Down-regulation of Runx1 Expression by TCR Signal Involves an Autoregulatory Mechanism and Contributes to IL-2 Production

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The Runx family of transcription factors is characterized by the presence of a Runt domain, a highly conserved 128-amino acid (aa) region (1). The Runt domain is required for heterodimerization with the CBFβ cofactor and binding to the DNA sequence 5′-PuACCPuCA-3′. Binding of Runx proteins to their targets can activate or repress gene expression, depending on the molecules involved (2). Mammalian Runx proteins are expressed in cells and tissues at specific developmental stages where they play distinct roles. In particular, Runx1 and Runx3 are involved in T cell differentiation (3, 4).

The Runx1 protein is highly expressed in T lymphocytes (5) and has been linked to diverse aspects of T cell differentiation, ranging from the early phase of thymocyte development to the peripheral phase of helper T (Th) differentiation (3, 4). During early thymocyte development, Runx1 promotes the transactivation of T-cell receptor (TCR) genes and inhibits CD4 expression, thereby promoting the differentiation of CD4/CD8 double-negative (DN) cells (6, 7). Runx1 appears to confer apoptotic resistance to double-positive (DP) thymocytes by modulating the expression of Fas and Bim, two pro-apoptotic molecules (8). In addition, Runx1 contributes to the positive selection of CD4 single-positive (SP) cells (7, 9). In peripheral lymphoid tissues, Runx1 has been implicated in the survival and homeostasis of CD4+ cells via regulation of IL-7Rα (7). During T cell differentiation, over-expression of Runx1 suppresses GATA3 (an essential Th2 transcription factor), thus promoting the differentiation of CD4+ cells to Th1 cells (10). In regulatory T cells (Treg), Runx1 induces Foxp3 gene expression and binds with Foxp3 protein to control Treg suppressive activity. Thus, deletion of Runx1 in Treg cells causes a severe autoimmune disease in a mouse model (11–13). The functions of Runx3 are similar to those of Runx1; Runx3 silences CD4 expression in CD8 single-positive thymocytes (14) and promotes the differentiation of CD4+ cells into the Th1 lineage (15–17).

Expression of Runx1 is controlled by proximal and distal promoters (18, 19). These promoters drive the expression of two Runx1 transcripts that differ in their 5′-untranslated region (UTR) and 5′ coding regions. Consequently, Runx1 proteins expressed from the proximal and distal promoters have identical aa sequences, with the exception of aa 1–5 of proximal Runx1 and aa 1–19 of distal Runx1 (20). Because of their different lengths, the proximal and distal Runx1 proteins can be distinguished from each other by immunoblot analyses (21). The proximal and distal Runx1 transcripts also differ with regard to their tissue expression patterns. Proximal Runx1 is expressed in a variety of tissues including the brain, pancreas, kidney, heart, and liver (where proximal Runx1 predominates over distal Runx1) (22). In contrast, distal Runx1 is highly expressed by T lymphocytes in the thymus and spleen, where proximal Runx1 is barely expressed. Therefore, distal Runx1 is thought to have multiple roles in T cells as mentioned above.
Expression of Runx1 is substantially down-regulated during two stages of T cell differentiation. The first down-regulation occurs when DN thymocytes advance to the DP stage (23), and we previously demonstrated that interference with Runx1 down-regulation at this stage severely inhibits the DN-to-DP transition (21). The second down-regulation occurs when naïve CD4+ cells differentiate into effector cells (10, 15, 17), the possible significance of which has yet to be determined. Interestingly, the DN-to-DP progression of thymocytes and the naïve-to-activated transition of CD4+ cells are both initiated by TCR activation. The TCR signal is known to activate/modulate a complicated signaling network (24). Thus, it is likely that TCR signals down-regulate Runx1, and that this regulation is an integral part of the T cell transitions mentioned above.

Here, we investigate the mechanisms and functional significance of the TCR-mediated down-regulation of Runx1 during the naïve-to-activated transition of CD4+ T cells. Our findings reveal that, in naïve cells, Runx1 plays an inhibitory role in the regulation of IL-2 transcription, whereas, in TCR-activated cells, the distal Runx1 promoter appears to be negatively self-regulated by Runx1 itself, resulting in a reduction of Runx1 that contributes to enhanced transcription of IL-2.

EXPERIMENTAL PROCEDURES

Plasmids—The pME18S (a backbone vector), pME18S-Lck WT (a vector expressing wild-type Lck), and pME18S-Lck Y505F (a vector expressing an active form of Lck) plasmids were provided by T. Yamamoto. NFAT was excised from the pMX-NFAT-IRESC-GFP plasmid (provided by M. Kubo) and inserted into an expression vector, pCDNA3 (Invitrogen, Carlsbad, CA). Runx1 expression vectors were constructed in a pCAGGS-Neo vector. Luciferase expression plasmids (pGL3Basic and pRL-TK) were purchased from Promega (Madison, WI). A luciferase reporter driven by the distal Runx1 promoter was then constructed. First, a genomic fragment spanning the promoter was cleaved from a corresponding mouse BAC clone via HindIII and BstBI restriction digestion and cloned into pGL3. Mutations were introduced into the promoter region by using a site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Mice and Cell Culture—Runx1+/−mice and conditional distal Runx1-tg mice were used as previously described (6, 21). CD4-Cre-transgenic (tg) mice were provided by L. Taniuchi with the consent of C. B. Wilson (25). Mice were housed under specific pathogen-free conditions and handled in accordance with Regulations for Animal Experiment and Related Activities at Tohoku University.

CD4+ (and CD8+) T cells were isolated from the spleens of 8–10 week-old C57BL/6 mice via negative selection using the CD4 (or CD8) Mouse T lymphocyte enrichment set DM (BD Biosciences, San Diego, CA). More than 95% of cells in the fraction were CD4 (or CD8) single-positive. The cells were briefly centrifuged at 700 × g for 10 min onto anti-CD3ε (5 μg/ml)- and anti-CD28 (2 μg/ml)-coated plates, then cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 10 mM Hepes (pH 7.4), 1 mM sodium pyruvate, 100 unit/ml penicillin, 100 μg/ml streptomycin, 1× non-essential amino acids, and 50 μM 2-mercaptoethanol. When necessary, IL-2 (Peprotech EC, London, England) was added to the medium at 20 ng/ml. In some cases, the isolated CD4+ T cells were first labeled with 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Dojindo, Kumamoto, Japan) in PBS containing 0.1% (w/v) BSA at 37 °C for 10 min before stimulation. Mouse anti-CD3ε antibody was purified from the supernatant of 145–2C11 hybridoma cells, and anti-CD28 (37.51) antibody was purchased from BD Biosciences. EL4 and Jurkat cells were cultured in the medium described above, whereas NIH3T3 cells were cultured in DMEM supplemented with 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Luciferase Reporter Assay—Jurkat cells were seeded at a density of 6 × 105 cells/ml in 24-well plates. Approximately 400 ng of firefly-luciferase reporter, Renilla-luciferase reporter, and effector DNA was transfected into the cells using the FuGENE HD reagent (Roche, Indianapolis, IN). After incubation for 24 h, the cells were harvested and lysed in passive lysis buffer (Promega). Luciferase activity of the lysates was measured using the Dual-Luciferase® Reporter Assay System (Promega) and a luminometer (Berthold, Bad Wildbad, Germany). In each transfection, firefly-luciferase activity was corrected by subtracting Renilla-luciferase.

Flow Cytometry—To detect surface molecules, T cells were labeled with anti-mouse TCR-β-PE, anti-CD24 (HSA)-FITC, anti-CD8a-PE/Cy7 (BioLegend, San Diego, CA), or anti-CD4-APC (eBioscience, San Diego, CA). For staining intracellular IL-2, TCR-stimulated T cells were treated with 20 ng/ml phorbol myristate acetate, 1 μM ionomycin, and 2 μM monensin (Fluka, Buchs, Switzerland) for 6 h, then fixed and permeabilized using a Fix and Perm Reagent (Caltag Laboratories, Berlingame, CA). After washing, cells were stained for 30 min with anti-mouse IL-2-APC or an isotype control antibody (eBioscience). To detect apoptosis, cells were processed using MEBCYTO-Apoptosis kit (MBL, Nagoya, Japan). Fluorescence intensity was analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA). Data were analyzed using Expo32 software (Beckman Coulter, Miami, FL).

Immunoblot Analysis—The immunoblot procedures were performed as described previously (26). The primary antibodies included anti-pan-Runx rabbit antiserum (27), anti-HA mAb (Roche Diagnostics, Indianapolis, IN), anti-α-tubulin rabbit IgG (Oncogene, Cambridge, MA), and anti-β-actin (Sigma).

Chromatin Immunoprecipitation (ChIP) Assay—Cells (5 × 106) were cross-linked with 1% (w/v) formaldehyde in PBS for 30 min, and the reaction was stopped by addition of 125 mM glycine, pH 3.0. The cells were washed and lysed in a buffer containing 1% (w/v) SDS, 5 mM EDTA, 50 mM Tris-HCl (pH 8.0), and 1× protease inhibitor mixture. Lysates were sonicated to fragment chromatin DNA into ∼500-bp pieces, preincubated with protein A agarose (Upstate, Temecula, CA), and anti-Runx1 antibodies overnight at 4 °C. These included anti-acetyl-histone H3 (Upstate, Temecula, CA), anti-NFATc2, anti-Runx1 N20 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Runx1 RHD (Calbiochem, San Diego, CA), anti-Runx3 3F12 (provided by Y. Ito) antibodies (28), and a control rabbit IgG antibody (Sigma). Subsequently, protein A agarose was added, and the mixture was incubated for an additional 1 h at 4 °C. Cross-linking was reversed by incubating the mixture at
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65 °C for 6 h. The mixture was then vigorously shaken in an elution buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% (w/v) SDS, and 0.1 M NaHCO₃, and DNA was recovered using the QiaQuick PCR purification kit (Qiagen, Valencia, CA). PCR was performed using primers specific to the distal Runx1 promoter (5'-GATCGTATCCCGTAGATGCACGACG-3' and 5'-CTGGTCTCTGGGATTTTTGTC-3'), the IL-2 promoter (5'-GATAGAAGCCCATCTTACCCCTG-3' and 5'-CAAAAACCAGCTTCAGACCCGTC-3') and the CD8 enhancer I (5'-CAGTTCCTTCTCCCAACC-3' and 5'-ATGGAGATGGATGAAGGACG-3').

RNA Isolation and RT-PCR Analysis—We isolated RNA from the cells using TRIzol Reagent (Invitrogen, Carlsbad, CA), and 5 μg of RNA was subjected to reverse transcription using Superscript™ II Reverse Transcriptase (Invitrogen). The cDNA was amplified by PCR using LA Taq polymerase (Takara, Shiga). Primers specific to distal cDNA was amplified by PCR using LA Taq polymerase (Takara, Shiga), GCAC-3'/H11032 mal Runx1, TCR Signal, and IL-2

Expression to high Runx3 and low Runx1.

We next examined whether the reduction in Runx1 expression after TCR/CD28 stimulation is regulated at the transcriptional level. As two distinct types of Runx1 transcripts exist (distal and proximal), primers were designed for each transcript and semi-quantitative RT-PCR analyses were performed (Fig. 1B). In CD4⁺ T cells, a substantial amount of distal Runx1 transcript was detected prior to stimulation. The level of this transcript remained unchanged up to 3 h, but began to decline after 6 h stimulation. In contrast, the proximal Runx1 (and IL-2) transcript was only marginally detected before activation, but was significantly increased after 1 h (and after 30 min) stimulation. Thus, compared with the increases in proximal Runx1 and IL-2 transcripts (after 1 h and 30 min, respectively), the decline in distal Runx1 transcript was a rather late response (after 6 h). However, once it was complete, it persisted for up to 48 h (supplementary Fig. S1). The results in Fig. 1, A and B and supplementary Fig. S1 suggest that the TCR-induced decline in Runx1 protein parallels that of the distal (but not proximal) Runx1 transcript.

We previously reported that EL4 and NIH3T3 cell lines express distal and proximal Runx1 transcripts, respectively and exclusively (21). Therefore, lysates from EL4 and NIH3T3 cells were used as controls in immunoblot analyses (Fig. 1C). Distal Runx1 from EL4 cells migrated slightly but definitely slower than proximal Runx1 from NIH3T3 cells. Notably, Runx1 pro-

RESULTS

TCR Stimulation Down-regulates Runx1 Expression in Splenic CD4⁺ T cells—We first examined Runx expression in T lymphocytes in response to TCR activation. After isolation of CD4⁺ T cells from mouse spleens, cells were continually incubated in anti-CD3/anti-CD28 antibody-coated plates. Cell lysates were prepared after varying lengths of incubation and processed for immunoblot analyses using an anti-pan-Runx antibody (Fig. 1A). A substantial amount of Runx1 expression was detected in unstimulated CD4⁺ T cells, whereas Runx3 was detected as a minor component. Expression of Runx1 remained unchanged up to 3 h, but began to decline at 6 h after stimulation, and only a trace amount of Runx1 was detected at 12 h. Reduction of Runx1 persisted for 48 h after stimulation. Expression of Runx3 was restored at 48 h and became the major Runx subtype. Thus, TCR activation overall shifted Runx expression to high Runx3 and low Runx1.

![FIGURE 1. TCR-induced modulation of Runx1 protein and transcript expression. A and B, CD4⁺ T cells were prepared from mouse spleen and stimulated with anti-CD3/anti-CD28 antibodies for the indicated time. In A, cell lysates were used in immunoblot analyses with anti-pan-Runx (detects both Runx1 and Runx3) and anti-α-tubulin antibodies. In B, RNA was isolated from cells for semiquantitative RT-PCR analyses of distal Runx1, proximal Runx1, IL-2, and β-actin. Triangle slopes indicate decreasing amounts (4:2:1) of template used. C, immunoblot analyses of Runx proteins expressed in EL4, NIH3T3, unstimulated (0 h), and anti-CD3/anti-CD28-incubated (48 h) CD4⁺ T cells. Anti-pan-Runx antibody was used as in A. Note the small but clear differential mobility of distal and proximal Runx1 proteins. In each of A, B, and C, representative data from three independent but reproducible experiments are shown.](image-url)
tein detected in untreated CD4+ T cells co-migrated with bands from E14 cells, a finding that was in good accordance with the abundant expression of distal Runx1 transcript in untreated CD4+ T cells. In contrast, a Runx1-derived protein band could hardly be detected in TCR-stimulated CD4+ T cells regardless of the distal/proximal subtype. The reason for the low expression of proximal Runx1 protein despite the induction of the proximal Runx1 transcript is not known at present, but may be due to the low translation efficiency of the proximal Runx1 transcript (29). In any case, the results in Fig. 1 collectively indicate that it was mainly distal Runx1/Runx1 expression that was reduced at both the transcript and protein levels in TCR-stimulated CD4+ T cells. Thus, we hereafter focused only on the regulation of distal Runx1 expression.

In unstimulated CD8+ T cells, it should be noted that Runx3 was the major Runx subtype expressed, and that Runx1 was detected only moderately (supplemental Fig. S2A). TCR activation reduced the amount of Runx1 at 6 h and only a trace amount of Runx1 was detected at 12 h, although Runx3 expression remained the same. The decline of Runx1 protein was accompanied by a decrease in the distal Runx1 transcript (Fig. S2B). Thus, modulation of Runx1/Runx1 expression in response to TCR-stimulation was similar in the two T cell subsets. We opted to use CD4+ T cells in the following experiments. Supplemental Fig. S3 shows that a TCR signal (represented by anti-CD3 treatment), and not a costimulatory signal (represented by anti-CD28 treatment), appears to be responsible for down-regulation of distal Runx1/Runx1 expression.

Activity of the Distal Runx1 Promoter Is Negatively Self-regulated by Runx1—As described above, distal Runx1 transcription was down-regulated by TCR stimulation. Distal Runx1 promoter sequences from different species were aligned and inspected (supplemental Fig. S4). Among various transcription factor-binding sites detected in the promoter region, a TCR-related, NFAT consensus sequence ((A/T)TTTCC) was detected at −187 nucleotides [nt] relative to the transcription initiation site. Thus, we first examined the responsiveness of the distal Runx1 promoter to TCR signaling. The distal (−579 to +393 nt) promoter was cloned, ligated into a luciferase reporter plasmid, and transfected into Jurkat T cells together with the Lck expression vector, which mimics the TCR signal. The effects of Lck on the distal Runx1 promoter, as determined by measuring luciferase activity 24 h after transfection, are shown in Fig. 2A. The wild type and a constitutively active form of Lck (Y505F) decreased promoter activity to 50 and 30%, respectively, compared with the control. Therefore, the distal Runx1 promoter likely contains an element or elements that permit TCR stimulation. The effects of NFAT1 or NFAT4 over-expression on the activity of the distal Runx1 promoter were then evaluated (Fig. 2B). Unexpectedly, neither NFAT1 nor NFAT4 appeared to alter the level of distal Runx1 promoter activity despite the presence of the NFAT consensus site. As a control, NFAT over-expression substantially enhanced the activity of an IL-2 promoter-driven reporter that harbors an NFAT site.4 Thus, NFAT is not likely to be involved in the TCR-mediated down-regulation of the distal Runx1 promoter.

Further inspection of the distal Runx1 promoter revealed that Runx consensus sequences (ACCACA) were also conserved at positions −300, +1, +9, and +93 nt, respectively (see supplemental Fig. S4 and Fig. 2C). A deletion mutant of the distal Runx1 promoter was constructed and transfected into Jurkat cells, together with active Lck (see the construct in Fig. 2C and the result in Fig. 2D). Interestingly, deletion of the 5’-sequence to −198 abolished the suppressive effect of Lck. Because one Runx binding site at −300 was located between −579 and −198, we introduced a mutation into this −300 site using the −579 version of the reporter. The mutation of most of the 5’-located Runx binding site ablated the repressive effect of Lck. Introduction of mutations into three other 3’-located Runx binding sites did not significantly affect reporter activity in the presence of Lck. Thus, it is likely that the TCR-induced suppression of distal Runx1 promoter is mediated through the −300 Runx-site.

We then investigated whether the distal Runx1 promoter is negatively regulated by Runx1 itself. A luciferase reporter gene, driven by the distal Runx1 promoter, was transfected into Jurkat cells, together with Runx1 expression vectors (Fig. 2E). Co-

4 K. Kohu, unpublished data.
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transfection of the distal (or proximal) forms of the Runx1 protein remarkably suppressed the promoter activity of distal Runx1 (note that there was no significant difference detected in the strength of repressive activity between the two forms of Runx1). The results in Fig. 2E are in line with those in Fig. 2D and collectively indicate that Runx1 negatively regulates its own distal promoter.

**Chromatin Configuration of the Distal Runx1 Promoter during TCR Activation of CD4+ T Cells**—ChIP analyses were then conducted to determine whether Runx1 protein bound to the Runx site of the distal Runx1 promoter. We employed PCR primers specific to the relevant Runx site and anti-Runx1 antibodies (N20 and RHD) from two different sources (Fig. 3A). In lysates from CD4+ T cells that had been stimulated with TCR for 24 h, both antibodies precipitated the Runx1 protein bound to the Runx site of the distal promoter. No PCR products were detected when unstimulated cells or control IgG was used. Thus, Runx1 appeared to bind to the distal promoter only after TCR stimulation. As a control, no significant binding of NFAT or Runx3 to the distal Runx1 promoter was detected (Fig. 3B and supplemental Fig. S5).

Furthermore, we checked the acetylation status of histone H3 adjacent to the distal Runx1 promoter (Fig. 3C). When chromatin fractions were precipitated by anti-acetylated histone H3 antibody, distal Runx1 promoter sequences were amplified from CD4+ T cells after no treatment or 3 h TCR stimulation, but not after 16 h TCR stimulation. This finding is consistent with the observations, described above, that the distal Runx1 transcript is easily detected between 0 and 3 h, but not after 16 h of TCR stimulation. Thus, the chromatin configuration of the distal Runx1 promoter is thought to be open both prior to TCR stimulation and at an earlier phase of TCR stimulation, but not at later phases. The results collectively suggest that TCR signal-

ing negatively regulates the distal Runx1 promoter via a self-regulatory mechanism. Binding of Runx1 to the Runx1 promoter and deacetylation of histone H3 appear to occur at a later phase of TCR stimulation. The ChIP analyses of the IL-2 promoter shown in Fig. 3 are described below.

**Transgenic Overexpression of Runx1 Modulates Interleukin Gene Transcription**—Next, we determined the physiological significance of TCR-mediated Runx1 down-regulation in CD4+ T cells. For this purpose, we employed transgenic overexpression of Runx1 to counteract Runx1 down-regulation. Conditional, distal Runx1-tg mice were mated with CD4-Cre-tg mice. The dRunx1-transgene was expressed in CD4+ T cells from dRunx1-tg; CD4-Cre-tg double-transgenic mice, but not in cells from single-transgenic dRunx1-tg mice. CD4+ T cells were isolated from the spleens of dRunx1-tg; CD4-Cre-tg and dRunx1-tg mice and stimulated with anti-CD3/anti-CD28 antibodies for 24 h. Extracts from these cells were processed for immunoblot analyses (Fig. 4A). Transgene-derived Runx1 protein harbors the HA epitope, which was detected by anti-HA antibody. At the protein level, the amount of dRunx1 in dRunx1-tg; CD4-Cre-tg cells was 28% higher than in dRunx1-tg cells under resting conditions (128% versus 100%). Similarly, even after TCR stimulation, the Runx1 level in dRunx1-tg; CD4-Cre-tg cells remained 25% higher than in dRunx1-tg cells (86% versus 61%).

At the transcriptional level, distal Runx1 transcription levels were analyzed by semi-quantitative RT-PCR using primers that detected both endogenous and exogenous transcripts (Fig. 4B). Distal Runx1 transcript levels were higher in dRunx1-tg; CD4-Cre-tg cells than in dRunx1-tg cells. TCR stimulation reduced Runx1 expression, but more dRunx1 transcript remained in dRunx1-tg; CD4-Cre-tg cells than in dRunx1-tg cells. Thus, we confirmed higher levels of Runx1 in dRunx1-tg; CD4-Cre-tg cells compared with dRunx1-tg cells both prior to and after TCR stimulation.

The TCR signal induces the expression of various interleukin genes. The expression kinetics of interleukin genes after TCR stimulation of wild-type CD4+ T cells is shown in the left panel of Fig. 4C. IL-2, IL-4, IFN-γ, IL-12A, IL-17A transcripts were barely detectable before TCR activation but were readily detected after stimulation. After observing that TCR stimulation down-regulates Runx1 (Fig. 1), we suspected that Runx1 might function negatively in the interleukin gene expression in resting cells. In turn, under the TCR stimulation, reduction of Runx1 expression would aid in sustaining interleukin gene transcription.

The TCR-induced expression of various interleukin transcripts was then compared in dRunx1-tg; CD4-Cre-tg and dRunx1-tg CD4+ T cells (Fig. 4D) as a quantification of the data in Fig. 4C, right panel). IFN-γ, IL-12A, and IL-17A expression was considerably higher in double-transgenic cells than in single-transgenic cells, indicating that Runx1 does not function negatively in these interleukins expression. In contrast, the relative amounts of IL-2 and IL-4 transcripts were substantially reduced in double-transgenic cells compared with single-transgenic cells. This pattern supports our hypothesis that Runx1 plays an inhibitory role in the interleukin expression. We previously reported that Runx1 represses IL-4 expression (10). The
putative role of Runx1 as a negative element in IL-2 regulation is examined below.

Runx1 Functions as an Inhibitor of IL-2 Production and Cell Proliferation—Expression of IL-2 after TCR stimulation was examined by flow cytometry analysis (Fig. 5A). The mean fluorescence intensity (MFI) of intracellular IL-2 was 1.8 in Runx1-overexpressing cells and 2.3 in non-overexpressing cells. A lower MFI value indicates that Runx1 over-expression suppresses IL-2 production. The effects of Runx1 over-expression on cell proliferation were then assayed in vitro (Fig. 5B). CD4+ T cells were isolated, labeled with CFSE, and stimulated with anti-CD3/anti-CD28 antibodies. On day 0, all cells were CFSE-hi. The number of CFSE-lo/med cells gradually decreased on subsequent days, indicating active cell division. On days 2 and 4, the percentages of CFSE-lo/med cells were substantially lower in double-transgenic than in single-transgenic cells. Thus, Runx1-overexpressing cells displayed a relatively poor proliferation capacity.

We then examined whether the poor proliferation of double transgenic cells was due to the low production of the T cell growth factor IL-2. In Fig. 5C, the numbers of cells were counted during the course of TCR stimulation. Supplement of IL-2 into the medium only slightly increased the number of single transgenic cells (compare the closed rectangle with the open rectangle). In contrast, exogenous addition of IL-2 substantially enhanced the extent of growth of double transgenic cells which otherwise showed limited activity of growth (compare the closed and open triangles). Notably, TCR-mediated induction of IL-2RA transcripts was similar in the two genotypes (supplemental Fig. S6). Collectively, these results suggest that Runx1 functions as an inhibitor of IL-2 production and, consequently, of cell proliferation.
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Absence of Runx1 Allows IL-2 Transcription in Naïve CD4+ T Cells—As stated above, persistent expression of Runx1 impeded the TCR-induced induction of IL-2 in activated T cells. Thus, we wondered whether Runx1 contributes to the repression of IL-2 during resting stages. This was examined by using Runx1f/f; CD4-Cre-tg and control Runx1f/+ mice (6). Splenocytes were isolated from respective mice, and their cellular characteristics were first examined by flow cytometry (Fig. 6A). The percentage of CD4+ population was substantially reduced in Runx1f/f; CD4-Cre-tg as compared with Runx1f/+ (3.2% versus 19%). When splenocytes were processed for apoptosis assay, the Runx1f/+; CD4-Cre-tg CD4+ cells contained more annexin V+ fraction than the corresponding Runx1f/+ cells (21% versus 4.0%). Therefore, one reason of reduced CD4+ population is an increased occurrence of apoptosis in the Runx1f/+; CD4-Cre-tg cells. It must be mentioned that the CD4+ cells from Runx1f/+; CD4-Cre-tg as well as Runx1f/+ mice retained high degree of TCRβ expression, indicating appropriate maturation of cells (as for the differentiation of thymocytes, see supplemental Fig. S7).

To examine the extent of Runx1 deletion, CD4+ splenocytes were isolated from Runx1f/+; CD4-Cre-tg and Runx1f/+ mice, and their lysates were processed for immunoblot analysis (Fig. 6B). As expected, Runx1 protein was scarcely detected in Runx1f/+; CD4-Cre-tg. Therefore, Runx1 deficiency does not appear to affect surface expression of TCRβ and CD4. RNA was then prepared and RT-PCR analysis was performed (Fig. 6C; the lower panel is a quantification of the data in the upper panel). Lack of Runx1 transcription was confirmed in Runx1-deficient cells. Interestingly, the levels of IL-2 and IL-4 transcripts in Runx1-deficient cells were substantially higher than in control cells. Runx1 is therefore likely to play an inhibitory role in the IL-2 (and IL-4) expression even in the unstimulated CD4+ cells.

Configuration of IL-2 Promoter—Finally, the IL-2 promoter status in wild type CD4+ T cells was examined by ChIP analyses (Fig. 3). Runx1 binding was detected in unstimulated CD4+ T cells, but not in cells activated by TCR for 24 h. In parallel, histone H3 adjacent to the IL-2 promoter was deacetylated prior to stimulation and acetylated after a 3-h stimulation with TCR, at which time NFAT binding was also detected. This feature of IL-2 promoter modulation is in accordance with the notion that Runx1 acts negatively in the IL-2 transcription.

**DISCUSSION**

The present study has demonstrated, firstly, that the regulation of Runx1 via T cell activation occurred at both the RNA and protein levels and, secondly, that TCR-mediated down-regulation of Runx1 is a physiological process to permit a higher level of induction of several cytokines including IL-2.

A substantial amount of Runx1 protein was detected in resting CD4+ T cells, and these proteins were predominantly derived from distal transcripts. Our data revealed that the two Runx1 promoters act differently in response to TCR activation. While TCR signaling initially (at 1 h) induces proximal Runx1 transcription, this proximal Runx1 scarcely contributes to efficient translation. Subsequently (at 6 h), distal Runx1 transcription was suppressed, resulting in the drastic reduction of the overall expression of Runx1 protein. Such differential promoter usage during distal and proximal Runx1 transcription has also been reported in mitogen-stimulated T cells and during thymocyte development (29, 30). Notably, our reporter assays using a distal Runx1 promoter revealed that Lck-mediated down-regulation involved a Runx consensus site in the Runx1 promoter, and that distal promoter activity was strongly suppressed by the over-expression of Runx1 protein itself. We propose, therefore, that the TCR-mediated down-regulation of distal Runx1 transcription involves self-inhibitory mechanisms by Runx1 (which of distal or proximal Runx1 is not known) via a Runx consensus site.

The question then arises whether endogenous distal Runx1 protein self-represses its own distal promoter in resting T cells. ChIP assays of resting cells revealed that Runx1 protein does not bind to the distal Runx1 promoter. The distal Runx1 promoter, which is highly active in resting T cells, may be preoccupied with other transcriptional factors and therefore inaccessible to Runx1. In contrast, the same ChIP assays on TCR-stimulated cells revealed that Runx1 protein had access to the distal promoter. Thus, Runx1 binding to the distal Runx1 promoter appeared to become possible only when the chromatin configuration of the distal promoter had been remodeled by TCR-activation. This situation was exemplified by the enhanced deacetylation of histone H3 adjacent to the promoter. Notably, the chromosome was converted into a compact...
configuration after more than 3 h of TCR stimulation, an observation that is in line with the decline of distal Runx1 transcription at only 6 h after TCR stimulation. Thus, the induction of proximal Runx1 (and IL-2) is categorized as an immediate response to the TCR signal, whereas the negative regulation of distal Runx1 promoter represents a rather late, and thus perhaps indirect/secondary, response.

The distal Runx2 promoter, which harbors multiple Runx sites, is regulated by a self-feedback mechanism (31). B cells that have been activated via infection with the Epstein-Barr virus or stimulation with phorbol myristate acetate are also subject to cross-regulation of the distal Runx1 promoter by the proximal Runx3 protein (32). However, we found that in activated CD4+ T cells, decrease in Runx1 expression occurred prior to the augmentation of Runx3. Furthermore, ChIP assays revealed Runx1- but not Runx3-binding to the distal Runx1 promoter. These observations indicate that it is not Runx3 but Runx1 that is involved in TCR-induced Runx1 down-regulation. In fact, in CD8+ T cells, which express very high levels of Runx3, the TCR signal similarly induced Runx1 down-regulation.

In light of the abundant Runx1 expression observed in resting T lymphocytes, it is likely that Runx1 helps to maintain a cellular resting state. A recent study demonstrated that Runx1 plays a critical role in the survival and maintenance of homeostasis in naive CD4+ T cells via the regulation of IL-7RA expression (7). However, this finding does not necessarily explain the down-regulation of Runx1 in response to TCR signaling. In the present study, analysis of a mouse model demonstrated that over-expression of distal Runx1 in activated T cells impairs IL-2 production and interferes with cell proliferation. On the other hand, Runx1 deficiency was shown to derepress IL-2 expression in resting T cells. It must be noted though that the enhanced transcription of IL-2 in the Runx1f/f; CD4-Cre-tg/Cre derived CD4+ cells did not appear to compensate an increased degree of cellular apoptosis.

Recently, Ono et al. (13) reported that Runx1 can bind to a Runx site in the core promoter and act as an activator or a repressor of IL-2, depending on the molecules involved. Specifically, Runx1 interacts with Foxp3 to suppress IL-2 expression in regulatory T cells, whereas Runx1 itself enhances IL-2 expression in conventional CD4+ T cells. It must be noted, however, that their latter observation was obtained by in vitro over-expression of Runx1 in TCR-activated Jurkat cells. Because the amount of Runx1 in CD4+ T cells declines during TCR activation, Runx1 is not likely to play a positive role in TCR-induced IL-2 expression. Instead, induction of IL-2 is likely orchestrated by several other positive regulators, including NFAT, AP-1, and NF-κB. Taken together, our data suggest that high levels of Runx1 protein contribute to repress IL-2 in resting T cells, and that the TCR-induced down-regulation of Runx1 helps enhanced production of IL-2 and cell proliferation, while Runx1 down-regulation itself is not likely a direct cause of IL-2 induction.

Runx1 and Runx3 proteins mediate T cell differentiation into Th1 or Th2 subtypes. Runx1 protein inhibits Th2 differentiation via the repression of GATA3 (10), while Runx3 directly inhibits IL-4 expression by binding to the HSIV silencer element (15). In CBF-β (Runx cofactor)-deficient mice, IL-4 production is elevated as a result of derepression (17). Consistent with these findings, we demonstrated that Runx1-transgenic cells express higher levels of IFN-γ and produce less IL-4. Higher and lower levels of IL-17 and IL-10, respectively, were also observed in Runx1-transgenic cells. IL-17 is a cytokine produced by Th17 cells, which modulates the inflammatory response (33, 34). In contrast, inflammation is suppressed by IL-10, a T-regulatory type 1 cytokine (35, 36). These results collectively suggest that, in the absence of Runx1 down-regulation, TCR-stimulated cells are biased to acquire the inflammatory Th1 and Th17 phenotypes.

In conclusion, our findings provide insights into the intricate regulatory mechanisms of Runx1 expression and illustrate the importance of maintaining precise intracellular levels of Runx1 in quiescent and TCR-activated CD4+ T lymphocytes.

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