Repression of interleukin-4 in T helper type 1 cells by Runx/Cbfβ binding to the Il4 silencer

Yoshinori Naoe,1 Ruka Setoguchi,1 Kaori Akiyama,1,2 Sawako Muroi,2 Masahiko Kuroda,3 Farah Hatam,4 Dan R. Littman,4 and Ichiro Taniuchi1,2

Upon encountering antigen, naive CD4+ Th cells differentiate into effector cell subsets that are defined by expression of distinct cytokines. Th1 cells produce IFNγ and mainly participate in cellular immune responses against intracellular pathogens, whereas Th2 cells produce IL-4, -5, and -13 and control infection with extracellular microbes (1). An inappropriate balance in Th1- and Th2-mediated responses has been proposed to be involved in various immune system disorders. For example, IL-4 and -5 are strongly implicated in atopic and allergic diseases, including asthma, through their enhancement of IgE-mediated and eosinophilic immune responses (2).

Cytokine signaling and transcription factor networks play essential roles in regulating differentiation of Th cell subsets. The transcription factors T-bet and GATA-3 are the central regulators in the induction of Th1 and Th2 differentiation, respectively (3, 4). In highly polarized Th1 and Th2 cells, each of the characteristic cytokines, IFNγ and IL-4, is reciprocally expressed. In Th1 cells, the stable repression of the Il4 gene has been ascribed to epigenetic regulation initiated by combined cis-regulatory elements (5, 6). Conserved noncoding sequences (CNSs) and DNaseI hypersensitive (HS) sites, which are often used to identify putative cis-regulatory regions, have been identified in the Il4 locus. The HS IV site is located toward the 3′ end of the Il4 locus and is well-conserved between species (7). Deletion of HS IV in the mouse genome led to increased Il4 transcription in naive CD4+ T cells and Th1 cells, but not in Th2 cells. Furthermore, enforced expression of GATA-3 in Th1 cells inhibited binding of Runx complexes to the Il4 silencer. Interestingly, T cell–specific inactivation of the Cbfβ gene in mice led to elevated serum immunoglobulin E and airway infiltration. These results demonstrate critical roles of Runx complexes in regulating immune responses, at least in part, through the repression of the Il4 gene.
Silencing of the \textit{Cd4} gene is another example of negative transcriptional regulation during differentiation of T lymphocytes. In thymocytes committed to differentiate toward the cytotoxic T cell lineage, the \textit{Cd4} locus is epigenetically silenced by an intronic \textit{Cd4} silencer whose function requires binding of Runx transcription factor complexes (8, 9). The Runx complexes are composed of two subunits, including one of the Runx proteins, which possess a conserved DNA-binding domain, and the unique Cbf\textbeta protein (10). Examination of mice lacking expression of either Runx1 or Runx3 in thymocytes revealed that Runx3 plays a major role in epigenetic \textit{Cd4} silencing (9, 11). Interestingly, Runx1 was suggested to be involved in repressing \textit{Gata-3} expression during differentiation of CD4\textsuperscript{+} Th cells (12). Moreover, a transient asthma-like disease, which was characterized by infiltration of eosinophilic cells into the lung, developed in Runx3-deficient mice (13, 14). In addition, the \textit{RUNX3} locus on human chromosome 1p36 maps to a region containing susceptibility genes for asthma (15). These results suggest the involvement of Runx family members in the differentiation of CD4\textsuperscript{+} Th cells. Hence, it is important to study the function of Runx complexes in CD4\textsuperscript{+} T cell differentiation programs in mouse models.

In this study, we show that T cell–specific inactivation of the Cbf\textbeta gene led to spontaneous development of asthma-related symptoms, including elevated serum IgE and airway infiltration. In cells cultured under Th1 differentiation conditions, derepressed IL-4 production was detected in IFN\gamma-producing Th1 cells in the absence of Cbf\textbeta or Runx3 protein. Furthermore, we show that binding of Runx complexes to the \textit{Il4} silencer correlated with IL-4 repression and was antagonized by GATA-3. These results demonstrate that Runx complexes play an important role in repressing IL-4 expression during Th cell differentiation and in the regulation of immune responses.

RESULTS AND DISCUSSION
Expression of Runx1 and Runx3 proteins during Th cell differentiation
We first examined expression of Runx proteins during differentiation of CD4\textsuperscript{+} Th cells. Purified CD4\textsuperscript{+}CD25\textsuperscript{−}CD62L\textsuperscript{+} naive T cells were stimulated with immobilized anti-CD3 antibody and soluble anti-CD28 antibody were cultured with no additional supplement (neutral) and with specific combinations of cytokine and antibody for inducing Th1 (Th1-skewed) or Th2 (Th2-skewed) differentiation. At 2 d (lane 1) and 6 d (lane 2) after stimulation, expression of Runx1, Runx3, Cbf\textbeta, T-bet, and Gata-3 proteins were examined (top). (bottom) Expression of distal promoter–derived Runx1 and Runx3 transcripts are shown. Data are representative of three independent experiments.
we generated Loxp-flanked Cbfβflx (Cbfβγ) and Runx3flx (Runx3γ) mutant alleles by gene targeting (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20062/DC1). Mice harboring either Cbfβγ or Runx3γ alleles were crossed with Lck-Cre or Cd4-Cre transgenic mice, to inactivate the targeted genes at CD4+CD8− DN or CD4+CD8+ DP stages, respectively. Whereas inactivation of Runx1 at the DN stage resulted in a more than fivefold reduction in the number of total thymocytes (9), the reduction was only approximately twofold in Cbfβγ/Lck mice, although development of mature thymocytes was severely impaired (Fig. 2A and Fig. S2). In contrast, the number of mature thymocytes was only moderately reduced in Cbfβγ/Cd4 mice (Fig. 2A and Fig. S2). Although Cre-mediated recombination of the Cbfβγ allele appeared to be very efficient in DP thymocytes by both Lck- and Cd4-Cre transgene, a significant amount of Cbfβγ protein could be detected in those cells from the Cbfβγ/Cd4 mice (Fig. 2B). However, in the peripheral TCRαβ cells from Cbfβγ/Cd4 mice, no Cbfβγ protein was detected (Fig. 2E), indicating that Cbfβγ protein was gradually lost after inactivation of the gene.

Generation of T cell–specific, Cbfβ-deficient mice

Because germline-null mutations of Cbfβ and Runx3 result in embryonic and neonatal lethality, respectively (16–18),

and soluble anti-CD28 antibodies. 2 d after stimulation, Runx1 protein was substantially decreased (Fig. 1). After another 4 d of culture, although expression of Runx1 protein was restored and detected in both Th1 and Th2 cells, Runx3 protein was detected almost specifically in Th1 cells (Fig. 1). Thus, both Runx1 and Runx3 proteins are expressed in polarized Th1 cells. Expression of distal (P1) promoter–derived Runx1 or Runx3 transcript was well correlated with that of Runx1 or Runx3 protein, suggesting that activation of a distal promoter is important for regulated expression of Runx proteins. Considering the redundant function of Runx1 and Runx3 in Cd4 silencing in CD8+ T cells (11), it is also possible that these two transcription factors function redundantly in CD4+ T cells. Because association with the nonredundant Cbfβ protein is essential for the function of both Runx1 and Runx3, we analyzed the effect of loss of Cbfβ on Th cell differentiation (10, 16).

Figure 3. Development of an asthma-like phenotype after T cell–specific inactivation of the Cbfβ gene. (A) Concentrations of serum IgA, IgG1, and IgE from 8–10–wk-old Cbfβγ (lane 1), Cbfβγ/Cd4 (lane 2), and Runx3γ/Cd4 (lane 3) mice. Horizontal lines represent averages from each group. (B) Representative results of H&E-stained sections of lung from the indicated 8–10–wk-old mice are shown using low (top) and high (bottom) magnification. Lymphocytes and eosinophils infiltrate the bronchioles, perivascular space, and alveolar septa in Cbfβγ/Cd4 mice, whereas lymphoid cells mainly infiltrate the perivascular space in Runx3γ/Cd4-Cre mice. Bars: (B, top) 50 μm; (B, bottom) 100 μm.
transgene in thymus resulted in a loss of Runx3<sup>fl/k</sup> allele in peripheral T cells (Fig. 2, C and F), which is consistent with loss of Runx3 protein in CD8<sup>+</sup> T cells from Runx3<sup>3/3</sup>: Cd4 mice (Fig. 1).

In peripheral lymphoid tissues from Cbfβ<sup>+/+</sup>: Cd4 mice, mature TCRAβ T cells consisted of two major subsets, CD4<sup>+</sup>CD8<sup>+</sup> and CD4<sup>+</sup>CD8<sup>−</sup> cells (Fig. 2 D). Perforin expression in CD4<sup>+</sup>CD8<sup>−</sup> cells was comparable to that in wild-type CD8<sup>+</sup> T cells (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20062456/DC1), which is consistent with the CD4<sup>+</sup>CD8<sup>−</sup> phenotype resulting from the loss of Cd4 silencing in CD8<sup>+</sup> cytotoxic-lineage cells in the absence of Cbfβ protein and Runx complexes (9).

Development of asthma-related symptoms after T cell–specific inactivation of the Cbfβ gene

It has been shown that outbred Runx3-deficient mice develop a transient inflammatory infiltrate in their lungs and elevated serum IgE (13, 14). In Cbfβ<sup>+/+</sup>: Cd4 mice, serum IgA, IgG1, and IgE titer were significantly elevated (Fig. 3 A). Numerous lymphocytes and eosinophils were found to infiltrate bronchioles, perivascular space, and alveolar septae in the lung from all Cbfβ<sup>+/+</sup>: Cd4 mice examined (Fig. 3 B). Mild infiltration of lymphoid cells in the bronchioles and perivascular space, but not in alveolar septae, was also observed in about one-third of Runx3<sup>3/3</sup>: Cd4 mice (Fig. 4). Thus, T cell–specific loss of Cbfβ protein led to spontaneous development of asthma-related features, and a similar, but milder, disease developed in mice lacking Runx3 in T cells. Because such asthma-related findings are often correlated with enhanced Th2 responses, we next examined cytokine production and differentiation of CD4<sup>+</sup> T cells in the absence of Cbfβ.

Derepression of the Il4 gene in nonpolarized and Th1 cells in the absence of Runx complexes

Purified naive T cells from Cbfβ<sup>+/+</sup>, Cbfβ<sup>/−</sup>: Cd4, and Runx3<sup>3/3</sup>: Cd4 mice were stimulated with anti-CD3 and -CD28 antibodies. After 48 h, the level of IL-4 secreted from Cbfβ-deficient cells was 10-fold higher than that from control cells (Fig. 4 A). After an additional 4 d of culture with IL-2, cells were analyzed by intracellular IL-4 and IFNγ staining. Consistent with the higher IL-4 production observed after 2 d, IL-4–producing cells, including IL4/IFNγ double producers, were differentiated efficiently from Cbfβ<sup>/−</sup>-deficient naive CD4<sup>+</sup> T cells (Fig. 4 B). The differentiation of IFNγ-producing Th1 cells was also enhanced by Cbfβ-deficiency by yet uncharacterized mechanisms.

When Cbfβ-deficient naive CD4<sup>+</sup> T cells were cultured under Th1 polarizing conditions, in the presence of IL-4 neutralizing antibody, cells producing both IL-4 and IFNγ were detected (Fig. 4 B). These IL-4/IFNγ double producers were, thus, differentiated independently of IL-4 signaling. In contrast, only IL-4–producing Th2 cells were differentiated under Th2-skewed conditions. These results indicate that expression of both IL-4 and IFNγ in the same cell is an outcome of IL-4 derepression in Th1 cells, rather than IFNγ derepression in Th2 cells. Previous studies showed that constitutive expression of Gata-3 induced derepression of the Il4 gene in Th1 cells (19, 20). However, we observed no difference in the level of induced T-bet or Gata-3 (Fig. 4 C), indicating that IL-4 derepression was not a consequence of dysregulation of these transcription factors.
Association of Runx complexes with the \( \text{Il4} \) silencer

Because derepression of the \( \text{Il4} \) gene was induced in Th1 cells upon deletion of the HS IV \( \text{Il4} \) silencer, which contains a putative Runx recognition motif \((5'\text{-ACCRCA-3')}\) (7), we next examined whether Runx complexes directly associate with the \( \text{Il4} \) silencer by chromatin immunoprecipitation (ChIP) assays. The \( \text{Il4} \) silencer region was efficiently amplified from DNA precipitated with anti-Cbf\(\beta\)2 antibody, but not with control antibody, from both naive CD4\(^+\) T cells and Th1 cells (Fig. 5 A). In sharp contrast, anti-Cbf\(\beta\)2 antibody failed to precipitate the \( \text{Il4} \) silencer from Th2 cells, although the antibody precipitated control \( \text{Tcf} \) enhancer, which is known to be regulated by Runx complexes, from both Th1 and Th2 cells. Thus, binding of Runx complexes is well correlated with the specificity of \( \text{Il4} \) silencer activity.

The level of IL-4 production and the severity of asthma-related symptoms were higher in Cbf\(\beta\)\(^{-}\)/Cd4 mice than in the Runx3\(^{-}\)/Cd4 mice (Figs. 3 and 4). This discrepancy suggests a compensatory function within Runx family members in the regulation of \( \text{Il4} \) silencer activity. Indeed, Runx1 protein is expressed in naive CD4\(^+\) T cells and Th1 cells (Fig. 1). Therefore, we analyzed whether Runx1 protein binds to the \( \text{Il4} \) silencer by using an anti-Runx1 antibody in ChIP assays. To eliminate possible cross-reactivity of the Runx1 antibody with Runx3 protein, we used Runx3-deficient cells as a control. The \( \text{Il4} \) silencer region was precipitated from both control cells and Runx3-deficient cells by the anti-Runx1 antibody (Fig. 5 B). Thus, it is likely that Runx1 is involved in regulating \( \text{Il4} \) silencer function, at least when Runx3 is not present.

Our results indicated that Runx complexes dissociate from the \( \text{Il4} \) silencer in Th2 cells, despite its expression. This result suggests that there is either a mechanism that inhibits Runx binding to the \( \text{Il4} \) silencer in Th2 cells or one that permits binding only in Th1 cells. Therefore, we examined the effect of enforced expression of Th2- and Th1-specific factors, Gata-3 and \( \text{T-bet} \), on the binding of Runx complex to the \( \text{Il4} \) silencer. Although Gata-3 expression in Th1 cells induced dissociation of Runx complexes from the \( \text{Il4} \) silencer, \( \text{T-bet} \) expression in Th2 cells only induced Runx3 expression, but not Runx complex association with the \( \text{Il4} \) silencer (Fig. 5, C and D). Furthermore, Gata-3 induced IL4 expression in polarized Th1 cells (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20062456/DC1), as previously reported (20). These results demonstrate that Gata-3 functions to inhibit binding of Runx complexes to the \( \text{Il4} \) silencer.

Our results are consistent with the recent description of \( \text{T-bet} \)-dependent Runx3 expression in Th1 cells and Runx3 binding to the \( \text{Il4} \) silencer (21). However, based on the expression patterns of Runx1 and Runx3 proteins, we propose that Runx1 is mainly involved in repressing the \( \text{Il4} \) gene in naive CD4\(^+\) T cells. When Runx1 expression is reduced after encounter with antigen, newly expressed Runx3 plays a role in initiating \( \text{Il4} \) repression in the early phase of Th1 differentiation. This would be followed by maintenance of \( \text{Il4} \) repression by both Runx1 and Runx3 proteins. In contrast, during Th2 cell differentiation, Gata-3 induces dissociation of Runx complexes from the \( \text{Il4} \) silencer by yet uncharacterized mechanism and induces IL4 expression.

Collectively, our results demonstrate that loss of Runx complex function in CD4\(^+\) T cells leads to spontaneous development of asthma-related symptoms caused by enhanced Th2 responses that are caused, at least in part, by failure of \( \text{Il4} \) silencing. It is not documented whether \( \text{Il4} \) silencer-deficient mice spontaneously develop similar symptoms, although impaired Th1-mediated immunity upon \textit{Leishmania major} infection of these mice was reported (7). It is possible that, in addition to loss of \( \text{Il4} \) silencing, additional mechanisms caused by loss of Runx complex function in T cells facilitate disease development. Alternatively, because an asthma-like phenotype was also attributed to loss of Runx3 function in dendritic cells (13, 14), we must consider the potential involvement of cells other than T cells in disease development. Because the human \( \text{RUNX3} \) locus is closely linked to one of the asthma-susceptibility loci (15),
dysfunction of Runx complexes may be involved in the human disease. A better understanding of Runx complex function during Th cell differentiation should provide important additional insights into the pathogenesis of allergic diseases.

MATERIALS AND METHODS

Mice. The targeting vectors for generating Cbfβlox and Runx3lox alleles were constructed in the pL-Neo plasmid (8), with genomic fragments containing the loxp-flanked coding exon and neo<sup>+</sup> gene inserted within the short 3′ side homology region (Fig. S1). The vector for the Cbfβ genomic fragment was obtained from S.-C. Bae (Chungbuk University, Cheongju, South Korea). Transfection into E14 ES cells was performed as previously reported (9). The Lek-Cre and Cd4-Cre transgenic mice were provided by C. Wilson (University of Washington, Seattle, WA). Mouse colonies were maintained in an animal facility in the Research Center for Allergy and Immunology RIKEN Institute, and experiments were performed according to the institutional guidelines for animal care.

Antibodies. Anti-Runx3 antibody was provided by Y. Ito (Institute of Molecular and Cell Biology, Singapore) (22). Anti-Runx1 and -Cbfβ2 antibodies were generated by immunizing rabbits with peptides corresponding to the N terminus of the distal promoter-derived Runx1 protein and to the C-terminal end of Cbfβ2, respectively. Anti-Gata3 (H3G-31) and -T-bet (4B10) antibodies were purchased from Santa Cruz Biotechnologies, and all monoclonal antibodies used for staining cells were obtained from BD Biosciences.

Isolation and culture of naive T cells. Naïve CD4<sup>+</sup>CD25<sup>-</sup>CD62L<sup>+</sup> T cells were sorted by flow cytometry. Differentiation of CD4<sup>+</sup> T cells was induced as previously described (12). In brief, for inducing Th1 or Th2 differentiation, the naive cells stimulated with 2 ng/ml of immobilized anti-CD3 and 2 ng/ml of soluble anti-CD28 antibody were cultured in the medium supplemented with 20 U/ml rIL-2 for an additional 4 d before staining for intracellular cytokines (12).

Western blot and ELISA. Whole-cell lysates were resolved by SDS-PAGE and transferred to Hybond-P membranes (GE Healthcare). The membranes were probed with an appropriate primary antibody, and immunocomplexes were detected using ECL reagents (GE Healthcare). Cytokine and serum immunoglobulin levels were assessed by ELISA using Quantikine (R&D Systems) and Mouse Ig ELISA Quantitation kit (Bethyl), respectively.

ChIP assay. ChIP assays were performed according to protocols provided for the ChIP Assay kit (Millipore). Chromatin DNA was fragmented by sonication to a mean length of 500 bp, and was immunoprecipitated with control, anti-Cbfβ2, or -Runx1 antibody. The precipitated DNA was subjected to PCR amplification. The primer sequences used in ChIP assays and RT-PCR are described in Fig. S5.

Retrovirus infection. The pMigRI-T-bet and the pMX-Gata-3 vectors were provided by S.L. Reiner (University of Pennsylvania, Philadelphia, PA) and M. Kubo (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan), respectively. Naïve T cells were stimulated in Th1 or Th2 conditions for 3 d, infected with retroviruses, and sorted 2 d after infection for analyses.

Online supplemental material. Fig. S1 shows the targeting strategy used to generate Cbfβ<sup>lox</sup> and Runx3<sup>lox</sup> alleles. Fig. S2 shows the decreased number of total and mature thymocytes in Cbfβ<sup>−/−</sup> Lek and Cbfβ<sup>−/−</sup> Cd4 mice. Fig. S3 shows the expression of perforin in peripheral T cells from Cbfβ<sup>−/−</sup> Cd4 mice. Fig. S4 shows the induction of IL4 expression in Th1 cells by Gata-3 transduction. Fig. S5 provides primer sequences used in this study. This online version of this article is available at http://www.jem.org/cgi/content/full/jem.20062456/DC1.

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