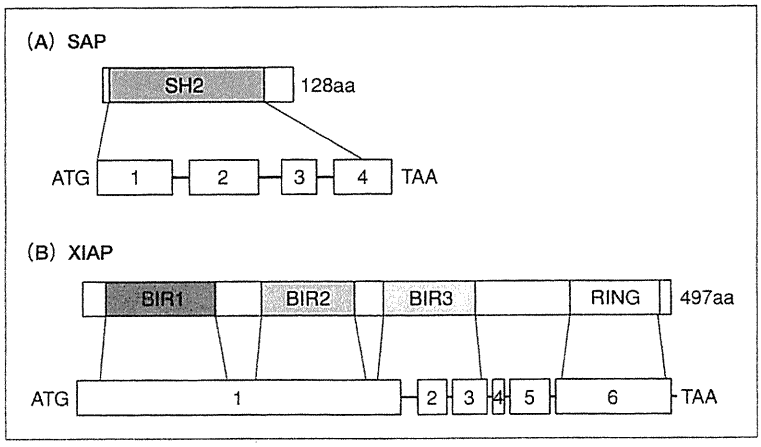

図 1　SAP(A)ならびにXIAP(B)の蛋白ならびに遺伝子構造

SAP 蛋白は 128 個のアミノ酸からなり，1 個の SH2 ドメインを有する(A)．XIAP 蛋白は 497 個のアミノ酸からなり，3 個の BIR ドメインと C 末端の RING ドメインから構成される(B)．

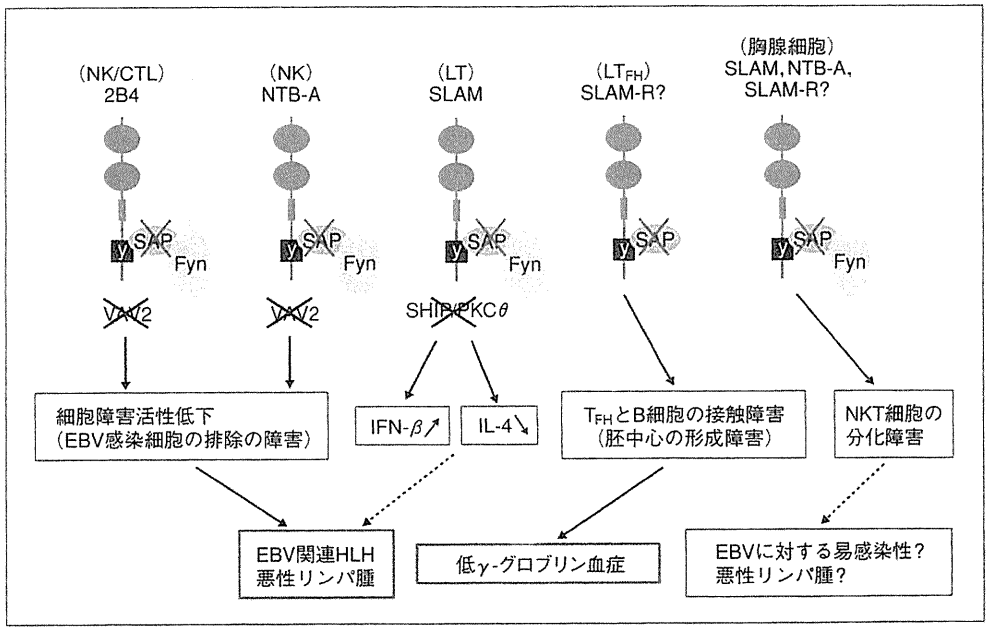

図 2　SLAMレセプターファミリーとSAP欠損症の病態形成

SAP の異常によって SLAM レセプターファミリーのシグナル伝達障害をきたし，SAP 欠損症の病態にかかわると考えられる．

作用し，シグナル伝達を制御している．

SAP の異常によって NK 細胞ならびに細胞障害性リンパ球(cytotoxic lymphocyte：CTL)における 2B4 や NTB-A のシグナルの異常をきたし，細胞障害活性の低下のために EBV 感染細胞の排除の障害が生じ，EBV 関連 HLH や EBV 陽性悪性リンパ腫を発症するものと思われる(図 2)．SLAM のシグナル異常によって IFN-β の産生増加や IL-4 の産生低下が生じ，これらのサイトカイン産生障害も EBV 関連 HLH や悪性リンパ腫の病態に関与しているかもしれない．また，濾胞ヘルパー T(follicular helper T：T_{FH})細胞のシグナル異

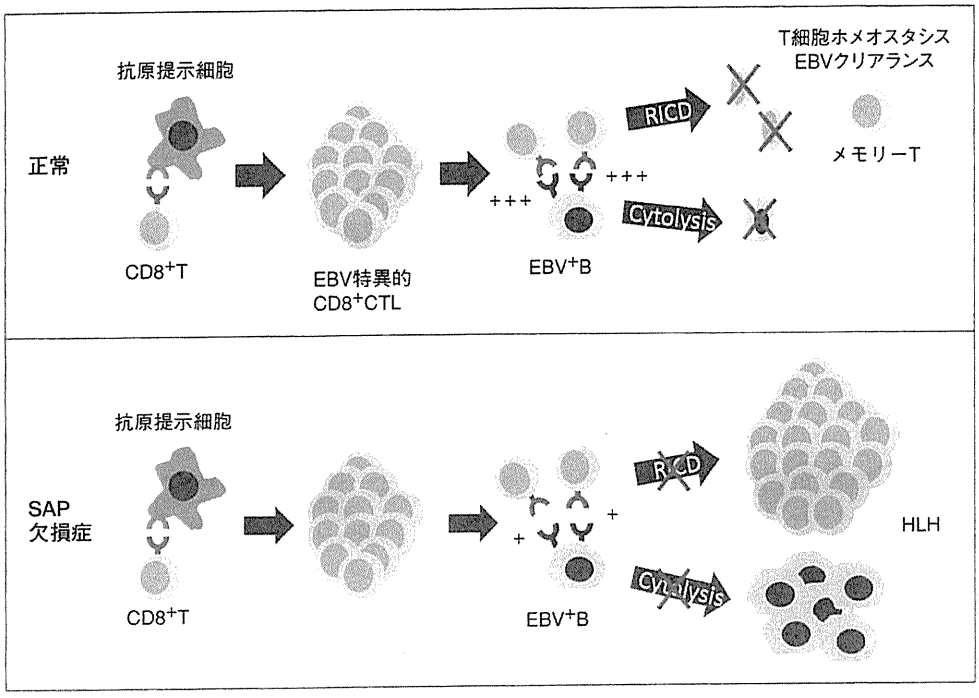

図 3　SAP欠損症におけるRICDの異常

SAP 欠損症の患者では，RICD(アポトーシス)の異常のために，EBV 感染症後に HLH を発症しやすいと考えられる．

常によって，T_{FH}と B 細胞の接触障害が生じ，リンパ節における胚中心の形成障害をきたし，低γ-グロブリン血症を呈すると思われる．NKT 細胞の分化障害も認められ，EBV に対する易感染性や悪性リンパ腫の発症にかかわっていると考えられている．

EBV感染に対する易感染性

EBV は普遍的なウイルスであり，通常は不顕性感染であるが，ときに一過性 T 細胞増殖症として伝染性単核症を発症する．しかし，SAP 欠損症では NK 細胞や $CD8^+$T 細胞における 2B4 を介するシグナル伝達異常により EBV 感染細胞の排除を行うことができずに致死的な伝染性単核症あるいは EBV 関連 HLH を発症する．SAP 欠損症では再活性化によって誘導される細胞死(reactivation-induced cell death：RICD)の障害があり，HLH の重症化をきたすと考えられている(図 3)[11]．すなわち，健常人では EBV 感染後に増殖した EBV 特異 $CD8^+$T 細胞は EBV 感染 B 細胞と反応することによって，B 細胞は細胞融解し，一方，増殖した T 細胞は RICD によって細胞死に至ることによって，EBV 感染細胞が排除され，少数のメモリー T 細胞が残存するという T 細胞のホメオスタシスが働く．しかし，SAP 欠損症では B 細胞の融解が生じないばかりか，RICD が生じないため，T 細胞の異常増殖がみられ，これが HLH の病態を形成すると考えられている．

また，EBV 関連リンパ増殖症として，近親婚のトルコ人姉妹例において *interleukin-2 inducible T cell kinase* (*ITK*)の変異が報告された[12]．SAP 欠損症と同様に NKT 細胞の減少が認められ，*ITK* 欠損マウスでも NKT 細胞の分化障害と T 細胞機能異常が報告されている．T 細胞と NKT 細胞の機能異常が EBV 感染細胞の排除に働かないため，EBV 関連リンパ増殖症を発症するものと考えられる．

異常γ-グロブリン血症

SAP 欠損症では低γ-グロブリン血症または高 IgM 血症が認められる．致死的 EBV 感染症をまぬがれた患者のほとんどで認められるが，EBV 陰性

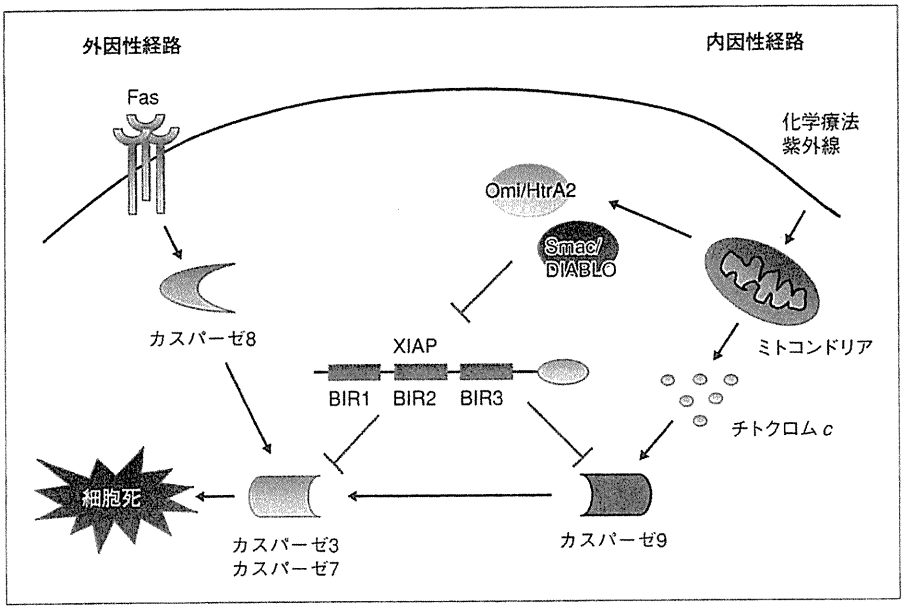

図 4　アポトーシス経路におけるXIAP

XIAP は，BIR ドメインを介してカスパーゼ 3，7 および 9 を抑制し，外因系および内因系のアポトーシスを制御している．

の患者でもときに観察される．

悪性リンパ腫

約 30%の患者で悪性リンパ腫を初発症状とする．多くの患者は EBV 感染 B 細胞の悪性転化の結果としてリンパ腫を発症するが，EBV 感染の既往が明らかでない患者も存在する．ほとんどが B 細胞リンパ腫であり，約半数は Burkitt タイプである．中枢神経などの節外性のリンパ腫もある．

XIAP欠損症の臨床的特徴

XLP と区別しがたい臨床的特徴を有する 3 家系 12 例の男性患者において，はじめて *XIAP* 変異が同定された[7]．最初の報告ではほとんどの患者が EBV 関連 HLH を発症し，約 1/3 の患者が低γ-グロブリン血症をきたすとされた．しかし，その後の報告例が増えるにつれて臨床的特徴がより明らかになってきた(表 1)[8,9]．HLH はほとんどの患者で認められるが，EBV 関連とは限らず，比較的軽症で反復するものも多い．低γ-グロブリン血症は認められるが，悪性リンパ腫の発症は 1 例も認められていない．また，SAP 欠損症に認められた再生不良性貧血や血管炎の報告もない．一方，持続する脾腫や出血性大腸炎が少なからず認められる点が SAP 欠損症と異なる点である．SAP 欠損症と XIAP 欠損症はオーバーラップするところは多少あるが，臨床的に区別しうる別の疾患と考えられるようになってきた．

XIAP欠損症の病態

XIAP 変異によってなぜ HLH 様の症状を呈するのか SAP 欠損症ほどにはわかっていない．*XIAP* は，カスパーゼ 3,7,9 の活性を抑制することによって内因性および外因性の細胞死を抑制している(図 4)．したがって，*XIAP* 変異によって細胞死が誘導されると考えられ，事実 *in vitro* の実験でも患者由来 T 細胞は健常人に比べて刺激後細胞死に陥りやすいがその差はわずかであり，細胞死の程度の違いのみで XIAP 欠損症の病態を説明することは困難であり，今後の解明が望まれる．

SAPおよびXIAP欠損症の診断

致死的あるいはそれに近い EBV 感染症，HLH，低γ-グロブリン血症の男児，とくに母方男性に家族例を有する場合には SAP または XIAP 欠損症の可能性が考えられる．当教室で開発された flow

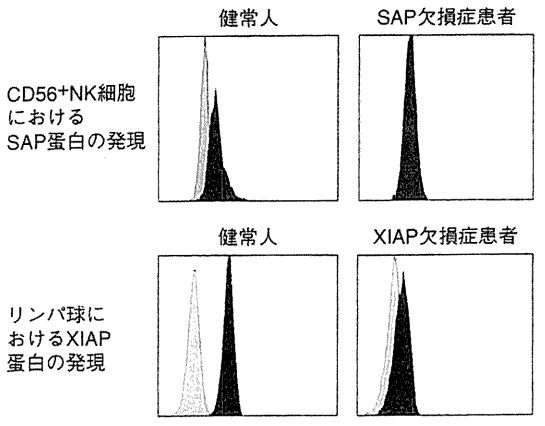

図 5　Flow cytometryによるXLPの診断
SAP 欠損症患者では，$CD56^{+}$NK 細胞内の SAP 蛋白の発現が健常人に比べて低下している(上段)．XIAP 欠損症患者では，リンパ球内の XIAP 蛋白の発現が健常人に比べて低下している(下段)．

cytometry により SAP または XIAP 蛋白の発現を調べる方法がもっとも効率よく診断できる(図5)[13-15]．臨床症状ならびに flow cytometry の結果から XLP が疑われた場合には *SH2D1A* または *XIAP* 遺伝子解析を行う．

SAPおよびXIAP欠損症の治療

臨床表現型に応じた治療が基本である．低γ-グロブリン血症に対しては，免疫グロブリン定期補充療法，悪性リンパ腫に対しては標準的化学療法を有効である．HLH に対しては HLH2004 プロトコールに準じた治療を行うが，EBV 感染 B 細胞を排除するために抗 CD20 モノクローナル抗体である rituximab が有効との報告がある[16]．根治的療法としては造血幹細胞移植であるが，XIAP 欠損症に対する移植例はまだ少ない．他の原発性免疫不全症と同様に，骨髄非破壊的前処置による造血幹細胞移植が有用かもしれない[17]．

おわりに

SAP と XIAP は，遺伝子の局在が近いといってもまったく異なる遺伝子変異によって，なぜよく似た臨床像をとるのかまだ明らかではない．今後の解析が期待される．

XLP はわが国にも少なからず存在し，これまで当教室では 32 例の SAP 欠損症，9 例の XIAP 欠損症を同定している．疾患の予後を左右するのは，早期診断による造血幹細胞移植も含めた早期治療介入と思われる．すこしでも XLP が疑われる症例があれば著者まで連絡いただければ幸いである．

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Overexpression of T-bet Gene Regulates Murine Autoimmune Arthritis

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Objective. **To clarify the role of T-bet in the pathogenesis of collagen-induced arthritis (CIA).**

Methods. **T-bet–transgenic (Tg) mice under the control of the CD2 promoter were generated. CIA was induced in T-bet–Tg mice and wild-type C57BL/6 (B6) mice. Levels of type II collagen (CII)–reactive T-bet and retinoic acid receptor–related orphan nuclear receptor γt (RORγt) messenger RNA expression were analyzed by real-time polymerase chain reaction. Criss-cross experiments using CD4+ T cells from B6 and T-bet–Tg mice, as well as CD11c+ splenic dendritic cells (DCs) from B6 and T-bet–Tg mice with CII were performed, and interleukin-17 (IL-17) and interferon-γ (IFNγ) in the supernatants were measured by enzyme-linked immunosorbent assay. CD4+ T cells from B6, T-bet–Tg, or T-bet–Tg/IFNγ$^{-/-}$ mice were cultured for Th17 cell differentiation, then the proportions of cells producing IFNγ and IL-17 were analyzed by fluorescence-activated cell sorting.**

Results. **Unlike the B6 mice, the T-bet–Tg mice did not develop CIA. T-bet–Tg mice showed overexpression of *Tbx21* and down-regulation of *Rorc* in CII-reactive T cells. Criss-cross experiments with CD4+ T cells and splenic DCs showed a significant reduction in IL-17 production by CII-reactive CD4+ T cells in T-bet–Tg mice, even upon coculture with DCs from B6 mice, indicating dysfunction of IL-17–producing CD4+ T cells. Inhibition of Th17 cell differentiation under an in vitro condition favoring Th17 cell differentiation was observed in both T-bet–Tg mice and T-bet–Tg/IFNγ$^{-/-}$ mice.**

Conclusion. **Overexpression of T-bet in T cells suppressed the development of autoimmune arthritis. The regulatory mechanism of arthritis might involve dysfunction of CII-reactive Th17 cell differentiation by overexpression of T-bet via IFNγ-independent pathways.**

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by autoimmunity, infiltration of the joint synovium by activated inflammatory cells, and progressive destruction of cartilage and bone. Although the exact cause of RA is not clear, T cells seem to play a crucial role in the initiation and perpetuation of the chronic inflammation in RA.

The Th1 cell subset has long been considered to play a predominant role in inflammatory arthritis, because T cell clones from RA synovium were found to produce large amounts of interferon-γ (IFNγ) (1). Recently, interleukin-17 (IL-17)–producing Th17 cells have been identified, and this newly discovered T cell population appears to play a critical role in the development of various forms of autoimmune arthritis in experimental animals, such as those with glucose-6-phosphate isomerase–induced arthritis (2) and collagen-induced arthritis (CIA) (3). Conversely, IFNγ has antiinflammatory effects on the development of experimental arthritis (4,5). IL-17 is spontaneously produced by RA synovium (6), and the percentage of IL-17–positive CD4+ T cells

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was increased in the peripheral blood mononuclear cells of patients with RA compared with healthy control subjects (7). It is therefore necessary to determine if autoimmune arthritis is a Th1- or a Th17-associated disorder.

The lineage commitment of each Th cell subset from naive CD4+ T cells is dependent on the expression of specific transcription factors induced under the particular cytokine environment. Differentiation of Th1 cells is dependent on the expression of the transcription factor T-bet, which is induced by IFNγ/STAT-1 signaling pathways and directly activates the production of IFNγ (8,9). Similarly, Th17 cell differentiation in mice is dependent on the transcription factor retinoic acid receptor–related orphan nuclear receptor γt (RORγt) induced by transforming growth factor β (TGFβ) and IL-6 (10). Previous studies showed that these transcription factors negatively regulate the differentiation of other T cell subsets by direct co-interaction and/or indirect effects of cytokines produced from each T cell subset (11,12). How the predominant differentiation of CD4+ T cells affects the development of autoimmune arthritis remains unclear, however.

In the present study, CIA was induced in C57BL/6 (B6) mice and T-bet–transgenic (Tg) mice under the control of the CD2 promoter. The results showed that CIA was significantly suppressed in T-bet–Tg mice as compared with B6 mice. IL-17 production was not detected in type II collagen (CII)–reactive T cells from T-bet–Tg mice, and a significant reduction in IL-17 production by CII-reactive CD4+ T cells from T-bet–Tg mice was observed even when they were cocultured with splenic dendritic cells (DCs) from B6 mice. IFNγ production was also reduced in T-bet–Tg mice as compared with B6 mice, and levels of IFNγ in CII-reactive CD4+ T cells from T-bet–Tg mice were not different from those in B6 mice. Inhibition of Th17 cell differentiation and predominant differentiation of Th1 cells under an in vitro condition favoring Th17 cell differentiation was observed in T-bet–Tg mice, and surprisingly, this inhibition was also observed in T-bet–Tg/IFN$\gamma^{-/-}$ mice. These results indicate suppression of Th17 cell differentiation by overexpression of T-bet, but not IFNγ. Our findings support the notion that the suppression of autoimmune arthritis in T-bet–Tg mice might be due to the direct inhibition of Th17 cell differentiation by T-bet overexpression in T cells.

MATERIALS AND METHODS

Mice. CD2 T-bet–Tg mice (12) were prepared by backcrossing mice on a C57BL/6 background. IFN$\gamma^{-/-}$ mice were obtained from The Jackson Laboratory. Littermates of T-bet–Tg mice were used as controls in all experiments. All mice were maintained under specific pathogen–free conditions, and the experiments were conducted in accordance with the institutional ethics guidelines.

Induction of CIA and assessment of arthritis. Native chicken CII (Sigma-Aldrich) was dissolved in 0.01*M* acetic acid and emulsified in Freund's complete adjuvant (CFA). CFA was prepared by mixing 5 mg of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco) and 1 ml of Freund's incomplete adjuvant (Sigma-Aldrich). Mice ages 8–10 weeks were injected intradermally at the base of the tail with 200 μg of CII in CFA on days 0 and 21. Arthritis was evaluated visually, and changes in each paw were scored on a scale of 0–3, where 0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced swelling, and 3 = ankylosis. The scores in the 4 limbs were then summed (maximum score 12).

Histopathologic scoring. For histologic assessment, mice were killed on day 42 after the first immunization, and both rear limbs were removed. After fixation and decalcification, joint sections were cut and stained with hematoxylin and eosin. Histologic features of arthritis were quantified by 2 independent observers (YK and IM) who were blinded with regard to the study group, and a histologic score was assigned to each joint based on the degree of inflammation and erosion, as described previously (13). The severity of inflammation was scored on a scale of 0–5, where 0 = normal, 1 = minimal inflammatory infiltration, 2 = mild infiltration with no soft tissue edema or synovial lining cell hyperplasia, 3 = moderate infiltration with surrounding soft tissue edema and some synovial lining cell hyperplasia, 4 = marked infiltration, edema, and synovial lining cell hyperplasia, and 5 = severe infiltration with extended soft tissue edema and marked synovial lining cell hyperplasia. The severity of bone erosion was also scored on a scale of 0–5, where 0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, and 5 = severe erosion with full-thickness defects in the cortical bone.

Analysis of cytokine profiles and cytokine and transcriptional factor gene expression. Inguinal and popliteal lymph nodes were harvested from each mouse on day 10 after the first immunization with CII. Single-cell suspensions were prepared, and lymph node cells (2×10^5/well on a 96-well round-bottomed plate) were cultured for 72 hours in RPMI 1640 medium (Sigma-Aldrich) containing 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 50 μM 2-mercaptoethanol in the presence of 100 μg/ml of denatured chicken CII. The supernatants were analyzed for IFNγ, IL-4, IL-10, and IL-17 by enzyme-linked immunosorbent assay (ELISA) using specific Quantikine ELISA kits (R&D Systems).

Lymphocytes harvested on day 10 after immunization were used to obtain complementary DNA (cDNA) by reverse transcription, using a commercially available kit. A TaqMan Assay-on-Demand gene expression product was used for real-time polymerase chain reaction (PCR; Applied Biosystems). The expression levels of *Ifng*, *Il17a*, *Tbx21*, *Rorc*, *Il12a*, and *Il23a* were normalized relative to the expression of *gapdh*. Analyses were performed with an ABI Prism 7500 apparatus (Applied Biosystems).

Criss-cross coculture with CD4+ T cells and CD11c+ splenic dendritic cells. Ten days after the first CII immunization, CD4+ cells in draining lymph nodes were isolated by

positive selection, using a magnetic-activated cell sorter (MACS) system with anti-CD4 monoclonal antibody (mAb; Miltenyi Biotec). After treatment with mitomycin C, CD11c+ cells were isolated from the spleen by positive selection, using a MACS system with anti-CD11c mAb (Miltenyi Biotec). Criss-cross coculture for 72 hours was performed with 1×10^5 CD4+ cells and 2×10^4 CD11c+ cells in 100 μg/ml of denatured CII-containing medium. Cytokine production and transcription factor expression were then analyzed.

Measurement of collagen-specific immunoglobulin titers. Serum was collected from the mice on day 56 after the first immunization. A total of 10 μg/ml of CII in phosphate buffered saline (PBS) was coated overnight at 4°C onto 96-well plates (Nunc MaxiSorp; Nalge Nunc). After washes with washing buffer (0.05% Tween 20 in PBS), the blocking solution, including 1% bovine serum albumin in PBS, was applied for 1 hour. After washing, 100 μl of diluted serum was added, and the plates were incubated for 1 hour at room temperature. After further washing, horseradish peroxidase–conjugated anti-mouse IgG, IgG1, IgG2a, or IgG2b (1:5,000 dilution) in blocking solution was added, and the plates were incubated for 1 hour at room temperature. After washing, tetramethylbenzidine was added, and the optical density was read at 450 nm using a microplate reader.

Purification of CD4+ cells and in vitro T cell cultures. CD4+ cells (1×10^6/well) were cultured in medium with 1 μg/ml of soluble anti-CD3ε mAb (eBioscience), 1 μg/ml of soluble anti-CD28 mAb (BioLegend), 10 μg/ml of anti-IFNγ mAb (BioLegend), and 10 μg/ml of anti–IL-4 mAb (BioLegend) for a neutral condition. For Th17 cell differentiation, CD4+ cells (1×10^6/well) were cultured in medium with 1 μg/ml of soluble anti-CD3ε mAb, 1 μg/ml of soluble anti-CD28 mAb, 3 ng/ml of human TGFβ (R&D Systems), 20 ng/ml of mouse IL-6 (eBioscience), 10 μg/ml of anti-IFNγ mAb, and 10 μg/ml of anti–IL-4 mAb. On day 4, cells were restimulated for 4 hours with 50 ng/ml of phorbol myristate acetate and 500 ng/ml of ionomycin and used in the experiments.

Surface and intracellular staining and fluorescence-activated cell sorter (FACS) analysis. GolgiStop (BD PharMingen) was added during the last 6 hours of each culture. Cells were stained extracellularly, fixed, and permeabilized with Cytofix/Cytoperm solution (BD PharMingen). Then, intracellular cytokine staining was performed according to the manufacturer's protocol, using fluorescein isothiocyanate (FITC)–conjugated anti-IFNγ (BD PharMingen) and phycoerythrin (PE)–conjugated anti–IL-17 (BD PharMingen) or FITC-conjugated anti–IL-17 (BioLegend). A Treg cell staining kit (eBioscience) was used to stain T-bet, RORγt, and FoxP3 in cultured cells according to the manufacturer's protocol, using PE-conjugated anti–T-bet (eBioscience), allophycocyanin-conjugated anti-RORγt (eBioscience), and PE-conjugated anti-FoxP3 (eBioscience). Samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson), and data were analyzed with FlowJo software (Tree Star).

Statistical analysis. Data are expressed as the mean ± SEM or the mean ± SD. Differences between groups were examined for statistical significance using Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Construction of the T-bet transgene and tissue distribution of transcription factors and cytokine production in naive mice. To generate transgenic mouse lines that express high levels of T-bet specifically in T cells, mouse T-bet cDNA was inserted into a VA vector containing a human CD2 transgene cassette (14). To confirm the expression of the transgene, reverse transcription–PCR (RT-PCR) was performed to monitor the expression of *Tbx21* (coding for T-bet) in organs from the T-bet–Tg mice. *Tbx21* messenger RNA (mRNA) expression was detected in the lymphatic system and in nonlymphatic organs in T-bet–Tg mice, and the expression levels were higher than those in B6 mice (data available upon request from the author). Analysis by semiquantitative RT-PCR and quantitative PCR (data not shown) revealed that the expression levels of other transcription factors (*Gata3*, *Rorc*, and *Foxp3*) in T-bet–Tg mice were not different from those in B6 mice. As previously reported by Ishizaki et al (14), high production of IFNγ was observed even when CD4+ T cells isolated from the spleen of T-bet–Tg mice were cultured under neutral conditions (data available upon request from the author).

Failure to induce CIA and low CII-specific IgG production in T-bet–Tg mice. To assess whether T cell–specific T-bet expression affects the development of arthritis, we induced CIA in T-bet–Tg mice and in wild-type B6 mice. The incidence and severity of arthritis in T-bet–Tg mice were markedly suppressed compared with those in B6 mice (Figure 1A). Surprisingly, the majority of T-bet–Tg mice were essentially free of arthritis, and even when arthritis was present, it was of the mild type. Consistent with these findings, histologic analyses of the joints obtained from each mouse 42 days after immunization revealed that joint inflammation and destruction were significantly suppressed in T-bet–Tg mice compared with B6 mice (Figures 1B and C). These results indicated that enforced expression of T-bet in T cells suppressed the development of CIA.

Because the levels of CII-specific IgG correlate well with the development of arthritis (15), we examined CII-specific IgG production in T-bet–Tg mice. CII-specific IgG, IgG1, IgG2a, and IgG2b levels were significantly lower in T-bet–Tg mice than in B6 mice, as determined by ELISA (Figure 1D). Thus, enforced expression of T-bet in T cells suppresses the development of CIA and CII-specific IgG production.

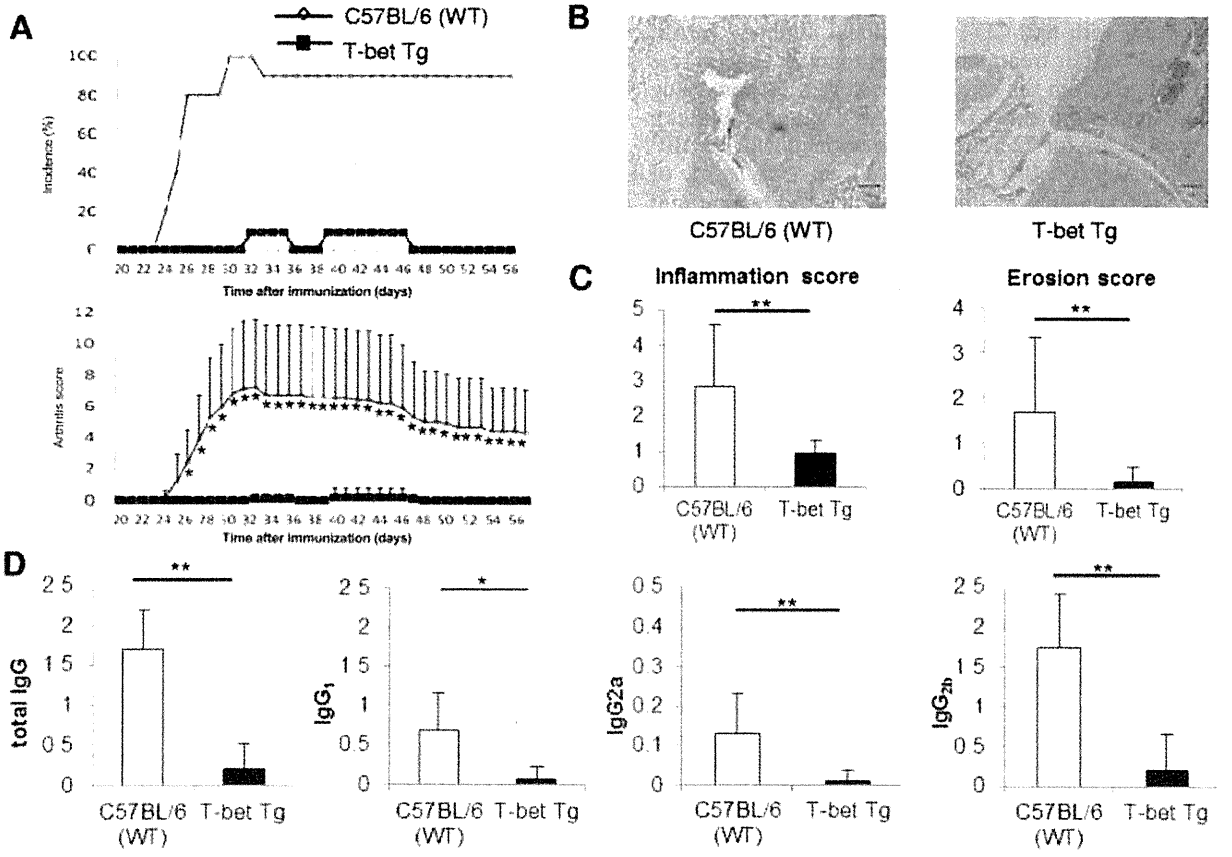

Figure 1. Significant suppression of collagen-induced arthritis (CIA) and type II collagen (CII)–specific IgG production in T-bet–transgenic (Tg) mice. On days 0 and 21, mice were immunized intradermally at several sites at the base of the tail with chicken CII emulsified with Freund's complete adjuvant. **A,** Incidence and severity of CIA. The arthritis score was determined as described in Materials and Methods. Data were obtained from 2 independent experiments involving 10 C57BL/6 (wild-type [WT]) mice and 11 T-bet–Tg mice. **B,** Hematoxylin and eosin–stained sections of the hind paws of mice obtained 6 weeks after the first immunization. Original magnification × 40. **C,** Inflammation and bone erosion scores in 7 C57BL/6 mice and 5 T-bet–Tg mice 6 weeks after the first immunization. Scores were determined as described in Materials and Methods. **D,** Serum levels of CII-specific IgG, IgG1, IgG2a, and IgG2b levels in 10 C57BL/6 mice and 11 T-bet–Tg mice 8 weeks after the first immunization, as measured by enzyme-linked immunosorbent assay. Values in **A, C,** and **D** are the mean ± SD. * = $P < 0.05$; ** = $P < 0.01$ by Student's *t*-test.

Suppression of CII-reactive IL-17 production and IL-17 mRNA expression in T-bet–Tg mice. Because enforced T-bet expression in T cells suppressed the development CIA, we examined antigen-specific cytokine production and transcription factor expression in mice with CIA. CD4+ T cells harvested from draining lymph nodes were stimulated with CII in vitro, and then various cytokine levels in the supernatants were measured by ELISA. IL-17 production by CII-reactive T cells was significantly reduced in T-bet–Tg mice as compared with B6 mice (Figure 2A). IFNγ production by CII-reactive T cells also tended to be decreased in T-bet–Tg mice.

We analyzed CII-reactive cytokine and transcription factor mRNA expression levels by real-time PCR (Figure 2B). Similar to the ELISA results, *Il17a* expression tended to be lower in T-bet–Tg mice than in B6 mice. No difference in *Ifng* expression was observed between B6 and T-bet–Tg mice (Figure 2B). *Tbx21* expression tended to be higher in T-bet–Tg mice, whereas *Rorc* expression was lower in T-bet–Tg mice than in B6 mice ($P < 0.05$). The level of expression of *Il12a* (coding for IL-12p35) was also higher in T-bet–Tg mice than in B6 mice ($P < 0.05$). However, there was no difference in the expression levels of *Il23a* (coding for IL-23p19) between B6 mice and T-bet–Tg mice. These

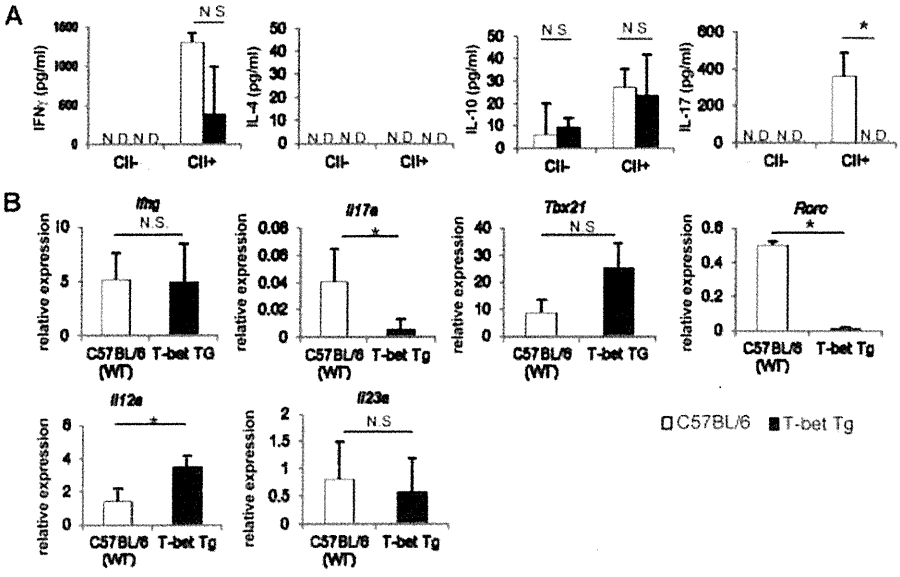

Figure 2. No production of interleukin-17 (IL-17) and low production of interferon-γ (IFNγ) in type II collagen (CII)–reactive CD4+ T cells. **A,** Ten days after the first CII immunization, lymphocytes derived from the draining lymph nodes of C57BL/6 (wild-type [WT]) mice and T-bet–transgenic (Tg) mice were cultured for 72 hours in the presence or absence of 100 μg/ml of denatured CII. Levels of IL-17, IFNγ, IL-4, and IL-10 in the supernatants were measured by enzyme-linked immunosorbent assay. **B,** After culture of lymphocytes with CII, cDNA was obtained, and levels of *Ifng*, *Il17a*, *Tbx21*, *Rorc*, *Il12a*, and *Il23a* expression were analyzed by real-time polymerase chain reaction. Values are the mean ± SD of 3 mice. * = $P < 0.05$ by Student's *t*-test. ND = not detected; NS = not significant.

results suggest that overexpression of T-bet on CD4+ T cells suppressed the expression of RORγt and IL-17.

No reduction of RORγt expression on CII-reactive CD4+ T cells in T-bet–Tg mice. CD4+ T cells from T-bet–Tg and B6 mice were cultured in vitro with CII, and analyses of T-bet and RORγt expression on CD4+ T cells were carried out by the intracellular staining method. T-bet expression on CII-reactive CD4+ T cells was significantly higher in T-bet–Tg mice than in B6 mice (Figure 3A). Surprisingly, the majority of T-bet+ CII-reactive T cells expressed RORγt in both the B6 mice and the T-bet–Tg mice (Figure 3A). Although there was no significant difference in the mean fluorescence intensity of RORγt between B6 mice and T-bet–Tg mice, the number of RORγt+ cells tended to be lower in T-bet–Tg mice (data available upon request from the author).

Moreover, in the case of CD4+ T cells examined under conditions favoring Th17 differentiation, RORγt expression on CD4+ T cells from T-bet–Tg mice was lower than that on cells from B6 mice (Figure 3B). Interestingly, most of the RORγt+ cells also expressed T-bet in the T-bet–Tg mice, and the proportion of IL-17–producing RORγt+ CD4+ T cells was lower in the T-bet–Tg mice than in the B6 mice. These findings support the notion that overexpression of T-bet not only suppresses RORγt expression on CD4+ T cells but also inhibits the production of IL-17 from RORγt+ T cells.

To investigate whether the suppression of arthritis and low antigen-specific cytokine production observed in T-bet–Tg mice was related to Treg cells, the next experiment analyzed FoxP3 expression on CD4+ T cells harvested from draining lymph nodes 10 days after immunization. There was no significant difference in the percentage of FoxP3+ cells among the CD4+ T cells between B6 mice and T-bet–Tg mice (data available upon request from the author). Thus, Treg cells do not seem to be involved in the suppression of CIA in T-bet–Tg mice.

Decreased numbers of T cells in the lymph nodes, spleen, and thymus of T-bet–Tg mice. To evaluate the low cytokine response and the low population of CII-reactive RORγt+CD4+ T cells in T-bet–Tg mice with CIA, we analyzed the lymphocyte subsets in the draining lymph nodes and spleen after immunization. The percentage and absolute number of CD3+ T cells were lower in both the draining lymph nodes and the

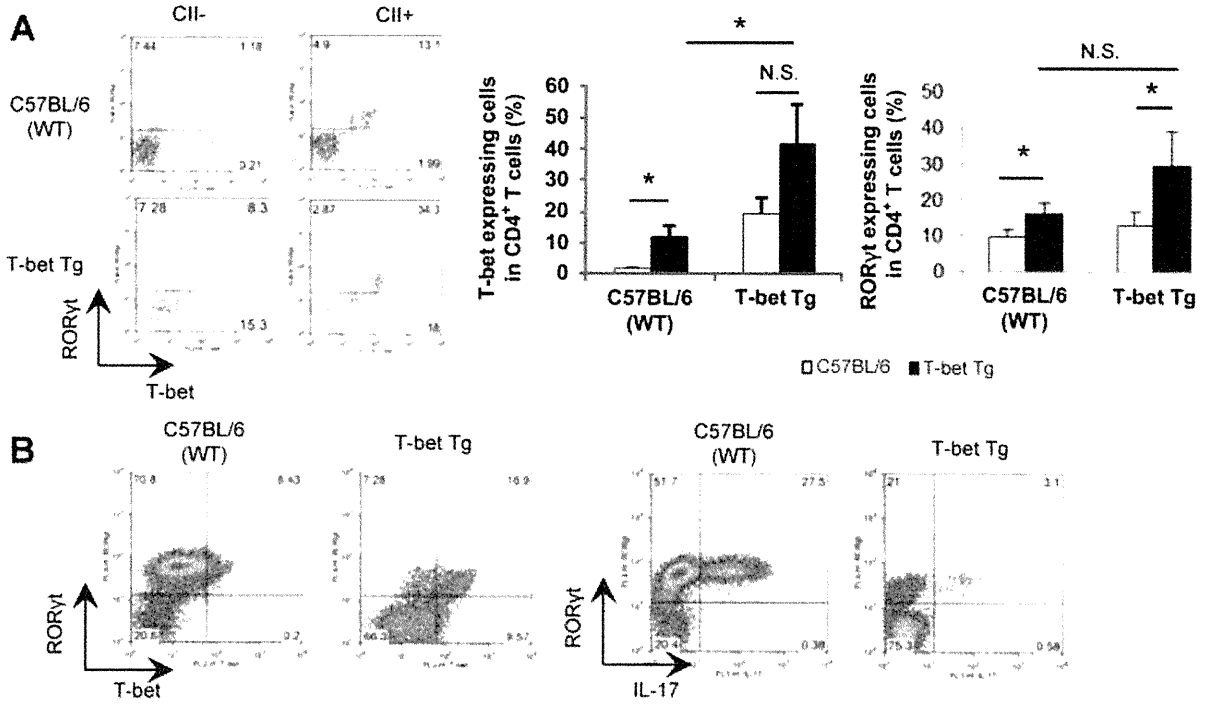

Figure 3. Suppression of Th17 cell differentiation by enforced expression of T-bet in T cells despite expression of retinoic acid receptor–related orphan nuclear receptor γt (RORγt). **A,** Ten days after the first type II collagen (CII) immunization, lymphocytes derived from the draining lymph nodes of C57BL/6 (wild-type [WT]) and T-bet–transgenic (Tg) mice were cultured for 72 hours in the presence or absence of 100 μg/ml of denatured CII. Levels of T-bet and RORγt expression on CD4+ T cells were analyzed by intracellular staining. Numbers in each compartment of the histograms are the percentage of transcription factor–expressing cells gated on CD4+ T cells. Values in the bar graphs are the mean ± SD of 3 mice per group. * = $P < 0.05$ by Student's *t*-test. NS = not significant. **B,** CD4+ T cells were isolated from the spleen of C57BL/6 and T-bet–Tg mice by magnetic-activated cell sorting and were then cultured for 96 hours with soluble anti-CD3 antibody, soluble anti-CD28 antibody, interleukin-6 (IL-6), and transforming growth factor β. Cytokine production and transcription factor expression on CD4+ T cells were analyzed by intracellular staining. Representative histograms from flow cytometric analysis of T-bet and RORγt expression with IL-17 production are shown. Numbers in each compartment are the percentage of positive cells gated on CD4+ T cells.

spleen of T-bet–Tg mice as compared with B6 mice (Figures 4A and B). The absolute number of CD4+ and CD8+ T cells also tended to be lower in T-bet–Tg mice (Figure 4B). Moreover, analysis of the thymus showed a significantly low number of total thymocytes in T-bet–Tg mice and the presence of an abnormal proportion of T precursor cells, such as a low number of double-positive T cells and CD4 single-positive T cells in T-bet–Tg mice (Figure 4C). These results suggest abnormal T cell development in the thymus of T-bet–Tg mice.

Inhibition of IL-17 production by CII-reactive CD4+ T cells in T-bet–Tg mice. To clarify whether T-bet overexpression on CD4+ T cells directly affects cytokine production, we performed criss-cross experiments using CD4+ T cells from B6 and T-bet–Tg mice, as well as DCs from B6 and T-bet–Tg mice in CII–containing medium, and measured IL-17 and IFNγ levels in the supernatants by ELISA. IL-17 production was detected in CII-reactive CD4+ T cells from B6 mice and in DCs from T-bet–Tg mice. Interestingly, IL-17 production was significantly reduced, even when CD4+ T cells from T-bet–Tg mice were cocultured with DCs from B6 mice (Figure 5A). These observations suggest that T-bet overexpression on CD4+ T cells is responsible for the inhibition of CII-reactive IL-17 production. No difference in IFNγ production was noted among the experimental conditions (Figure 5A), suggesting that reduced IFNγ production by CII-reactive CD4+ T cells from T-bet–Tg mice (Figure 2) was probably related to the reduced numbers of CD4+ T cells in draining lymph nodes. Moreover, intracellular staining revealed that RORγt expression was suppressed and T-bet expression was increased, even when CD4+ T cells from T-bet–Tg

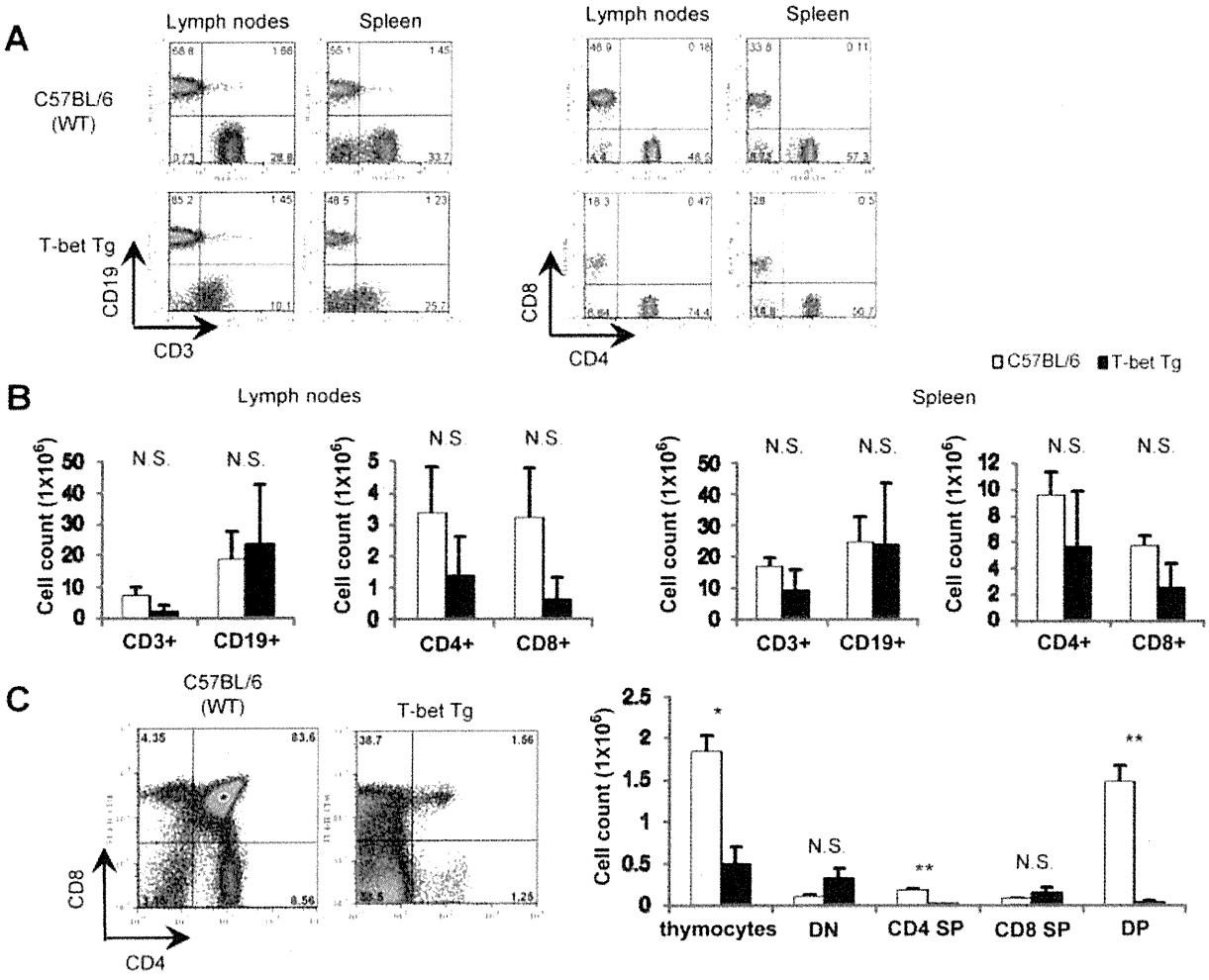

Figure 4. Decreased number of CD3+ T cells in spleen and lymph nodes and abnormal development of T precursor cells in the thymus in T-bet–transgenic (Tg) mice. **A,** Ten days after first immunization, the proportion of lymphocytes in draining lymph nodes and spleen were analyzed by fluorescence-activated cell sorting (FACS), and the absolute numbers of cells were calculated. Numbers in each compartment are the percentage of the parent population. **B,** The absolute numbers of CD3+, CD19+, CD4+, and CD8+ T cells in the lymph nodes and spleen of C57BL/6 (wild-type [WT]) and T-bet–Tg mice were determined. Values are the mean ± SD of 3 mice per group. NS = not significant. **C,** The proportion of T precursor cells in the thymus of nonimmunized mice was analyzed by FACS, and the absolute numbers of thymocytes, double-negative (DN) T cells, CD4 and CD8 single-positive (SP) T cells, and double-positive (DP) T cells were determined. Values in the bar graphs are the mean ± SD of 3 mice per group. * = $P < 0.05$; ** = $P < 0.01$ by Student's *t*-test.

mice were cocultured with DCs from B6 mice (Figure 5B). These results indicate that T-bet overexpression on CD4+ T cells suppressed CII-reactive IL-17 production by inhibition of the expression of RORγt.

Overexpression of T-bet directly suppresses Th17 cell differentiation via IFNγ-independent mechanisms. To clarify whether IFNγ production influences Th17 cell differentiation, we generated T-bet–Tg/IFNγ$^{-/-}$ mice. CD4+ T cells were isolated from the spleen of T-bet–Tg, T-bet–Tg/IFNγ$^{-/-}$, and B6 mice and were then cultured for Th17 cell differentiation. FACS analysis demonstrated that the proportion of IL-17–producing CD4+ T cells was lower in T-bet–Tg mice than in B6 mice, whereas the proportion of IFNγ-producing CD4+ T cells was higher in T-bet–Tg mice. Similarly, the proportion of IL-17–producing CD4+ T cells was also lower in T-bet–Tg/IFNγ$^{-/-}$ mice, although no IFNγ-producing CD4+ T cells were detected in

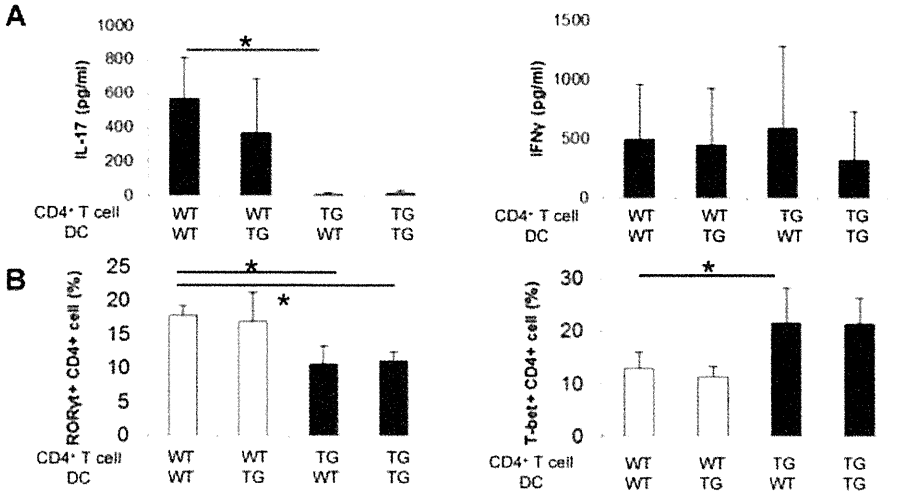

Figure 5. Impaired antigen-specific Th17 cell responses in T-bet–transgenic (Tg) mice with collagen-induced arthritis (CIA). Ten days after the first type II collagen (CII) immunization, CD4+ cells were isolated from draining lymph nodes of C57BL/6 (wild-type [WT]) mice and T-bet–Tg (TG) mice by positive selection using magnetic-activated cell sorting (MACS) with anti-CD4 monoclonal antibody (mAb). After treatment with mitomycin C, CD11c+ cells were isolated from the spleen by positive selection using a MACS system with anti-CD11c mAb. Criss-cross coculture for 72 hours was performed with 1×10^5 CD4+ cells and 2×10^4 CD11c+ cells in 100 μg/ml of denatured CII–containing medium. **A,** Levels of interleukin-17 (IL-17) and interferon-γ (IFNγ) in culture supernatants were measured by enzyme-linked immunosorbent assay. **B,** Expression of retinoic acid receptor–related orphan nuclear receptor γt (RORγt) and T-bet expression on CD4+ T cells were analyzed by intracellular staining. Representative data from flow cytometric analysis of the percentage of RORγt+ or T-bet+ cells in the CD4+ T cell subset are shown. Values are the mean ± SD of 3 mice per group. * = $P < 0.05$ by Student's *t*-test. DC = dendritic cells.

T-bet–Tg/IFNγ$^{-/-}$ mice (Figure 6). These results strongly support the view that inhibition of Th17 cell differentiation in T-bet–Tg mice cannot be due to overproduction of IFNγ, indicating that overexpression of T-bet directly suppresses Th17 cell differentiation in T-bet–Tg mice.

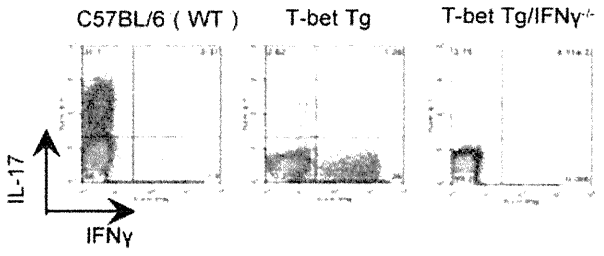

Figure 6. Suppressed expression of interleukin-17 (IL-17) by T-bet overexpression independently of interferon-γ (IFNγ) in T-bet–transgenic (Tg) mice. CD4+ T cells were isolated from the spleen of C57BL/6 (wild-type [WT]), T-bet–Tg, and T-bet–Tg/IFNγ$^{-/-}$ mice by magnetic-activated cell sorting and then cultured for 96 hours with soluble anti-CD3 monoclonal antibody (mAb), soluble anti-CD28 mAb, IL-6, and transforming growth factor β. IFNγ and IL-17 production by CD4+ cells was analyzed by intracellular cytokine staining. Numbers in each compartment are the percentage of cells secreting cytokines.

DISCUSSION

Recent studies showed that IL-17 plays a crucial role in the development of CIA (3) and other types of experimental arthritis (2). In contrast, it has been reported that IFNγ can suppress IL-17 production in vitro (16) and has antiinflammatory effects on the development of experimental arthritis (4,5). T-bet is a transcription factor known to induce the differentiation of naive CD4+ T cells to Th1 cells (8). Although the absence of T-bet can result in severe IL-17–mediated experimental autoimmune myocarditis via dysregulation of IFNγ (17), several studies have shown that T-bet is essential for the development of several models of autoimmunity, such as experimental autoimmune encephalitis (18,19), colitis (20), and diabetes mellitus (21). Nevertheless, the effect of T-bet expression on Th17 cell differentiation and function during arthritis remains unclear.

T-bet–Tg mice overexpress T-bet and mainly produce IFNγ in their T cells (14). Previous studies in T-bet–Tg mice suggested that overexpression of T-bet and a predominant Th1 response affect the pathogenesis of various diseases (14,22,23). To examine whether T-bet overexpression on T cells affects the regulation of

autoimmune arthritis, we induced CIA in T-bet–Tg mice and found marked suppression of CIA in T-bet–Tg mice.

To determine the reason for the low incidence of CIA in T-bet–Tg mice, we measured CII-reactive cytokine production and expression in vitro. IL-17 production from CII-reactive CD4+ T cells and *Il17a* expression were reduced in T-bet–Tg mice as compared with B6 mice. Although a predominant Th1 cell response was reported by Ishizuka et al (14), CII-specific IFNγ production was reduced in T-bet–Tg mice, and no significant difference was observed in *Ifng* expression between B6 mice and T-bet–Tg mice. Furthermore, *Il12a* expression was significantly higher in T-bet–Tg mice than in B6 mice, suggesting that overexpression of T-bet on T cells seems to affect innate immune cells, because the main producers of IL-12 are DCs and macrophages, not CD4+ T cells.

In criss-cross coculture experiments with CD4+ T cells and splenic DCs from B6 mice and T-bet–Tg mice, CII-reactive IL-17 production was also reduced even when CD4+ T cells from T-bet–Tg mice were cocultured with DCs from B6 mice, although there was no significant difference in IL-17 production by CD4+ T cells from B6 mice cocultured with DCs from either B6 mice or T-bet–Tg mice. In contrast, no difference in IFNγ production was observed under all coculture conditions examined. Moreover, suppression of RORγt expression and high expression of T-bet on CD4+ T cells were observed even when CD4+ T cells from T-bet–Tg mice were cocultured with DCs from B6 mice. These findings indicate that T-bet overexpression on CD4+ T cells might suppress CII-reactive IL-17 production resulting from suppression of RORγt expression in an IFNγ-independent manner, and that overexpression of T-bet has no direct effect on DC function.

CII-specific IgG levels correlate well with the development of arthritis (15). We observed significant suppression of CII-specific IgG production in the T-bet–Tg mice as compared with the B6 mice. A previous study showed that IL-17 is required for anti-CII antibody production (3). Therefore, the suppression of anti-CII antibody formation might be due to lower CII-reactive IL-17 production in T-bet–Tg mice.

To evaluate the low cytokine response to CII in T-bet–Tg mice, we analyzed lymphocytes obtained after immunization from draining lymph nodes and spleen. The percentage and absolute number of T cells tended to be lower in both the draining lymph nodes and spleen of T-bet–Tg mice compared with B6 mice. Moreover, significantly lower numbers of total thymocytes and an abnormal proportion of T precursor cells were observed in T-bet–Tg mice. The latter phenomenon could be due to T-bet transgene expression on double-negative thymic cells in T-bet–Tg mice. Because previous observations showed that T-bet interferes with GATA-3 function (11) and that GATA-3 was required for the development of early thymic T cells (24), one of the reasons for abnormal T cell development in the thymus might be the dysfunction of GATA-3 by overexpression of T-bet. These results suggest that overexpression of T-bet in thymic T cells affects T cell development, is responsible for the low number of T cells in spleen and lymph nodes, and is related to the low cytokine production against CII in T-bet–Tg mice.

To assess the effect of T-bet on CD4+ T cell differentiation in T-bet–Tg mice, we performed in vitro induction of Th17 cells. Analysis of T-bet–Tg mice showed a reduction in IL-17–producing CD4+ T cells and an increase in IFNγ-producing CD4+ T cells in spite of the condition favoring Th17 differentiation, which indicates suppression of Th17 cell differentiation and predominance of Th1 cell differentiation in vitro in T-bet–Tg mice. These results did not contradict the previous findings that the phenotype of polarized Th1 cells was not affected by Th cell–polarizing conditions (25). It is possible that suppression of CII-reactive IL-17 production in T-bet–Tg mice was not associated with IFNγ. For this reason, we generated T-bet–Tg/IFNγ$^{-/-}$ mice and performed in vitro induction of Th17 cells in these mice. Surprisingly, in T-bet–Tg/IFNγ$^{-/-}$ mice, the levels of IL-17–producing CD4+ T cells were also markedly reduced under Th17 cell differentiation–favoring conditions, indicating an IFNγ-independent suppressive pathway against Th17 cell differentiation. Although previous studies showed that suppression of Th17 cell differentiation was mediated through IFNγ signal transduction (16), our findings allow us to propose a new hypothesis: Th17 cell differentiation is regulated by a pathway that is distinct from the IFNγ signaling pathway. Therefore, we suggest that T-bet expression either directly or indirectly suppresses Th17 cell differentiation via an IFNγ-independent mechanism.

Tbx21 expression was significantly higher in T-bet–Tg mice as compared with B6 mice, and FACS analysis of CII-reactive CD4+ T cells revealed a significantly higher percentage of T-bet+ cells among the CD4+ T cell subset in T-bet–Tg mice. While there was no significant difference in the percentage of RORγt+ cells among the CD4+ T cell subset in T-bet–Tg mice as compared with B6 mice, *Rorc* expression was down-regulated on CII-reactive CD4+ T cells in T-bet–Tg mice. In the case of CD4+ T cells under

conditions favoring Th17 cell differentiation, RORγt expression on CD4+ T cells from T-bet–Tg mice was lower than that on cells from B6 mice. Interestingly, most of the RORγt+ cells also expressed T-bet in T-bet–Tg mice, and the proportion of IL-17–producing RORγt+ T cells in the CD4+ cell subset was lower in T-bet–Tg mice than in B6 mice. These findings support the notion that overexpression of T-bet not only suppresses RORγt expression on CD4+ T cells, but also inhibits the production of IL-17 from RORγt+ T cells.

Previous studies showed that RORγt expression is positively regulated by several transcription factors, such as runt-related transcription factor 1 (RUNX-1), interferon regulatory factor 4, and STAT-3 (26–28). Lazarevic et al (29) recently reported that T-bet prevented RUNX-1–mediated activation of the gene encoding RORγt, followed by the suppression of Th17 cell differentiation. In addition to direct promotion of RORγt expression, RUNX-1 also acts as a coactivator, together with RORγt, and induces the expression of *Il17a* and *Il17f* (26); therefore, T-bet inhibits IL-17 production by RORγt+ cells induced by RUNX-1 (29). Although further studies will be required to identify the effect of T-bet overexpression on the function of RUNX-1, it might be associated with the suppression of Th17 cell differentiation that was observed in the T-bet–Tg mice.

In conclusion, our results demonstrated that overexpression of T-bet in T cells suppressed the development of autoimmune arthritis. The regulatory mechanism of CIA might involve dysfunction of CII-reactive Th17 cell differentiation by overexpression of T-bet via IFNγ-independent pathways. These findings should enhance our understanding of the pathogenesis of autoimmune arthritis and help in the development of new therapies for RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sumida had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Sugihara, Hayashi, Yoh, Takahashi, Matsumoto, Sumida.

Acquisition of data. Kondo, Yao, Tahara.

Analysis and interpretation of data. Kondo, Iizuka, Wakamatsu, Tsuboi, Matsumoto.

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In conclusion, the associations among asthma, biofilm-forming bacteria, and revision ESS are strong and robust after adjusting for other factors in patients with CRS from a tertiary medical center. Despite its limitations, this study may improve our understanding of refractory CRS pathogenesis, possibly leading to more effective treatment strategies, such as incorporating the treatments of asthma and biofilm infection into conventional CRS therapies. Prospective cohort studies in diverse populations are needed to assess the causality of these associations.

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Quantification of κ-deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects

To the Editor:

X-linked agammaglobulinemia (XLA) is a primary immunodeficiency caused by severely decreased numbers of mature peripheral B lymphocytes as a result of a mutation in the *BTK* gene. Non-XLA is characterized by hypogammaglobulinemia with decreased B-cell counts (less than 2% of mature B cells) in the absence of the *BTK* gene mutation. Both XLA and non-XLA are caused by an early B-cell maturation defect.[1] In patients with XLA and non-XLA, recurrent infections appear between 3 and 18 months of age, whereas the mean age at diagnosis is 3 years.[2] This delayed diagnosis results in frequent hospitalization because of pneumonia, sepsis, meningitis, and other bacterial infections, which frequently require intravenous administration of antibiotics and can be fatal. Frequent pneumonia results in a high incidence of chronic lung diseases.[3] Thus, early diagnosis and early treatment, including periodical intravenous immunoglobulin replacement therapy, is essential to improve the prognosis and the quality of life of patients with XLA and non-XLA.

In the process of B-cell maturation, immunoglobulin κ-deleting recombination excision circles (KRECs) are produced during κ-deleting recombination allelic exclusion and isotypic exclusion of the λ chain.[4] Coding joint (cj) KRECs reside within the chromosome, whereas signal joint (sj) KRECs are excised from genomic DNA. cjKREC levels remain the same after B-cell division, whereas sjKREC levels decrease, because sjKRECs are not replicated during cell division.[5] Because the B-cell maturation defects in XLA and non-XLA occur before κ-deleting recombination, KRECs are not supposed to be produced. Therefore, measurements of KRECs have the potential to be applied to the identification of these types of B-cell deficiencies in patients, which consist of around 20% of all B-cell defects.[6] In addition, some types of combined immunodeficiencies show an arrest in B-cell maturation and can also be identified by this method. The success of newborn screening for T-cell deficiencies by measuring T-cell–receptor excision circles[7] prompted us to develop a newborn screening method for XLA and non-XLA by measuring KRECs derived from neonatal Guthrie cards.

The study protocol was approved by the National Defense Medical College institutional review board, and written informed consent was obtained from the parents of normal controls, the affected children, and adult patients, in accordance with the Declaration of Helsinki.

First, we determined the sensitivity of detection levels of cjKRECs and sjKRECs in Guthrie cards using real-time quantitative PCR.[5] Normal B cells from a healthy adult were isolated from peripheral blood (PB; mean purity, 88.5%). PB was also obtained from 1 patient with XLA (P20) whose B-cell number was 0.09 in 1 μL whole blood and who was negative for sjKRECs ($<1.0 \times 10^2$ copies/μg DNA). Various numbers of normal B cells were serially added to 1 mL whole PB obtained from this patient with XLA. The B-cell–added XLA whole blood was then applied to filter papers, and 3 punches (3 mm in diameter) of dried blood spots were used for DNA extraction. At least 3 DNA samples containing the same B-cell concentrations (0.09-400 B cells/μL) were used for the real-time quantitative PCR of cjKRECs and sjKRECs. The percentages of the positive samples ($>1.0 \times 10^2$ copies/μg DNA) of cjKRECs and sjKRECs increased constantly

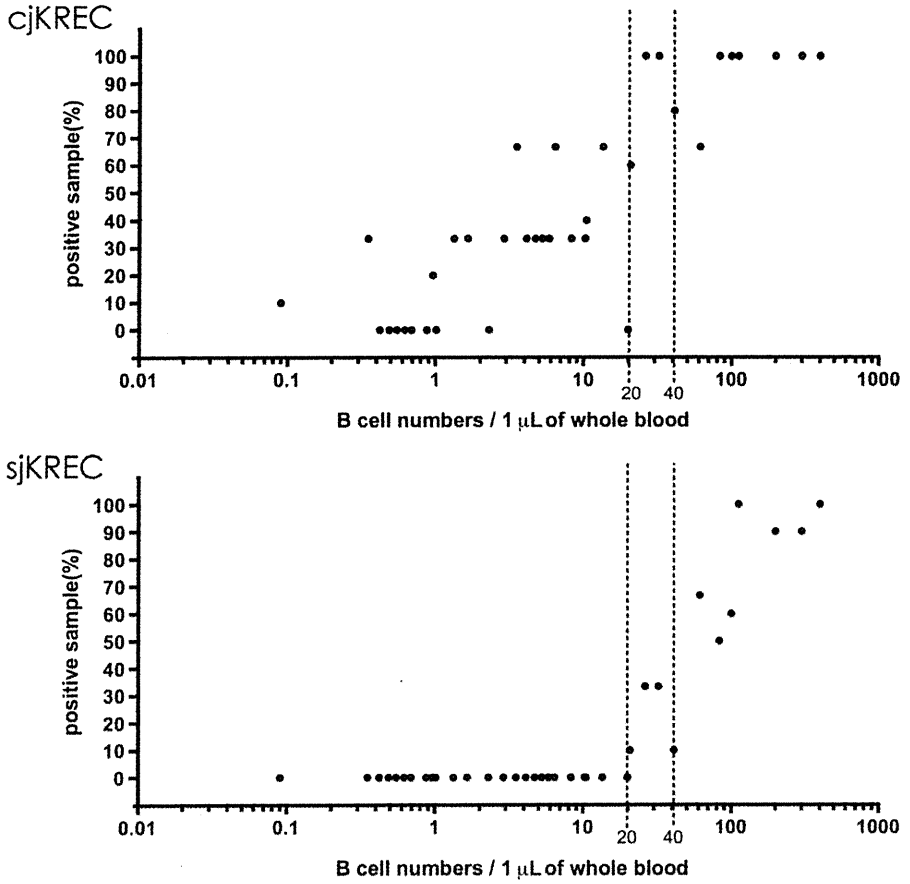

FIG 1. Sensitivity levels of cjKRECs and sjKRECs. Various numbers of purified normal B cells were serially added to whole PB from a patient with XLA (P20) to obtain B-cell–added XLA whole blood. cjKRECs and sjKRECs were measured in 3 to 10 samples of each concentration in triplicate. In all analyses, RNaseP (internal control) was positive ($2.3 \pm 0.2 \times 10^5$ copies/μg DNA). *X-axis,* B-cell numbers in 1 μL whole blood from a patient with XLA. *Y-axis,* Percentages of the KREC-positive results in the tests.

as the B-cell concentrations increased (Fig 1). None of the samples were positive for sjKRECs when the B-cell numbers were less than 20/μL, but cjKRECs were often positive. It has been reported that 90% of patients with XLA have less than 0.2% B cells in the PB at diagnosis.[1] Because peripheral lymphocyte numbers in neonates range from 1200 to 9800/μL,[8] the absolute B-cell numbers of 90% of patients with XLA are estimated to be 2.4 to 19.6/μL at the time of blood collection for Guthrie cards, although exact B-cell numbers of XLA in neonatal periods are not known at this moment. Because neonates are known to have fewer B cells than infants,[9] and we observed that B-cell numbers are constantly low in patients with XLA throughout infancy (Nakagawa, unpublished data, June 2010), which is consistent with the fact that BTK plays an essential role in B-cell maturation. It is likely that neonates with XLA also have severely decreased B cells. On the other hand, all samples obtained from 400 B cells/μL were positive for both cjKRECs and sjKRECs. We also observed that all healthy infants (1-11 months old; n = 15) were sjKREC-positive (Nakagawa, unpublished data, June 2010) and might have at least 600 B cells/μL whole blood.[9] From these data, it is assumed that at least 90% of patients with XLA are sjKREC-negative, and healthy neonates are positive for sjKRECs on neonatal Guthrie cards.

Next, we measured cjKRECs and sjKRECs in dried blood spots in filter papers or Guthrie cards from 30 patients with XLA and 5 patients with non-XLA and from 133 neonates born at the National Defense Medical College Hospital during this study period (August 2008 to October 2009) and 138 healthy subjects of various ages (1 month to 35 years old) to investigate the validity of this method. The levels of B cells of the patients ranged from 0.0% to 1.1% of total lymphocytes and 0.0 to 35.78/μL. IgG levels were 10 to 462 mg/dL (see this article's Tables E1 and E2 in the Online Repository at www.jacionline.org). Patients with leaky phenotypes[1,10] were included; 1 patient (P30) had more than 1% B cells and 34.22/μL total B cells, and 4 patients had more than 300 mg/dL serum IgG (P12, P30, P31, P33). All of the normal neonatal Guthrie cards were positive for both cjKRECs and sjKRECs ($7.2 \pm 0.7 \times 10^3$ and $4.8 \pm 0.6 \times 10^3$ copies/μg DNA, respectively). All healthy subjects of various ages were also positive for both cjKRECs and sjKRECs (Nakagawa, unpublished data, June 2010). In contrast, specimens from all 35 B-cell–deficient patients were sjKREC-negative ($<1.0 \times 10^2$ copies/μg DNA; Fig 2). All 5 patients with leaky phenotypes were also sjKREC-negative, which might be explained by the hypothesis that leaky B cells of patients with XLA are long-lived B cells that divided several times and have fewer sjKRECs than naive B cells.

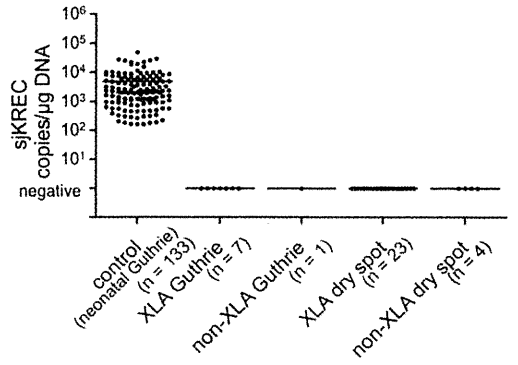

FIG 2. Copy numbers of sjKRECs measured in neonatal Guthrie cards or dried blood spots obtained from B-cell–deficient patients. On all samples from control, neonatal Guthrie cards (n = 133) were sjKREC-positive (4.8 ± 0.6 × 10^3 copies/μg DNA). B-cell–deficient patients were negative for sjKRECs in neonatal Guthrie cards (XLA, n = 7; non-XLA, n = 1) and dried blood spots (XLA, n = 23; non-XLA, n = 4).

One patient (P27) was positive for cjKRECs, but other patients were negative for it. *RPPH1* (internal control) was detectable at the same level as in normal controls in all samples.

These results indicate that sjKRECs are undetectable in XLA and non-XLA and suggest that measurement of sjKRECs in neonatal Guthrie cards has the potential for the use of newborn mass screening to identify neonates with early B-cell maturation defects. Greater numbers of neonatal Guthrie cards should be examined to confirm this potential, and the data obtained from dried blood spots on filter papers must be examined to prove that they truly reflect the data obtained from neonatal Guthrie cards. We should also examine whether screening can reduce the cost of treatment of the bacterial infections and chronic lung diseases in patients with XLA and non-XLA and increase the benefits for these patients. An anticipated pilot study using a large cohort of newborns must address these problems. We also found that T-cell–receptor excision circles and sjKRECs can be measured simultaneously on the same plate. Thus, a pilot study of neonatal screening for both T-cell and B-cell deficiencies could be performed simultaneously.

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TABLE E1. Characteristics of patients with XLA

Patient no.	Unique patient no.	Age (y)	Sex	Serum Ig (mg/dL)			$CD19^+$		*BTK* mutation			Source	
				IgG	IgA	IgM	% Lymph	/μL	Genomic DNA	cDNA	Amino acid	Guthrie	Dry spot
P1	670	0	M	87	<6	10	0.21	12.99	29269G>T	1178-1G>T	Splice acceptor defect	x	
P2	718	0	M	215	<10	<10	0.07	7.04	11593_11594 insA	144_145insA	Arg49 frameshift	x	
P3	722	0	M	80	<1	1	<1.00	NA	25644C>T	763C>T	Arg255X	x	
P4	727	8	M	295	59	57	0.11	3.52	29269G>T	1178-1G>T	Splice acceptor defect		x
P5	732	34	M	1140*	<6	8	0.02	0.24	11631T>A	182T>A	Ile61Asn		x
P6	811	24	M	458*	0	13	0.50	5.32	23570T>G	426T>G	Tyr142X		x
P7	813	18	M	628*	109	6	0.60	6.87	23570T>G	426T>G	Tyr142X		x
P8	814	19	M	260	0	NA	0.20	3.01	16180C>T	344C>T	Ser115Phe		x
P9	815	13	M	600*	<10	<5	0.08	1.72	11590G>T	142-1G>T	Splice acceptor defect		x
P10	816	11	M	12	0	5	0.00	0.00	150kb deletion of *BTK, TIMM8A, TAF7L, DRP2*				x
P11	817	10	M	10	2	24	0.80	35.78	36288C>T	1928C>T	Thr643Ile		x
P12	824	13	M	462	6	27	0.41	14.49	27518C>A	895-11C>A	Splice acceptor defect		x
P13	834	5	M	<237	<37	43	0.00	0.00	25715_26210del	776+57_839+73del	Exon 9 deletion		x
P14	838	21	M	<50	<5	7	0.00	0.00	31596G>C	1631+1G>C	Splice donor defect		x
P15	839	16	M	604*	<1	<2	0.04	0.66	31596G>C	1631+1G>C	Splice donor defect		x
P16	847	11	M	698*	26	11	0.08	1.86	25536delG	655delG	Val219 frameshift		x
P17	877	14	M	20	19	8	0.21	NA	32357T>C	1750+2T>C	Splice donor defect		x
P18	880	5	M	233	39	41	0.06	NA	10941-?_14592+?del	1-?_240+?del	Exon 1-3 deletion		x
P19	888	8	M	<212	<37	150	0.15	6.60	11023G>A	83G>A	Arg28His		x
P20	891	21	M	195	<6	37	0.02	0.09	32243C>G	1638C>G	Cys502Trp		x
P21	958	0	M	<50	<10	9	0.80	27.14	31544_31547 delGTTT	1580_1583del GTTT	Cys527 frameshift		x
P22	701	2	M	115	<2	4	0.09	1.99	16172C>A	336C>A	Tyr112X		x
P23	911	0	M	<10	<6	<4	0.00	0.00	29955A>C	1350-2A>C	Splice acceptor defect	x	
P24	937	0	M	60	<2	58	0.00	0.00	11022C>T	82C>T	Arg28Cys	x	
P25	938	0	M	<20	<4	<6	0.00	0.00	36269-?_36778+?del	1909-?_2418+?del	Exon 19 deletion	x	
P26	939	0	M	60	<2	22	0.00	0.00	11022C>T	82C>T	Arg28Cys	x	
P27	890	12	M	<237	<37	<20	0.03	NA	36261G>A	1909-8G>A	Splice acceptor defect		x
P28	944	6	M	12	<1	1	0.02	NA	36281C>T	1921C>T	Arg641Cys		x
P29	948	5	M	<237	<37	<20	0.01	0.70	36261G>A	1909-8G>A	Splice acceptor defect		x
P30	1053	5	M	386	5	113	1.10	34.22	32259A>C	1654A>C	Thr552Pro		x

Age, Age at analysis of KRECs; *$CD19^+$ % Lymph,* CD19-positive cell percentage in lymphocytes; *$CD19^+$ /μL,* CD19-positive cell number in 1 μL whole peripheral blood; *M,* male; *NA,* not available; *Serum Ig,* serum levels of immunoglobulins at diagnosis.

BTK mutation's reference sequences are NCBI NC_000023.9, NM_000061.2, and NP_000052.1.

*Trough level during intravenous immunoglobulin therapy.