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Runx1 Deficiency in CD4+ T Cells Causes Fatal Autoimmune Inflammatory Lung Disease Due to Spontaneous Hyperactivation of Cells

Won Fen Wong,* Kazuyoshi Kohu,* Akira Nakamura,†‡ Masahito Ebina,§ Toshiaki Kikuchi,§ Ryushi Tazawa,¶ Keisuke Tanaka,* Shunsuke Kon,* Tomo Funaki,* Akiko Sugahara-Tobinai,† Chung Yeng Looi,† Shota Endo,† Ryo Funayama,‖ Mineo Kurokawa,‡ Sonoko Habu,** Naoto Ishii,†† Manabu Fukumoto,‡‡ Koh Nakata,¶ Toshiyuki Takai,‖ and Masanobu Satake*

The Runx1 transcription factor is abundantly expressed in naive T cells but rapidly downregulated in activated T cells, suggesting that it plays an important role in a naive stage. In the current study, Runx1−/−/Bcl2flox mice harboring Runx1-deleted CD4+ T cells developed a fatal autoimmune lung disease. CD4+ T cells from these mice were spontaneously activated, preferentially homed to transgenic; BM, bone marrow; CBFb

A ctivation of peripheral T cells by Ag engagement triggers their rapid expansion and the gain of effector functions. However, after Ag elimination, these cells are exhausted and destined to apoptotic cell death. When in a quiescent stage without any Ag stimulation, naive T cells consume less energy and are capable of existing for long periods in peripheral tissues, thus maintaining the diversity of their Ag-recognizing repertoire (1).

To maintain the quiescence state of T cells, intricate controls by intrinsic transcription factors, such as Klf2, Tob, Slfn2, Foxo, Foxp1, and Tsc1 (2–8), or extrinsic factors, such as regulatory T cells (Treg) (9), are pivotal. Failure of the quiescence controls can be caused by the deletion of quiescence-associated transcription factors or by defects in Treg activity. Under these circumstances, T cells are spontaneously hyperactivated and release excessive amounts of cytokines, which can cause a cytokine storm and often develop into systemic inflammatory response syndrome (SIRS) (10). Such a breakdown of immune tolerance is deleterious to the host. However, a full picture of intrinsic quiescence-control mechanisms for T cells remains elusive.

The Runx1 transcription factor is one of the key factors that drives various aspects of T cell differentiation through interplay with distinct molecules (11). In T cell differentiation, interaction of Runx1 with Gata3 suppresses IL-4 secretion and induces IFN-γ production (12). In addition, Runx1 transactivates IL-17 through cooperative binding with ROR-γt (13), but it also inhibits IL-17 when forming a complex with T-bet or Foxp3 (13, 14). In the differentiation of Treg, the interaction of Runx1 with Foxp3 is important for the continuous expression of the Foxp3 gene, which ensures maintenance of a Treg phenotype (15, 16). Treg-specific deletion of Runx1 or core-binding factor β (CBFβ; a cofactor of the Runx family) in two independent mice models caused colitis or pneumonitis, respectively (17, 18).

We previously observed that Runx1 is highly expressed in naive CD4+ T cells but is rapidly turned off upon T cell activation (19). Runx1 downregulation during T cell activation appears crucial for

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the maximal production of cytokines and cell expansion, because Runx1-transduced CD4+ cells show a reduction in both IL-2 production and cell proliferation in vitro upon stimulation (19). Conversely, deleting Runx1 in naive CD4+ T cells induces IL-2 production and cell proliferation, as noted using Runx1ΔRunx1Δ+CD4 cells (19, 20). These observations led to the investigation of the potential role of Runx1 in maintaining quiescence of naive CD4+ T cells. However, mice with Runx1-deficient T cells suffer from lymphopenia, which is likely due to the enhancement of cell apoptosis and impairment of cell homeostasis (19, 20). The occurrence of lymphopenia in Runx1ΔRunx1Δ mice makes it difficult to analyze the role of Runx1 in quiescence control by using these mice. In CBFΔBΔ+CD4 cells, CBFβ is deleted in CD4+ T cells, and mice develop asthma-like symptoms (21). However, the CBFβ cofactor is shared by three members of the Runx family; thus, CBFΔBΔ+CD4 cells are not suitable for analyzing the specific function of Runx1 in CD4+ T cells.

In the current study, Runx1ΔRunx1Δ+CD4 cells were crossed with Bcl2-transgenic (Bcl2Δb) mice to improve the survival and total number of Runx1-deleted CD4+ T cells. Strikingly, Runx1 deficiency caused spontaneous hyperactivation of CD4+ T cells, their preferential homing to the lungs, and the increased production of cytokines, such as IL-17 and IL-21, from cells. Mice eventually developed a fatal autoimmune lung disease and severe systemic inflammation. Our observations indicate that Runx1 plays an essential role in repressing cytokine expression and, thereby, maintaining CD4+ T cells in a quiescent stage.

Materials and Methods

Mice

Conditional Runx1-knockout (Runx1ΔRunx1Δ) mice were prepared, as previously described (22). To delete Runx1 specifically in CD4+ T cells, Runx1ΔRunx1Δ mice were crossed with CD4-Cre-tg mice (23). Bcl2 expression in T lymphocytes was enforced by crossing mice with Bcl2Δb mice (B6.Cg-Tg [Bcl1.2]25Wheii/J) (24) to generate Runx1ΔRunx1Δ, CD4-Cre-tg, Bcl2Δb mice (denoted as Runx1ΔRunx1ΔΔb). CD4+ T cell-deficient mice (B6.129S2- Cd4tm1Mak) (25) and C57BL6 mice were from The Jackson Laboratory and CLEA, respectively. Ly5.1ΔCD45.1ΔC57BL6/6 mice were as described previously (26). All mice were kept in a pathogen-free environment and handled in accordance with the Regulations for Animal Experiments and Related Activities at Tokoh University.

Flow cytometry analyses

Cell suspensions were prepared from spleens, lymph nodes, lungs, or thymuses of mice, and 1 × 106 cells were stained with the following Abs: FITC–B220, FITC–heat-stable Ag, PE–TCRβ, PE–CCR5, PE–CCR3, allophycocyanin–B220, and PE–CD5-CD69 (BioLegend, San Diego, CA); FITC–CD69, FITC–Fas, PE–CD4–CD40L, PE–CD21, PE–CD44, PE–CD11a, PE–CD103, allophycocyanin–Mac-1, and biotin–syndecan-1 (BD Pharmingen, San Jose, CA); FITC–CCR9, FITC–CD23, FITC–NK1.1, PE–CD11a, PE–CD44, PE–CD62L, PE–Cy7–IgM, and allophycocyanin–CD4 (eBiosciences, San Diego, CA); PE–Thyl (Cell Laboratory, Fullerton, CA); and biotin–c–pea agglutinin (PNA) (Biomeda, Burlingame, CA). The Abs used were described previously (19).

Immunoblotting and RT-PCR

Expression vectors of Runx1-hemagglutinin or dominant-negative Runt domain were constructed by inserting the respective sequences into a C-terminal hemagglutinin vector. To synthesize a reporter driven by the mouse IL-21 promoter-luciferase (P-Luc), the mouse genomic sequence was amplified using the primers 5′-GGAGATCTGCTGACAAACACCTGAGGTTGC-3′ and 5′-CCCAAGCTTCTAGGTCTCCAGGACCTGATAGA-3′. Underlined

Adaptive transfer and mixed bone marrow chimera experiments

CD4+ T cells were collected from spleens of 16–24-wk-old donor mice using anti-mouse CD4 Magnetic Particles-DM (BD Biosciences), and 3 × 106 cells were injected into tail veins of 8–12-wk-old CD4+ T cell-deficient mice. Recipient mice were sacrificed for histological analyses after 5 or 25 wk of injection. In chimera experiments, cells were collected from bone marrow (Bm) of CD4–Tg (CD45.1ΔC57BL6/6 and Runx1ΔRunx1ΔΔb (CD45.2)) mice and depleted of CD4+ T cells using anti-mouse CD4 Magnetic Particles-DM. A 1:1 mixture of each genotype of cells (total 5 × 106) was injected i.v. into tail veins of C57BL6/6 mice that had been lethally irradiated (9 Gy). Recipient mice were given 2 mg/ml G418 (Sigma, St. Louis, MO) in drinking water for the first 2 wk and were sacrificed for analyses at 8 wk after transplantation.

Histology

Mouse tissues were fixed in 3.7% (w/v) parafomaldehyde in PBS and kept at 4°C. Tissues were weighed after they were drained on a tissue tower. For analysis of lung tissues, inflation with formalin was performed before paraffin sections were prepared and stained with H&E, Elastica-Masson (EM), and periodic acid-Schiff (PAS), according to standard procedures. In certain cases, paraffin sections were counterstained by an anti-sulfactant protein A Ab (Millipore, Bedford, MA). Lung histology was scored as follows: grade 0, normal lung; grade 1, mild to limited peribronchovascular infiltration of lymphocytes; grade 2, severe/frequent peribronchovascular infiltration of lymphocytes; and grade 3, severe/frequent peribronchovascular infiltration of lymphocytes with massive accumulation of exudate in the alveoli. Crystat sections were prepared, blocked with 5% (w/v) BSA in PBS, and stained with anti-mouse IgG (H+L) Fab′-a,488 (Cell Signaling, Danvers, MA), FITC–anti-CD4 (eBioLegend), or biotin–c–PNA (Biomaeda), followed by FITC–streptavidin in 1 h in the dark and viewed through a Zeiss LSM 5 PASCAL confocal microscope. In certain cases, frozen sections were stained with Sudan III.

ELISA of serum Ig

A 96-well plate was coated with mouse serum (1000-fold diluted) at 37°C for 2 h, blocked with 1% (w/v) BSA in PBS, and incubated with goat anti-mouse IgG-His/GlutG2a (HRP-labeled Abs (2000-fold dilution) (Bethyl, Montgomery, TX) for 1 h. Color was developed by tetramethylbenzidine peroxidase substrate (Bethyl), stopped by 1 M HCl, and analyzed on a SpectraMax M2e plate reader. Anti-dsDNA Abs in sera were measured using an anti-mouse dsDNA ELISA kit (Shibayagi, Gunma, Japan).

Immunohistoblotting and RT-PCR

CD4+ T cells isolated from splenocytes using the respective anti-mouse Magnetic Particles-DM (BD Biosciences), and 1 × 106 cells were lysed in SDS sample buffer and sonicated. The lysate was centrifuged, and the supernatant was mixed with SDS sample buffer. Denatured samples were then run on SDS-polyacrylamide gels and transferred onto membranes. Filters were incubated with Abs, followed by the AP-conjugated secondary Ab (at 1:4000 dilution), and immune complexes were detected using NBT/BCIP substrate (Promega, Madison, WI). The Runx1 Runx1 Ab was as described previously (27). Bcl2 and β-actin Abs were from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma, respectively. For RT-PCR analysis, RNA was extracted from isolated cells using TRizol reagent and reverse transcribed using SSRT II (Invitrogen, Carlsbad, CA). The primers used were described previously (19).

Plasmid construction

Expression vectors of Runx1-hemagglutinin or dominant-negative Runt-hemagglutinin were constructed by inserting the respective sequences into a pCAGGSneo plasmid. To synthesize a reporter driven by the mouse IL-21 promoter-luciferase (P-Luc), the mouse genomic sequence was amplified using the primers 5′-GGAGATCTGCTGACAAACACCTGAGGTTGC-3′ and 5′-CCCAAGCTTCTAGGTCTCCAGGACCTGATAGA-3′. Underlined...
sequences represent restriction enzyme sites for BglII and HindIII. The PCR products containing a promoter region from -398 to +43 were digested and ligated into the BglII and HindIII sites of pGL3 reporter. To insert a conserved noncoding sequence (CNS) region, a mouse genomic sequence was amplified using the primers 5′-AAAGTTACGCGGATCTCAAGGAG-3′ and 5′-GAGAAGATGCTGACTTCAAGGAGACAA-TCAG-3′. Underlined sequences represent KpnI and BglII sites. The PCR products containing a CNS region from +2890 to +3437 were digested and ligated into the KpnI and BglII sites of P-Luc to generate a CNS+P-Luc reporter. For mutagenesis, the Runx sites at locations +3114 and +3162 were amplified using the primers 5′-GAAAGAGAAAAGAAAAAAACTTCAACAAACATGAACA-3′ and 5′-TTTTCTCTTCTTTTTG-3′ and ligated into the BglII and HindIII sites of the pGL3 reporter. To insert a reporter, for mutagenesis, the Runx sites at locations +3114 and +3162 were amplified using the primers 5′-GAAGAGAAAAGAAAAAAACTTCAACAAACATGAACA-3′ and 5′-CTTAGATGTTCATGTT-3′ and ligated into the KpnI and BglII sites of P-Luc to generate a CNS+P-Luc reporter.

Luciferase reporter and chromatin immunoprecipitation assay

Jurkat cells were transfected with a total of 500 ng plasmid DNA using FuGENE HD (Roche, Indianapolis, IN). After 24 h, cells were stimulated with 200 ng/ml PMA and 1 μM ionomycin for 6 h before harvest. Luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega), as described (28). pRL-TK (5 ng) was included in each transfection as a normalization control for transfection efficiency. A chromatin immunoprecipitation (ChIP) assay was performed, as described previously (19). Briefly, CD4+ T cells were purified from splenocytes of C57BL/6 mice, fixed, sonicated, and precipitated with control IgG or an anti-Runx1 Ab (Abcam). The following primers were used for amplification of the IL-21 CNS region: for the Runx site at +3114 (CNS-1): 5′-AGGTAGCTTGGCTGTACTAGGGCAAGGT-3′ and 5′-TTTCTCCAGTAAGTTAAGCCGGTTTGTAGGTC-3′; and for the Runx site at +3162 (CNS-2): 5′-TTTTGACCAACACCGCTTAACTTACCGG-3′ and 5′-TACGCCCCCCCAAGTCTTTTTGAACAGG-3′. A ChiP library was constructed from the precipitate using TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA). Quantitative PCR was performed using SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA) in a real-time PCR CFX96 machine.

Statistical analysis

All statistical data were evaluated using an unpaired two-tailed Student t test and were considered significant if p < 0.05.

Results

Generation of mice harboring a Runx1-deleted CD4+ T cell population

To examine the function of Runx1 in naive CD4+ T cells, Runx1 expression in CD4+ T cells was disrupted by breeding Runx1fl/fl mice, in which the Runx1 exon 5 is flanked by the loxP sites (details of targeting vector were described previously) (22), with CD4-Cre-tg mice (23) to yield Runx1fl/fl;CD4-Cre-tg mice (hereafter referred to as Runx1−/−). Immunoblot analyses of splenocytes were used to confirm that Runx1 protein amounts were reduced by ∼90% in Runx1−/− CD4+ T cells (Fig. 1A, note that in a CD8+ subset, Runx3, not Runx1, was dominant; therefore the reduction of Runx1 was not apparent). In Runx1−/− mice, spleens were of a smaller size and weight (Fig. 1B) and contained significantly lower numbers of CD4+ T cells compared with control littermates (0.41 ± 0.22 × 107 versus 2.0 ± 0.47 × 107; Fig. 1C).

Because Runx1−/− mice suffered from a severe reduction in the CD4+ T cell population, this population was boosted by crossing them with Bcl2tg mice (24) to yield Runx1+/−;CD4-Cre-tg;Bcl2tg mice (hereafter referred to as Runx1+/−;Bcl2tg). The expression of transduced Bcl2 protein in T cells from control or Runx1+/−;Bcl2tg spleens was confirmed by immunoblot analyses (Fig. 1A). Bcl2 did not affect the efficiency of the Runx1 deletion in Runx1+/−;Bcl2tg CD4+ T cells. Spleens with a Bcl2 background displayed an increase in size and weight (Fig. 1B) due to the expansion of the TCR-β, CD4+, and CD8+ T populations (Fig. 1C). This is consistent with a previous report showing an increased lymphocyte number in Bcl2tg mice due to enhanced cell viability and resistance to apoptosis (24). As a consequence, Runx1−/−;Bcl2tg spleens contained an increased number (2.0 ± 1.4 × 107 versus 0.41 ± 0.22 × 107) and percentage of CD4+ T cells compared with those of Runx1−/− mice without a Bcl2 background (Fig. 1C, data not shown).

Splenocytes were then stained with annexin V and propidium iodide (Fig. 1D). The percentages of apoptotic (annexin V+) cells among the CD4+ cells in the control Runx1−/−;Bcl2tg mice were 2.8-fold higher in Runx1−/− mice than in Runx1+/− mice (18.7 ± 4.4% versus 6.8 ± 2.3%). The presence of the Bcl2 transgene significantly reduced the percentages of apoptotic CD4+ T splenocytes to 7.6 ± 4.5% in Runx1−/−;Bcl2tg mice. Results similar to those above were also obtained by staining cells with anti-ssDNA Ab (data not shown). This indicates that the reduction of Runx1 in CD4+ T cells induced apoptosis, which can be prevented by Bcl2 transduction. Given that Runx1−/−;Bcl2tg mice contained substantial numbers of Runx1-deleted peripheral CD4+ T cells, these mice were used to explore the possible role of Runx1 in maintaining the quiescence status of T cells. Runx1−/−;Bcl2tg mice were used as controls.

Runx1−/−;Bcl2tg mice develop severe lung inflammation

The development and growth of Runx1−/−;Bcl2tg mice were apparently normal, and no recognizable phenotypes were detected during young adulthood. However, after >28 wk, Runx1−/−;Bcl2tg mice inevitably showed tachypnea, took a hunched posture (data not shown), and suffered from general weakness and loss of body weight (Fig. 2A). More than 70% of Runx1−/−;Bcl2tg mice died between 28 and 36 wk of age (Fig. 2B). None of them had a life span >56 wk, whereas all of the control mice survived this observation period.

To explore the cause(s) of death, young (16–24-wk-old) and aged (28–36-wk-old) Runx1−/−;Bcl2tg mice, together with age-matched control mice, were sacrificed, and the internal organs were examined. Macroscopically, the lungs from Runx1−/−;Bcl2tg mice were substantially large, diffusely red, and 2.3-fold heavier than those from control mice (0.61 ± 0.07 g versus 0.26 ± 0.02 g) (Fig. 2C). Histological sections of the lungs were stained by H&E, EM, and PAS (Fig. 2D). With all three staining methods, the infiltration and accumulation of lymphoid cells into peribronchovascular interstitial regions were observed in the lungs from both young and aged Runx1−/−;Bcl2tg mice. This infiltration was not seen in control Runx1+/−;Bcl2tg mice. In accordance with lymphoid infiltration in the lungs, peripheral lymph nodes (pLN) and lung-draining mediastinal lymph nodes (mLN) from Runx1−/−;Bcl2tg mice were also markedly enlarged compared with those from control littermates (data not shown).

In the lungs of aged Runx1−/−;Bcl2tg mice (Fig. 2D), many alveolar spaces were filled with exudates that contained eosinophilic granular materials and a vast amount of immune cells (predominantly neutrophils and foamy macrophages). Fig. 2E is a higher magnification of such alveolar spaces. Exudates were stained positive for eosin and surfactant protein A, whereas alveolar macrophages were stained positive for surfactant protein A and Sudan III, indicating their engulfing activity. Based on these hallmarks, the pathology seen in the Runx1−/−;Bcl2tg lungs was considered similar to pulmonary alveolar proteinosis (PAP) in humans (29). Note that this PAP-like pathology was detected focally in the young Runx1−/−;Bcl2tg lungs as well.

The disease score of mice was determined by observing histological sections (Fig. 2F), as described in Materials and Methods.
Lungs from young Runx1−/−Bcl2tg mice scored between 1 and 2, whereas those from aged mice scored up to 3, reflecting the more severe pathology in aged mice. In contrast, lungs from age-matched control mice showed no pathological phenotype and scored 0.

To monitor airway-residing immune cells, BALF was recovered, and the cells in it were analyzed (Fig. 3A, 3B). Strikingly, in aged (but not young) Runx1−/−Bcl2tg mice, ∼5-fold greater numbers of BALF cells were detected compared with control littersmates. BALF cells from Runx1−/−Bcl2tg mice were composed predominantly of Gr-1+Mac-1+ granulocytes and, to lesser degrees, TCR-β+ T cells, B220+ B cells, and Gr-1+Mac-1+ macrophages. Also, the levels of proinflammatory cytokines, such as IFN-γ and TNF, were ∼3-fold higher in BALF from aged Runx1−/−Bcl2tg mice compared with control mice (Fig. 3C). This suggests the occurrence of a cytokine storm in the lungs of aged Runx1−/−Bcl2tg mice.

**Systemic inflammation in aged Runx1−/−Bcl2tg mice**

Organs other than the lungs were examined next. In young Runx1−/−Bcl2tg mice, tissues from the kidneys, liver, pancreas, or the digestive tract exhibited normal histology (data not shown). In contrast, aged Runx1−/−Bcl2tg mice developed a wasting disease of various organs, including muscles. For example, in the liver of aged mice (Fig. 4A), hepatocytes were atrophic, whereas sinusoids were enlarged and contained immune cells. Glycogenesis appeared insufficient, probably due to ischemia. The hemocyte count of peripheral blood (Table I) revealed slightly increased numbers of RBC and increased hemoglobin concentration and hematocrit percentage, suggesting compensatory erythrocytosis, whereas the numbers of WBC were decreased to less than half. In addition, the percentages of Gr-1medMac-1+ monocytes were remarkably increased in peripheral blood (monocytosis in Fig. 4B), suggesting the presence of a chronic systemic inflammation in aged Runx1−/−Bcl2tg mice. Consistently, the levels of IFN-γ and TNF were elevated in sera from aged Runx1−/−Bcl2tg mice compared with control mice (Fig. 4C).

Altogether, the phenotypes described above suggest that active inflammation, such as lymphocyte infiltration, was initially limited to local areas in the lung of young Runx1−/−Bcl2tg mice. Subsequently, chronic and exacerbated immune responses resulted in SIRS in the aged Runx1−/−Bcl2tg mice, as exemplified by symptoms such as wasting disease, organ dysfunction, and lethality. In addition, aged Runx1−/−Bcl2tg mice developed a pathology similar to PAP.

**Runx1-deleted CD4+ T cells traffic to the lung and initiate lung inflammation**

To examine whether the lung-infiltrating cells in Runx1−/−Bcl2tg mice were Runx1-deleted CD4+ T cells, immunofluorescent de-
PAP-like histology in the lungs of aged Runx1

Reduced survival of Runx1+/−/Bcl2tg mice. Survival rate percentages of control and Runx1+/−/Bcl2tg mice within 1 y of age (n = 13). (C) A representative photograph of lungs derived from >28-wk-old control (+/+) or Runx1+/−/Bcl2tg (−/−) mice (top panel). The lungs from Runx1+/−/Bcl2tg mice became enlarged and diffusively red. Weights of lungs from >28-wk-old control (+/+ ) and Runx1+/−/Bcl2tg (−/−) mice (n = 5–6; bottom panel). **p = 0.000003.

Histology of lung tissue sections of control and Runx1+/−/Bcl2tg mice. Tissues were stained with H&E or Sudan III or counterstained with an Ab to surfactant protein A (SPA). n = 3. Scale bars, 10 μm. (E) Lung disease scores of control (+/+ ) and Runx1+/−/Bcl2tg (−/−) mice at the indicated ages (n = 5).

The mechanism by which the Runx1-deleted CD4+ T cells preferentially targeted the lungs was investigated by assessing whether Runx1 deletion caused deregulation of the expression of integrins. CD4+ gated fractions from Runx1+/−/Bcl2tg splenocytes showed reduced expression of CD62L, a marker of homing to lymphoid organs (Fig. 5B). Expression of the gastrointestinal homing markers CD103 and CCR9 was subtle in lymphoid organs (Fig. 5B). Expression of the gastrointestinal homing markers CD103 and CCR9 was subtle in lymphoid organs (Fig. 5B). Expression of the gastrointestinal homing markers CD103 and CCR9 was subtle in lymphoid organs (Fig. 5B).

Runx1 was reported to regulate CD11a expression by binding to a Runx site in the promoter (30). CD11a is a subunit of LFA-1, which interacts with ICAM1 expressed on the vessel wall in bronchial mucosa (31). We also examined chemokine receptors, such as CXCR3 and CCR5, which are important for lung infiltration (32, 33). Interestingly, the cell surface level of CXCR3 was increased, whereas CCR5 was not markedly changed in Runx1+/−/Bcl2tg CD4+ T cells. Therefore, increased expression of CD11a and CXCR3 might cause the retention of Runx1-deficient CD4+ cells in the lung.

The detection of various types of immune cells in the aged Runx1+/−/Bcl2tg lungs suggested that the infiltrating Runx1-deleted CD4+ T cells may be responsible for the subsequent inflammation in the lungs. To address this possibility, Runx1+/−/Bcl2tg CD4+ T cells were adoptively and intravenously transferred into CD4+ T cell-deficient mice, and the lungs of these recipient mice were examined. Interestingly, recipient mice injected with Runx1+/−/Bcl2tg CD4+ T cells showed lung phenotypes similar to those of donor mice, and disease scores were 1–2 at 5 wk and 2–3 at 25 wk postinjection (Fig. 5C, 5D). Infiltration of lymphocytes to the peribronchovascular region of recipient mouse lungs strongly suggested that the Runx1-deleted CD4+ T cells were capable of homing to the lungs, activating an immune response and causing inflammation.

**Runx1-deleted CD4+ T cells are hyperactivated**

To better understand the mechanisms underlying the aggressive immune responses of Runx1+/−/Bcl2tg mice, spleens, pLN, and mLN were excised from nonimmunized control mice and Runx1+/−/Bcl2tg mice and examined by flow cytometry (Fig. 6A). As seen in the summary of Fig. 6B, naive cells (CD44loCD62Lhi) constituted only a small proportion (13 ± 9.3%) of the CD44+ gated population in Runx1+/−/Bcl2tg lymphatic tissues compared with control tissues (49 ± 14%). Meanwhile, the majority of CD44+ gated cells exhibited an active/memory phenotype (CD44hi) in Runx1+/−/Bcl2tg tissues compared with control tissues (76 ± 13% versus 41 ± 10%). In addition, a 1.5–2.0-fold increase in the CD69+ and CD40L+ fractions was observed in the CD44+ gated
Runx1<sup>−/−</sup>Bcl12<sup>−/−</sup> tissues compared with control tissues (Fig. 6C, 6D). These findings indicated the continuous activation of Runx1-deleted CD4<sup>+</sup> T cells.

One possible explanation for the presence of autoactivated T cells is the escape of immature, self-reactive thymocytes into the periphery. Examination of thymocyte differentiation (Supplemental Fig. 1) revealed that the percentage of CD4 single-positive cells was reduced to half in Runx1<sup>−/−</sup>Bcl12<sup>−/−</sup> mice (2.4% compared with 4.4% in the control). However, the percentage of HSA<sup>low</sup> TCR-β<sup>+</sup> mature cells in the CD4<sup>+</sup> gate did not differ significantly between control and Runx1<sup>−/−</sup>Bcl12<sup>−/−</sup> thymuses (37% versus 33%). Furthermore, most of the CD4<sup>+</sup> cells in the two spleen genotypes belonged to a mature stage (89% versus 88%). Therefore, despite the delay in early thymocyte development in Runx1<sup>−/−</sup>Bcl12<sup>−/−</sup> mice, CD4<sup>+</sup> cells appeared to be released into the periphery as fully mature T cells.

Another explanation for the presence of autoactivated CD4<sup>+</sup> T cells could be the expansion of a particular T cell clone capable of recognizing a specific Ag. To address this possibility, the expression of TCR V region β-chains (Vβ) in control and Runx1<sup>−/−</sup>Bcl12<sup>−/−</sup> CD4<sup>+</sup> T cells was analyzed (Supplemental Fig. 2). Similar distribution patterns of TCR Vβ were observed in the two cell genotypes, confirming the polyclonality of cells.

A third possible explanation for autoactivated T cells is the dysfunction of Treg. As seen in Supplemental Fig. 3A, the percentage of Foxp3<sup>+</sup> cells among the CD4<sup>+</sup> subset was increased in Runx1<sup>−/−</sup>Bcl12<sup>−/−</sup> spleens compared with control spleens (37%...
recipient mice at 25 wk postinjection. Sections were

increase in Foxp3+ Treg might function to circumvent the robust

versus 14%). In support of this observation, increased CTLA4 and

FIGURE 5. Runx1−/− T cells produce elevated levels of cytokines

To examine whether the Runx1−/− T cells that showed an activated phenotype produced cytokines, the mRNA levels of cytokines were measured by RT-PCR (Fig. 7A). In the Runx1−/− mice, IL-21 mRNA levels were 5-fold higher than in control cells (2.0 ± 0.09 versus 0.41 ± 0.24 folds). Similarly, the levels of proinflammatory cytokine IL-17A mRNA were also increased (1.9 ± 0.18 versus 0.29 ± 0.11). Consistent with our previous study (19), Runx1 deletion caused increased expression of the T cell growth factor cytokine IL-2 (2.4 ± 0.68 versus 0.31 ± 0.38), which suggested actively dividing Runx1−/− T cells. Furthermore, IL-10 expression levels were increased (2.1 ± 0.45 versus 0.08 ± 0.05), which was in accordance with the increased percentage of Treg in Runx1−/− mice. These data suggested that the Runx1 deletion in naive CD4+ T cells led to spontaneous hyperactivation of cells, as reflected in the transcriptional induction of a wide range of cytokines. No significant increase in the IFN-γ mRNA level was detected in Runx1−/− cells.

The expression of cytokines was next examined by flow cytometry after in vitro stimulation of CD4+ T cells with PMA plus into irradiated C57BL/6 recipient mice. Reconstituted cells in spleen, pLN, mLN, and lung of recipient mice were examined after 8 wk (Fig. 6E). CD4+ T cells derived from Runx1−/− mice contained a substantially greater percentage of memory/effector cells and fewer naive cells compared with the corresponding cells derived from wild-type BM. Notably, CD4+ T cells from Runx1−/− mice contained the highest percentage of effector/memory cells (84 ± 2%). The observations strongly suggest that Runx1 deficiency-mediated hyperactivation of CD4+ T cells reflects a cell-intrinsic process.

Runx1-deleted CD4+ T cells infiltrate into the lung and initiate lung inflammation. (A) Accumulation of CD4+ T cells in the lung interstitial region. Immunofluorescence staining of lung sections from >28-wk-old control and Runx1−/− mice with an FITC-CD4 Ab. Scale bar, 100 μm. Data are representative of three independent experiments (n = 3). (B) Expression profiles of integrins and chemokine receptors in Runx1-deficient CD4+ T cells. Surface expression of CD62L, CD103, CCR9, CD11a, CXCR3, and CCR5 in Runx1−/− mice compared with control CD4+ T cells. Data are representative of three independent experiments. (C) Adoptive-transfer experiments of Runx1-deficient CD4+ T cells. Control or Runx1−/− mice were injected with CD4+ T cell-deficient mice. At 5 or 25 wk postinjection, recipient mice were sacrificed for lung examination. Representative histology of lung sections from recipient mice at 25 wk postinjection. Sections were stained with H&E; scale bars, 100 μm. (D) Scoring of lung disease (n = 3–5).
FIGURE 6. Runx1-deficient CD4+ T cells are hyperactivated. (A) Runx1-deleted CD4+ T cells gained active/memory phenotypes. Flow cytometry analyses of CD44 and CD62L expression on CD4+ or CD8+ T cells derived from spleens (SP), pLN, and mLN of 24–32-wk-old control and Runx1−/− Bcl2+ mice. The percentages of naive (CD62LhiCD44hi) and active/memory (CD62LloCD44hi) fractions among the CD4+ or CD8+ T-gated cells are indicated. Data are representative of four independent experiments. (B) Bar graphs show the percentages (mean ± SD) of naive and active/memory cells among the CD4+ fractions from control or Runx1−/− Bcl2+ mice. The average percentage was calculated from SP, pLN, and mLN (n = 4). (C and D) Surface expression of the activation markers CD69 and CD40L in CD4+ T cells from spleens and pLN of 24–32-wk-old control or Runx1−/− Bcl2+ mice. Data are representative of three independent experiments (n = 3). (E) Flow cytometry analyses of naive versus active/memory phenotypes in CD4+ T cells reconstituted by mixed-BM chimera experiment. C57BL/6 mice were lethally irradiated and transplanted by a mixture of wild-type (WT; CD45.1+) and transplanted by a mixture of wild-type (WT; C57BL/6) and BALB/c (CD45.1−) and BALB/c (CD45.2−) BM cells. After 8 wk, spleen (SP), pLN, mLN, and lung were prepared and analyzed. Numbers shown are mean ± SD from two independent experiments (n = 4).

Runx1 suppresses the transcription of IL-21

Runx1 is reported to regulate the transcription of IL-2, IL-4, and IL-17 (12, 13, 19). The role of Runx1 in IL-21 expression is not known, although the role of IL-21 in both inflammation and the formation of IgG-secreting plasma cells is well established. This prompted an examination of the transcriptional regulation of IL-21 by Runx1. Transcription of IL-21 is controlled by two DNase-hypersensitive sites (HS) designated promoter (P)/HS1 and HS2 (34). Using Vista comparative genomic tools, an additional CNS was identified in intron 2 (Fig. 8A). This ~500-bp region of CNS was 99% identical between human and mouse, suggesting that it has a potentially important function. The P, HS2, and CNS sequences from different species were aligned and searched for Runx binding sites. Notably, two Runx binding sites were identified in the CNS region (Supplemental Fig. 4) but not in the P or HS2 regions. To test the functional significance of the CNS region, the P and CNS regions of IL-21 were ligated to a luciferase reporter (Fig. 8B). When transfected into Jurkat cells, both P-Luc and CNS+P-Luc plasmids showed only minimal basal activity. However, PMA plus ionomycin treatment of cells markedly induced P activity (27 ± 3-fold), as previously reported (34, 35). The addition of the CNS region further enhanced the reporter activity (42 ± 4-fold), indicating positive regulation by a response element in the CNS.

To examine whether Runx1 is involved in the regulation of CNS activity, the reporters were cotransfected with a Runx1-expressing vector, which was induced by PMA plus ionomycin (Fig. 8C). Runx1 reduced CNS+P-Luc activity to 50%, whereas it did not affect P-Luc activity. As a control, the cotransfection of Runt, a dominant-negative form of Runx1, did not reduce the CNS+P-Luc activity. In the case of CNS+SV40P-Luc, in which the P element in the CNS. The addition of the CNS region further enhanced the reporter activity (42 ± 4-fold), indicating positive regulation by a response element in the CNS.

ChIP assay was carried out to examine Runx1 binding to the IL-21 CNS region. Lysates prepared from unstimulated CD4+ T cells from C57BL/6 mice were immunoprecipitated with a Runx1 Ab. Sequences spanning each Runx site in the CNS, but not the promoter, were recovered as enriched (Fig. 8E). Mutations of the Runx site at +3114 (m1), +3162 (m2), or both sites (m1&m2) partially or completely abolished the Runx1-mediated reduction in CNS activity.

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ionomycin (Fig. 7B). The percentages of IL-21+ and IL-17+ cells were several-fold higher in the spleens and pLN of Runx1−/− Bcl2+ mice compared with controls, suggesting that the Runx1−/− Bcl2+ CD4+ T cells were more or less committed to differentiate into cytokine-producing effector T cells.
a tively indicated that Runx1, if present, functions negatively to repress IL-21 expression through binding to the CNS region.

**Germinal center formation and Ab secretion in Runx1<sup>−/−</sup>Bcl2<sup>tg</sup> mice**

Increased expression of IL-21 was observed in Runx1-deleted CD4<sup>+</sup> T cells. An increase in IL-21 expression is associated with the development of inflammatory and autoimmune diseases in mice (36). For example, IL-21 can induce the differentiation of activated CD4<sup>+</sup> T cells into proinflammatory Th17 cells (37–39). In addition, IL-21 is important in promoting the formation of the germinal center (GC) and in the differentiation of B cells into Ig-secreting plasma cells (40–43).

Immunofluorescence was used to examine the effect of Runx1 deletion on GC formation, and the results showed that T (CD4<sup>+</sup>) and B (B220<sup>+</sup>) cell zones in white pulps were disrupted in Runx1<sup>−/−</sup>Bcl2<sup>tg</sup> spleens (Fig. 9A). Furthermore, in white pulps of Runx1<sup>−/−</sup>Bcl2<sup>tg</sup> spleens, IgD<sup>+</sup> naive B cells were not detected in the follicle region; PNA (a GC marker)-positive cells were detected instead (Fig. 9B). Analysis by flow cytometry (Fig. 9C) revealed a 2-fold increase in PNA<sup>+</sup>Fashi cells (both are GC markers) in the Runx1<sup>−/−</sup>Bcl2<sup>tg</sup>-derived CD4<sup>+</sup> fraction compared with controls (28 ± 6% versus 14 ± 6%), whereas a 1.5-fold increase in PNA<sup>+</sup>Fashi cells was detected in the Runx1<sup>−/−</sup>Bcl2<sup>tg</sup>-derived B220<sup>+</sup> fractions (13 ± 9.6% versus 8.2 ± 4.1%). These observations indicate that GC formation is accelerated spontaneously in Runx1-deleted spleens.

Because GC formation is associated with the expansion of B cells and Ig class switching, B cell phenotypes were examined further. A 2-fold increase in syndecan-1+B220<sup>med</sup> Ig-secreting plasma cells was observed in Runx1<sup>−/−</sup>Bcl2<sup>tg</sup> spleens compared with controls (3.7 ± 0.6% versus 1.8 ± 0.4%; Fig. 9D).

The possible development of hyperimmunoglobulinemia in Runx1<sup>−/−</sup>Bcl2<sup>tg</sup> mice was examined by measuring titers of Ig isotypes in sera. IgM and IgG2a levels (but not IgG1) were moderately increased in Runx1<sup>−/−</sup>Bcl2<sup>tg</sup> mice compared with...
control mice (Fig. 9E). Interestingly, the titers of anti-dsDNA Ab were also higher in Runx1−/− Bcl2tg mice than in control mice. However, the titer of anti-dsDNA in Runx1−/− Bcl2tg mice was a few-fold lower than in aged MPL−/−/− mice (data not shown). Finally, frozen sections of lung were stained by fluorescein-tagged anti-IgG (Fig. 9F). Some interstitial lymphoid cells were positive for IgG staining, an indication of plasma cells. Altogether, the above observations suggest that plasma cell-associated humoral responses, including autoantibodies, might be involved in lung pathogenesis.

Discussion

Runx1−/− Bcl2tg mice generated in this study developed severe lung disease in the absence of Ag challenge. Mixed pathological phenotypes were observed, such as lymphoid infiltration into peribronchovascular interstitial regions and granulocyte-, foamy macrophage-, and surfactant protein A-containing exudates into alveolar spaces. Also, high titers of proinflammatory cytokines in BALF suggested the existence of severe inflammatory responses in the lungs. Additionally, the mice suffered from systemic inflammatory responses and died at 6–7 mo of age.

It is noteworthy that some of the pathology seen in Runx1−/− Bcl2tg lungs resembled PAP in humans. In 90% of cases of human PAP, the emergence of neutralizing autoantibodies against GM-CSF in sera appears to be responsible for the pathogenesis (44). In mouse models, PAP is generated by targeting GM-CSF, and mice exhibit pulmonary lymphoid hyperplasia, as well as alveolar proteinosis (45, 46). In humans and mice that lack GM-CSF signaling, the accumulation of exudates in alveolar spaces is attributed to a dysfunction of alveolar macrophages in clearing surfactant proteins (29). In our Runx1−/− Bcl2tg mice, anti-GM-CSF autoantibodies were not detected in sera (data not shown), and alveolar macrophages were found to be positive for surfactant protein A and Sudan III, an indication of cellular engulfing activity. Although there are no reports linking T lymphocyte abnormality to PAP, a possible cytokine storm in the lung might somehow cause macrophage dysfunction and the consequent failure to digest the incorporated materials.

It must be noted that although GM-CSF−null mice suffer from lung disease, the mice are apparently healthy and have a normal life span. In contrast, Runx1−/− Bcl2tg mice died before they reached 6–7 mo old, suggesting a much more severe and complicated pathogenesis in our mice. At a terminal stage in Runx1−/− Bcl2tg mice, systemic inflammation developed, probably due to the leakage of cytokines into the circulation. As signs of SIRS, various complications, such as monocytosis, blood coagulation, muscle wasting syndrome, and liver failure, commonly occur (10). Indeed, at least some of the above signs were confirmed in older Runx1−/− Bcl2tg mice. In addition, the chronic reactivity of CD4+ T cells is reported to drive autoimmune and destructive inflammation (47). Thus, the characteristic pathology of Runx1−/− Bcl2tg mice would be the development of lung-localized inflammation as well as systemic inflammation.

Circulating T lymphocytes, while preserving their ability to fight invading pathogens, are maintained in a quiescent stage and prevented from unnecessary autoactivation. In early studies, quiescence was considered a default stage of mature T cells before encountering a cognate Ag. Subsequently, increasing numbers of

FIGURE 8. Runx1 controls IL-21 transcription as revealed by reporter assays. (A) Homology of IL-21 gene sequences between humans and mice, as detected by Vista browser. Location of the IL-21 promoter (P), DNase-hypersensitive sites (HS1 or HS2), and CNS are indicated. (B) Jurkat cells were transfected with IL-21 CNS+P-Luc or P-Luc reporters and treated or not with PMA plus ionomycin. (C–E) Jurkat cells were cotransfected with an IL-21 reporter and an empty, Runx1- or Runt-expressing vector and stimulated with PMA plus ionomycin. (F) Mutations introduced into the Runx sites at +3114 or +3162 are indicated by “x.” In (B)–(E), the reporter activities recovered are shown as fold induction (mean ± SD). In one experiment, samples were run in triplicate; representative results of three independent experiments are shown. (F) Runx1 binds to the IL-21 CNS region inside cells. CD4+ T cells from C57BL/6 mice were subjected to ChIP with anti-Runx1 Ab or control IgG, and the precipitates were processed for PCR. (G) Relative amounts of input, anti-Runx1 ChIP, or control in a concentrated library, as quantified by real-time PCR. In (F) and (G), the precipitated DNA was amplified with primers to recognizing the Runx site at +3162, and negative control P (promoter harboring no Runx site), n.s., not significant.
FIGURE 9. Enhancement of GC formation, plasma cell mobilization, and serum Ig levels in Runx1−/− Bcl2tg mice. (A and B) Immunofluorescence staining of spleens from 24–32-wk-old control and Runx1−/− Bcl2tg mice with anti-CD4, anti-B220, anti-IgD, or anti-PNA Abs. Representative images from two independent experiments are shown. White pulps and follicle areas (F) are indicated by solid and dashed white lines, respectively. Original magnification ×10. Flow cytometry analyses of PNA+ Fas+ GC cells in CD4+ and B220+ splenocytes (C) and Syn-1+B220med plasma B cells in splenocytes (D). Control (−/−) and Runx1−/− Bcl2tg (−/−) 24–32-wk-old mice were used. Data are representative of five (C) or three (D) independent experiments. (E) Levels of Ig subtypes in sera. ELISA analyses of IgM, IgG1, IgG2a, and anti-dsDNA in sera from 24–32-wk-old control and Runx1−/− Bcl2tg mice. Data are representative of five (E) or three (F) independent experiments. (F) Immunofluorescence staining of IgG on frozen lung sections from 24–32-wk-old Runx1−/− Bcl2tg mice. Scale bar, 100 μm. Data are representative of three independent experiments (n = 3).

studies suggested that the maintenance of quiescence in T cells required the activity of transcription factors, such as Klf2, Tob, Foxo, Slfn2, Tsc1, and Foxp1 (2–8). However, an IL-17–independent role for IL-21, if any, in the induction of cell proliferation to compensate for lymphopenia, and a quiescence control mechanism remains a controversial issue. We avoided this complexity by protecting the ectopic expression of Runx1 suppressed the PMA plus ionomycin-induced CNS activity of IL-21. Multiple Runx and NFAT binding sites were identified in the CNS region. One possibility is that Runx1 binding to the CNS suppresses IL-21 transcription by masking the NFAT binding sites. Conversely, the lack of Runx1, as in the case of Runx1−/− Bcl2tg CD4+ T cells, is likely to cause an induction of IL-21 transcription through a derepression mechanism.

IL-21 plays important roles in inflammation through its ability to induce IL-17 expression (37–39), whereas IL-17, in turn, mediates immunopathogenesis in experimental hypersensitivity pneumonia and bronchiolitis obliterans syndrome [e.g. (49, 50)]. In a mouse model of experimental autoimmune encephalitis, IL-21 deficiency slowed disease progression as the result of a secondary effect of IL-17 reduction (38). In Runx1-deficient CD4+ T cells, expression of both IL-17 and IL-21 were increased. Augmentation of IL-21 might exacerbate lung inflammation indirectly through the enhancement of IL-17 expression in Runx1−/− Bcl2tg mice. However, an IL-17–independent role for IL-21, if any, in the inflammatory responses seen in Runx1−/− Bcl2tg lungs remains to be elucidated.

The known, direct effects of IL-21 are the enhancement of GC formation and the generation of IgG-secreting plasma cells (40–43). As seen in Runx1−/− Bcl2tg mice, an increased percentage of B cells became plasma cells in the spleen GC. IgG+ plasma cells were detected in the lung, although it is not clear whether they produced autoantibodies that contributed to pathogenesis in the Runx1−/− Bcl2tg lungs. Levels of IgM, IgG2a, and anti-dsDNA Ab
in sera were moderately increased, indicating the mobilization of humoral immune responses. It is possible that Abs produced by IgG plasma cells might cooperate with other immune cells and exacerbate the localized immune responses in the lung, as well as systemic inflammation.

In conclusion, the current study suggests a novel role for the Runx1 transcription factor in maintaining the quiescent stage of mature CD4+ T cells in peripheral lymphoid tissues. Deletion of Runx1 in naïve CD4+ T cells caused spontaneous cellular activation and cytokine production that eventually led to a catastrophic autoimmune inflammatory disease. The pathology seen in Runx1−/−/Bcl2−/− lungs was similar to that of human PAP. This study also implies a therapeutic potential of the Runx1 molecule for the suppression of inflammatory disease mediated by hyperactivated CD4+ T cells.

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Disclosures

The authors have no financial interests of conflict.

References


Supplemental Figure 1. Maturation of CD4+ T cells in thymus and spleen was not affected in Runx1^{+/-}Bcl2^{tg} mice. Flow cytometry analyses of thymocytes and splenocytes from 16-24 weeks old, control and Runx1^{+/-}Bcl2^{tg} mice. Percentages of mature (HSA^{lo}TCR {\beta}^{hi}) cells in total and CD4^{+} T-gated fractions are indicated. Data are representative from two independent experiments.
Supplemental Figure 2

Supplemental Figure 2. Polyclonality of CD4+ T cells appears not perturbed in Runx1^{+/−}-Bcl2^{tg} mice. Histogram shows the repertoire of TCR Vβ expression in CD4+ and CD8+ splenocytes. Splenocytes were prepared from 16-24 week old control and Runx1^{+/−}-Bcl2^{tg} mice. Shown are mean ± s.d. from two independent experiments (n=2).
Supplemental Figure 3. Increased percentage of Treg cells in Runx$^{1+/+}$Bcl2$^{tg}$ mice. (A) Flow cytometry analysis of Foxp3 expression on CD4+ T-gated splenocytes from control and Runx$^{1+/+}$Bcl2$^{tg}$ mice. Shown are representative data from two independent experiments. (B) mRNA expression level of CD25 and CTLA4 gene on CD4+ T cells purified from control or Runx$^{1+/+}$Bcl2$^{tg}$ spleens. β-actin served as a loading control.
Supplemental Figure 4. The CNS region of *IL-21* contains two conserved Runx-sites. The CNS regions of *IL-21* from indicated species are aligned, and the conserved nucleotides are highlighted. The binding sites for Runx and other transcription factors are indicated as boxed.