

Figure 2. Chest computed tomography (CT) revealed peripheral ground-glass opacities (A), which worsened after oral prednisolone and cyclosporine regime (B) and improved markedly after 6-month GM-CSF inhalation therapy (C).

Table 1. Laboratory Data on Admission

<Hematology>				<Arterial blood gas>	
WBC	11,600	/ μ L	BUN	13	mg/dL
Seg	80	%	Cr	0.7	mg/dL
Lym	14	%	Na	146	mEq/L
Eos	0	%	K	3.7	mEq/L
Bas	0	%	Cl	109	mEq/L
Mon	6	%	<Serology>		AaDO ₂
RBC	4.88×10^6	/ μ L	CRP	0.1	mg/dL
Plt	257×10^3	/ μ L	KL-6	1,176	U/mL
<Blood chemistry>			SP-D	151.2	ng/mL
TP	6.2	g/dL	<BAL cell findings>		%DLco
T-Bil	3.2	mg/dL	Total cell	58.5×10^5	cells/mL
AST	19	IU/L	AM	87	%
ALT	16	IU/L	Lym	10	%
LDH	252	IU/L	Neu	1	%
ALP	121	IU/L	CD4/CD8	8.35	
γ -GTP	23	IU/L			

ued, the opacities did not improve during the subsequent 3-month observation. The patient was treated with GM-CSF inhalation of high-dose administration (sargramostim, 125 μ g twice daily on Days 1-8, and none on Days 9-14) for six 2-week cycles and consequent low dose administration months (125 μ g once daily on Days 1-4, none on Days 5-14) for six 2-week cycles, using a jet nebulizer (LC Plus; PARI GmbH, Starnberg, Germany) which generates particles

of mean mass aerodynamic diameter of 3.97-5.25 μ m at a flow rate of 3-6 L/min, according to the manufacturer's instructions (11). The peripheral shadows on the chest radiograph and CT as well as bronchoscopic findings showed marked improvement (Fig. 1D, 2C, 3B), and serum levels of SP-D and KL-6 decreased. Amorphous materials had markedly decreased in BALF (macrophages, 89%; lymphocytes, 10%; neutrophils, 1%). No recurrence was observed during

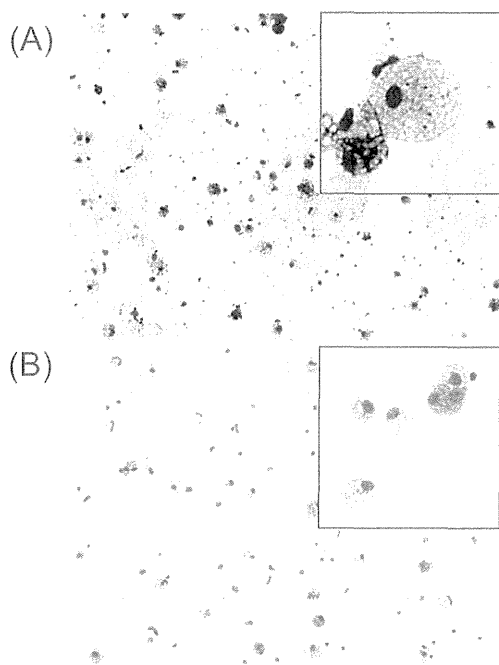


Figure 3. Wright-Giemsa staining of the cells in the bronchoalveolar lavage fluid before (A) and after (B) GM-CSF inhalation therapy ($\times 200$). Insets show the cells at a higher magnification ($\times 400$). The extracellular proteinaceous material and cell debris decreased markedly after 6-month GM-CSF inhalation therapy. The number of foamy macrophages decreased, although that of smaller alveolar macrophages increased after treatment.

the five years following treatment. However, the serum level of GM-CSF antibody was still elevated ($19.5 \mu\text{g/mL}$) five years after the end of the GM-CSF inhalation therapy.

Discussion

A typical CT finding of PAP is a “crazy-paving appearance,” distributed centrally in both lungs. Instead, the present case showed bilateral GGOs localized in peripheral subpleural areas from the basal to the apical segments. Ten Japanese cases of PAP with subpleural infiltrates on CT findings have been reported in the Japanese and English literature. Most patients had no symptoms, and their condition did not worsen without treatment (Table 2) (9, 10, 12-19). Eight out of ten patients had never been a smoker.

The 10 cases yielded CT images of two types. One type showed solitary pulmonary nodule (20), as in Cases 5 (a nodule of 30 mm in diameter), 8 (nodules, not referred), and 10 (a nodule of 40×20 mm). In these cases, lung cancer was suspected. Then they underwent surgical resection under video-assisted thoracoscopy. Cases 5, 8 and 10 were diagnosed pathologically as PAP. However, GM-CSF antibody was not measured in two of the cases. Case 8 demonstrated multiple nodular lesions, of which one lesion was diagnosed as bronchoalveolar carcinoma; the rest were pulmonary al-

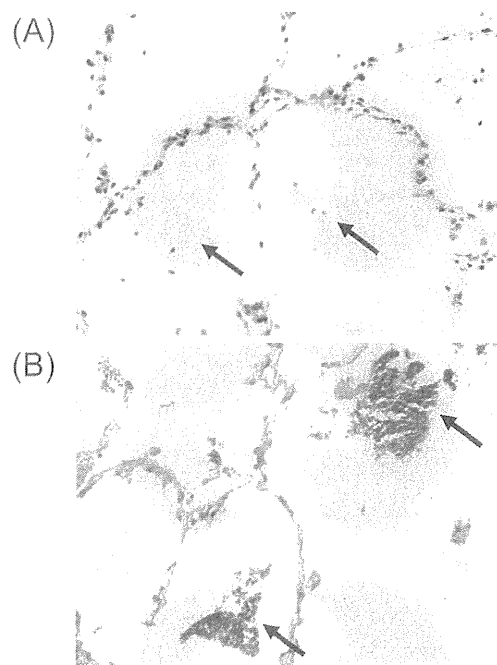


Figure 4. Hematoxylin and Eosin staining of lung tissue during transbronchial lung biopsy (TBLB; $\times 200$) (A), showing accumulation of proteinaceous materials within alveolar spaces. Periodic acid-Schiff (PAS) staining of lung tissue during TBLB ($\times 200$) shows that the amorphous material is PAS-positive (B). Arrows indicate PAS-positive materials.

veolar proteinosis. It is particularly interesting that Case 8 demonstrated positive GM-CSF antibody. The other was GGO distributed in the subpleural area, which the remaining seven cases showed. Six of the seven were female. GM-CSF antibody was measured in five of the seven cases: all five were positive. Case 4 worsened two years later, but the others remained stable.

In patients with PAP, infiltrates often disappear from the peripheral region to the central region (1). Mohri et al. (14) speculated that the GGOs might extend from the periphery to the central region and develop into air space consolidation as respiratory symptoms occur, suggesting that patients with peripheral GGOs might be exhibiting early stages of PAP. In this regard, the present case developed GGOs that extend from the peripheral to the central region and which disappeared from the central area toward the peripheral region. Recently, it has been demonstrated that GM-CSF administration by subcutaneous injection (21, 22) or inhalation (2, 4, 11) improved the respiratory function as well as CT findings of autoimmune PAP patients. Inhaled GM-CSF might first reach the mildly-impaired, proximal region in the lungs and improve the function of the macrophages present in those locations. The restored function of these alveolar macrophages may contribute to improving the clearance in the adjacent, distal regions. The present report describes exacerbation and improvement of peripheral infiltrates of a PAP patient in a series of CT, supporting the speculations

Table 2. Summary of Japanese Pulmonary Alveolar Proteinosis (PAP) Patients Showing Peripheral Ground-glass Opacities on CT Images

Case No.	Age (y)	Gender	Smoking history	Dust exposure	Symptoms	BAL appearance Lymphocyte	GM-CSF antibody	Therapeutic history	Prognosis	Reference
1	39	F	Never	None	No	n.a. 11.5%	n.a.	None	7 mo stable	Imui <i>et al.</i> (9)
2	38	F	n.a.	n.a.	No	n.a.	n.a.	None	12 mo stable	Mita <i>et al.</i> (12)
3	55	F	Never	n.a.	No	Milk like 20%	Positive (BAL)	None	Recurrence after 2 y	Sugimoto <i>et al.</i> (13)
4	32	F	Never	None	No	Milk like 25.6%	Positive (BAL&Serum)	None	15 mo partially improved	Mohri <i>et al.</i> (14)
5	70	M	Current	Asbestos	No	n.a.	n.a.	(Resection for diagnosis)	3 y stable	Norikane <i>et al.</i> (15)
6	56	F	Never	n.a.	No	n.a.	Positive (Serum)	AM	No recurrence	Yamasaki <i>et al.</i> (16)
7	45	M	Never	None	No	n.a. 82%	Positive (Serum)	None	No recurrence for 3 y	Toyama <i>et al.</i> (17)
8	58	F	Never	None	No	n.a.	Positive (Serum)	(Resection for diagnosis)+AM	No recurrence for 3 y	Taniguchi <i>et al.</i> (18)
9	65	F	Never	None	No	n.a.	Positive	None	18 mo stable	Haga <i>et al.</i> (10)
10	57	M	Never	n.a.	No	n.a.	n.a.	(Resection for diagnosis)	No recurrence for 1 y	Sunadome <i>et al.</i> (19)

Key: n.a., not available; AM, ambroxol hydrochloride

described by Mohri *et al.* (14).

Although whole-lung lavage (WLL) has been the standard therapy for PAP, the recent perspective of the pathophysiology of PAP is leading us to innovative treatment options including GM-CSF administration, plasmapheresis and B-cell depletion. GM-CSF inhalation could be considered as an alternative in patients refractory to WLL, with a contraindication to general anesthesia during WLL, or under the effects of immunosuppressants, as in the present case. The treatment options will become more defined, as our knowledge in this field including CT patterns advances.

The present case worsened after steroid therapy. Cyclosporine, which was added on the expectation of improving organizing pneumonia refractory to 1-year steroid treatment (23), might also be associated with the worsening in chest radiographs during its administration. It is notable that Case 3 worsened as well after prednisolone therapy for autoimmune hemolytic anemia as well (17). Careful consideration should be devoted to steroid administration in the treatment of autoimmune PAP because of its lack of efficacy and its associated risk. Reportedly, steroid therapy increases the production of phospholipids (24) and down-regulates monocyte function (25). To prevent inappropriate application of immunosuppressants, it is important to consider PAP in the differential diagnosis of such peripheral opacities, which requires detailed characterization of HRCT findings and serum markers. In this regard, GM-CSF antibody might be helpful for diagnosis.

In conclusion, PAP should be considered in the differential diagnosis of peripheral subpleural opacities. Measure-

ment of GM-CSF antibody might be a useful diagnostic option. GM-CSF inhalation therapy improved the peripheral opacities observed on CT images of PAP patients.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

The authors thank Dr. Bruce C. Trapnell for critical reading of the manuscript.

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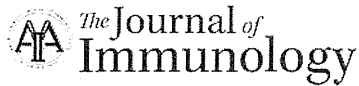
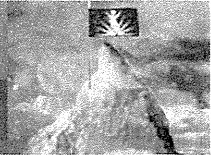
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This information is current as of May 20, 2015.

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J Immunol 2012; 188:5408-5420; Prepublished online 2 May 2012;

doi: 10.4049/jimmunol.1102991

<http://www.jimmunol.org/content/188/11/5408>

Supplementary Material <http://www.jimmunol.org/content/suppl/2012/05/03/jimmunol.1102991.DC1.html>

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

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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*^{-/-}*Bcl2*^{tg} 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 the lung, and expressed various cytokines, including IL-17 and IL-21. Among these, the deregulation of *IL-21* transcription was likely to be associated with Runx binding sites located in an *IL-21* intron. IL-17 produced in Runx1-deleted cells mobilized innate immune responses, such as those promoted by neutrophils and monocytes, whereas IL-21 triggered humoral responses, such as plasma cells. Thus, at an initial stage, peribronchovascular regions in the lung were infiltrated by CD4⁺ lymphocytes, whereas at a terminal stage, interstitial regions were massively occupied by immune cells, and alveolar spaces were filled with granular exudates that resembled pulmonary alveolar proteinosis in humans. Mice suffered from respiratory failure, as well as systemic inflammatory responses. Our data indicate that Runx1 plays an essential role in repressing the transcription of cytokine genes in naive CD4⁺ T cells and, thereby, maintains cell quiescence. *The Journal of Immunology*, 2012, 188: 5408–5420.

Activation 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, Sln2, 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 Th 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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Received for publication October 17, 2011. Accepted for publication March 28, 2012.

This work was supported by grants-in-aid for scientific research from the Japan Society for the Promotion of Science and a research grant from the Ministry of Education, Science, Sports, Culture and Technology, Japan. W.F.W. is a Japan Society for the Promotion of Science postdoctoral fellow. N.I., T.T., and M.S. are members of the Global Center of Excellence program “Network Medicine” at Tohoku University.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; *Bcl2*^{tg}, *Bcl2* transgenic; BM, bone marrow; CBFβ, core binding factor β; ChIP, chromatin immunoprecipitation; CNS, conserved noncoding sequence; EM, Elastica-Masson; GC, germinal center; HS, hypersensitive site; mLN, mediastinal lymph node; P, promoter; PAP, pulmonary alveolar proteinosis; PAS, periodic acid-Schiff; pLN, peripheral lymph node; P-Luc, promoter-luciferase; PNA, peanut agglutinin; SIRS, systemic inflammatory response syndrome.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1102991

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 *Runx1^{fl/fl}CD4^{cre}* mice (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 *Runx1^{fl/fl}CD4^{cre}* mice makes it difficult to analyze the role of Runx1 in quiescence control by using these mice. In *CBFβ^{fl/fl}CD4^{cre}* mice, 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, *CBFβ^{fl/fl}CD4^{cre}* mice are not suitable for analyzing the specific function of Runx1 in CD4⁺ T cells.

In the current study, *Runx1^{fl/fl}CD4^{cre}* mice were crossed with Bcl2-transgenic (*Bcl2^{tg}*) 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 quiescence stage.

Materials and Methods

Mice

Conditional Runx1-knockout (*Runx1^{fl/fl}*) mice were prepared, as previously described (22). To delete Runx1 specifically in CD4⁺ T cells, *Runx1^{fl/fl}* mice were crossed with CD4-Cre-tg mice (23). Bcl2 expression in T lymphocytes was enforced by crossing mice with *Bcl2^{tg}* mice (B6.Cg-Tg [BCL2]25Wehi/J) (24) to generate *Runx1^{fl/fl};CD4-Cre-tg;Bcl2^{tg}* mice (denoted as *Runx1^{-/-}Bcl2^{tg}*). CD4⁺ T cell-deficient mice (B6.129S2-Cd4tm1Mak) (25) and C57BL/6 mice were from The Jackson Laboratory and CLEA, respectively. Ly5.1⁺ (CD45.1)-C57BL/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 Tohoku University.

Flow cytometry analyses

Cell suspensions were prepared from spleens, lymph nodes, lungs, or thymuses of mice, and 1×10^6 cells were stained with the following Abs: FITC-B220, FITC-heat stable Ag, PE-TCRβ, PE-CXCR3, PE-CCR5, allophycocyanin-B220, and PECy7-CD8a (BioLegend, San Diego, CA); FITC-CD69, FITC-Fas, PE-CD21, PE-CD40L, PE-Gr-1, 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, PECy5-CD62L, PE-Cy7-IgM, and allophycocyanin-CD4 (eBiosciences, San Diego, CA); PE-Thy1 (Cell Laboratory, Fullerton, CA); and biotin-c-peanut agglutinin (PNA) (Biomed, Burlingame, CA). Cells stained with biotin-conjugated Abs were subjected to secondary incubation with streptavidin-PE (BD Pharmingen). For intracellular staining of cytokines, CD4⁺ T cells were isolated from splenocytes using anti-mouse CD4 Magnetic Particles-DM (BD Biosciences). The purity of isolated cells was >93%. Cells were incubated in RPMI 1640/10% FBS for 4 h in the presence of 200 ng/ml PMA, 1 μM ionomycin, and 2 μM monensin. Intracellular staining was performed using a Fix and Perm kit (Invitrogen, Carlsbad, CA). The Abs used were FITC-IFN-γ, PE-IL-17 (BD Biosciences), and PE-IL-21 (R&D Systems, Minneapolis, MN). For the detection of nuclear Foxp3, a PE-Foxp3 Ab and staining buffer set (eBiosciences) were used. Apoptosis was assayed using the Mebcyto Apoptosis kit (MBL, Nagoya, Japan). TCR polyclonality was analyzed with a Mouse Vβ TCR Screening kit (BD Biosciences). Flow cytometry analyses were carried out in a Cytomics FC500 and analyzed with CXP analysis software.

Bronchoalveolar lavage cells and cytokine analyses

Mouse lungs were lavaged three times with 700 μl PBS, and the recovered bronchoalveolar lavage fluid (BALF) was centrifuged at $300 \times g$ for 5

min. For the pellets, the number of cells was counted by a hemocytometer, and cell types were identified by flow cytometry analyses of cell surface markers. For the supernatants, the amount of cytokines was measured using a Cytometric Bead Array Mouse Th1/Th2 Cytokine Kit (BD Biosciences, San Jose, CA). A 50-μl volume was incubated with mixed capture beads for 2 h in the dark, washed, and processed for flow cytometry analyses. Mean fluorescence intensities were measured. The concentrations of each cytokine were extrapolated by the equation of each standard curve ($R^2 \geq 0.99$).

Adoptive 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 3×10^6 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 congenic Ly5.1⁺ (CD45.1)-C57BL/6 and *Runx1^{-/-}Bcl2^{tg}* (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 5×10^6) was injected i.v. into tail veins of C57BL/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) paraformaldehyde 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 excision. 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-surfactant protein A Ab (Millipore, Bedford, MA). Lung histology was scored as follows: grade 0, normal lung; grade 1, mild/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. Cryostat sections were prepared, blocked with 5% (w/v) BSA in PBS, and stained with anti-mouse IgG (H+L) F(ab')₂-488 (Cell Signaling, Danvers, MA), FITC-anti-CD4 (eBiosciences), FITC-anti-IgD, FITC-anti-B220, PE-anti-CD4 (all from BioLegend), or biotin-c-PNA (Biomed), followed by FITC-streptavidin for 1 h in the dark and viewed through a Zeiss LSM5 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 IgM/IgG1/IgG2a 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).

Immunoblotting and RT-PCR

CD4⁺ T or CD8⁺ T cells were isolated from splenocytes using the respective anti-mouse Magnetic Particles-DM (BD Biosciences), and 1×10^6 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 *Pan-Runx* Ab was as described previously (27). Bcl2 (100) 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 Runx1-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'-GAAGATCTGTGACACAAACCAGGTGAGGTG-3' and 5'-CCCAAGCTTCTGAGTCTCCAGGAGCTGATGA-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 the pGL3 reporter. To insert a conserved noncoding sequence (CNS) region, a mouse genomic sequence was amplified using the primers 5'-AAAGGTACCGGATAGTACAGGG-AGTTTGTCT-3' and 5'-AAAAGATCTGAATCCTCTCAGGGACAA-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 mutated from TGTGGT to TCTAAG using a PCR-amplification method. Primers used for the construct were m1: 5'-AAGCCGGTTC-TAAGCAAAAAGAAG-3' and 5'-CTTCTTTTTGCTTAGAACCCGGCTT-3'; m2: 5'-AACATGAACATCTAAGTTTCAAGG-3' and 5'-CCTTGAA-ACTTAGATGTTTCATGTT-3'; and m1&2: 5'-CGGTTCTAAGCAAAA-GAAGAAAAGAAAAAAGCTTCAACAAAACATGAACATCTAAGT-TTC-3' and 5'-GAAACTTAGATGTTTCATGTTTGTGAAGTTTTTTC-TTTTCTTCTTTTTGCTTAGAACCCG-3'. The sequences of mutated Runx sites are underlined.

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 μ M 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'-AGGTAGCTTGCCTGCTACTAGGGCAAAGTG-3' and 5'-TTTTTCC-AGTAAGTTAAGCCGGTTGTTGTC-3' and for the Runx site at +3162 (CNS-2): 5'-TTTTTGACCACAACCCGGCTTAACCTTACTGG-3' and 5'-TACGACCCCTCCCAAGTCTTCTTGGAACG-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 *Runx1^{fl/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 *Runx1^{fl/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 ($0.41 \pm 0.22 \times 10^7$ versus $2.0 \pm 0.47 \times 10^7$; Fig. 1C).

Because *Runx1^{-/-}* mice suffered from a severe reduction in the CD4⁺ T cell population, this population was boosted by crossing them with *Bcl2^{tg}* mice (24) to yield *Runx1^{fl/fl};CD4-Cre-tg;Bcl2^{tg}* mice (hereafter referred to as *Runx1^{-/-}Bcl2^{tg}*). The expression of transduced Bcl2 protein in T cells from control or *Runx1^{-/-}Bcl2^{tg}* spleens was confirmed by immunoblot analyses (Fig. 1A). Bcl2 did not affect the efficiency of the Runx1 deletion in *Runx1^{-/-}Bcl2^{tg}* CD4⁺ T cells. Spleens with a *Bcl2^{tg}* 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 *Bcl2^{tg}* mice due to enhanced cell viability and resistance to apoptosis (24). As a consequence, *Runx1^{-/-}Bcl2^{tg}* spleens contained an increased number ($2.0 \pm 1.4 \times 10^7$ versus $0.41 \pm 0.22 \times 10^7$) and percentage of CD4⁺ T cells compared with those of *Runx1^{-/-}* mice without a *Bcl2^{tg}* 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⁺ fraction were 2.8-fold higher in *Runx1^{-/-}* mice than in *Runx1^{+/+}* mice ($18.7 \pm 4.4\%$ versus $6.8 \pm 2.3\%$). The presence of the *Bcl2* transgene significantly reduced the percentages of apoptotic CD4⁺ T splenocytes to $7.6 \pm 4.5\%$ in *Runx1^{-/-}Bcl2^{tg}* 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^{-/-}Bcl2^{tg}* 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^{+/+}Bcl2^{tg}* mice were used as controls.

Runx1^{-/-}Bcl2^{tg} mice develop severe lung inflammation

The development and growth of *Runx1^{-/-}Bcl2^{tg}* mice were apparently normal, and no recognizable phenotypes were detected during young adulthood. However, after >28 wk, *Runx1^{-/-}Bcl2^{tg}* 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^{-/-}Bcl2^{tg}* 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^{-/-}Bcl2^{tg}* mice, together with age-matched control mice, were sacrificed, and the internal organs were examined. Macroscopically, the lungs from *Runx1^{-/-}Bcl2^{tg}* mice were substantially large, diffusively red, and 2.3-fold heavier than were those from control mice (0.61 ± 0.07 g versus 0.26 ± 0.02 g) (Fig. 2C). Histological sections of the lung 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^{-/-}Bcl2^{tg}* mice. This infiltration was not seen in control *Runx1^{+/+}Bcl2^{tg}* mice. In accordance with lymphoid infiltration in the lungs, peripheral lymph nodes (pLN) and lung-draining mediastinal lymph nodes (mLN) from *Runx1^{-/-}Bcl2^{tg}* mice were also markedly enlarged compared with those from control littermates (data not shown).

In the lungs of aged *Runx1^{-/-}Bcl2^{tg}* 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^{-/-}Bcl2^{tg}* 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^{-/-}Bcl2^{tg}* lungs as well.

The disease score of mice was determined by observing histological sections (Fig. 2F), as described in *Materials and Methods*.

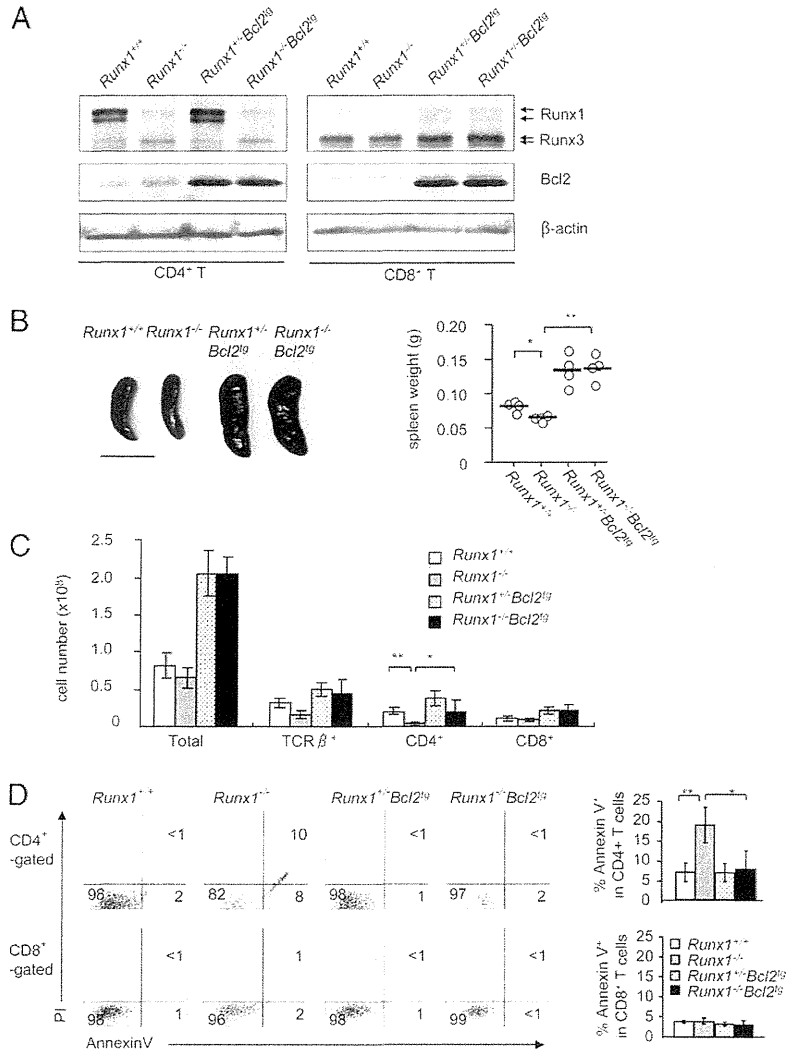


FIGURE 1. Overexpression of *Bcl2* rescues apoptotic *Runx1*-deleted CD4⁺ T cells. **(A)** Confirmation of *Runx1* deletion and *Bcl2* transgene expression by immunoblot analyses. CD4⁺ and CD8⁺ T cells were purified from spleens of 8–12-wk-old *Runx1*^{+/+}, *Runx1*^{-/-}, *Runx1*^{+/-}*Bcl2*^{tg}, or *Runx1*^{-/-}*Bcl2*^{tg} mice. Lysates were prepared and processed. β -actin served as a loading control. **(B)** A representative photograph of spleens from 8–12-wk-old mice (left panel). Scale bar, 1 cm. Spleen weights of 8–12-wk-old mice (right panel; *n* = 4). **p* = 0.01, ***p* = 0.0003. **(C)** The number of CD4⁺ T cells increased after *Bcl2* transgene expression. Cell numbers of total, TCR β ⁺, CD4⁺, or CD8⁺ splenocytes from 8–12-wk-old mice (*n* = 4). **p* = 0.046, ***p* = 0.0004. **(D)** The percentages of apoptotic cells were reduced in the presence of *Bcl2* transgene expression. Flow cytometry analyses of splenocytes derived from 8–12-wk-old mice. Bar graphs show mean (\pm SD) percentages of annexin V⁺ cells in the CD4⁺- or CD8⁺-gated populations derived from each mouse (*n* = 4). **p* = 0.013, ***p* = 0.0012.

Lungs from young *Runx1*^{-/-}*Bcl2*^{tg} 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*^{-/-}*Bcl2*^{tg} mice, ~5-fold greater numbers of BALF cells were detected compared with control littermates. BALF cells from *Runx1*^{-/-}*Bcl2*^{tg} 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*^{-/-}*Bcl2*^{tg} mice compared with control mice (Fig. 3C). This suggests the occurrence of a cytokine storm in the lungs of aged *Runx1*^{-/-}*Bcl2*^{tg} mice.

Systemic inflammation in aged Runx1^{-/-}*Bcl2*^{tg} mice

Organs other than the lungs were examined next. In young *Runx1*^{-/-}*Bcl2*^{tg} mice, tissues from the kidneys, liver, pancreas, or the digestive tract exhibited normal histology (data not shown). In contrast, aged *Runx1*^{-/-}*Bcl2*^{tg} 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

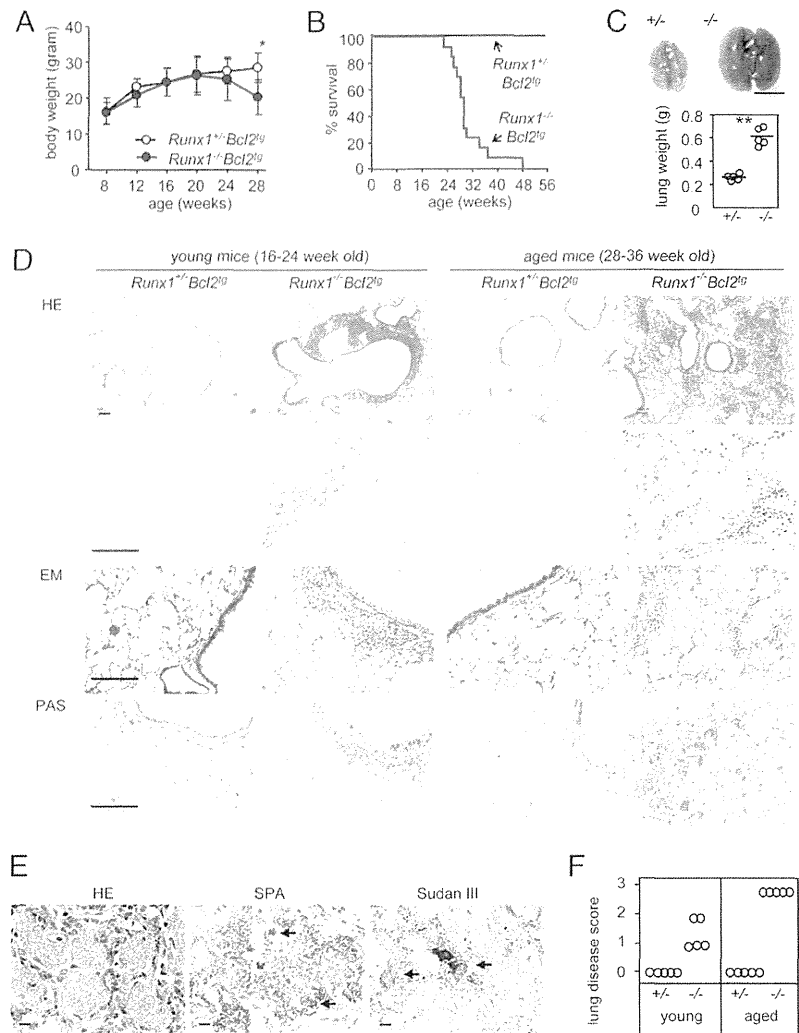
were enlarged and contained immune cells. Glycogenesis appeared insufficient, probably due to ischemia. The hemocyte count of peripheral blood (Table I) revealed slightly increased numbers of RBC and increased hemoglobin concentration and hematocrit percentage, suggesting compensatory erythrocytosis, whereas the numbers of WBC were decreased to less than half. In addition, the percentages of Gr-1^{med}Mac-1⁺ monocytes were remarkably increased in peripheral blood (monocytosis in Fig. 4B), suggesting the presence of a chronic systemic inflammation in aged *Runx1*^{-/-}*Bcl2*^{tg} mice. Consistently, the levels of IFN- γ and TNF were elevated in sera from aged *Runx1*^{-/-}*Bcl2*^{tg} 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*^{-/-}*Bcl2*^{tg} mice. Subsequently, chronic and exacerbated immune responses resulted in SIRS in the aged *Runx1*^{-/-}*Bcl2*^{tg} mice, as exemplified by symptoms such as wasting disease, organ dysfunction, and lethality. In addition, aged *Runx1*^{-/-}*Bcl2*^{tg} 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*^{-/-}*Bcl2*^{tg} mice were *Runx1*-deleted CD4⁺ T cells, immunofluorescent de-

FIGURE 2. Mice harboring *Runx1*-deficient T cells develop lethal lung inflammation. **(A)** Weight loss in aged *Runx1*^{-/-}*Bcl2*^{tg} mice. Body weights of *Runx1*^{-/-}*Bcl2*^{tg} and control mice between 8 and 28 wk of age. Shown are mean \pm SD ($n = 6$ – 8 mice per each age group). * $p = 0.0038$. **(B)** Reduced survival of *Runx1*^{-/-}*Bcl2*^{tg} mice. Survival rate percentages of control and *Runx1*^{-/-}*Bcl2*^{tg} mice within 1 y of age ($n = 13$). **(C)** A representative photograph of lungs derived from >28 -wk-old control (+/+) or *Runx1*^{-/-}*Bcl2*^{tg} (-/-) mice (top panel). The lungs from *Runx1*^{-/-}*Bcl2*^{tg} mice became enlarged and diffusively red. Weights of lungs from >28 -wk-old control (+/+) and *Runx1*^{-/-}*Bcl2*^{tg} (-/-) mice ($n = 5$ – 6 ; bottom panel). *** $p = 0.000003$. **(D)** Histology of lung tissue sections of control and *Runx1*^{-/-}*Bcl2*^{tg} mice. Tissues were stained with H&E, EM, or PAS ($n = 5$). Scale bars, 100 μ m. **(E)** PAP-like histology in the lungs of aged *Runx1*^{-/-}*Bcl2*^{tg} mice. Lung sections were stained with H&E or Sudan III or counterstained with an Ab to surfactant protein A (SPA). $n = 3$. Scale bars, 10 μ m. **(F)** Lung disease scores of control (+/+) and *Runx1*^{-/-}*Bcl2*^{tg} (-/-) mice at the indicated ages ($n = 5$).



tection of the CD4 Ag was performed in frozen lung sections (Fig. 5A). As expected, CD4⁺ T cells concentrated in the peribronchovascular regions of *Runx1*^{-/-}*Bcl2*^{tg} (but not control) lungs.

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*^{-/-}*Bcl2*^{tg} 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 *Runx1*^{-/-}*Bcl2*^{tg} cells; in contrast, CD11a expression was substantially enhanced. *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*^{-/-}*Bcl2*^{tg} 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*^{-/-}*Bcl2*^{tg} lungs suggested that the infiltrating *Runx1*-deleted CD4⁺ T cells may be responsible for the subsequent inflam-

mation in the lungs. To address this possibility, *Runx1*^{-/-}*Bcl2*^{tg} 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*^{-/-}*Bcl2*^{tg} 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 mice 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*^{-/-}*Bcl2*^{tg} mice, spleens, pLN, and mLN were excised from nonimmunized control mice and *Runx1*^{-/-}*Bcl2*^{tg} mice and examined by flow cytometry (Fig. 6A). As seen in the summary of Fig. 6B, naive cells (CD44^{lo}CD62L^{hi}) constituted only a small proportion (13 \pm 9.3%) of the CD4⁺-gated population in *Runx1*^{-/-}*Bcl2*^{tg} lymphatic tissues compared with control tissues (49 \pm 14%). Meanwhile, the majority of CD4⁺-gated cells exhibited an active/memory phenotype (CD44^{hi}) in *Runx1*^{-/-}*Bcl2*^{tg} tissues compared with control tissues (76 \pm 13% versus 41 \pm 10%). In addition, a 1.5–2.0-fold increase in the CD69⁺ and CD40L⁺ fractions was observed in the CD4⁺-gated

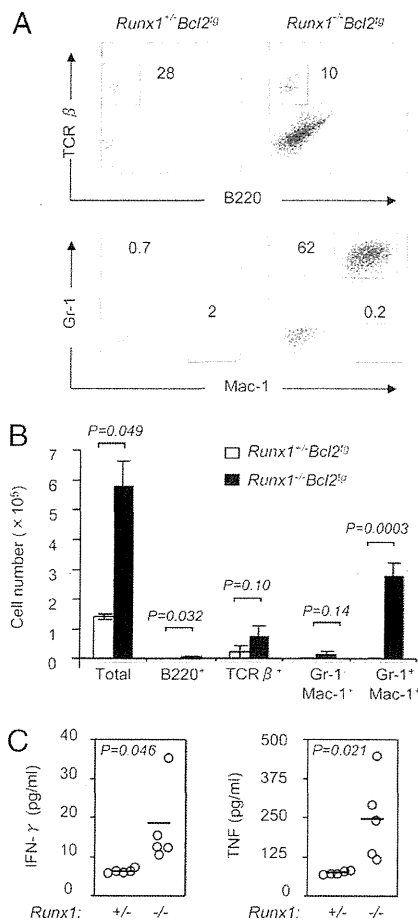


FIGURE 3. Increase in immune cells and proinflammatory cytokines in BALF of *Runx1*^{-/-}*Bcl2*^{tg} mice. (A) Flow cytometry analyses of cells in BALF. Representative results from three independent experiments are shown. (B) Bar graphs show mean (± SD) cell counts in BALF prepared from 24–32-wk-old control and *Runx1*^{-/-}*Bcl2*^{tg} mice (*n* = 3). Immune cells, including lymphocytes (B220⁺ and TCRβ⁺) and neutrophils (Gr-1⁺Mac-1⁺), were increased in *Runx1*^{-/-}*Bcl2*^{tg} mice. The B220⁺ fraction constituted a small population in BALF but was increased in *Runx1*^{-/-}*Bcl2*^{tg} mice (1329 ± 1681 versus 7725 ± 106). (C) Amounts of proinflammatory cytokines IFN-γ and TNF in BALF derived from >28-wk-old control (+/+) or *Runx1*^{-/-}*Bcl2*^{tg} (-/-) mice (*n* = 5).

Runx1^{-/-}*Bcl2*^{tg} tissues compared with control tissues (Fig. 6C, 6D). These findings indicated the continuous activation of Runx1-deleted CD4⁺ 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*^{-/-}*Bcl2*^{tg} mice (2.4% compared with 4.4% in the control). However, the percentage of HSA^{low} TCR-β⁺ mature cells in the CD4⁺ gate did not differ significantly between control and *Runx1*^{-/-}*Bcl2*^{tg} thymuses (37% versus 33%). Furthermore, most of the CD4⁺ cells in the two spleen genotypes belonged to a mature stage (89% versus 88%). Therefore, despite the delay in early thymocyte development in *Runx1*^{-/-}*Bcl2*^{tg} mice, CD4⁺ cells appeared to be released into the periphery as fully mature T cells.

Another explanation for the presence of autoactivated CD4⁺ T cells could be the expansion of a particular T cell clone capable of recognizing a specific Ag. To address this possibility, the expres-

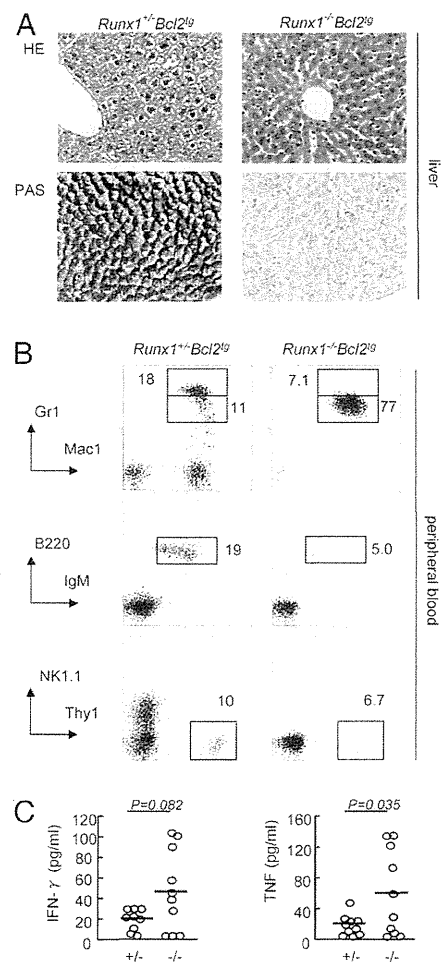
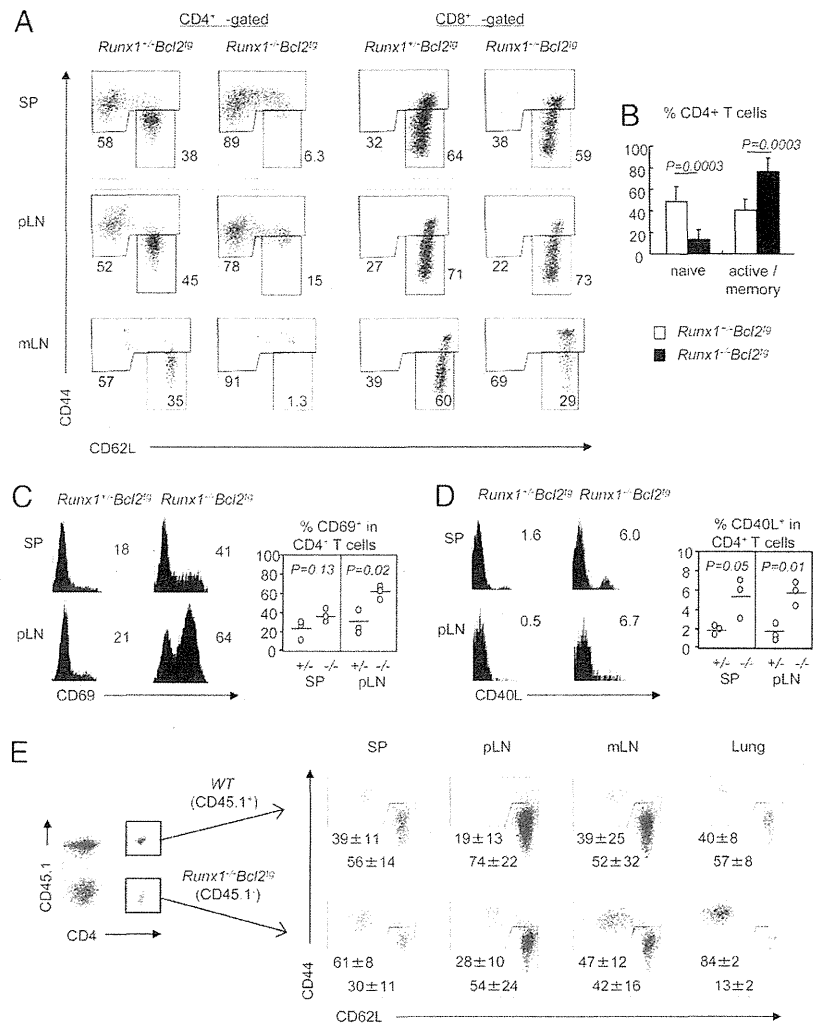


FIGURE 4. Aged *Runx1*^{-/-}*Bcl2*^{tg} mice develop SIRS. (A) Glycogenesis insufficiency in livers of aged *Runx1*^{-/-}*Bcl2*^{tg} mice. Liver sections from 28–36-wk-old control and *Runx1*^{-/-}*Bcl2*^{tg} mice were stained. H&E staining showed atrophy of hepatocytes, as well as enlargement of sinusoids. PAS-positive materials were abundant in the periphery of hepatocytes of control mice but were not detected in the *Runx1*^{-/-}*Bcl2*^{tg} hepatocytes, suggesting poor glycogenesis. Original magnification ×40. (B) Monocytosis in aged *Runx1*^{-/-}*Bcl2*^{tg} mice. Peripheral blood was collected from aged (>28-wk-old) control and *Runx1*^{-/-}*Bcl2*^{tg} mice and analyzed by flow cytometry staining. The percentage of monocytes (Gr-1^{int}Mac-1^{hi}) was dramatically increased in *Runx1*^{-/-}*Bcl2*^{tg} mice, indicating a presence of chronic inflammation and suggesting an increased demand for, for example, phagocytotic activity in the inflamed tissues. Percentages of other lineages, including B (B220⁺IgM⁺), NK (NK1.1⁺), or T (Thy1⁺) cells, were relatively reduced as the result of an increase in monocytes. Data are representative of two independent experiments. Note that monocytosis was not observed in the peripheral blood of young *Runx1*^{-/-}*Bcl2*^{tg} mice. (C) Elevated levels of proinflammatory cytokines in sera from aged *Runx1*^{-/-}*Bcl2*^{tg} mice. Sera were collected from aged (>28-wk-old) control (+/+) and *Runx1*^{-/-}*Bcl2*^{tg} (-/-) mice (*n* = 10 for each genotype). Amounts of IFN-γ (left panel) and TNF (right panel) were measured by cytokine bead array, using 5-fold diluted sera. The *p* values were evaluated by the unpaired Student *t* test.

sion of TCR V region β-chains (Vβ) in control and *Runx1*^{-/-}*Bcl2*^{tg} CD4⁺ 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⁺ cells among the CD4⁺ subset was increased in *Runx1*^{-/-}*Bcl2*^{tg} spleens compared with control spleens (37%

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*^{tg} mice. The percentages of naive (CD62L^{hi}CD44^{lo}) and active/memory (CD62L^{lo}CD44^{hi} or CD62L^{hi}CD44^{hi}) 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*^{tg} 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*^{tg} 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 *Runx1*^{-/-}*Bcl2*^{tg} (CD45.1⁻) 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).



ionomycin (Fig. 7B). The percentages of IL-21⁺ and IL-17⁺ cells were several-fold higher in the spleens and pLN of *Runx1*^{-/-}*Bcl2*^{tg} mice compared with controls, suggesting that the *Runx1*^{-/-}*Bcl2*^{tg} CD4⁺ T cells were more or less committed to differentiate into cytokine-producing effector T cells.

Runx1 suppresses the transcription of *IL-21*

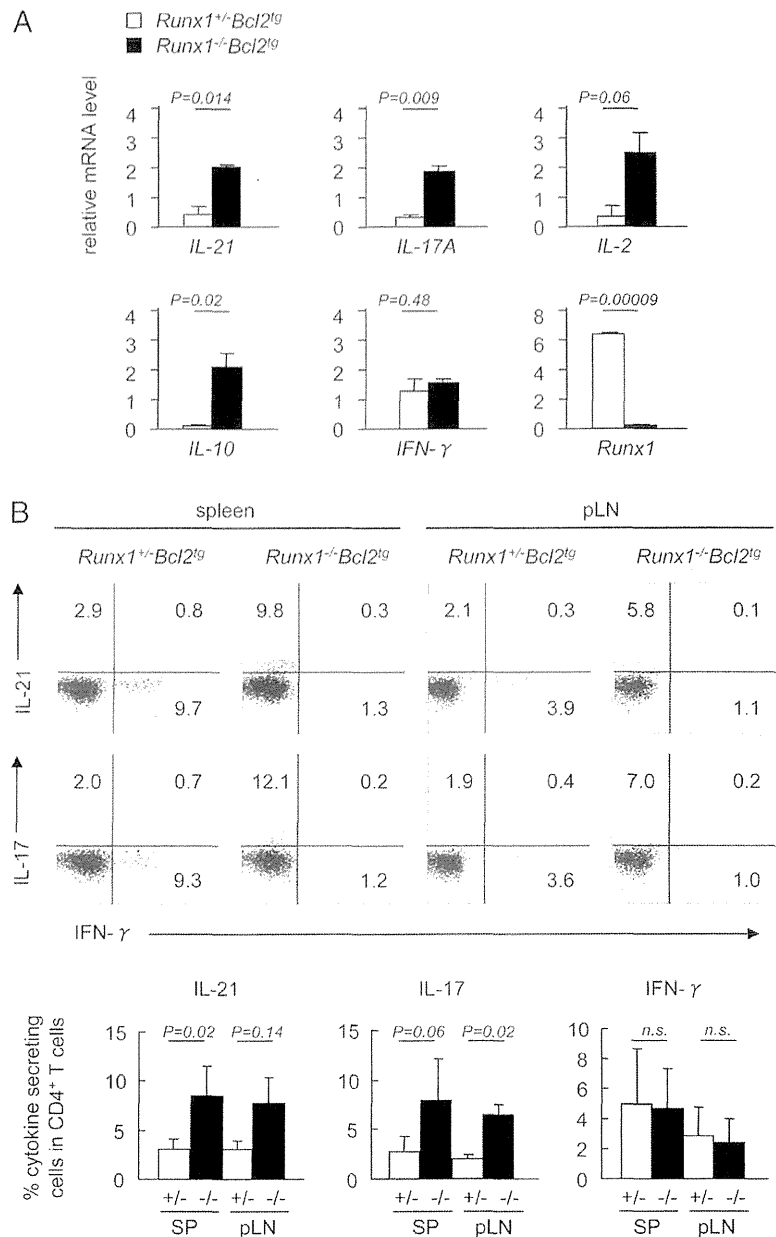
Runx1 is reported to regulate the transcription of *IL-2*, *IL-4*, and *IL-17* (12, 13, 19). The role of *Runx1* in *IL-21* expression is not known, although the role of *IL-21* in both inflammation and the formation of IgG-secreting plasma cells is well established. This prompted an examination of the 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 in-

duced 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 *IL-21* promoter was replaced by the SV40 promoter, *Runx1* reduced the activity to 30%, confirming the negative role of the CNS region in *IL-21* regulation (Fig. 8D). Mutations were then introduced into two *Runx* sites in the CNS, individually or simultaneously (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.

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. 8F). To further confirm the *Runx1* binding, a concentrated DNA library was prepared and processed for quantitative real-time PCR. The CNS-1 and CNS-2 regions were successfully enriched (Fig. 8G). The results collec-

FIGURE 7. Enhanced expression of IL in *Runx1*-deficient CD4⁺ T cells. **(A)** Relative amounts of IL transcripts compared with those of β -actin. CD4⁺ T cells were purified from 24–32-wk-old control and *Runx1*^{-/-}*Bcl2*^{tg} spleens. RNA was prepared and processed for semiquantitative RT-PCR analyses. Band intensities were compared and quantified, using β -actin as a control. Bar graphs are mean \pm SD from two independent experiments. **(B)** CD4⁺ T cells were purified from the spleens or pLN of 24–32-wk-old control and *Runx1*^{-/-}*Bcl2*^{tg} mice. Cells were stimulated in vitro with PMA plus ionomycin and processed for intracellular staining of indicated IL and flow cytometry analyses. Representative data from three independent experiments are shown. Bar graphs show the percentages of cytokine-positive CD4⁺ T cells in spleen or pLN from the control (+/-) and *Runx1*^{-/-}*Bcl2*^{tg} (-/-) mice. Mean \pm SD from three independent experiments is shown.



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*^{-/-}*Bcl2*^{tg} mice

Increased expression of IL-21 was observed in *Runx1*-deleted CD4⁺ 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⁺ 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).

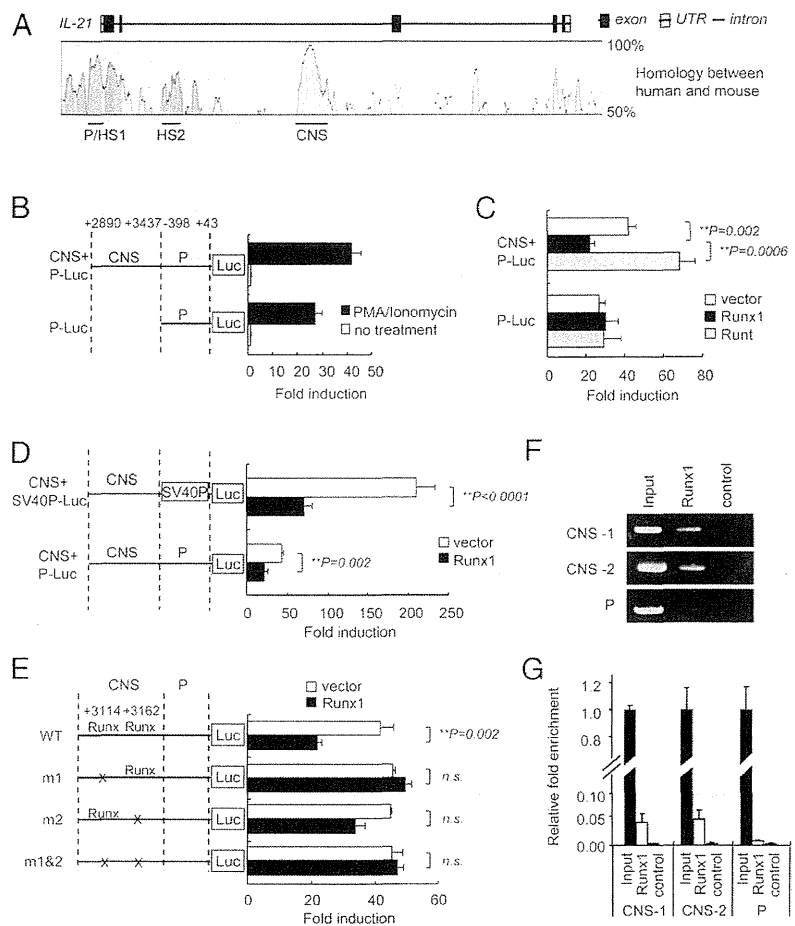
Immunofluorescence was used to examine the effect of *Runx1* deletion on GC formation, and the results showed that T (CD4⁺) and B (B220⁺) cell zones in white pulps were disrupted in *Runx1*^{-/-}*Bcl2*^{tg} spleens (Fig. 9A). Furthermore, in white pulps of *Runx1*^{-/-}*Bcl2*^{tg} spleens, IgD⁺ naive 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⁺Fas^{hi} cells (both are GC markers) in the *Runx1*^{-/-}*Bcl2*^{tg}-derived CD4⁺ fraction compared with the control (28 \pm 10% versus 14 \pm 6%), whereas a 1.5-fold increase in PNA⁺Fas^{hi} cells was detected in the *Runx1*^{-/-}*Bcl2*^{tg}-derived B220⁺ fractions (13 \pm 9.6% versus 8.2 \pm 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^{med} Ig-secreting plasma cells was observed in *Runx1*^{-/-}*Bcl2*^{tg} spleens compared with controls (3.7 \pm 0.6% versus 1.8 \pm 0.4%; Fig. 9D).

The possible development of hyperimmunoglobulinemia in *Runx1*^{-/-}*Bcl2*^{tg} mice was examined by measuring titers of Ig isotypes in sera. IgM and IgG2a levels (but not IgG1) were moderately increased in *Runx1*^{-/-}*Bcl2*^{tg} mice compared with

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. **(E)** 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 \pm 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 CNS-1 (recognizing the Runx-site at +3114), CNS-2 (recognizing the Runx site at +3162), and negative control P (promoter harboring no Runx site). n.s., not significant.



control mice (Fig. 9E). Interestingly, the titers of anti-dsDNA Ab were also higher in *Runx1*^{-/-}*Bcl2*^{tg} mice than in control mice. However, the titer of anti-dsDNA in *Runx1*^{-/-}*Bcl2*^{tg} mice was a few-fold lower than in aged *MPL*^{pr/pr} 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^{-/-}*Bcl2*^{tg} 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*^{-/-}*Bcl2*^{tg} 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 attrib-

uted to a dysfunction of alveolar macrophages in clearing surfactant proteins (29). In our *Runx1*^{-/-}*Bcl2*^{tg} 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*^{-/-}*Bcl2*^{tg} 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*^{-/-}*Bcl2*^{tg} 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*^{-/-}*Bcl2*^{tg} mice. In addition, the chronic reactivity of CD4⁺ T cells is reported to drive autoimmunity and destructive inflammation (47). Thus, the characteristic pathology of *Runx1*^{-/-}*Bcl2*^{tg} 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

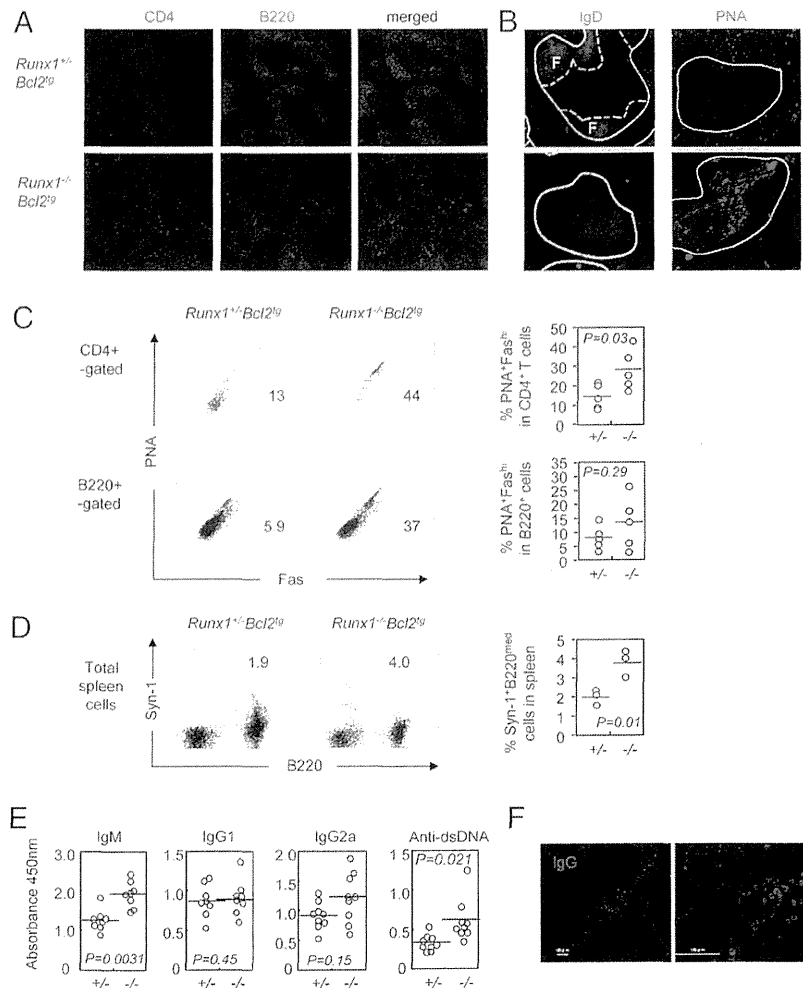


FIGURE 9. Enhancement of GC formation, plasma cell mobilization, and serum Ig levels in *Runx1*^{-/-} *Bcl2*^{tg} mice. **(A and B)** Immunofluorescence staining of spleens from 24–32-wk-old control and *Runx1*^{-/-} *Bcl2*^{tg} mice with anti-CD4, anti-B220, anti-IgD, or anti-PNA Abs. Representative images from two independent experiments are shown. White pulps and follicle areas (F) are indicated by solid and dashed white lines, respectively. Original magnification $\times 10$. Flow cytometry analyses of PNA^{hi}Fas⁺ GC cells in CD4⁺ and B220⁺ splenocytes **(C)** and Syn-1^{hi}B220^{med} plasma B cells in splenocytes **(D)**. Control (+/-) and *Runx1*^{-/-} *Bcl2*^{tg} (-/-) 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*^{-/-} *Bcl2*^{tg} (-/-) mice ($n = 8-9$). **(F)** Immunofluorescence staining of IgG on frozen lung sections from 24–32-wk-old *Runx1*^{-/-} *Bcl2*^{tg} 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, Sifn2, Tsc1, and Foxp1 (2–8). However, the thus-far reported quiescence-related molecules are also involved in the regulation of cell homeostasis, cell survival, and/or cell trafficking, and their targeting often results in a lymphopenic situation in mice. Therefore, the loss of cell quiescence seen in targeted mice might be due to the induction of cell proliferation to compensate for lymphopenia, and a quiescence control mechanism remains a controversial issue. We avoided this complexity by protecting cells from apoptosis with the use of the *Bcl2* transgene and observed that *Runx1*^{-/-} *Bcl2*^{tg} CD4⁺ T cells exhibited hyperactivated phenotypes, as judged by the expression of activation markers (CD62L^{lo}CD44^{hi}CD69⁺), the lung-homing integrin molecule CD11a, and various cytokines. The present results suggest that *Runx1* may function as a guardian of naive CD4⁺ T cells in their quiescence stage.

Then how does a *Runx1* deficiency lead to the disruption of cell quiescence? The spontaneous expression of various cytokines in naive *Runx1*^{-/-} *Bcl2*^{tg} CD4⁺ T cells suggests that *Runx1* might contribute to quiescence by intrinsically suppressing the expression of various cytokines in resting T cells. Regulation of cytokine genes, such as *IL-2*, *IL-4*, or *IL-17*, by *Runx1* was reported (12, 13, 19). In this study, we explored the regulation of *IL-21* transcription by *Runx1* because *IL-21* is closely associated with both inflammatory (37–39) and autoimmune diseases (41, 48). We found that 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*^{-/-} *Bcl2*^{tg} 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 pneumonitis 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*^{-/-} *Bcl2*^{tg} mice. However, an *IL-17*-independent role for *IL-21*, if any, in the inflammatory responses seen in *Runx1*^{-/-} *Bcl2*^{tg} 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*^{-/-} *Bcl2*^{tg} 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*^{-/-} *Bcl2*^{tg} 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 naive 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*^{tg} 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.

Acknowledgments

We thank I. Taniuchi (RIKEN, Yokohama, Japan) for CD4-Cre-tg mice and Y. Yoshikai and H. Yamada (Kyushu University, Kyushu, Japan) for *Bcl2*^{tg} mice. We thank M. Kuji for secretarial assistance.

Disclosures

The authors have no financial conflicts of interest.

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We believe that our study is the first to identify different proportions of obstructive and restrictive airway disease between the main ethnic groups in sarcoidosis, and also the first to report an association of functional impairment with age. Despite a weak evidence base, sarcoidosis patients are often prescribed inhaled corticosteroids and β -agonists for respiratory symptoms regardless of their pattern of lung function at presentation [12]. Large airway obstruction in sarcoidosis is associated with increased mortality [13] and our data suggest that these patients may represent an under-diagnosed group. Further studies are needed to confirm these demographic associations, as well as to assess disease progression and response to inhaled therapies in this particular clinical phenotype of sarcoidosis.

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Statement of Interest: None declared.

Acknowledgements: We would like to thank S. Martin and the lung function team at St Mary's Hospital (Imperial College London NHS Healthcare Trust, London, UK) for their assistance with data collection.

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DOI: 10.1183/09031936.00103811

Reduced GM-CSF autoantibody in improved lung of autoimmune pulmonary alveolar proteinosis

To the Editors:

Pulmonary alveolar proteinosis (PAP) is a rare lung disease characterised by excessive accumulation of surfactant materials within alveolar spaces [1]. Patients with autoimmune PAP (aPAP) present a high level of granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibodies (GM-Ab) in the serum as well as in bronchoalveolar lavage fluid (BALF) [2]. GM-Ab neutralise the biological activity of GM-CSF in the lung [3], impairing terminal differentiation of alveolar macrophages and macrophage-mediated pulmonary surfactant clearance [4].

Based on the aetiology, clinical trials of exogenous GM-CSF supplementation have been carried out by a number of

physicians with variable response rates ranging from 40 to 62% [5–9]. Previously, we reported that in three patients who received a pilot GM-CSF inhalation therapy, oxygenation was improved and the concentration of GM-Ab in BALF was reduced [7]. BONFIELD *et al.* [8] also reported that the serum titre of GM-Ab was reduced during successful treatment of aPAP with subcutaneously injected GM-CSF. However, our recent phase II trial of GM-CSF inhalation involving 35 patients revealed that serum levels of GM-Ab remained unchanged throughout the therapy, suggesting that GM-CSF inhalation therapy did not affect the production of GM-Ab [9]. Thus, the effect of exogenous GM-CSF administration on GM-Ab levels in the serum remains controversial. This discrepancy may be due to differences in the route

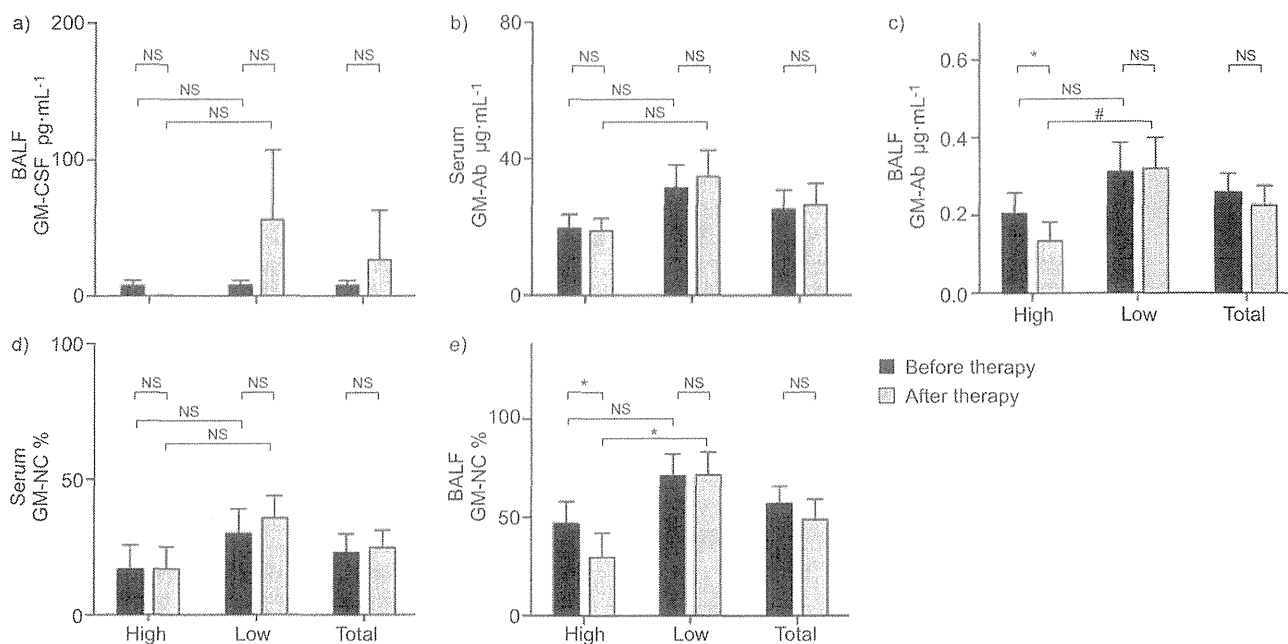


FIGURE 1. The titres of granulocyte-macrophage colony-stimulating factor (GM-CSF) and GM-CSF autoantibodies (GM-Ab) in bronchoalveolar lavage fluid (BALF) and serum obtained from high and low responders and total patients before and after GM-CSF inhalation therapy. a) GM-CSF in BALF, GM-Ab b) in serum and c) in BALF, and GM-CSF-neutralising capacity (GM-NC) d) in serum and e) in BALF are shown. Data are presented as mean and SE. *: $p < 0.05$; #: $p = 0.054$; NS: nonsignificant; p-values calculated using paired t-test for comparison between normally distributed data before and after therapy, and unpaired t-test for group comparison between high and low responders.

of administration and/or the dose of GM-CSF. Aerosolised GM-CSF reaches the lower respiratory tract and may stimulate immature alveolar macrophages directly to promote terminal differentiation and improve the local clearance of the accumulated surfactant and GM-Ab, although it does not affect the production of systemic GM-Ab.

To test this hypothesis, we performed a retrospective study using preserved BALF obtained through an optional evaluation procedure from the patients that participated in a pilot study ($n=1$) [7], an early phase II trial ($n=6$) [9] and a multicentre phase

II trial ($n=12$) [9] of GM-CSF inhalation therapy. Importantly, each patient underwent the bronchoalveolar lavage procedure on the same bronchus of the right middle lobe by the same operator of the same institute within 1 week of the start of, and after the end of, the GM-CSF inhalation therapy period according to the unified standard procedure protocol described previously [9]. BALF was sent to the Niigata University Medical and Dental Hospital (Niigata, Japan) and subjected to centralised analysis. As the median alveolar-arterial oxygen tension difference (P_{A-a,O_2}) improvement was 13 mmHg, we classified the participants into two groups, high responders with an improvement >13 mmHg ($n=10$) and low responders with an improvement <13 mmHg ($n=9$), in order to evaluate the relationship between therapeutic response and changes in the level of GM-CSF or GM-Ab in the serum and BALF. There were no significant differences between the two groups in symptoms, including cough and dyspnoea, demographic data, lung function tests, except for P_{A-a,O_2} or BALF recovery rates.

To determine the stoichiometry of GM-CSF during the inhalation treatment, we measured the concentrations of total GM-CSF (*i.e.* autoantibody-bound plus free GM-CSF) in BALF according to a method described previously [10] in order to rule out the possibility that GM-CSF inhalation may augment the production of intrinsic GM-CSF in the lung. The concentration of total GM-CSF did not change during the inhalation therapy in both high and low responders (fig. 1a). Thus, GM-CSF inhalation was not associated with an increase of GM-CSF in the lung. Notably, BALF of low responders tended to contain a high level of GM-CSF that might derive from GM-Ab-GM-CSF complex residing in the alveolar space, as $>99\%$ of serum GM-CSF was bound to

TABLE 1 Correlation between levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibodies (GM-Ab) and GM-CSF-neutralising capacity (GM-NC) in serum and bronchoalveolar lavage fluid (BALF)

Variable	Before treatment		After treatment	
	$r^{\#}$	p-value [†]	$r^{\#}$	p-value [†]
BALF GM-NC and BALF GM-Ab	0.7556	0.0005	0.8430	<0.0001
BALF GM-Ab and serum GM-Ab	0.8616	<0.0001	0.7780	0.0002
BALF GM-NC and serum GM-NC	0.5345	0.0271	0.6087	0.0073

[#]: Pearson's correlation coefficient; [†]: values calculated using the Pearson's correlation test.