Responsiveness of Naive CD4 T Cells to Polarizing Cytokine Determines the Ratio of Th1 and Th2 Cell Differentiation

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The intrinsic features of naive CD4 T cells that affect their ability to respond to polarizing signals for Th cell differentiation are not well understood. In this study, we show that naive CD4 T cells from mice transgenic for the Hlx gene expressed lower levels of IL-4Rα. The down-regulation of IL-4Rα diminished IL-4 signaling and the Th2 response and enhanced the Th1 response under suboptimal polarizing conditions. In nontransgenic CD4 T cells, blocking IL-4Rα with Abs had the same effect in an Ab dose-dependent manner. Conversely, Hlx haploinsufficiency caused higher expression of IL-4Rα to favor Th2 cell differentiation. Thus, the IL-4Rα level on naive CD4 T cells is genetically controlled by Hlx and determines the ratio of Th1 and Th2 cell differentiation. The Journal of Immunology, 2006, 176: 1553–1560.

The dichotomy of type 1 and type 2 Th cells stands as a central paradigm in cellular immunology that provides the framework to understand the nature of an immune response that is either beneficial or detrimental to the host (1, 2). Th1 and Th2 cells are characterized by their mutually exclusive expression patterns of cytokines. Th1 cells produce IFN-γ, whereas Th2 cells produce IL-4, IL-5, and IL-13 (2). Functionally, Th1 responses are required for the clearance of intracellular infections, and Th2 responses are required for the clearance of helminth infection. The failure to generate the appropriate Th cell responses is often the cause of chronic infectious diseases. Under autoimmune conditions, polarized Th1 and Th2 responses are associated with organ-specific autoimmune diseases and allergies, respectively. Therefore, the mechanisms for controlling the differentiation of Th1 and Th2 cells from their naive precursors are crucial to our understanding of the complex regulation of immune responses under these pathological conditions.

Studies in the past have established an instruction model of Th cell differentiation. In this model, surface molecules on the APC or cytokines in the surrounding tissue environment deliver polarizing signals to naive CD4 T cells upon Ag stimulation. Such polarizing signals induce up-regulation of transcription factors that drive the differentiation of Th1 and Th2 cells. During Th1 cell differentiation, the transcription factor T-bet is up-regulated (3), whereas GATA-3 is up-regulated during Th2 cell differentiation (4). These two transcription factors, respectively, function as master regulators of the differentiation of Th1 and Th2 cells (3–9). Additionally, Th1 cells specifically express the transcription factor Hlx and Erm (10–12), and Th2 cells specifically express c-Maf (13), to enhance differentiation and effector cytokine expression.

Cell surface molecular interaction is an important means of delivering the initial polarizing signals. Early studies have shown that different strengths of TCR ligation and duration could dictate Th1 or Th2 cell differentiation (14). Numerous studies have also documented costimulatory and accessory interactions of CD28/B7, ICOS/B7h, OX40/OX40 ligand, and LFA-1/ICAM to regulate Th cell differentiation. More recently, the Notch ligands Delta and Jagged expressed on APC are found to induce Th1 and Th2 cell differentiation, respectively (15).

Apart from the surface molecules, cytokines in the environment in which naive CD4 T cells encounter their specific Ags provide potent polarizing signals for Th subset differentiation (16). Although other cytokines have been reported to regulate Th cell differentiation, the best understood and perhaps the most important polarizing cytokines are IFN-γ, IL-4, and IL-12. Interestingly, IFN-γ and IL-4 are both the effector cytokines and the inducers of the differentiation of their own producers (17–21). IFN-γ, through Stat1 activation, induces T-bet expression (18, 22, 23), whereas IL-4, through Stat6 activation, up-regulates GATA-3 expression (4, 6, 24–26). IL-12 activates Stat4, which plays an important role in Th1 cell differentiation because Th1 response in mice deficient in Stat4 or IL-12 is severely diminished (27, 28). In fact, IL-12 and IL-4 are the polarizing cytokines typically used for in vitro differentiation of Th1 and Th2 cells, respectively.

The polarizing cytokines are derived from multiple sources. NK cells (29, 30) and a special population of acute responding CD8 T cells (31) could provide the initial IFN-γ for Th1 cell differentiation. An important source of early IL-4 is the naive CD4 T cells themselves, which can produce IL-4 immediately upon TCR stimulation to induce their own differentiation to Th2 cells (17, 21, 32). Other potential sources of the early IL-4 include the NK1.1+ CD4 T cells (33), a special population of NKL1.1+ CD4 T cells (34, 35), mast cells (36), and eosinophils (37, 38). IL-12 is produced by APC, such as macrophages and dendritic cells (39–43). Studies using infectious disease models have shown that at least some
infectious agents can induce acute production of polarizing cytokines to skew the Th response toward Th1 or Th2. For example, Th2 responses to Leishmania major and Schistosoma mansoni infection could be significantly attributed to an early burst of IL-4 secretion from a population of CD4 T cells expressing the Vβ4/Vα8 TCR and eosiophils, respectively (34, 44). In contrast, intracellular infection of Listeria or Toxoplasma stimulates IL-12 expression in macrophages and IFN-γ expression in NK cells to instruct Th1 cell differentiation (39, 40, 45).

As supported by these studies, the current instruction model of Th cell differentiation emphasizes the creation of a strong polarizing cytokine environment to induce a dominant Th1 or Th2 response. Less attention has been devoted to the contribution of the intrinsic features of the responding naive CD4 T cells to the differentiation outcome. However, in many immune responses, polarizing cytokines for one subset may not always dominate over those for the other subset. Under such weak polarizing conditions, polarizing cytokines for Th1 or Th2 cell differentiation probably coexist and compete to influence Th cell differentiation. In previous studies (10, 12), we and others found that the homeobox transcription factor Hlx is specifically expressed in Th1 cells and not in Th2 cells. Consistent with its specific expression in Th1 cells, overexpression of Hlx in CD4 T cells promoted Th1 cell differentiation. However, Hlx is also expressed in naive CD4 T cells at low levels. In this study we show that Hlx in naive CD4 T cells genetically down-regulates IL-4Rα expression in naive CD4 T cells. The genetically determined IL-4Rα level dictates the ratio of Th1 and Th2 cell differentiation under weak polarizing conditions.

Materials and Methods

Mice

CD4-Hlx transgenic mice were described previously (12) and maintained as heterozygous by crossing to C57BL/6 mice. FVB Hlx+/− mice were derived from the originally reported Hlx+/− mice (46) by crossing to the wild-type FVB mice for >10 generations. Inbred mice were purchased from the National Institutes of Health. All animal studies were conducted in accordance with procedures approved by the University of Rochester committee for animal research.

Preparation of CD4 T cells and APC

Total CD4 T cells were isolated by negative selection with magnetic beads as previously described (12). APC were prepared by depleting T and NK cells in spleen and lymph node cells and were irradiated with 3000 rad. As specified in the experiments, naive CD4 T cells were isolated from total CD4 T cells either by MACS sorting of the CD62Llow population (12) or FACS sorting of the CD4+CD45+CD62Llow population from the total CD4 T cell preparations.

Th cell differentiation

Cultures for Th cell differentiation were set up essentially as previously described (4). Briefly, equal numbers of naive CD4 T cells and APC (5 × 10⁶/ml) were stimulated with Con A (2.5 μg/ml; Roche) in RPMI 1640 plus 10% FBS and 50 U/ml IL-2 (Chiron). As specified in each experiment, anti-IL-4 Abs (11B11) or exogenous IL-4 were added to the cultures at the CD4 T cell preparations.

Retroviral infection of T cells

The retroviral vector MigR1 is gift from Drs. W. Pear and S. Reiner (Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA). The IL-4Rα-MigR1 for bicistronic expression of IL-4Rα and GFP is a gift from Dr. M. Boothby (Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN). The packaging cell line Phoenix Eco was originally developed in Dr. G. Nolan’s laboratory (Department of Molecular Pharmacology, Stanford University Medical Center, Stanford, CA) and was purchased from American Type Culture Collection. The packaging cells were transfected with retroviral vectors using LipofectaMINE 2000 (Invitrogen Life Technologies) according to the manufacturer’s instructions. Forty-eight hours after transfection, culture supernatants were harvested and centrifuged at 6000 × g to concentrate the viruses. For T cell infection, naive CD4 T cells were stimulated for 12 h. Culture supernatants were removed and kept at 4°C. Cells were then mixed with freshly prepared viruses plus 8 μg/ml polybene (American Bioanalytical) 0.5 ng/ml IL-4, and 50 U/ml IL-2, then centrifuged at 1800 rpm for 45 min at room temperature. After 12 h of incubation at 37°C, viruses were removed, and the saved culture supernatants with fresh IL-4 were added back to the cells.

Flow cytometry

For intracellular cytokine staining, differentiated CD4 T cells were activated with PMA (50 ng/ml) and ionomycin (1 μM), and stained for IL-4, IFN-γ, and CD4 or other surface Ags as previously described (12). For analysis of cell surface expression of IL-4Rα, total CD4 T cells prepared by negative selection with magnetic beads were first incubated with FC block (BD Pharmingen), followed by incubation with biotinylated anti-mouse IL-4Rα Ab. After washing, the cells were incubated with streptavidin-PE (BD Pharmingen), anti-CD4-allophycocyanin, anti-CD44-PE-Cy5, and anti-CD62L-FTTC (eBioscience). All stained cells were analyzed on a FACS Calibur.

Real-time RT-PCR

Total RNA were isolated from FACS-sorted naive CD4 T cells using an Ultraspec-II RNA isolation system (Biotex) and reverse transcribed with SuperScript II reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer’s instructions. Primers were purchased from Intergen (Nyack, NY). Sequences of the primers are as follows: gac cga atc tct gtc tat aat g and cca gcc gta cgg aaa acg aag t for β-tubulin, gtt act tca gta cca aga tta ag and agt ttg cca gtg ggg cta g for IL-4Rα, tgc cat gat gcc acc acc tta tg and gtt agg ctc ctg aa aag ta for Stat6, cat cgg cat tta gga cgt gat and gcg aag cta agt tct tc for IL-4, and gta cta cgc gcc tca tta tat cca and gtt cta agg aag tgc ttc tg for GATA-3. PCR were set up with the 2× SYBR Green real-time PCR Master Mix (Applied Biosystems). The reactions were run on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Data were analyzed with the SDS2.0 software. Transcripts of each gene relative to the β-tubulin V gene were determined with the 2−ΔΔCT method (47).

Western blot

Cells were lysed in lysis buffer (300 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.2 mM Na3VO4, 1 mM sodium fluoride, 50 mM β-glycerol phosphate, 1% Triton X-100, and 0.5% Nonidet P40). The cell lysates were directly used for Western blot analyses. Primary Abs are goat anti-mouse actin (Santa Cruz Biotechnology), rabbit anti-mouse Stat6 Ab (Cell Signaling Technology), and rabbit anti-Hlx Abs. The rabbit polyclonal anti-Hlx Abs were raised against a synthetic peptide of Hlx and cross-reacted to the human Hlx homologue HB24. After the primary Ab incubation, the protein blots were incubated with HRP-conjugated rabbit anti-goat IgG (Sigma-Aldrich) or goat anti-rabbit IgG (Santa Cruz Biotechnology). The proteins were then detected using Western Lightening Chemiluminescence Reagent Plus (PerkinElmer).

Immunoprecipitation

Cell lysates were diluted with an equal volume of dilution buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 50 mM β-glycerol phosphate, and 20% glycerol) plus protease inhibitors. The diluted lysates were incubated with anti-Stat6 Ab. The protein-Ab complexes were pulled down with protein G beads (Amersham Biosciences) and washed with the diluted lysis buffer. Protein blots were probed with HRP-conjugated anti-phosphotyrosine Ab 4G10 (Upstate Biotechnology).

Results

Effect of the Hlx transgene on Th cell differentiation in the absence of exogenous polarizing cytokines

The transcription factor Hlx is expressed in naive CD4 T cells and specifically up-regulated during Th1 cell differentiation (10, 12). In an earlier study, we found that transgenic mice expressing Hlx under a CD4 promoter generated more Th1 cells than their wild-type littermates when immunized with the protein Ag keyhole limpet hemocyanin. However, when naive CD4 T cells were stimulated to differentiate in vitro under extreme polarizing conditions for Th1 cell differentiation, no differences in Th1 cell differentiation
were observed between wild-type and transgenic CD4 T cells (12). We reasoned that the extreme polarizing conditions used in the in vitro cultures might have masked the effect of Hlx on Th cell differentiation under less polarizing conditions, which was probably the case for the in vivo environment. To better mimic the in vivo conditions, we compared in vitro Th cell differentiation of wild-type and transgenic naive CD4 T cells in the absence of exogenous IL-4, IL-12, and neutralizing Abs. After differentiation, the cells were restimulated for intracellular cytokine staining to detect Th1 and Th2 cells (Fig. 1a, upper panel). A markedly higher percentage of Th1 cells was detected in the Hlx transgenic CD4 T cells than in the wild-type CD4 T cells. Conversely, a significant number of Th2 cells were observed in the wild-type CD4 T cells, but only a minimum number of Th2 cells in the transgenic CD4 T cells. Thus, the Hlx transgene not only enhanced Th1, but also diminished Th2 cell differentiation. This effect was naive CD4 T cell intrinsic because the same wild-type APC were used in the differentiation cultures of both wild-type and transgenic CD4 T cells.

We suspected that the use of anti-IL-4-neutralizing Abs in the in vitro cultures for extreme polarization of Th1 cell differentiation might be responsible for the lack of difference between transgenic and wild-type T cells after differentiation. To test this, we set up cultures with anti-IL-4 neutralizing Abs, but without exogenous IL-12. Anti-IL-4 Abs completely blocked the generation of IL-4 single-producer and IL-4, IFN-γ double-producer cells in both wild-type and transgenic cells (Fig. 1a, lower panel), so that only Th1 cells were detected. The percentages of Th1 cells were essentially the same between wild-type and transgenic CD4 T cells differentiated in the presence of anti-IL-4 Abs. Therefore, anti-IL-4 Ab is sufficient to abolish the differences between wild-type and transgenic CD4 T cells differentiated under unmanipulated conditions.

To verify the difference in Hlx expression between wild-type and transgenic naive CD4 T cells, we performed Western blot analysis of Hlx expression. Rabbit polyclonal Abs were raised against an Hlx peptide. The specificity of the Ab was confirmed by Western blot analysis of 293T cells transfected with Hlx expression vector. As shown in Fig. 1b (left), a strong band was detected in the lysate of 293T cells transfected with the Hlx expression vector. A weak band was also detected in 293T cells transfected with the empty vector due to endogenous expression of the human Hlx homologue HB24, which could also be detected by RT-PCR (data not shown). When the Abs were used to examine the expression of Hlx in Hlx transgenic naive CD4 T cells, a relatively high level of expression was detected. Consistent with previous analysis with RT-PCR (10, 12), a relatively low level of Hlx was also detected in the wild-type naive CD4 T cells (Fig. 1b, right).

**Down-regulation of IL-4Ra in Hlx transgenic naive CD4 T cells**

Our data have indicated that endogenous IL-4 signaling is key to the difference in the ratio of Th1 and Th2 cells between transgenic and wild-type CD4 T cells differentiated under the unmanipulated, weak, polarizing condition. To uncover the underlying mechanism, we examined whether the early expression of IL-4 would be different and responsible for the different outcomes. Naive CD4 T cells were stimulated in the absence of exogenous IL-4 and IL-12. Thirty-four hours later, CD4 T cells were sorted for RNA extraction and real-time RT-PCR. Albeit at low levels, IL-4 messages were detected in both wild-type and transgenic CD4 T cells. The IL-4 message level of wild-type CD4 T cells was not higher, but instead slightly lower than the transgenic CD4 T cells (Fig. 2a).

Based on these data, we believed that the amount of early endogenous IL-4 was unlikely to be responsible for the different outcomes of differentiation between wild-type and transgenic naive CD4 T cells. We then suspected that signaling potentials of the wild-type and transgenic naive CD4 T cells might be different. To test this possibility, we analyzed the expression of IL-4Ra and Stat6 in wild-type and Hlx transgenic naive CD4 T cells by real-time RT-PCR. Although no difference in Stat6 expression was detected between wild-type and transgenic naive CD4 T cells, the IL-4Ra level in the transgenic naive CD4 T cells was ~4-fold lower.

![Figure 1](image-url)

**FIGURE 1.** Comparison of Th cell differentiation in the absence of exogenous polarizing cytokines and Hlx expression between wild-type and Hlx transgenic naive CD4 T cells. *a*, MACS-sorted naive CD4 T cells (CD4+CD62L<sup>hi</sup>) from wild-type and Hlx transgenic mice were stimulated with Con A (2.5 μg/ml) plus T and NK cell-depleted APC and IL-2 (50 U/ml) either without (upper panel) or with anti-IL-4 Abs (lower panel) in the absence of exogenous IL-4 or IL-12. After 4 days of differentiation, the cells were rested for 1 day and restimulated with PMA and ionomycin. Dot plots of intracellular staining of IL-4 and IFN-γ on gated CD4 T cells are shown. Numbers in the plots are percentages of each cell populations. *b*, Detection of Hlx proteins in naive CD4 T cells. The specificity of rabbit anti-mouse Hlx polyclonal Abs was tested by Western blot using cell lysates of 293T transfected with MigR1 GFP, or MigR1 Hlx. Proteins were separated by PAGE. Actin and Hlx proteins were detected using the anti-Hlx and anti-actin Abs (left panel). Right panel, Naive CD4 T cells were isolated by FACS sorting of the CD4+CD44<sup>low</sup>CD62L<sup>hi</sup> population. Cell lysates were analyzed as described in the left panel.
lower than in wild-type naive CD4 T cells (Fig. 2b). We also analyzed the surface expression of IL-4Rs by flow cytometry. Consistent with the real-time RT-PCR results, the surface IL-4Rα level on the Hlx transgenic naive CD4 T cells was dramatically lower than in wild-type naive CD4 T cells (Fig. 2c, left). In contrast, Western blot analysis confirmed that similar protein levels of Stat6 in wild-type and transgenic CD4 T cells (Fig. 2c, right).

IL-4 dose response of Hlx transgenic and wild-type naive CD4 T cells

The gene expression data suggested that the responsiveness of Hlx transgenic naive CD4 T cells to IL-4 might be impaired. To functionally analyze IL-4 responsiveness, we compared IL-4 dose-response curves of transgenic and wild-type naive CD4 T cells. Despite the low level of IL-4Rα, Hlx transgenic naive CD4 T cells remained responsive to IL-4. In the differentiation cultures of both transgenic and wild-type naive CD4 T cells stimulated with Con A and APC, we observed an IL-4 dose-dependent increase in Th2 cells and a decrease in Th1 cells (Fig. 3a), demonstrating the Th2-promoting and Th1-suppressing activities of IL-4. However, at each concentration of exogenous IL-4, a lower percentage of Th2 and a higher percentage of Th1 cells were detected in transgenic cells than in wild-type cells. Therefore, Hlx transgenic naive CD4 T cells showed weaker responses to IL-4 in terms of both promoting Th2 and suppressing Th1 cell differentiation. Similar results were obtained when the cells were stimulated with anti-CD3 and anti-CD28 (Fig. 3b). In subsequent experiments we will use only Con A and APC for T cell stimulation.

When the differentiated cells were analyzed for Hlx expression by Western blot, we found only a weak signal in nontransgenic CD4 T cells, which was further diminished in the presence of IL-4 (Fig. 3c, left). As expected, much stronger signals were detected in Hlx transgenic CD4 T cells (Fig. 3c, right). The difference between Hlx transgenic CD4 T cells differentiated in the absence or the presence of IL-4 was somewhat masked by the constitutive expression of Hlx driven by the CD4 promoter.

Stat6 activation in wild-type and Hlx transgenic CD4 T cells

We also measured the strength of IL-4 signaling in Hlx transgenic and wild-type CD4 T cells by detecting Stat6 activation. Transgenic and wild-type naive CD4 T cells were activated with Con A and APC in the presence of suboptimal concentration of IL-4. Cells were harvested after a relatively short culture time of 20 h. Cell lysates were immunoprecipitated with anti-Stat6 Abs. The precipitated proteins were analyzed with anti-phospho-tyrosine Abs in Western blot to detect phosphorylated Stat6. When equal amounts of immunoprecipitated Stat6 were loaded, as determined by Western blot analysis with anti-Stat6 Abs, less phosphorylated Stat6 was detected in Hlx transgenic cells than in wild-type cells (Fig. 4a, left). We also examined the difference in IL-4 signaling between Hlx transgenic and nontransgenic naive CD4 T cells (Fig. 4a, right). In this case, fresh isolated naive CD4 T cells were stimulated with IL-4. Cell lysates were analyzed in the same way as before. As expected, less Stat6 activation was detected in Hlx transgenic naive CD4 T cells than in nontransgenic naive CD4 T cells. To determine whether IL-4Rα expression could enhance Th2 cell differentiation in Hlx transgenic CD4 T cells, Hlx transgenic CD4 T cells were stimulated to differentiate in the presence of IL-4. After 12 h of T cell stimulation, the cells were infected with retrovirus expressing a bicistronic IL-4Rα-GFP or GFP alone. After infection, the cells were returned to differentiation in the presence of IL-4. The differentiated cells were analyzed by intracellular cytokine staining. As shown in Fig. 4b, more Th2 and fewer Th1 cells were detected in cells infected with retrovirus expressing IL-4Rα. These results directly demonstrated that a lower IL-4Rα level in Hlx transgenic CD4 T cells not only led to less IL-4 signaling, but also to less Th2 cell differentiation and more Th1 cell differentiation.

Effect of anti-IL-4Rα Ab on Th cell differentiation

The study of Hlx transgenic mice clearly showed that the IL-4Rα level is a key factor that controls the ratio of Th1 and Th2 cell differentiation under suboptimal polarizing conditions. However, an alternative explanation, such as changes in T cell development in the transgenic mice, could be responsible for the effect. To demonstrate that the IL-4Rα level indeed determines the ratio of Th1 and Th2 cell differentiation under suboptimal conditions, we used anti-IL-4Rα neutralizing Ab to titrate down the IL-4Rα level on normal CD4 T cells (Fig. 5). Thus, different concentrations of anti-IL-4Rα Abs were added to Th cell differentiation cultures either without exogenous IL-4 or with a suboptimal concentration of exogenous IL-4. As expected, exogenous IL-4 increased Th2 and decreased Th1 cell differentiation compared with cultures without exogenous IL-4. In both cases, the addition of anti-IL-4Rα
Abs effectively reduced Th2 cell differentiation and enhanced Th1 cell differentiation proportionally to the Ab concentration. Therefore, modulating the IL-4Rα level on the cell surface alone is indeed sufficient to change the ratio of Th1 and Th2 cell differentiation under weak or suboptimal polarizing conditions.

**T cell development in Hlx \(^{+/−}\) heterozygous mice**

We have presented evidence that overexpression of Hlx in transgenic mice diminishes the responsiveness of naive CD4 T cells to IL-4. A logical prediction would be that the loss of Hlx would have the opposite effect on naive CD4 T cells. However, Hlx deficiency is embryonically lethal and causes miniature fetal liver development (46). We therefore opted to use Hlx \(^{+/−}\) heterozygous mice to study genetic regulation of IL-4 responsiveness of naive CD4 T cells by Hlx. We first examined whether there were any abnormalities in T cell development in Hlx \(^{+/−}\) heterozygous mice. Staining of thymocytes for CD4 and CD8 showed essentially the same distribution of all four populations of thymocytes in heterozygous and wild-type mice (Fig. 6a). Likewise, similar numbers of CD4 and CD8 single-positive cells were detected in the periphery in heterozygous and wild-type mice (Fig. 6b). The percentages of peripheral CD4 T cells with the naive phenotype (CD4\(^{+}\)CD62L\(^{high}\)) were also similar in heterozygous and wild-type mice (Fig. 6c). Thus, T cell development in Hlx \(^{+/−}\) heterozygous mice appeared to be normal.

**IL-4Rα expression and IL-4 responsiveness of Hlx \(^{+/−}\) naive CD4 T cells**

We compared IL-4Rα expression on naive CD4 T cells of wild-type and heterozygous mice (Fig. 7a). Flow cytometric analysis showed a surprisingly clear up-regulation of IL-4Rα on Hlx \(^{+/−}\) heterozygous naive CD4 T cells compared with wild-type naive CD4 T cells. Consistently, real-time RT-PCR results showed a higher mRNA level of IL-4Rα in heterozygous than wild-type naive CD4 T cells. Real-time RT-PCR analysis also confirmed the expected lower expression of Hlx in heterozygous naive CD4 T cells (Fig. 7a, right). To functionally analyze the responsiveness to IL-4, we compared the IL-4 dose responses of heterozygous and wild-type naive CD4 T cells (Fig. 7b). As expected, an inverse relationship between Th1 cell differentiation and exogenous IL-4 concentration was observed, whereas a positive correlation existed between Th2 cell differentiation and exogenous IL-4 concentration. At each exogenous IL-4 concentration tested, heterozygous naive CD4 T cells gave rise to more Th2 cells and fewer Th1 cells than wild-type naive CD4 T cells. Real-time RT-PCR analysis showed higher GATA-3 expression in Hlx heterozygous CD4 T cells differentiated in the presence of IL-4 than wild-type CD4 T cells (Fig. 7c), consistent with the fact that there were more Th2 cells in heterozygous CD4 T cells. Therefore, heterozygosity of Hlx gene caused a heightened responsiveness of naive CD4 T cells to IL-4, a phenotype opposite to that of the Hlx transgene.

**Discussion**

Studies of Th1 and Th2 cell differentiation have greatly benefited from in vitro models that can provide a nearly homogeneous population of Th1 or Th2 cells. Using such in vitro differentiation models, much progress has been made toward understanding the development of the transcription machineries responsible for effector cytokine expression in Th1 and Th2 cells. However, one of the ultimate goals of studying Th cell differentiation is to understand how genetic and physiological factors influence the outcome of differentiation in vivo. Under in vivo conditions, neutralizing...
Cell lysates were prepared and analyzed as described in the nontransgenic mice were stimulated with IL-4 (0.5 ng/ml) only for 20 min. Western blot using the phosphotyrosine-specific Ab 4G10 (top panel). Immunoprecipitation containing equal amounts of Stat6 were analyzed by Western blot for Stat6 (lower panel). The immunoprecipitated proteins were analyzed by Western blot for Stat6 (lower panel). The immunoprecipitated proteins containing equal amounts of Stat6 were analyzed by Western blot using the phosphotyrosine-specific Ab 4G10 (upper panel). In the right panel, fresh isolated naive CD4 T cells from Hlx transgenic and nontransgenic mice were stimulated with IL-4 (0.5 ng/ml) only for 20 min. Cell lysates were prepared and analyzed as described in the left panel. Effect of retroviral expression of IL-4Rα on the outcome of Th cell differentiation. Naive CD4 T cells from Hlx transgenic mice were stimulated with Con A and APC in the presence of IL-4 (0.5 ng/ml). Twelve hours later, the cells were infected with retrovirus expression bicistronic IL-4Rα-GFP or GFP alone. After infection, cells were stained for CD4 and CD8, and the percentages of single positive cells are shown. Numbers in the quadrants show the percentages of the cell populations.

Abs against polarizing cytokines used in the in vitro cultures to create extreme polarizing conditions do not exist, and polarizing cytokines for one subset may not always dominate over those for the other subset. Therefore, it is important to understand the factors that influence Th cell differentiation under such weak or competitive conditions. In this study we investigated an earlier observation that Hlx transgenic mice generated elevated Th1 response to a protein Ag in vivo (12). This study led us to uncover a naive CD4 T cell intrinsic element that influences the outcome of Th cell differentiation. Our study demonstrates that Hlx modulates the responsiveness of naive CD4 T cells to the polarizing cytokine IL-4. The relative responsiveness of naive CD4 T cells to IL-4 is a critical factor that determines the ratio of Th1 and Th2 cells generated from differentiation under weak polarizing conditions.

The cytokine milieu in which naive CD4 T cells respond to Ag has long been considered to be the predominant factor that drives Th cell differentiation toward either a Th1- or Th2-dominant outcome (16). Some infectious agents are known to induce cytokine environment that overwhelmingly favors either Th1 or Th2 cell differentiation. However, this mode of regulation may not be applicable to many other immune responses, such as those against autoantigens and environmental Ags, where no microbial products are present to stimulate innate immune responses to create a dominantly polarizing cytokine environment. Under these circumstances, the immediate production of IL-4 by the responding naive CD4 T cells upon Ag stimulation is especially important because many studies have shown that this source of IL-4 is sufficient to drive Th2 cell differentiation. In this study we found that the level of IL-4Rα in Hlx transgenic naive CD4 T cells was markedly reduced. The reduction of IL-4Rα expression led to less activation of Stat6 and ultimately less Th2 and more Th1 differentiation under weak or suboptimal polarizing conditions. In contrast, Hlx haploinsufficiency caused more IL-4Rα expression and Th2 cell differentiation and concomitantly less Th1 cell differentiation.
Therefore, we have established that IL-4Rα expression on naive CD4 T cells is genetically controlled by Hlx. Furthermore, the level of IL-4Rα is a determining factor that controls the ratio of Th1 and Th2 cell differentiation under weak polarizing conditions.

Supporting the idea of IL-4Rα as a key factor for regulating Th2 cell differentiation in vivo is the genetic lineage of atopic diseases to polymorphism of IL-4Rα amino acid sequences. Atopic diseases are caused by biased Th2 responses to environmental Ags. Allelic polymorphism at certain amino acid positions of the IL-4Rα correlates with the risk level of developing atopic diseases (48, 49). Our study shows that not only the allelic amino acid sequence variation, but also other transcription factors such as GATA-3, are a determining factor for regulating Th2 cell differentiation in vivo. The level of IL-4Rα expression on naive CD4 T cells is not surprising, because high levels of Hlx promote IFN-γ expression. Therefore, Hlx must be kept at low levels to avoid aberrant expression of IFN-γ in naive CD4 T cells. Thus, the requirement for a threshold level of Hlx to regulate IL-4Rα expression is much lower than that of Hlx to regulate IFN-γ gene expression. Such dose-dependent regulation of gene expression is reminiscent of that of GATA-3, for which different threshold levels seem to be required for activating different Th2 cytokine genes (4). The biochemical bases for such quantitative requirements are of great biological significance and remain a challenge for future studies.

In conclusion, our study has modified the instruction model of Th cell differentiation to include the intrinsic difference in the responsiveness of naive CD4 T cells to polarizing cytokine as a key factor in determining the outcome of differentiation. We provided definitive evidence that the responsiveness of naive CD4 T cells to polarizing cytokine is genetically controlled. The genetically determined variations of the responsiveness predispose naive CD4 T cells to generate quantitative differences in Th1 and Th2 responses.

FIGURE 7. IL-4Rα expression and IL-4 dose response of wild-type and Hlx<sup>−/−</sup> heterozygous naive CD4 T cells. a. Staining for IL-4Rα was performed as described in Fig. 2. Overlay histograms of IL-4Rα-PE staining and isotype IgG control of gated naive CD4 T cells (CD4<sup>+</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup>) of wild-type and heterozygous mice are shown (left). FACS-sorted naive CD4 T cells (CD4<sup>+</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup>) from wild-type and Hlx<sup>−/−</sup> heterozygous mice were analyzed for the expression of IL-4Rα and Hlx by real-time RT-PCR. Transcripts relative to the Tubb5 gene are shown (right). b, MACS-sorted naive CD4 T cells were activated by Con A and wild-type APC in the presence of different concentrations of exogenous IL-4. After 4 days of differentiation, the cells were rested and reactivated by PMA and ionomycin. The cells were stained for CD4 and intracellular IL-4 and IFN-γ. The percentages of Th1 or Th2 cells on gated CD4 T cells were plotted against the initial concentration of the exogenous IL-4. c, GATA-3 expression in differentiated CD4 T cells. Naive CD4 T cells from wild-type or Hlx heterozygous mice were differentiated in the presence of IL-4 (1 ng/ml). On day 4, RNA was extracted and analyzed for GATA-3 expression by real-time RT-PCR.
Genetic Control of IL-4Rα Expression in Naive CD4+ T Cells

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The authors have no financial conflict of interest.

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