at the whole-cell level in CD4⁺ NKT cells, not just up-regulated in the nucleus. Normal Th2 cells also express very high levels of NFAT2, which correlates with the fact that these cells also produce high levels of IL-4. We next addressed whether NFAT2 also was up-regulated in CD4⁺ NKT cells from Stat6-deficient mice (Fig. 5B). NFAT2 was expressed at much higher levels in Stat6-deficient CD4⁺ NKT cells, compared with Stat6-deficient CD4⁻ NKT cells, suggesting that the increased NFAT2 could promote the Stat6-independent IL-4 expression seen in these cells. We also wanted to test whether NFAT2 contributed to NFAT DNA binding in CD4⁺ NKT cells. Experiments with gel shifts testing NFAT binding and Ab to NFAT2 showed that overall NFAT binding was up-regulated in CD4⁺ NKT cells, compared with CD4⁻ NKT cells, and that a significant amount of NFAT binding was shifted with Ab to NFAT2 (Fig. 5C). Overall, CD4⁺ NKT cells displayed an NFAT DNA binding pattern similar to that of normal Th2 cells. We then tested the expression of the two isoforms of NFAT that have been described, NFAT2α and NFAT2β (33), to determine whether there was differential expression of these two gene products between CD4⁺ and CD4⁻ NKT cells (Fig. 5D). Using RT-PCR and primers specific for the two differently spliced forms of NFAT2, we found that mRNA for the common isoform, NFAT2α, was expressed at about 2-fold higher

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**FIGURE 3.** GATA-3 is expressed at low levels by NKT cells, compared with conventional TH2 cells. NKT cell populations were isolated and grown as described in Fig. 1. Primary TH2 cells from WT and Stat6-deficient (SKO) mice were cultured under standard TH1 or TH2 conditions to obtain the indicated populations. Cells were activated with or without anti-CD3 Ab for 3 h. Whole-cell lysates were prepared and GATA-3, and β-actin levels were analyzed by Western blot. The data shown are representative of three different experiments for A and two different experiments for B.
levels in CD4+ NKT cells than CD4− NKT cells before stimulation, and that after stimulation, both cell types up-regulated NFAT2α mRNA by 3-fold. After stimulation, CD4+ NKT cells expressed NFAT2α mRNA at slightly less than 2-fold higher levels than the CD4− NKT cells. The mRNA for the NFAT2β isoform was expressed at higher levels in resting CD4+ NKT cells, compared with resting CD4− NKT cells. Similar to previously published data, expression of NFAT2β mRNA was not altered by stimulation of CD4− NKT cells (33), although NFAT2β mRNA was decreased by stimulation of CD4+ NKT cells. NFAT1 mRNA was expressed at slightly higher levels in CD4− NKT cells than in CD4+ NKT cells. These data show that increased NFAT2α and NFAT2β expression correlates with IL-4 expression in CD4+ NKT cells.

Specific inhibition of IL-4 expression in NKT cells by cyclosporin A

The immunosuppressive drug cyclosporin A is known to inhibit activation of the NFAT transcription factor (46), and thus we tested whether cyclosporin A could affect IL-4 expression by CD4+ NKT cells. As shown in Fig. 6, cyclosporin A strongly inhibited IL-4 expression from CD4+ NKT cells in a dose-dependent manner but did not significantly affect IFN-γ expression from the same cells. Although cyclosporin A can inhibit other transcription factors besides NFAT, such as NF-xB, our data show that IL-4 expression by NKT cells is preferentially inhibited by cyclosporin A and support a model wherein NFAT activity is critical for IL-4 expression by NKT cells.

A direct role for NFAT2 in IL-4 expression by NKT cells

We next wanted to test whether NFAT2 overexpression in total NKT cells and in CD4− NKT cells could result in increased Th2 cytokine expression. We therefore used a high-titer retrovirus system to infect NKT cells with a retrovirus expressing NFAT2. We also tested the role of GATA-3 in NKT cells by use of a GATA-3 expressing retrovirus (42). After sorting marker-positive cells (GFP in the case of NFAT2 and human CD4 in the case of GATA-3), we stimulated the NKT cells with anti-CD3 Abs and tested cytokine expression by ELISA (Fig. 7). We found that NFAT2 was able to increase IL-4 expression by roughly 50% in CD4− NKT cells, but that NFAT2 did not markedly affect IL-5, IL-10, IL-13, or IFN-γ. These data support the idea that increased expression of NFAT2 in CD4+ NKT cells can promote Stat6-independent IL-4 transcription in these cells. Because other Th2 cytokines were not significantly affected by NFAT2, the role of NFAT2 in Th2 cytokine expression by NKT cells may be limited to IL-4. Overexpression of GATA-3 in CD4+ NKT cells by retrovirus led to a similar increase in IL-4 production as NFAT2. GATA-3 also promoted IL-5 expression in CD4+ NKT cells, which supports the idea that NFAT2 and GATA-3 have different roles in Th2 cytokine expression. Like NFAT2, GATA-3 did not alter IL-10, IL-13, or IFN-γ production.

Lastly, we tested the effects of NFAT2 and GATA-3 overexpression on conventional T cells. We infected nonpolarized lymph node T cells with our NFAT2 and GATA-3 retroviruses, sorted out marker-positive cells, stimulated the infected cells with anti-CD3 Abs, and again tested cytokine secretion by ELISA. Strikingly, we found that the effects of NFAT2 and GATA-3 were different in conventional T cells, compared with NKT cells (Fig. 8). In conventional T cells, GATA-3 very strongly promoted IL-4, IL-5 and IL-13 expression, but it did not affect IL-10 expression. NFAT2 also was able to activate IL-4 in conventional T cells but not as strongly as GATA-3. In contrast to what was seen in NKT cells, NFAT2 increased IL-10 expression in conventional T cells. As in NKT cells, neither NFAT2 nor GATA-3 affected IFN-γ in conventional T cells. These data show that NFAT2 and GATA-3 play markedly different roles in Th2 cytokine expression, and that the functions of these two transcription factors vary between T cell types.

Discussion

The differential expression of Th2 cytokine expression by NKT cells is critical for their function in many different disease states, and very little is known about this process. There is evidence that cytokine mRNA expression by NKT cells is constitutive and controlled at the posttranscriptional level, yet very little is known about this mode of regulation (29). In this study, we investigated how Th2 cytokine expression was controlled in NKT cells at the transcriptional level. We focused on three major transcription factors known to be involved in Th2 cytokine expression: Stat6, GATA-3, and NFAT2. Stat6 and GATA-3 were analyzed because of their known role in Th2 cytokine transcription, whereas we identified NFAT2 in this study as a unique transcription factor that is up-regulated in Th2 cytokine-expressing CD4+ NKT cells. We have found that Stat6, GATA-3, and NFAT2 all behave very differently in NKT cells vs conventional T cells.
Stat6, which is activated in response to IL-4 signaling, is a central transcriptional mediator of Th2 differentiation (24). We have found that, although NKT cells require Stat6 for normal expression of Th2 cytokines, NKT cells can produce high levels of IL-4 independent of Stat6. Thus, NKT cells use an alternative pathway from conventional T cells for IL-4 expression. As a major mechanism by which Stat6 activates Th2 cytokine expression is the up-regulation of GATA-3, we wondered whether NKT cells might induce GATA-3 by a different mechanism than Stat6. However, we found that GATA-3 is expressed at extremely low levels in NKT cells, compared with Th2 cells, suggesting that NKT cells do not rely on GATA-3 to the same extent as conventional Th2 cells. Moreover, there was little difference in GATA-3 expression between WT and Stat6-deficient NKT cells, indicating that Stat6 did not induce GATA-3 as in conventional T cells. These results prompted us to search for other transcription factors that could

**FIGURE 5.** Up-regulation of NFAT2 in CD4⁺ NKT cells. NKT and Th2 cell populations were isolated and grown as described above. Cells were then left untreated, treated with P/I for 6 h, treated with ionomycin alone for 3 h, or stimulated with anti-CD3 Abs for 3 h. Whole-cell lysates, nuclear extracts, or RNA were then prepared. A, Analysis of NFAT2 and NFAT1 expression by Western blot, either in nuclear extracts or whole-cell extracts. β-actin is a loading control. B, Up-regulated NFAT2 in nuclear extracts from CD4⁺ NKT cells of both WT and Stat6-deficient mice. C, Analysis of NFAT DNA binding by gel shift using nuclear extracts. Cells were stimulated with P/I for 6 h before preparation of the nuclear extracts. D, Analysis of NFAT2 isoform and NFAT1 mRNA expression in CD4⁺ and CD4⁺ NKT cells. Cells were activated with or without anti-CD3 Ab for 3 h. Semi quantitative RT-PCR was used to analyze mRNA levels for the indicated genes. Gene expression was analyzed by 3-fold serial dilutions of each input cDNA.
promote Th2 cytokine transcription in NKT cells. Using a transcription factor array system, we identified NFAT2 as being selectively up-regulated in Th2 cytokine-expressing CD4⁺ NKT cells.

A direct test of the effects of NFAT2 on cytokine expression in NKT cells showed that it could up-regulate IL-4 but not other Th2 cytokines. GATA-3 gave a similar result to NFAT2 when overexpressed in NKT cells, although GATA-3 was also able to weakly up-regulate another Th2 cytokine, IL-5. Tests with NFAT2 and GATA-3 expression in conventional T cells showed clearly divergent results from that obtained in NKT cells: NFAT2 selectively increased IL-4 and IL-10 expression in conventional T cells, and GATA-3 very strongly activated expression of IL-4, IL-5 and IL-13 in conventional T cells. Thus, Stat6, GATA-3, and NFAT2 all behave very differently in NKT cells than in conventional T cells. Taken together, our data support a model wherein the Stat6 signaling pathway is important for the expression of IL-5, IL-10, and IL-13 in NKT cells and NFAT2 can promote IL-4 expression by a Stat6-independent pathway. Although GATA-3 can activate IL-4 and IL-5 in NKT cells, the precise role for GATA-3 in NKT cell cytokine expression is somewhat unclear.

One very striking finding from our work is that GATA-3 can activate IL-4, IL-5, and IL-13 strongly in conventional T cells but not in NKT cells. These data support a model wherein the Stat6 signaling pathway is important for the expression of IL-5, IL-10, and IL-13 in NKT cells and NFAT2 can promote IL-4 expression by a Stat6-independent pathway. Although GATA-3 can activate IL-4 and IL-5 in NKT cells, the precise role for GATA-3 in NKT cell cytokine expression is somewhat unclear.

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GATA-3 has no extra effect on chromatin remodeling. If the IL-4-IL-5-IL-13 locus is already open in NKT cells, a further question would be what keeps Th2 cytokine expression off in CD4⁺ NKT cells. One possibility is that Th2 cytokine expression is regulated at the posttranscriptional level in NKT cells (29). This idea also may explain why overexpression of NFAT2 and GATA-3 by retrovirus had only a minor effect on IL-4, IL-5, and IL-13 expression in NKT cells. Another possibility is that low levels of GATA-3 in CD4⁺ NKT cells are important to shut off Th2 cytokine expression, and that other transcription factors besides GATA-3 turn on Th2 cytokine expression in CD4⁺ NKT cells. Yet another possibility is that NKT cells express a gene, such as repressor of GATA (ROG) (51), that inhibits activation of Th2 cytokine expression by GATA-3 in these cells. Because GATA-3 is an autoactivator of its own expression, a GATA-3 inhibitor may explain the relatively low levels of GATA-3 in NKT cells. Strikingly, we found that Stat6 function did not affect GATA-3 expression levels in NKT cells, and that CD4⁺ NKT cells, which transcribe high levels of Th2 cytokines, had relatively little expression of GATA-3. These data indicate that GATA-3 is regulated very differently in NKT cells from conventional T cells, and that CD4⁺ NKT cells use another mechanism to express high levels of Th2 cytokines. Because full expression of IL-5, IL-10, and IL-13 is Stat6 dependent in NKT cells, our data suggest that another factor that is regulated by Stat6 besides GATA-3 may regulate these cytokines in NKT cells.

Our data suggest that NFAT2 may be a key regulator of IL-4 transcription in NKT cells, and this findings fits with other studies correlating increased NFAT2 and IL-4 expression (38, 52, 53). Unexpectedly, we found that NFAT2 is specifically up-regulated in CD4⁺ NKT cells. This finding is reminiscent of the up-regulation of NFAT2 (NFATc) observed in activated Th2 cells (54). Whereas much of the up-regulation of NFAT2 can be accounted for by increased mRNA expression and protein in the cells, we cannot rule out that NFAT2 is preferentially activated to enter the nucleus over other NFAT family members following cell stimulation. Activation of NFAT proteins is regulated by the strength of T cell stimulation, and NFAT2 appears to be induced by weaker signaling than NFAT1. Thus NFAT2-activated IL-4 transcription is associated with weaker TCR signaling in conventional T cells (38). It is unknown whether this increased sensitivity of NFAT2 to T cell activation is conserved in NKT cells. A key question is what up-regulates overall NFAT2 expression in CD4⁺ NKT cells and Th2 cells. Currently, very little is known about what regulates expression of the NFAT genes themselves. Interestingly, NFAT1 up-regulates NFAT2 during T cell activation (55), and NFAT2 expression also is regulated by mRNA splicing (56), indicating an intricate regulation of NFAT2 activity. Future experiments will address whether these pathways affect the up-regulation of NFAT2 seen in CD4⁺ NKT cells.

During the course of these studies, we found that CD4⁺ NKT cells are much more difficult to expand in vitro than CD4⁺ NKT cells. Although CD4⁺ NKT cells were transduced at a similar frequency to CD4⁺ NKT cells, it was difficult to isolate significant numbers of transduced CD4⁺ NKT cells for cytokine expression analysis. However, in two experiments, we found that CD4⁺ NKT cells transduced with NFAT2 showed a greater induction of IL-4 expression than CD4⁺ NKT cells transduced with NFAT2 (data not shown). These data indicate that CD4⁺ NKT cells are more receptive to the effects of NFAT2 than CD4⁺ NKT cells. Experiments with transduction of total NKT cells also supported this idea (data not shown). One explanation for this finding may be that CD4⁺ NKT cells express higher levels of inhibitory NFAT proteins, such as NFAT1 and NFAT4, and that more NFAT2 is required to overcome this inhibitory effect. Our data in Fig. 5D show somewhat more NFAT1 in CD4⁺ NKT cells than in CD4⁺ NKT cells, which is consistent with this idea. Other explanations are that CD4⁺ NKT cells have other inhibitors of NFAT function or that CD4⁺ NKT cells express proteins that cooperate with NFAT2 to induce IL-4 expression.

One caveat to our experiments is that, to have enough NKT cells for biochemical and molecular analysis, as well as for retroviral infection, we had to expand the NKT cells in vitro in IL-2-containing medium for several days, typically for 7 days. In vitro expansion of NKT cells with IL-2 has been used in other studies with NKT cells (28), and our NKT cells, in general, behave as typical NKT cells. However, it is possible that in vitro expansion in IL-2 alters the expression of and/or dependence on certain transcription factors that regulate cytokine expression in NKT cells. Nonetheless, our results clearly show that NKT cells regulate cytokine gene expression differently than conventional T cells, and it was already established that NKT cells do not require Stat6 for high-level IL-4 production (29, 31), showing that NKT cells have unusual mechanisms for cytokine regulation.

In conclusion, we have found that Th2 cytokine expression in NKT cells is regulated by strikingly different mechanisms than in conventional T cells, and this information is useful for understanding how NKT cells modulate Th1 vs Th2 responses. However, much more work is required to fully understand how NKT cells control Th2 cytokine expression.

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Disclosures

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References


