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High prevalence of autoantibodies to muscarinic-3 acetylcholine receptor in patients with juvenile-onset Sjögren syndrome

Sjögren syndrome (SS) is an autoimmune disease characterised pathologically by lymphocytic infiltration into the lacrimal and salivary glands, and clinically by dry eyes and mouth. Lymphocytic infiltration is also found in the kidneys, lungs, thyroid, and liver. Immunohistochemical studies have shown that most infiltrating lymphocytes around the labial salivary and lacrimal glands and the kidneys are CD4-positive αβT cells.[1] Candidate autoantigens recognised by T cells that infiltrate the labial salivary glands of SS have been analysed and Ro/SS-A 52 kDa,[2] α-amylase, heat shock protein, and TCR BV6[3] have been identified, although Ro/SS-A 52 kDa reactive T cells were not increased in peripheral blood.[4]

In contrast, various autoantibodies (autoAbs) have been identified in the sera of patients with SS, and some of these autoAbs, such as anti-SS-A antibody (Ab) and anti-SS-B Ab, are used as diagnostic markers. Muscarinic-3 acetylcholine receptor (M3R) is involved in activation of salivary and lacrimal glands. This receptor is G-protein-linked and its activation triggers a second-messenger cascade that culminates in a rise in intracellular calcium and activation of K^+ and Cl^- channels that drive fluid secretion.[5] Although autoAbs to M3R have been demonstrated in patients with SS,[6] the location of B cell epitopes on M3R remain controversial.[7 8] We previously reported the presence of autoAbs against the second loop domain of M3R in 11.2% of patients with adult SS.[9] Anti-M3R Ab is specific for SS because it is not present in patients with other autoimmune diseases such as rheumatoid arthritis and systemic lupus erythaematosus. Based on these early findings, we hypothesised that the presence of anti-M3R Ab may be directly related to defective salivary and lacrimal secretion in SS patients. The prevalence of M3R Ab in juvenile SS is still unknown. To examine this issue, we screened sera of patients with juvenile SS for anti-M3R Ab.

Serum samples were collected from 38 Japanese paediatric patients with juvenile-onset SS (JSS) followed-up at the Departments of Pediatrics of Graduate School of Medicine, Chiba University and Yokohama City University School of Medicine, Yokohama. We recruited 76 healthy control subjects from the Division of Clinical Immunology, Major of Advanced Biological Applications, Graduate School Comprehensive Human Science, University of Tsukuba. The mean (SD) age of the patients was 15 (5) and 22 (2) years for the JSS and control groups, respectively. The 25mer synthetic amino acid encoding the second extracellular domain of M3R was used as the antigen, because this portion plays an important role in intracellular signalling. Figure 1 shows that the mean titre of anti-M3R Ab in patients with JSS (0.329 (0.189)) was significantly higher than that of controls (0.105 (0.089), $p<0.001$). Moreover, the prevalence of anti-M3R Ab in patients with JSS (52.6%) was significantly higher than that

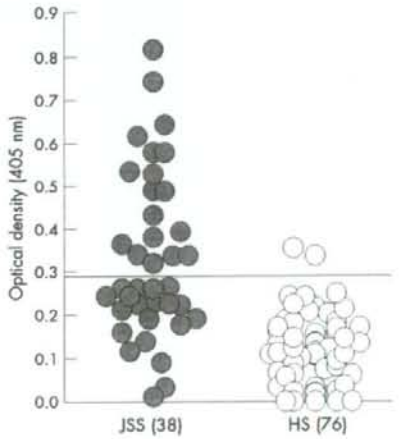

Figure 1 Comparison of anti-M3R Abs in patients with juvenile Sjögren syndrome (JSS) and control. A 25mer peptide (KRTVPPGECFIQFLSEPTITFGTAI) corresponding to the sequence of the second extracellular loop domain of the human M3R was synthesised (Sigma-Aldrich Japan, Ishikari, Japan). A 25mer peptide (SGSGSGSGSGSGSGSGSGSGSGS) was also synthesised as a negative control (Sigma-Aldrich Japan). Peptide solution (100 µl/well at 10 µg/ml) in 0.1M Na_2CO_3 buffer, pH 9.6, was adsorbed onto a Nunc-Immuno plate (Nalge Nunc International, Rochester, New York, USA) overnight at 4°C, and blocked with 5% bovine serum albumin (Wako Pure Chemical Industries, Osaka, Japan) in phosphate buffered saline (PBS) for 1 h at 37°C. Serum at 1:50 dilution in blocking buffer was incubated for 2 h at 37°C. The plates were then washed twice with 0.05% Tween 20 in PBS, and 100 µl of alkaline phosphatase-conjugated goat antihuman IgG (Fc; American Qualex, San Clemente, California, USA) diluted 1:000 in PBS was added for 1 h at room temperature. After three washes, 100 µl of *p*-nitrophenyl phosphate (Sigma) solution (final concentration 1 mg/ml) was added as alkaline phosphate substrate. Plates were incubated for 30 min at room temperature and the optical density at 405 nm was measured by plate spectrophotometry (Bio-Rad Laboratories, Hercules, California, USA). Optimal density was used to express the titre of anti-M3R Abs. Measurements were performed in triplicate and standardised between experiments. Numbers in parentheses represent the number of patients in each group.

in controls (2.9%, p<0.001). These results indicate the high prevalence of anti-M3R in JSS patients, compared to adult-onset SS patients. The presence of anti-SS-A Ab or anti-SS-B Ab were not associated with the presence of anti-M3R Ab in patients with JSS.

In conclusion, the high titre and prevalence of anti-M3R Abs in patients with JSS suggest that anti-M3R Ab could be potentially useful as a diagnostic marker for JSS.

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Invariant NKT cells produce IL-17 through IL-23-dependent and -independent pathways with potential modulation of Th17 response in collagen-induced arthritis

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Abstract. Invariant natural killer T (iNKT) cells play a protective role in the development of certain autoimmune diseases. However, their precise role in the pathogenesis of autoimmune arthritis remains unclear. In this study, we examined the possible contribution of iNKT cells in collagen-induced arthritis (CIA) by using iNKT cell-deficient mice ($J\alpha281^{-/-}$ mice). CIA in these mice was markedly suppressed and interleukin (IL)-17 production was reduced in a native type II collagen (CII)-specific T cell response. Draining lymph nodes of CII-immunized $J\alpha281^{-/-}$ mice contained a significantly low number of IL-17-producing T helper cells. To determine whether iNKT cells produce IL-17, we measured IL-17 by enzyme-linked immunosorbent assay in iNKT cells stimulated with the ligand, α-galactosylceramide (α-GalCer). Notably, splenocytes from $J\alpha281^{-/-}$ mice stimulated in this way were negative for IL-17, whereas those from C57BL/6 mice produced IL-17. Immunostaining for IL-17 in iNKT cells confirmed intracellular staining of the protein. RT-PCR analysis showed that iNKT cells expressed retinoid-related orphan receptor γT and IL-23 receptor. Moreover, cell sorting demonstrated that $NK1.1^-$ iNKT cells were the main producers of IL-17 compared with $NK1.1^+$ iNKT cells. IL-17 production by iNKT cells was induced by IL-23-dependent and -independent pathways, since iNKT produced IL-17 when stimulated with either IL-23 or α-GalCer alone. Our findings indicate that iNKT cells are producers and activators of IL-17 via IL-23- dependent and -independent pathways, suggesting that they are key cells in the pathogenesis of CIA through IL-17.

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Key words: natural killer T cells, interleukin-17, collagen-induced arthritis, interleukin-23, T helper 17 cells

Introduction

Natural killer T (NKT) cells are a subset of T lymphocytes that express NK-cell markers, such as NK1.1 in mice. The majority of NKT cells express an invariant T cell receptor (TCR) encoded by Vα14Jα281 associated with highly skewed sets of Vβs, mainly Vβ8.2 in mice, and they are therefore called invariant NKT (iNKT) cells. The receptor recognizes glycolipid antigen presented by CD1d, a nonclassical antigen-presenting molecule (1,2). TCR stimulation induces iNKT cells to rapidly secrete large amounts of pro- and anti-inflammatory cytokines, such as interleukin (IL)-4 and interferon (IFN)-γ (3). iNKT cells are therefore known as immune regulators.

Deficiency of iNKT cells is associated with various autoimmune diseases (4-7). In animal models, iNKT cells suppress the development and progression of diabetes mellitus (8), experimental autoimmune encephalitis (EAE) (9), and systemic lupus erythematosus (10). These cells also act as effector cells in autoimmune arthritis by promoting T helper (Th) 1-type immune responses, inducing the production of autoantibodies, and suppressing the production of transforming growth factor (TGF)-β (11-14). The evidence thus suggests a dual function for iNKT cells in autoimmunity.

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by chronic inflammation of the synovial tissues in multiple joints leading to joint destruction (15). Although the etiopathogenesis of RA remains unclear, pro-inflammatory cytokines including tumor necrosis factor (TNF)-α, IL-1β, and IL-6 play a major role in this process (16). It is generally accepted that IFN-γ-producing Th1 cells play a pathogenic role in the development of autoimmune diseases. In addition, IL-17 from $CD4^+$ effector T cells, but not IFN-γ, was recently implicated in the pathogenesis of EAE and collagen-induced arthritis (CIA) (17,18).

IL-17 is a T cell-derived cytokine associated with a variety of autoimmune diseases. The trigger for IL-17 release

has not been fully elucidated, but IL-23 is known to promote the production of IL-17 (19). Specifically, differentiated Th17 cells express IL-23 receptor (IL-23R) as well as orphan nuclear receptor retinoid-related orphan receptor (ROR) γT (20,21). Furthermore, IL-23-dependent IL-17-producing $CD4^+$ effector T cells, which develop via a lineage distinct from Th1 and Th2 cells, play a pathogenic role in EAE and CIA (17,18,22). IL-17 is a potent inducer of various cytokines such as TNF-α, IL-1, and receptor activator of NF-κB ligand (RANKL), and is involved in synovial inflammation, cartilage destruction, and bone erosion (23). Moreover, IL-17-deficient or -suppressed mice are resistant to the development of CIA (24-26).

The present study was designed to assess the role of iNKT cells in the development of one form of autoimmune arthritis, CIA, by using iNKT cell-deficient mice ($J\alpha281^{-/-}$ mice). The results demonstrated that production of IL-17 was significantly reduced in iNKT deficient mice. The results also showed that iNKT cells produce IL-17 in IL-23-dependent and -independent pathways. The majority of IL-17-producing iNKT cells were of the $NK1.1^-$ population. The findings implicate iNKT cells as producers and activators of IL-17, making them key players in the pathogenesis of CIA via IL-17.

Materials and methods

Mice. C57BL/6 (B6) mice were purchased from Charles River Japan Inc. (Tokyo, Japan). Jα281-knockout mice were kindly provided by Dr Masaru Taniguchi (Riken Research Center for Allergy and Immunology, Yokohama, Japan). The animals were kept under specific pathogen-free conditions and studied at 6-9 weeks of age. The Committee on Institutional Animal Care and Use at Tsukuba University approved all experimental plans.

Induction of CIA. Mice were immunized intradermally at the base of their tails with 100 μg of chicken CII emulsified with an equal volume of complete Freund's adjuvant (CFA) and containing 250 μg of H37Ra *Mycobacterium tuberculosis* (Difco, Detroit, MI). The animals were boosted by intradermal injection with the same antigen preparation on day 21. Joint swelling was monitored and scored as follows: 0, no swelling or redness, 1; swelling or redness in one joint, 2; two or more joints involved, 3; severe arthritis of the entire paw and joint. The score for each animal was the sum of the score for all four paws. The clinical score was calculated from all mice in the group.

Flow analysis and sorting of iNKT cells. Prior to antibody staining, cells were preincubated with anti-CD16/32 to block nonspecific FcγR binding. The following antibodies were used in this study: FITC-conjugated anti-CD19 (BD Pharmingen, San Diego, CA), FITC-conjugated anti-CD4 (BD Pharmingen), FITC-conjugated anti-NK1.1 (eBioscience, San Diego, CA), PE- or APC-conjugated CD1d tetramer (PE, Medical & Biological Laboratories Co., Nagoya, Japan; APC, Proimmune), and Per-CP-conjugated anti-CD3 (BD Pharmingen). Samples were acquired on a FACScalibur instrument (Pharmingen) and data were analyzed with FlowJo software.

Intracellular cytokine staining. Standard intracellular cytokine staining was performed as described previously (BD Bioscience, San Jose, CA). Cells were first stained for a cell surface marker, then fixed and permeabilized with Cytofix/Cytoperm solution (Pharmingen), before further immunostaining with PE-conjugated anti-IL-17 (Pharmingen) or FITC-conjugated anti-IFN-γ (eBioscience), FITC-conjugated anti-IL-4 (eBioscience), PE-conjugated Isotype Rat IgG (Pharmingen), and FITC-conjugated Isotype Rat IgG (eBioscience).

Measurement of ex vivo CII-specific T cell response. B6 and $J\alpha281^{-/-}$ mice were immunized intradermally with 100 μg chicken CII emulsified in CFA containing 250 μg of inactivated *M. tuberculosis* H37Ra. At 10 days after CII/CFA immunization, draining lymph node (DLN) cells were restimulated in triplicate with 100 μg/ml chicken CII for 72 h. IL-17 concentrations in the culture supernatant were measured by enzyme-linked immunosorbent assay (ELISA). Analysis of cytokine production by DLN cells was performed by first isolating these cells from B6 and $J\alpha281^{-/-}$ mice at 10 days after CII immunization, and then their culture ($1x10^6$ cells/ml) for 72 h in 96-well plates with 100 μg/ml of CII. Golgistop (BD Pharmingen) was added for the final 6 h of culture before cytokine staining to enhance intracellular protein accumulation.

Activation of iNKT cells in vitro. Splenocytes from B6 and $J\alpha281^{-/-}$ mice were collected and stimulated with or without 100 ng/ml or sequential amounts (0, 1, 10, 100, 1000 ng/ml) of α-galactosylceramide (α-GalCer), for 72 h in 96-well round-bottom plates. Cytokine concentrations in the culture supernatants were measured by ELISA. The cells were used for intracellular cytokine staining. In experiments shown in Fig. 4, sorted total iNKT cells, $NK1.1^+$iNKT cells or $NK1.1^-$ iNKT cells ($2x10^3$ cells/well) were cultured with mitomycin-C-treated $CD11c^+$ cells ($2x10^3$ cells/well), which were selected by MACS CD11c-beads from splenocytes as antigen-producing cells (APC), in the presence or absence of 10 ng/ml IL-23 (eBioscience) and/or 10 ng/ml α-GalCer (Kirin Brewery Co.). For neutralization of IL-23, splenocytes were stimulated with 10 ng/ml α-GalCer or 100 pg/ml rIL-23 (eBiosciences) plus 100 IU/ml IL-2 for 72 h in the presence of sequential amounts of anti-IL-23 antibody (0, 0.3, 1, 3 μg/ml; eBiosciences) or isotype-matched control IgG (eBiosciences).

RT-PCR. Total RNA was extracted from purified iNKT cells, B cells and Th17 cells, and was reverse transcribed into cDNA using RevertAid™ First-strand cDNA Synthesis kit (Fermentas, Burlington, ONT, Canada) according to the protocol supplied by the manufacturer. iNKT cells ($CD1d$ tetramer$^+CD3^+CD19^-$ cells) were sorted from naive or α-GalCer stimulated splenocytes. B cells were purified from α-GalCer-stimulated splenocytes using anti-CD19 magnetic microbeads as recommended by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). For induction of Th17 cells, MACS-purified $CD4^+$ T cells were cultured with 20 ng/ml IL-6 (eBioscience) and 5 ng/ml TGF-ß (R&D Systems, Minneapolis, MN) in 96-well plates containing plate bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (1 μg/ml)

for 3 days. The following primers were used for PCR: GAPDH: forward AACTTTGGCATTGTGGAAGG, reverse ACACATTGGGGGTAGGAACA; IL-17: forward TCCAG AAGGCCCTCAGACTA, reverse AGCATCTTCTCGAC CCTGAA; and RORγT: forward TGCAAGACTCATC GACAAGG, reverse AGGGGATTCAACATCAGTGC. The results were normalized with GAPDH expression.

Statistical analysis. The Mann-Whitney U Test was used for comparisons of clinical score of arthritis and *ex vivo* T cell response. The Student's t-test was used for *in vitro* iNKT cell response. $P<0.05$ denoted the presence of a statistically significant difference.

Results

iNKT cells promote joint inflammation. iNKT cells act as regulatory cells in some autoimmune diseases. However, in the development of autoimmune arthritis, an effector function has been assigned to these cells (11-14). To confirm the specific role of iNKT cells in the development of collagen-induced arthritis (CIA), we examined the incidence and severity of arthritis in iNKT cell-deficient Jα281$^{-/-}$ mice and B6 controls. The incidence of arthritis and disease severity in mice lacking iNKT cells was markedly lower than in B6 mice (Fig. 1A and B). These results suggest that iNKT cells promote joint inflammation in the development of CIA.

iNKT cells enhance IL-17 production. We examined whether the presence or absence of iNKT cells altered the T cell response to type II collagen (CII). We isolated the draining lymph node (DLN) cells from B6 mice and iNKT cell-deficient mice 10 days after the first immunization with CII, and then further stimulated the DLN cells with CII *in vitro*. There was significantly less IL-17 in the culture supernatant of Jα281$^{-/-}$ cells than in cultures of B6 DLN cells. However, the concentrations of IL-4, IL-10, and IFN-γ did not differ between these mice (Fig. 2A). It was suggested that IL-17-producing CD4$^+$ T cells (Th17 cells) play a crucial role in the generation of autoimmune diseases (17,18,20). To determine whether iNKT cells accelerate IL-17 production by Th17 cells in CIA, we measured cytokine production by CD4$^+$ T cells in DLN. The percentage of IL-17-producing CD4$^+$ T cells (Th17 cells) was lower in Jα281$^{-/-}$ mice than in B6 mice (Fig. 2B, $P<0.01$), suggesting that iNKT cells enhance Th17 cell activation in the development of CIA.

iNKT cells synthesize and secrete IL-17. We hypothesized that iNKT cells directly produced IL-17 and that IL-17 promoted the pathogenesis of CIA. We stimulated iNKT cells obtained from naive B6 and iNKT cell-deficient mice with the iNKT cell ligand, α-GalCer. Isolated splenocytes were further stimulated with α-GalCer, and the amount of IL-17 in the culture supernatant was assayed. iNKT cells from B6 mice stimulated with α-GalCer showed dose-dependent IL-17 production, while iNKT cell-deficient mice were negative for IL-17 (Fig. 3A and B). The α-GalCer-stimulated splenocytes were then immunostained to localize IL-17 and identify the IL-17-producing cells (Fig. 3C). Notably, a small population of IL-17$^+$ iNKT cells was observed in B6 mice, but none were

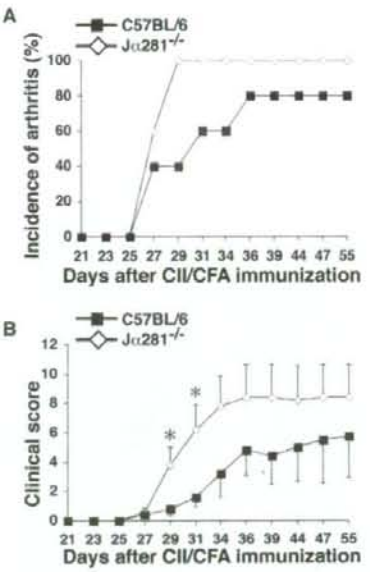

Figure 1. iNKT cells promote joint inflammation in collagen-induced arthritis (CIA). Incidence of arthritis (A) and clinical scores (B) in Jα281$^{-/-}$ (solid symbols) and B6 (open symbols) mice. Data are representative of two experiments. Error bars, SEM; n=5 mice per group. *$P<0.05$.

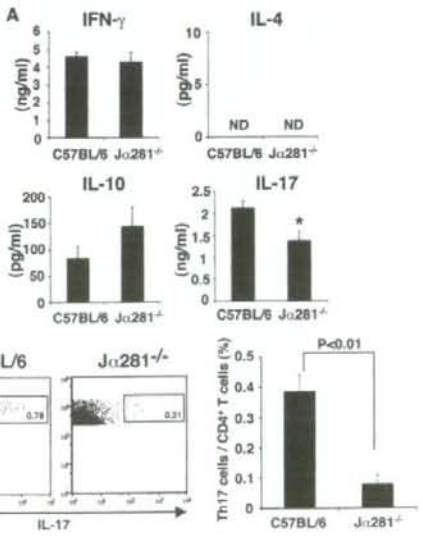

Figure 2. Low proportion of Th17 cells in draining lymph node (DLN) cells from type II collagen (CII)-immunized Jα281$^{-/-}$ mice. At 10 days after CII/complete Freund's adjuvant (CFA) immunization, DLN cells were restimulated with CII for 72 h. (A) IFN-γ, IL-4, IL-10, and IL-17 concentrations in the culture supernatant were measured by ELISA. (B) Left: representative flow cytometry demonstrating IL-17 expression in CD4$^+$ T cells (CD4$^+$CD3$^+$ population) of DLN cells from B6 and Jα281$^{-/-}$ mice. Right: proportion of IL-17-producing CD4$^+$ T cells among DLN cells from B6 and Jα281$^{-/-}$ mice expressed as a percentage of total CD4$^+$ T cells. Data are representative of at least two experiments with similar results. Error bars, SEM; n=5 mice per group. *$P<0.05$. ND, not detected.

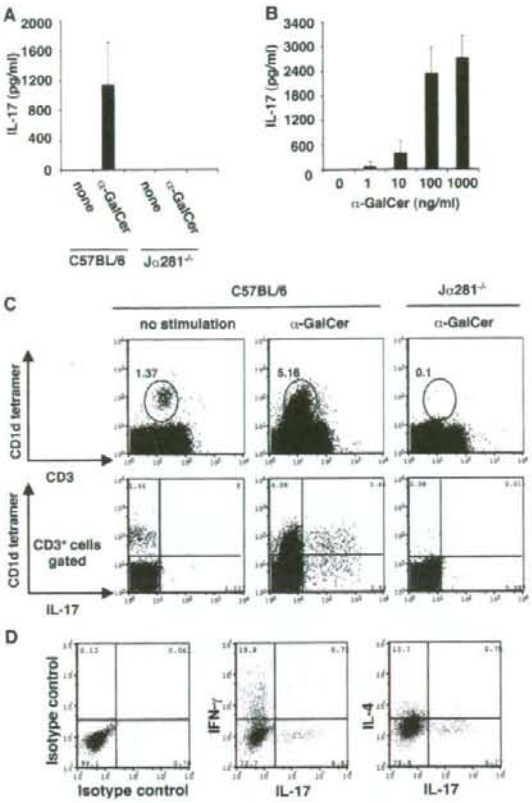

Figure 3. Glycolipid antigen-stimulated iNKT cells synthesize and secrete IL-17. (A) Splenocytes from naive B6 and Jα281$^{-/-}$ mice were collected and stimulated with or without α-GalCer for 72 h in triplicate. IL-17 levels in the supernatant were measured by ELISA. (B) Splenocytes from B6 mice were stimulated with various concentrations of α-GalCer (0, 1, 10, 100, 1000 ng/ml), and IL-17 levels in the supernatants were then measured by ELISA. (C) Top panels: population of splenocytes from B6 or Jα281$^{-/-}$ mice stimulated with or without α-GalCer for 72 h. The CD19$^-$CD1d tetramer$^+$ CD3$^+$ cells were counted as iNKT cells. Bottom panels: IL-17 production by splenocytes from B6 or Jα281$^{-/-}$ mice stimulated with or without α-GalCer for 72 h. CD19$^-$CD3$^+$ cells were gated and IL-17$^+$CD1d tetramer$^+$ cells were estimated as IL-17-producing iNKT cells. (D) Intracellular IL-17, IFN-γ and IL-4 staining was performed after *in vitro* stimulation of splenocytes at 72 h and analyzed among gated iNKT cells (CD1d tetramer$^+$CD3$^+$) by flow cytometry. Data are representative of three independent experiments.

evident in the Jα281$^{-/-}$ mice (Fig. 3C), suggesting that iNKT cells themselves produce IL-17. Moreover, IL-17-producing iNKT cells did not produce IFN-γ or IL-4 (Fig. 3D). The above experiments indicate the presence of IL-17-producing iNKT cells, although there is no evidence that IL-17-producing iNKT cells are specifically Th17 activators.

NK1.1$^-$ iNKT cell-mediated IL-17 production is dependent on and independent of IL-23. IL-23 is a heterodimeric protein consisting of common IL-12p40 and IL-23p19 and is essential for maintenance of Th17 cells (18,19). To establish whether the same effect was seen in iNKT cells, we analyzed the expression of IL-23R as well as RORγT by RT-PCR. As shown in Fig. 4A, naive and activated iNKT cells expressed

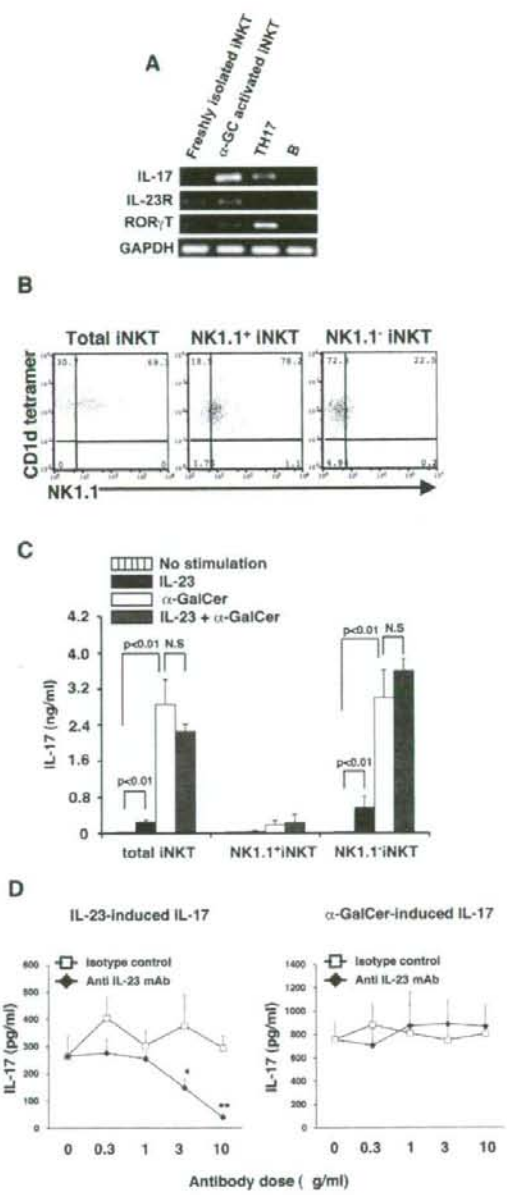

Figure 4. Phenotype of IL-17-producing iNKT cells. (A) IL-17, RORγT and IL-23R expression in purified iNKT cells was determined by using RT-PCR. Lane 1, iNKT cells from naive splenocytes; Lane 2, iNKT cells from α-GalCer-stimulated splenocytes; Lane 3, Th17 cells; Lane 4, B cells from α-GalCer-stimulated splenocytes. (B) Representative FACS profiles of sorted total iNKT cells, NK1.1$^+$iNKT cells and NK1.1$^-$iNKT cells. The purity of iNKT cells in this experiment was >95% (total iNKT), >78% (NK1.1$^+$iNKT) and >72% (NK1.1$^-$iNKT). (C) Sorted total iNKT cells, NK1.1$^+$iNKT cells, and NK1.1$^-$iNKT cells were co-cultured with mitomycin-C-treated CD11c as APC and stimulated with IL-23, α-GalCer, or IL-23 plus α-GalCer. IL-17 was measured in the supernatants after 72 h. No IL-17 was detected in unstimulated total iNKT, NK1.1$^+$iNKT, and NK1.1$^+$iNKT cells. (D) Splenocytes were stimulated with α-GalCer or rIL-23 plus rIL-2 in the presence of anti-IL-23 mAb or control Ig. IL-17 was measured in the supernatants after 72 h. *P<0.01; **P<0.01. Data are representative of three independent experiments. N.S, not significant.

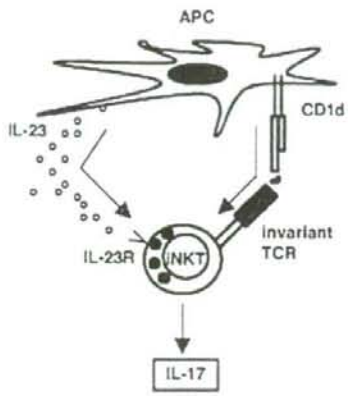

Figure 5. IL-17 production by iNKT cells is regulated through two distinct pathways, IL-23-dependent and IL-23-independent. Left: IL-23-dependent IL-17 production by iNKT cells through IL-23R. Right: IL-23-independent IL-17 production by iNKT cells through invariant T cell receptor (TCR). APC, antigen presenting cells.

IL-23R and RORγT similar to Th17 cells. Notably, IL-17 expression was detected in activated iNKT cells and not in naive iNKT cells, suggesting that IL-17 gene was inducible in the iNKT cell activation. Recently, Michel *et al* (27) reported that only NK1.1$^-$iNKT cells could produce IL-17. To examine whether the production of IL-17 by iNKT cells was dependent or independent of IL-23, splenocytes from naive B6 mice were sorted into either NK1.1$^-$iNKT cells, NK1.1$^+$iNKT cells, and total iNKT cells (Fig. 4B), and then each group was co-cultured with CD11$^+$ dendritic cells (DC) as antigen-producing cells in the presence or absence of IL-23 and α-GalCer. Fig. 4C clearly shows that total iNKT cells produced IL-17 by either IL-23 or α-GalCer alone, indicating that iNKT cells can generate IL-17 through IL-23-dependent and IL-23-independent pathways. The effect of α-GalCer on IL-17 production was not through IL-23 production, as no IL-23 was found in the culture supernatant from iNKT cells stimulated with α-GalCer (data not shown) and anti-IL-23 mAb did not block α-GalCer-induced IL-17 production (Fig. 4D). No additional effect for IL-23 and α-GalCer stimulation was observed (Fig. 4C). These results suggest that NK1.1 iNKT cells produced IL-17 through IL-23-dependent and -independent pathways.

Discussion

iNKT cells act as regulatory cells in certain autoimmune diseases. However, in the development of autoimmune arthritis, an effector function has been assigned to these cells (11-14). Both the severity of arthritis and autoantibody production was reduced in iNKT cell-deficient mice with CIA (11,12). In addition, the T cell response to autoantigens shifted from Th1 to Th2 in these mice (11). Furthermore, in antibody-induced arthritis, iNKT cells suppressed TGF-ß expression in joints, suggesting an effector-cell function (13,14). The present results indicate that iNKT cells function as IL-17 producers and activators in autoimmune arthritis. This is the first time that the role of iNKT cells through IL-17 production in the development of CIA has been reported. Moreover, we propose two functional roles for iNKT cells in the development of CIA. The first is upregulation of IL-17 production by CII-reactive Th17 cells, while the second is direct production of IL-17 (Fig. 5).

IL-17 is a pathogenic cytokine in autoimmune diseases such as EAE and CIA, since its neutralization or deficiency reduces the severity of disease (24-26). Our study demonstrated that deficiency of iNKT cells reduced CII-reactive IL-17 production in CIA mice. These findings suggest that iNKT cells are associated with IL-17 production and that these cells play a crucial role in the development of CIA.

Previous studies showed that Th17 cells are distinct from the traditional Th1- and Th2-cell subsets, and that they are important in regulating tissue inflammation and the development of disease in several animal models of autoimmunity (20,22,28,29). TGF-ß promotes Th17-cell development in the presence of IL-6 (30-32), and IL-23 is critical for the maintenance of Th17-cell activation in chronic inflammation (20). Our study demonstrated that iNKT cells enhance IL-17 production by Th17 cells in CIA. Some reports have shown that activated iNKT cells induce maturation of DC, and thereby, enhance the antigen-specific T cell response (33,34). In addition, Eberl *et al* (35) suggested that iNKT cells contribute to the maintenance and persistent stimulation of memory T cells through cytokine secretion. Recent reports suggested that IL-21, which activates Th17 cells (36-38), is produced by iNKT cells (39,40). Thus, we speculate that iNKT cells might maintain or activate Th17 cells in CIA mice.

In this study, we clearly demonstrated that iNKT cells synthesize and release IL-17 in addition to accelerating IL-17 production by Th17 cells. Moreover, the main cell population of IL-17-producing iNKT cells is NK1.1$^-$ cells. In recent study, Michel *et al* (27) reported a similar finding in relation to IL-17-producing iNKT cells. Although the function of NK1.1 in IL-17 production is not clear at present, NK1.1 may be a key regulatory molecule in IL-17 production by iNKT cells. In our study, we demonstrated that iNKT cells expressed RORγT as well as Th17 cells, indicating that RORγT might also be involved in IL-17 production by iNKT cells as well as Th17 cells.

Several IL-17-producing cells have been identified, such as CD4 T cells (Th17) and γδT cells, with IL-17 production dependent on IL-23. In contrast, Mangan *et al* (32) detected IL-17 in IL-23-deficient mice and Liu *et al* (41) showed short-term production of IL-17 induced by TCR stimulation in the absence of IL-23. Thus, IL-17 might be regulated in an IL-23-dependent or -independent manner. In our system, iNKT cells produced IL-17 not only by IL-23 but also by α-GalCer alone. Therefore, IL-17 production by iNKT cells is regulated by two distinct pathways (Fig. 5).

In conclusion, we demonstrated that iNKT cells can produce IL-17 by themselves and enhance Th17 cell activity, thus playing a significant role in the development of collagen-induced arthritis (CIA). Moreover, we also showed that iNKT cells (mainly the NK1.1$^-$ cell population) can secrete IL-17 through two stimulation pathways, the IL-23-IL-23R pathway and the glycolipid antigen-TCR pathway.

Acknowledgements

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Critical role of class IA PI3K for c-Rel expression in B lymphocytes

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The fact that the *Xid* mutation of Btk impairs the ability of pleckstrin homology domain of Btk to bind phosphatidylinositol-(3,4,5)-trisphosphate, a product of class IA phosphoinositide-3 kinases (PI3Ks), has been considered strong evidence for the hypothesis that Btk functions downstream of PI3Ks. We demonstrate here that the *Xid* mutation renders the Btk protein unstable. Furthermore, class IA PI3K- and Btk-deficient mice show different phenotypes in B-cell development, collectively indicating that PI3Ks and Btk differentially function in BCR signal transduction. Nevertheless, both PI3K and Btk are required for the activation of NF-κB, a critical transcription factor family for B-cell development and function. We demonstrate that PI3Ks maintain the expression of NF-κB proteins, whereas Btk is known to be essential for IκB degradation and the translocation of NF-κB to the nucleus. The loss of PI3K activity results in marked reduction of c-Rel and to a lesser extent RelA expression. The lentivirus-mediated introduction of c-Rel corrects both developmental and proliferative defects in response to BCR stimulation in class IA PI3K-deficient B cells. These results show that the PI3K-mediated control of c-Rel expression is essential for B-cell functions. (Blood. 2009;113:1037-1044)

Introduction

The nuclear factor κB (NF-κB) family of transcription factors plays an essential role in the expression of genes required for proliferation, survival, and development in many cell types. NF-κB functions as a homodimer or heterodimer consisting of a variety of combinations of 5 different Rel proteins: RelA (p65), c-Rel, RelB, NF-κB1 (p50), and NF-κB2 (p52). The latter 2 are generated through proteolytic processing from their precursor molecules p105 and p100.[1] In most resting cells, NF-κB dimers are sequestered in the cytoplasm through binding to the ankyrin repeats of molecules termed inhibitor of NF-κBs (IκBs).[2] The precursors of NF-κB1 and NF-κB2 also contain ankyrin repeats and therefore act as inhibitors for Rel proteins. The activation of NF-κB signaling pathway ultimately leads to IκB kinase (IKK)–mediated phosphorylation and subsequent degradation of IκBs, releasing NF-κBs to translocate to the nucleus where they induce NF-κB–dependent gene expression.[3,4]

NF-κB family proteins play pivotal roles in the diverse functions of B cells such as proliferation, isotype switching, and cytokine production.[5,6] Furthermore, the NF-κB family is indispensable for the development of B-cell lineage, as demonstrated by the analyses of genetically manipulated mice deficient for more than one of the NF-κB subunits. In p50/p52 double-knockout mice, B-cell development is blocked at the immature transitional stage, shortly after B cells exit from the bone marrow (BM).[7] Adoptive transfer experiments using mixed fetal liver cells showed that $p50^{-/-}RelA^{-/-}$ B cells cannot develop into marginal zone (MZ) B cells,[8] whereas $RelA^{-/-}c\text{-}Rel^{-/-}$ fetal liver cells fail to give rise to mature follicular (FO) B cells.[9] In addition, a recent report revealed that the survival of mature B cells depends on the IKK-mediated activation of NF-κB.[10]

Another set of molecules important in B-cell development and function are the class IA phosphoinositide 3-kinases (PI3Ks), which are a family of heterodimeric lipid kinases consisting of a catalytic (p110α, p110β, and p110δ) and a regulatory (p85α, p55α, p50α, p85β, and p55γ) subunit, and generate phospholipid second messengers that signal downstream of tyrosine kinases in the immune system.[11,12] These PI3K products (phosphatidylinositol-(3,4)-bisphosphate PIP_2 or phosphatidylinositol-(3,4,5)-trisphosphate [PIP_3]) are bound by various proteins containing pleckstrin homology (PH) domains, such as Akt and phosphoinositide-dependent kinase 1 (PDK1). Thus, these PH-containing proteins function downstream of PI3K.[12]

Mice deficient for the gene encoding p85α, the most abundantly and ubiquitously expressed regulatory subunit of class IA PI3K, show the loss of B1 cells, reduced numbers of mature B cells, and failure to proliferate following BCR cross-linking.[13] This phenotype is similar to that of $Btk^{-/-}$ mice or mice with the *Xid* mutation (a natural mutation in the PH domain of Btk in which an arginine residue critical for binding PIP_3 is replaced with cysteine).[14] Btk, an essential tyrosine kinase in BCR signaling, facilitates the activation of PLCγ2, which triggers NF-κB signaling through IKK activation.[15,16] The BCR-induced activation of NF-κB is indeed blocked in $Btk^{-/-}$ B cells.[17,18] $p85\alpha^{-/-}$ B cells also show a defect in the BCR-induced NF-κB activation.[19] In addition, LY294002, a potent PI3K inhibitor, blocks NF-κB activation induced by simultaneous stimulation with BCR and LPS.[20] These observations are consistent with the dogma that PI3K functions upstream of Btk by recruiting Btk to the plasma membrane through the binding of the PH domain of Btk to PIP_3 generated by PI3K.[21] However, we have demonstrated that Btk activation upon BCR stimulation is unaffected by

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the inhibition of PI3K or the lack of p85α, raising the possibility that PI3K regulates the NF-κB signaling pathway independent of Btk.[19] Here we show that *Xid* mutation renders the Btk protein unstable and *Xid* B cells express greatly reduced amounts of Btk protein that can still be activated upon BCR stimulation, likely resulting in the impaired B-cell signaling. We also show that PI3K activity is essential for maintaining the expression of NF-κB proteins such as c-Rel and RelA in B cells, whereas previous studies have shown that Btk is essential for IκB degradation.[17,18] Forced expression of c-Rel restored the development and proliferative responses of $p85\alpha^{-/-}$ B cells. Our results indicate that PI3K is involved in the B-cell homeostasis through the expression of NF-κB proteins.

Methods

Mice

$p85\alpha^{-/-}$ mice[13] were backcrossed to C57BL/6 or BALB/c mice for 12 generations before intercrossing heterozygous mice. Rag-$2^{-/-}$ mice on a BALB/c background (stock no. 000601) and B6.SJL, a C57BL/6 congenic strain expressing the CD45.1 allele (stock no. 004007), were obtained from Taconic (Germantown, NY). $Btk^{-/-(y)}$ mice on a (C57BL/6 × 129/Sv) mixed background (stock no. 002536) and *Xid* mice on a CBA background (stock no. 001011) were obtained from The Jackson Laboratory (Bar Harbor, ME). Although Btk is encoded on the X chromosome and Btk-deficient female and male mice have the $Btk^{-/-}$ and $Btk^{-/y}$ genotypes, respectively, we designate here Btk-deficient mice as $Btk^{-/-}$ mice. All mice were maintained at Taconic or in our animal facility under specific pathogen-free conditions. All experiments were approved by the animal care and use committee at Keio University and were performed in accordance with institutional guidelines.

Reagents

Antibodies to c-Rel (sc-70), RelA (sc-109), ERK2 (sc-154), Btk (sc-1107), and IκBα (sc-371) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GFP (JL-8) was obtained from Clontech (Mountain View, CA). Anti–phospho-ERK (E10) was purchased from Cell Signaling Technology (Beverly, MA). Anti-Btk mAb, 43-3B,[22] was a generous gift from Dr S. Tsukada (Osaka University, Osaka, Japan). Antiphosphotyrosine mAb (4G10)[23] was a generous gift from Dr T. Roberts (DFCI, Boston, MA). Wortmannin was purchased from Calbiochem (La Jolla, CA).

Cell stimulation and immunoblotting

B cells were purified from total splenocytes using anti-B220–coated magnetic beads and AutoMACS (Miltenyi Biotec, Sunnyvale, CA), where the purity was more than 95%. Purified B cells were stimulated with or without the $F(ab')_2$ fragment of a goat polyclonal antibody against mouse IgM (20 μg/mL anti-IgM $F(ab')_2$; Jackson ImmunoResearch, West Grove, PA) at 37°C for the indicated times. The cell lysates were prepared and subjected to either immunoblot analysis or immunoprecipitation as described previously.[19]

Flow cytometric analysis

Fluorescein isothiocyanate (FITC)–conjugated anti-mouse IgM, FITC-conjugated anti-CD21, FITC-conjugated anti-mouse IgD, phycoerythrin (PE)–conjugated anti-CD23, PE-conjugated anti-IgM, PE-conjugated anti-CD21, biotinylated anti-CD45.2, biotinylated anti-CD23, and allophycocyanin-cyanin 7 (APC-Cy7)–conjugated anti-B220 were purchased from BD Biosciences (San Jose, CA). PE-conjugated anti-AA4.1 was purchased from eBioscience (San Diego, CA). The binding of biotinylated mAbs was detected with streptavidin-APC (BD Biosciences). One to 2 million cells were stained with the designated antibodies in HBSS with 2% fetal calf serum (FCS) and subjected to analysis on a FACSAria using FACSDiva software (BD Biosciences). Dead cells were eliminated from the analysis by staining with 7-amino-actinomycin D.

Generation of recombinant lentiviral vectors

A lentiviral vector system[24] consisting of pCAG-HIVgp, pCMV-VSVG-RSV-Rev, and CSII-EF1α-IRES2-Venus was kindly provided by Dr H. Miyoshi (RIKEN, Tsukuba, Japan). Mouse c-Rel cDNA was amplified by polymerase chain reaction (PCR) using pMSCVIRES-c-Rel (kindly provided by Dr T. Kurosaki, RIKEN RCAI, Yokohama, Japan) as a template, whereas human p85α cDNA was amplified by reverse-transcription (RT)–PCR using total RNA from Jurkat T lymphocytes as a mRNA source, followed by cloning into the *Bam*HI site of CSII-EF1α-IRES2-Venus. Resultant vectors along with pCAG-HIVgp and pCMV-VSVG-RSV-Rev were transiently transfected into 293T cells to generate HIV-1–based lentivirus vectors pseudotyped with the vesicular stomatitis virus G glycoprotein. BM cells were collected from the femurs of $p85\alpha^{+/-}$ or $p85\alpha^{-/-}$ mice 2 days after the intraperitoneal injection of 5-fluorouracil (Sigma-Aldrich, St Louis, MO) at a dose of 3 mg per mouse, and were cultured in RPMI-1640 medium containing 10% FCS, 50 μM 2-mercaptoethanol, 100 U/mL each of penicillin and streptomycin, nonessential amino acids (Invitrogen, Carlsbad, CA), 10 mM Hepes, and 1 mM sodium pyruvate supplemented with 10 ng/mL mouse IL-3, 5 ng/mL mouse IL-6, and 10 ng/mL stem cell factor (all from Peprotech, London, United Kingdom). After 48-hour incubation, nonadherent BM cells were collected and seeded on 24-well plates at 5×10^6 cells/mL in the medium supplemented with IL-3, IL-6, and stem cell factor. Lentivirus vectors were then infected by centrifugation (700*g*) for 2 hours in the presence of 5 μg/mL polybrene (Sigma-Aldrich). At 48 hours after infection, BM cells were injected into sublethally (3 Gy) irradiated Rag-$2^{-/-}$ mice.

Btk expression

A mammalian expression vector, pcDNA3.1(+) (Invitrogen) was modified by the introduction of the IRES-EGFP fragment, derived from pIRES-EGFP (Clontech), resulting in a vector named pcDNA3.1-IRESEGFP. A cDNA clone for human Btk[25] was generously provided by Dr S. Tsukada (Osaka University). Three different cDNAs (WT, wild type; R28P, a mutant where arginine 28 was substituted with proline; and R28C, a mutant where arginine 28 was substituted with cysteine) tagged with a Myc-epitope (EQKLISEEDL) at their C-termini were generated and cloned into the pcDNA3.1-IRESEGFP vector. The expression vector for Btk was in vitro transcribed and translated using TNT T7 system (Promega, Madison, WI) in the presence of Transcend tRNA (Promega) to generate biotinylated Btk protein.

Cell-cycle analysis

Purified splenic B cells (5×10^6 cells/mL in 24-well plates) were stimulated with or without 20 μg/mL anti-IgM $F(ab')_2$. Lentivirus vectors were then infected by centrifugation (700*g*) for 2 hours in the presence of 5 μg/mL polybrene. At 48 hours after infection, the cells were incubated with 10 μg/mL Hoechst33342 (Calbiochem), followed by the evaluation of cell-cycle progression on a FACSAria according to the manufacturer's instruction.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney *U* test with Microsoft Excel software (Redmond, WA), with a *P* value less than .05 considered significant.

Results

Reduced expression of Btk in *Xid* B cells

The presence of *Xid* mutation in both mice[14] and humans[25] has been considered strong evidence for the hypothesis that Btk is activated

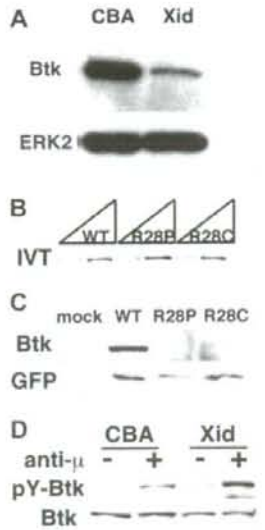

Figure 1. *Xid* mutation affects the stability of Btk protein. (A) Expression levels of Btk protein in *Xid* splenic B cells. The whole-cell lysates of splenic resting B lymphocytes obtained from control CBA/J (CBA) or *Xid* (Xid) mice were subjected to immunoblot analysis with an anti-Btk mAb (43-38). The membrane was reblotted with anti-ERK2. Data are representative of 3 independent experiments with similar results. (B) The expression vector (0.5 μg and 1.0 μg per reaction, respectively) for either human wild-type Btk (WT) or XLA-derived mutants (R28P and R28C) was in vitro transcribed and translated in the presence of Transcend tRNA (Promega). The amounts of biotinylated Btk proteins were evaluated with streptavidin-HRP. (C) Jurkat T lymphocytes were transiently transfected with the expression vector for either human wild-type Btk (WT) or XLA-derived mutants (R28P and R28C), which contains EGFP downstream of an IRES. Whole-cell lysates were subjected to immunoblot with an anti-Btk antibody (top panel), followed by reblot with an anti-GFP antibody (bottom panel). (D) In vivo activation of Btk protein with *Xid* mutation. Splenic B cells obtained from 2 control CBA or 20 *Xid* mice were stimulated with or without 20 μg/mL anti-IgM $F(ab')_2$ (anti-μ) at 37°C for 3 minutes. Btk was then immunoprecipitated with an anti-Btk antibody, followed by immunoblot analysis with 4G10 (pY-Btk). The membrane was reblotted with 43-38 (Btk). Data in panels B through D are representative of 2 independent experiments with similar results.

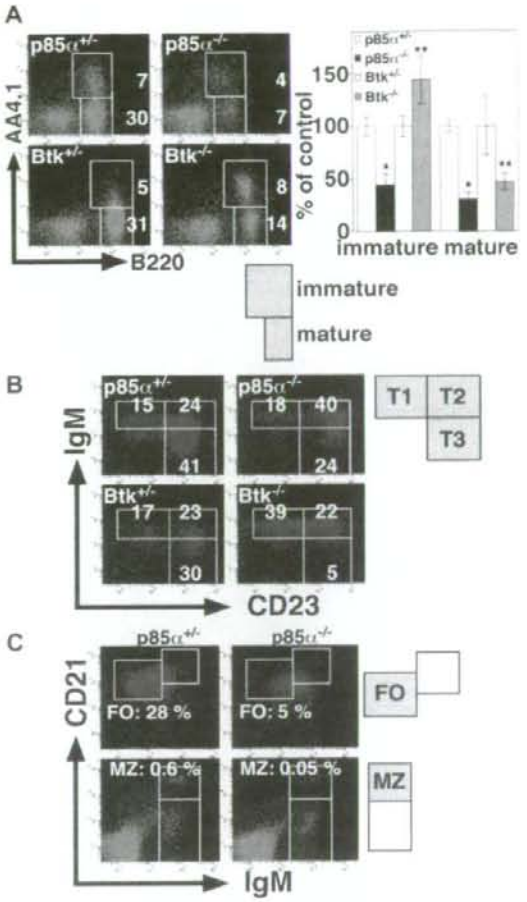

Figure 2. Phenotypes of p85α$^{-/-}$ and Btk$^{-/-}$ mice. (A) Splenocytes from the indicated mice were stained with anti-B220 and anti-AA4.1, and analyzed on a FACSAria. The percentages of immature B cells ($B220^+AA4.1^+$) and mature B cells ($B220^+AA4.1^-$) are indicated (left). Results are presented as mean plus or minus SD (percentages of heterozygotes) (right). Significant differences between p85α$^{+/-}$ and p85α$^{-/-}$ mice or Btk$^{+/-}$ and Btk$^{-/-}$ mice are indicated. $^*P < .01$; $^{**}P < .05$. (B) Analysis of $B220^+AA4.1^+$ immature B cells for surface expression of IgM and CD23: T1 represents IgM^+CD23^{lo}; T2, IgM^+CD23^+; and T3, $IgM^{hi}CD23^+$. Among $B220^+AA4.1^+$ cells, the percentages of the T1, T2, and T3 subsets are indicated. (C) Splenocytes from the indicated mice were stained with anti-CD23, anti-CD21, and anti-IgM. Among $CD23^+$ (top panels) and $CD23^-$ (bottom panels) lymphocyte populations, the percentages of $CD23^+CD21^{int}IgM^{lo}$ FO B cells identified as the cells in the square region (top panels) or $CD23^-CD21^{hi}IgM^{hi}$ MZ B cells identified as the cells in the square region (bottom panels) are indicated. Data are representative of 6 mice each for p85α$^{+/-}$ and p85α$^{-/-}$ mice on a BALB/c background or 4 mice each for Btk$^{+/-}$ and Btk$^{-/-}$ mice on a (C57BL/6 × 129/Sv) mixed background.

downstream of PI3K through interaction between the PH domain of Btk and PIP_3, a PI3K product (reviewed in Satterthwaite et al[21]). However, our previous study demonstrated that the interaction between PIP_3 and the PH domain of Btk is dispensable for the Btk function,[19] raising a fundamental question: how does the *Xid* mutation in the PH domain perturb Btk function? Since a point mutation often renders the protein unstable within cells,[26,27] we considered the possibility that the *Xid* mutation affects the expression of Btk. This was indeed the case with splenic B cells as B cells from *Xid* mice contained reduced amounts of Btk protein compared with control CBA mice (Figure 1A). We next examined the expression of human Btk proteins containing a point mutation found in XLA patients[25] at the same arginine residue critical for PIP_3 binding in the PH domain. Mutant and wild-type Btk proteins were expressed similarly when translated in an in vitro translation system (Figure 1B). However, mutant Btk constructs showed reduced expression of mutant proteins compared with wild-type Btk protein when transfected into Jurkat T lymphocytes (Figure 1C top panel). On the other hand, the amounts of EGFP protein translated from the same mRNA via the internal ribosomal entry site (IRES) were comparable (Figure 1C bottom panel), indicating that similar amounts of mRNA were transcribed from all 3 constructs. These results suggest that the mutations in the PH domain decrease the stability of Btk proteins within a cell. Despite its lower expression, the *Xid* mutant Btk was tyrosine-phosphorylated upon BCR-stimulation as wild-type Btk (Figure 1D). These results taken together suggest that the defect of BCR signaling in *Xid* mice is likely due to the reduced amounts of Btk protein but not a lack of Btk activation.

Differential defects in B-cell development between p85α$^{-/-}$ and Btk$^{-/-}$ mice

We next examined the phenotypes of p85α$^{-/-}$ and Btk$^{-/-}$ mice by comparing splenic B-cell subsets (Figure 2; Table 1). Note that p85α$^{-/-}$ and control mice were on a BALB/c background, whereas

Table 1. Splenic B-cell subsets in p85α$^{-/-}$ and Btk$^{-/-}$ mice

Genotype*	Total, ×10^6	T1, ×10^6	T2, ×10^6	T3, ×10^6	FO, ×10^6	MZ, ×10^6
p85α$^{+/-}$, n = 6	88.3	0.92	1.54	2.80	24.1	0.47
p85α$^{-/-}$, n = 6	68.0†	0.42‡	0.91§	0.57‡	3.62‡	0.09‡
Btk$^{+/-}$, n = 4	67.0	0.69	0.77	0.86		
Btk$^{-/-}$, n = 4	28.0†	0.63†	0.32†	0.09‡		

*Mice are on a BALB/c background (p85α$^{+/-}$ and p85α$^{-/-}$ mice) or on a mixed background between C57BL/6 and 129/Sv (Btk$^{+/-}$ and Btk$^{-/-}$ mice).

Significance between knockout and control mice was examined by the Mann-Whitney *U* test: †no significance; ‡$P < .01$ from control heterozygous mice; §$P < .05$ from control heterozygous mice.

Btk$^{-/-}$ and control mice were on a (C57BL/6 × 129/Sv) mixed background. We stained splenocytes with antibodies to B220 and AA4.1, the latter of which is a complement C1q receptor and serves as an immature B-cell marker.[28] Consistent with the previous reports demonstrating that PI3K deficiency leads to a decrease in the number of mature B cells in the periphery,[11-13] the percentage of B220$^+$AA4.1$^-$ cells corresponding to mature B cells was greatly decreased in p85α$^{-/-}$ mice (Figure 2A). In the p85α$^{-/-}$ spleen, the percentage of B220$^+$AA4.1$^+$ immature B cells was also decreased. In marked contrast, Btk$^{-/-}$ mice had an increased proportion of B220$^+$AA4.1$^+$ immature B cells compared with wild-type mice, whereas the percentage of B220$^+$AA4.1$^-$ mature B cells was decreased (Figure 2A). Both p85α$^{-/-}$ and Btk$^{-/-}$ mice had significantly reduced number of splenic B cells compared with wild-type mice (Suzuki et al[19] and data not shown). The present study further demonstrates that p85α$^{-/-}$ mice have reduced numbers of both mature and immature B cells, whereas Btk$^{-/-}$ mice have defects in only mature B cells. These results are consistent with the previous report showing that splenic B-cell development in mice with the *Xid* mutation is arrested at the immature stage.[29]

B220$^+$AA4.1$^+$ immature B cells can be subdivided into 3 transitional subsets, T1 (IgM$^+$CD23^{lo}), T2 (IgM$^+$CD23$^+$), and T3 (IgM^{hi}CD23$^+$), according to the surface expression of IgM and CD23.[28] p85α$^{-/-}$ mice had a reduction in the size of all 3 populations (Table 1). In addition, the proportion of T2 B cells in the p85α$^{-/-}$ immature B-cell compartment was increased, whereas the proportion of T3 B cells was decreased (Figure 2B). On the other hand, the proportion of T1 B cells, but not that of T2 B cells, was increased in the splenic immature B-cell compartment of Btk$^{-/-}$ mice, whereas the proportion of T3 B cells was markedly decreased (Figure 2B). The reduction in cell size of T3 population in Btk$^{-/-}$ mice was significant (Table 1). Considering the possibility that some of the phenotypic differences we observed in p85α$^{-/-}$ mice might be strain specific, we examined B-cell development in the spleen of p85α$^{-/-}$ mice on a C57BL/6 background and obtained essentially the same results (Figure S1 [available on the *Blood* website; see the Supplemental Materials link at the top of the online article]; Figure 3). We thus conclude that the defects in the development of splenic B-cell subsets are different between p85α$^{-/-}$ and Btk$^{-/-}$ mice.

To further analyze mature B-cell populations in p85α$^{-/-}$ mice, splenocytes were stained with antibodies to IgM, CD21, and CD23, and then separated into CD23$^+$ (Figure 2C top panels) and CD23$^-$ (Figure 2C bottom panels) populations. Among the CD23$^+$ cells, p85α$^{-/-}$ mice had a markedly decreased proportion of FO B cells (CD23$^+$CD21^{int}IgM^{lo}) compared with wild-type mice (Figure 2C top panels). In the CD23$^-$ cell subset, p85α$^{-/-}$ mice had a reduced percentage of MZ B cells (CD23$^-$CD21^{hi}IgM^{hi}) compared with wild-type mice (Figure 2C bottom panels). The reduction of FO and MZ B cells in p85α$^{-/-}$ mice was significant (Table 1). These results strongly indicate that the development of the most mature FO and MZ B cells is severely impaired in p85α$^{-/-}$ mice. In contrast to the defects in B-cell development, p85α$^{-/-}$ mice had no apparent defect in T-cell development in either CD8/CD4 expression profile in the thymus[13] or CD44/CD62L expression profile in splenic CD4$^+$ T cells (Figure S2), the latter of which reflects the spontaneous activation of T cells and the generation of memory-type cells during aging, indicating that p85α deficiency impairs mostly B-cell development without apparent defects in T-cell development.

B cell–intrinsic developmental defect in p85α$^{-/-}$ mice

Immature B cells that enter the spleen express high levels of IgM and very little IgD (IgM^{hi}IgD^{lo}), and undergo maturational progression through an IgM^{hi}IgD^{hi} stage to the most mature IgM^{lo}IgD^{hi} stage that is reportedly equivalent to the FO B-cell subset.[30] In accordance with the data shown in Figure 2C, the percentage of the most mature IgM^{lo}IgD^{hi} cells was severely decreased in p85α$^{-/-}$ mice on a C57BL/6 background (Figure 3A top panels). In

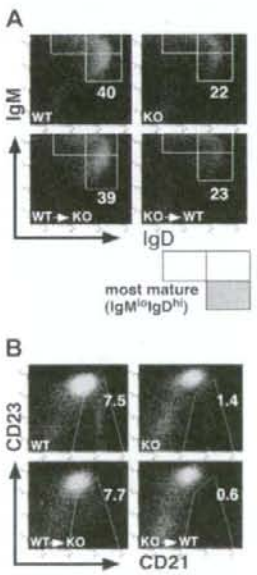

Figure 3. Impaired development of splenic B cells in p85α$^{-/-}$ mice is B-cell intrinsic. BM cells from the wild-type (p85α$^{+/+}$CD45.1$^+$) and p85α-deficient (p85α$^{-/-}$CD45.2$^+$) mice on a C57BL/6 background were transplanted into sublethally (3 Gy) irradiated PI3K-deficient (CD45.2$^+$) and wild-type (CD45.1$^+$) mice, respectively (bottom panels). Eight weeks after transplantation, splenocytes from recipient mice were stained with a combination of antibodies against B220, IgM, IgD, and CD45.2 (A) or B220, CD21, CD23, and CD45.2 (B), and analyzed on a FACSAria. Wild-type (WT) and p85α$^{-/-}$ (KO) mice were also analyzed (top panels). Among B220$^+$CD45.2$^+$ cells (for p85α$^{-/-}$ mouse–derived cells) or B220$^+$CD45.2$^-$ cells (for the wild-type mouse-derived cells), the percentages of the most mature IgM^{lo}IgD^{hi} subset (A) and CD21^{hi}CD23^{lo} MZ B cells (B) are indicated. Data are representative of 3 mice in each type of transplantation.

addition, expression profile of CD21 and CD23 in the $B220^+$ cell population, another criteria for MZ B cells, revealed that $p85\alpha^{-/-}$ mice had a reduced percentage of MZ B cells compared with wild-type mice (Figure 3B top panels). It is of interest to note that the amount of CD23 was higher in $p85\alpha^{-/-}$ B cells compared with wild-type B cells (Figure 3B), although the molecular mechanism for this increase remains to be determined.

Since PI3K is involved in various signal transduction pathways,[11,12] PI3K deficiency may affect a variety of cells including stroma cells that can influence B-cell development. To determine whether the defect of B-cell development in $p85\alpha^{-/-}$ mice is due to an intrinsic abnormality of the B cells, an abnormality of the splenic microenvironment, or a combination, we transplanted BM cells from wild-type ($CD45.1^+$ on a C57BL/6 background) mice into sublethally irradiated $p85\alpha^{-/-}$ ($CD45.2^+$ on a C57BL/6 background) mice and vice versa. We analyzed B-cell development 8 weeks after transplantation. $p85\alpha^{-/-}$ recipients that received wild-type $CD45.1^+$ BM cells showed normal B-cell development, whereas wild-type $CD45.1^+$ recipients that received $p85\alpha^{-/-}$ BM cells had reduced percentages of MZ B cells and mature $IgM^{lo}IgD^{hi}$ B cells as observed with $PI3K^{-/-}$ mice (Figure 3A,B bottom panels). These data demonstrate that the splenic microenvironment in $p85\alpha^{-/-}$ mice is able to support the development of $p85\alpha$-sufficient B cells, and that the defects in B-cell development observed in $p85\alpha^{-/-}$ mice are B-cell autonomous.

Requirement for PI3K in the expression of NF-κB proteins

Previous studies have shown that BCR-induced NF-κB activation is impaired in both $p85\alpha^{-/-}$ and $Btk^{-/-}$ B cells.[17-19] Since the differences in phenotypes between $p85\alpha^{-/-}$ and $Btk^{-/-}$ mice as well as our previous study[19] show that PI3K and Btk function independently, PI3K and Btk likely have distinct roles in the NF-κB activation. Btk is essential for PLCγ2 activation and sustained elevation in intracellular Ca^{2+} following BCR engagement.[15] PLCγ2 activation then triggers PKCβ activation,[16] followed by the activation of signaling cascade consisting of CARMA1,[31,32] Bcl10,[33] and paracaspase (also termed MALT1)[34] leading to the degradation of IκBs and the release of active NF-κB. Indeed, IκB degradation is impaired in $Btk^{-/-}$ B cells.[17,18] Since the molecular mechanism(s) underlying the impairment of BCR-induced NF-κB activation in $p85\alpha^{-/-}$ B cells remains obscure, we examined whether PI3K shares this signaling pathway with Btk. Consistent with the previous report,[35] BCR-induced Ca^{2+} mobilization in $p85\alpha^{-/-}$ B cells was nearly intact, albeit the peak intensity was slightly decreased (data not shown). ERK phosphorylation upon BCR stimulation, which is regulated downstream of PLCγ2 through the activation of the RasGRP3/Ras pathway,[36] was also induced in $p85\alpha^{-/-}$ B cells to a level comparable to wild-type B cells (Figure 4A). In addition, PI3K deficiency had little effect on the BCR-induced tyrosine phosphorylation of PLCγ2 and PKCβ-mediated Btk phosphorylation at Ser180[37] (Figure S3). Since PKCβ is another downstream effector of PLCγ2, these results taken together indicate that PLCγ2 activation in response to BCR stimulation is unaffected in $p85\alpha^{-/-}$ B cells. Considering that PKCβ plays a critical role in BCR-induced IκBα degradation,[16] we next examined whether PI3K deficiency affects this process. As shown in Figure 4B, BCR stimulation triggered the degradation of IκBα in $p85\alpha^{-/-}$ B cells in a time course similar to wild-type B cells.

The fact that PI3K deficiency has little effect on the signaling pathway leading to IκBα degradation, which is impaired in $Btk^{-/-}$ B cells,[17,18] raises the possibility that PI3K regulates BCR-induced

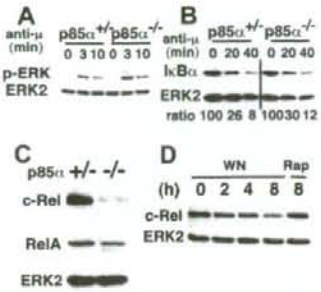

Figure 4. PI3K activity is essential for maintenance of c-Rel expression. (A) PI3K-independent activation of ERK. Splenic B cells obtained from $p85\alpha^{-/-}$ ($p85\alpha^{-/-}$) or control ($p85\alpha^{+/+}$) mice on a BALB/c background were stimulated with or without 20 μg/mL anti-IgM $F(ab')_2$ (anti-μ) at 37°C for the indicated times. BCR-induced activation was evaluated by immunoblotting with a specific antibody against phospho-ERK (p-ERK). The membrane was reblotted with an anti-ERK2 antibody (ERK2). (B) PI3K-independent degradation of IκBα. Splenic B cells were stimulated as in panel A. The degradation of IκBα was evaluated by immunoblotting with an anti-IκBα antibody. The membrane was reblotted with an anti-ERK2 antibody. IκBα levels were normalized by ERK2 levels and indicated as percentage relative to that of the unstimulated lysate (ratio). (C) The whole-cell lysates of splenic B cells obtained from $p85\alpha^{-/-}$ (−/−) or control (+/−) mice on a BALB/c background were subjected to immunoblot analysis with antibodies against c-Rel, RelA, and ERK2. (D) PI3K-dependent maintenance of c-Rel expression. Splenic B cells of C57BL/6 mice were cultured at 5×10^6 cells/mL in the presence or absence of either 100 nM wortmannin (WN) or 10 μg/mL rapamycin (Rap) for the indicated times, and evaluated for the expression level of c-Rel by immunoblotting with an anti-c-Rel antibody. Rapamycin, a potent inhibitor for mTOR, was chosen as a control since wortmannin is known to suppress mTOR activity besides PI3K. The membrane was reblotted with an anti-ERK2 antibody. Data in panels A-D are representative of 3 independent experiments with similar results.

NF-κB activation in a different way from Btk. Immunoblot analysis indeed revealed that c-Rel expression was markedly decreased in $p85\alpha^{-/-}$ B cells compared with wild-type B cells (Figure 4C top panel). In addition, a slight reduction of RelA expression was observed in $PI3K^{-/-}$ B cells as well (Figure 4C middle panel). To exclude the possibility that the decrease of c-Rel reflects the developmental defects of $p85\alpha^{-/-}$ B cells, we further compared c-Rel expression levels between $p85\alpha^{+/-}$ and $p85\alpha^{-/-}$ mature B cells ($B220^+AA4.1^-$) and found that c-Rel protein was severely reduced in PI3K-deficient mature B cells (Figure S4A). On the other hand, Btk deficiency had little effect on the expression of these NF-κB components (Figure S4B). As shown in Figure 4D, treatment of wild-type B cells with wortmannin, a potent inhibitor of PI3K, also results in the reduction of c-Rel expression, whereas the amounts of ERK2 were unaffected. Although wortmannin also inhibits mTOR downstream of PI3K, rapamycin, an inhibitor for mTOR, showed no effect on the expression of c-Rel (Figure 4D). These results indicate that the PI3K activity is essential for the maintenance of c-Rel expression in splenic B cells.

Restoration of B-cell defects in $p85\alpha^{-/-}$ mice by c-Rel expression

Considering that the decreased amounts of c-Rel may underlie the defects in B-cell development and the blockade of cell cycle progression upon BCR stimulation in $p85\alpha^{-/-}$ B cells, we examined whether the forced expression of c-Rel could overcome the defects due to the loss of PI3K. We used a lentivirus-mediated approach, in which we injected sublethally irradiated $Rag\text{-}2^{-/-}$ mice with $p85\alpha^{-/-}$ BM cells transfected with c-Rel or $p85\alpha$ as a positive control. We cloned genes of interest into a lentivirus vector[24] that contains Venus, a derivative of EGFP downstream of IRES, thus ensuring that Venus-positive cells also expressed

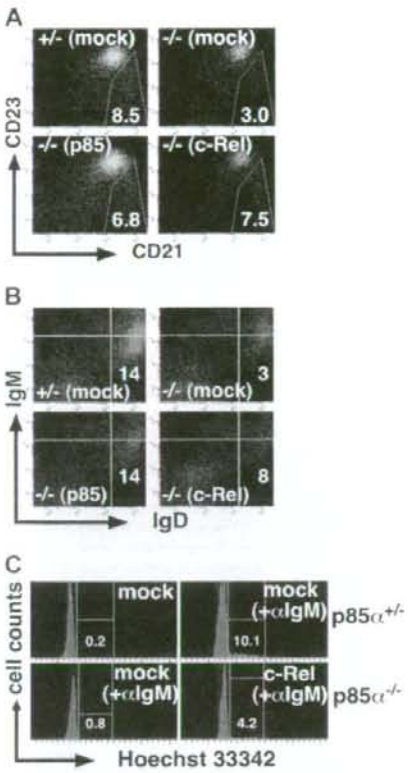

Figure 5. Forced expression of c-Rel partially rescues B-cell defects in p85α$^{-/-}$ mice. Recipient Rag-2$^{-/-}$ mice on a BALB/c background were reconstituted with BM cells from p85α$^{-/-}$ (−/−) or control (+/−) mice on a BALB/c background expressing either c-Rel (c-Rel), p85α (p85), or Venus alone (mock). Spleen cells were analyzed 10 weeks after transplantation. Among Venus$^+$B220$^+$ cells, the percentages of CD21^{hi}CD23^{lo} MZ B cells are shown (A). Among Venus$^+$ cells, the percentages of IgM^{lo}IgD^{hi} most mature B cells identified as the cells in the lower right quadrant are indicated (B). (C) Splenic B cells from p85α$^{-/-}$ (p85α$^{-/-}$) or control (p85α$^{+/-}$) mice on a BALB/c background were unstimulated or stimulated with anti-IgM F(ab')$_2$ (+ αIgM) and infected with lentivirus vector encoding c-Rel (c-Rel) or Venus alone (mock). Among Venus$^+$ cells, BCR-induced cell-cycle progression was evaluated on a FACSAria using Hoechst 33342 as an indicator for DNA content. Shown are percentages of cells in S + G2/M. Mice are all on a BALB/c background. Data are representative of 3 (A,B) or 5 (C) independent experiments with similar results.

lentivirally produced c-Rel or p85α. We analyzed splenocytes of reconstituted mice 10 weeks after transplantation. As shown in Figure 5A, p85α$^{-/-}$ cells reconstituted with p85α had MZ B cells (B220$^+$CD21^{hi}CD23^{lo}) to the level comparable with mock-infected wild-type cells, whereas control vector failed to restore the phenotype of p85α$^{-/-}$ cells, demonstrating the feasibility of this system. The introduction of c-Rel into p85α$^{-/-}$ BM cells also resulted in the restoration of MZ B cells to normal percentages, indicating that the developmental defect of p85α$^{-/-}$ MZ B cells is due to the reduced amount of c-Rel expression. On the other hand, c-Rel expression failed to fully restore p85α$^{-/-}$ B-cell maturation to IgM^{lo}IgD^{hi} cells (Figure 5B). It is likely that the overexpression of c-Rel alone is insufficient to fully compensate the lack of PI3K signaling pathway during B-cell development.

Since c-Rel is involved in cell-cycle progression,[38] it is possible that the defect in proliferation upon BCR stimulation in p85α$^{-/-}$ B cells is also attributed to the reduced amount of c-Rel expression. We thus investigated whether the forced expression of c-Rel can overcome the proliferative defect in p85α$^{-/-}$ B cells. It was technically difficult to sort out lentivirally transfected B cells with high purity on the basis of Venus expression because of the limited transfection efficiency into primary B cells. Therefore, we estimated the BCR-induced cell-cycle progression through flow cytometric analysis using Hoechst 33342. As indicated by the percentage of cells in S and G2/M phases of the cell cycle, mock virus–infected p85α$^{-/-}$ B cells failed to proliferate in response to BCR stimulation compared with the mock-infected wild-type B cells (Figure 5C). In contrast, the percentage of p85α$^{-/-}$ B cells undergoing cell cycling was markedly increased by the forced expression of c-Rel, although it was still lower than that of wild-type cells. We noted that uninfected cells (Venus$^-$ cells) failed to enter the S and G2/M phases (data not shown). It is well established that the BCR stimulation of mature B cells leads to activation and proliferation, whereas the same signals on developing B cells result in an arrest in an unresponsive state or cell death.[39,40] Therefore, the partial blockade of BCR-induced proliferation in p85α$^{-/-}$ B cells with lentivirally transfected c-Rel may reflect the reduced percentage of the most mature IgM^{lo}IgD^{hi} population (Figure 5B).

Discussion

Present observations that NF-κB activation is differentially regulated by PI3K and Btk and that p85α$^{-/-}$ mice and Btk$^{-/-}$ mice show different developmental defects support the conclusion of our previous study that PI3K and Btk independently function in BCR signal transduction.[19] The presence of *Xid* mutation has long been considered strong evidence for the hypothesis that Btk functions downstream of PI3K.[14,25] On the other hand, as shown here, both mouse and human Btk proteins with *Xid* mutation are unstable and degraded. As a result, BCR cross-linking is unable to transmit sufficient signals in *Xid* B cells. It is thus likely that the interaction of PIP_3 with the PH domain of Btk is dispensable for the BCR-induced activation of Btk, although PH domain does interact with PIP_3 as shown previously by fusion proteins between GFP and the PH domain of Btk.[41]

We have demonstrated here that p85α$^{-/-}$ mice exhibit impaired development of 2 major populations of mature splenic B cells: FO and MZ B cells in a cell-autonomous manner. We also found that PI3K deficiency leads to significant decreases in immature B-cell subsets in agreement with the observation by Donahue et al.[35] On the other hand, as previously reported, Btk-deficient mice had increased percentages of immature B-cell populations.[30] Consistent with these differences between p85α$^{-/-}$ and Btk$^{-/-}$ mice, PI3K deficiency affects B-cell activation in a way distinct from Btk deficiency: the former perturbs the expression of NF-κB components such as c-Rel, whereas the latter causes the impairment of BCR-induced IKK activation. Since the inhibition of PI3K by wortmannin also resulted in the loss of c-Rel expression in wild-type B cells, the PI3K-Akt signaling pathway at its basal activity seems essential for the maintenance of c-Rel expression. It is possible that the BCR in p85α$^{-/-}$ B cells fails to transmit a basal signal without apparent ligand binding, so-called tonic signaling, which is required for maintaining NF-κB expression.[42]

Our previous observation that p85α$^{-/-}$ B cells fail to induce the target genes of the NF-κB pathway, such as cyclin D2 and Bcl-xL, upon BCR stimulation[19,43] can be explained by the loss of c-Rel expression in p85α$^{-/-}$ B cells. However, the molecular mechanism underlying the reduction of c-Rel and/or RelA expression is yet to be determined. It has been shown that a lack of BCAP, an adaptor

protein functioning downstream of the BCR, leads to a reduction of c-Rel expression at the transcriptional level,[44] prompting us to examine *c-Rel* gene expression in p85α$^{-/-}$ B cells. Our preliminary experiments, however, suggest that the reduced amounts of c-Rel in p85α$^{-/-}$ B cells are likely due to perturbations in posttranscriptional regulation (data not shown). Consistent with this, there are several differences between p85α$^{-/-}$ B cells and BCAP$^{-/-}$ B cells. First, BCAP-deficient mice show a defect in B-cell development of the most mature IgM^{lo}IgD^{hi} subset but not of MZ B cells. Second, the forced expression of c-Rel in BCAP$^{-/-}$ BM cells corrects the developmental defect nearly completely, unlike in p85α$^{-/-}$ BM cells. BCR-induced Ca^{2+} flux is impaired in BCAP$^{-/-}$ cells, whereas p85α$^{-/-}$ B cells show only slight decrease in Ca^{2+} flux. Finally, BCAP$^{-/-}$ B cells exhibit BCR-induced PI3K activation as strong as wild-type B cells.

Reduced numbers of FO and MZ B cells in p85α$^{-/-}$ mice is likely due to the impairment of the NF-κB pathway. Accordingly, forced expression of c-Rel significantly, but not completely, restored the defect in B-cell development as well as the proliferative defect upon BCR stimulation in p85α$^{-/-}$ mice. One can argue that defective c-Rel protein stability in p85α$^{-/-}$ B cells may attenuate the effect of lentiviral c-Rel overexpression. Since PI3K deficiency also results in the reduction of RelA expression, incomplete restoration by c-Rel overexpression may also reflect the remaining defect caused by the reduced amounts of RelA. Alternatively, developmental defects in p85α$^{-/-}$ B cells could be ascribed, at least in part, to signaling defects other than the NF-κB pathway. For instance, the PI3K-Akt pathway is involved in the regulation of forkhead family transcription factors such as FOXO1[45] as well as cell motility in response to chemokines,[46] which play a critical role during B-cell development.

Class IA PI3K regulatory subunits function as a molecular chaperone to bind and stabilize their cognate catalytic subunits.[11,12] We have previously shown that the expression of p110δ, the most abundantly expressed catalytic subunit in lymphocytes, is nearly completely lost in p85α$^{-/-}$ B cells.[19] It has been shown, however, that a lack of p110δ activity results in reduced numbers of MZ B cells, but not of FO B cells.[12] Moreover, p110δ deficiency has, if any, only a marginal effect on LPS-induced B-cell proliferation, whereas p85α$^{-/-}$ B cells fail to respond to LPS.[11-13] It seems reasonable to assume that the lack of p85α affects the expression level of other catalytic subunits such as p110α, which could be more important for cell proliferation than p110δ. Consistently, in our preliminary experiment, the expression level of p110α in PI3K-deficient B cells was estimated as approximately 50% of that in wild-type B cells using an anti-p110α mAb (kindly provided by Dr L. T. Williams; data not shown). Interestingly, it has been reported by microinjecting neutralizing antibodies specific for p110α, p110β, and p110δ that in macrophages p110α, but not p110β or p110δ, is critical for cell proliferation and survival, whereas p110β and p110δ are important for cell migration.[47] The contribution of p110α to B-cell function, especially in LPS-induced proliferation, will be clarified in a future study using a conditional knockout strategy.

Given that the NF-κB signaling pathway plays a critical role in B-cell development, survival, and activation, a comprehensive understanding of the PI3K signaling essential for maintenance of c-Rel expression in B cells will provide the molecular basis for explaining immunologic disorders such as XLA-like common-variable immunodeficiency and B-cell lymphomas.

Acknowledgments

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Authorship

Contribution: S.M. designed and performed research and wrote the paper; Y.M., M.O., M.F., Y.H., and A.M. performed research; Y.T. and T.K. contributed vital new reagents or analytical tools; and S.K. designed research and wrote the paper.

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Critical role of dendritic cells in determining the T_h1/T_h2 balance upon *Leishmania major* infection

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Keywords: antigen-presenting cell, infectious disease, protozoan parasite, T_h

Abstract

The onset of T_h1 immunity is in part regulated by genetic background. To elucidate the cell type carrying critical factors determining the T_h1 response, we employed Rag-$2^{-/-}$ mice on *Leishmania major*-susceptible BALB/c and -resistant B10.D2 backgrounds. By using bone marrow (BM) chimeras generated by the transplantation of B10.D2 BM cells into BALB/c-Rag-$2^{-/-}$ mice, and *vice versa*, it was shown that hematopoietic cells carry factors determining the disease outcome and T_h1 response against *L. major* infection. B10.D2-Rag-$2^{-/-}$ mice reconstituted with BALB/c $CD4^+$ T cells exhibited a T_h1 response and controlled *L. major* infection. Wild-type BALB/c mice inoculated with *L. major*-parasitized B10.D2-Rag-$2^{-/-}$ splenocytes also exhibited a T_h1 response and a mild disease outcome, whereas such a T_h1 response was not induced when $CD11c^+$ dendritic cells (DCs) were depleted from parasitized B10.D2-Rag-$2^{-/-}$ splenocytes. T_h1 response was reconstituted by the addition of *L. major*-parasitized B10.D2 DCs but not *L. major*-parasitized BALB/c DCs to DC-depleted parasitized B10.D2-Rag-$2^{-/-}$ splenocytes. These results indicate that DCs determine the outcome of the disease upon *L. major* infection.

Introduction

The balance between two distinct $CD4^+$ T_h responses, namely T_h1 and T_h2 immune responses, is tightly correlated with the outcome of various diseases including tumor, autoimmune diseases and infectious diseases (1). Understanding the basis of the genetic control of T_h1/T_h2 differentiation is important for the development of vaccines and therapeutic strategies. The infection of mice with *Leishmania major*, a macrophage-tropic intracellular protozoan parasite, is widely employed as a model for the functional analyses of T_h1 and T_h2 responses upon microbial infection (2). After infection with *L. major*, most strains of wild-type (WT) mice exhibit a T_h1 immune response that is essential in controlling the intracellular micro-organism. In contrast, BALB/c mice exhibit a T_h2 response and succumb to *L. major* infection (2–4).

Previous studies have reported that a fraction of $CD4^+$ T cells recognizing a specific *Leishmania* antigen produce a large amount of IL-4 during the early phase of infection in naive BALB/c mice (5, 6). Such IL-4 production is considered to be the exacerbation factor of *L. major* infection by instructing naive T cells to elicit a T_h2 response in BALB/c mice (6–8). On the other hand, other reports have shown that B10.D2 mice also produce a large amount of IL-4 but are able to control *L. major* infection and BALB.B mice sharing the same MHC with C57BL/6 and C57BL/10 mice produce only a small amount of IL-4 yet succumb to infection (9, 10). In addition, BALB/c mice mount a T_h2 response upon infection with *L. major* lacking an immunodominant epitope that induces early IL-4 production from $CD4^+$ T cells (11). Furthermore, IL-4-deficient mice on a BALB/c background are still susceptible to *L. major* (12). These findings suggest that cells other than the specific subset of T cells producing IL-4 are crucial in determining the outcome of *L. major* infection in BALB/c mice.

To this end, we performed a series of experiments to determine the cell type carrying the genetic factors that

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controls the outcome of the disease upon *L. major* infection by using Rag-$2^{-/-}$ mice on *L. major*-susceptible BALB/c and -resistant B10.D2 backgrounds. Our results demonstrate that dendritic cells (DCs) play a critical role in determining the T_h1/T_h2 balance as well as the outcome of disease upon *L. major* infection.

Methods

Mice

WT BALB/c and B10.D2 mice were purchased from Japan SLC (Shizuoka, Japan) and Rag-$2^{-/-}$ mice on BALB/c (N12) and B10.D2 (N10) backgrounds (13, 14) were obtained from Taconic (Germantown, NY, USA). Mice were maintained in our specific pathogen-free facility and all experiments were performed in accordance with our Institutional Guidelines.

Antibodies

FITC-, PE- and biotin-labeled mAbs were purchased from BD PharMingen (San Diego, CA, USA). FITC-F4/80 and Red670-streptavidin were purchased from Caltag Laboratories (Burlingame, CA, USA) and GIBCO BRL (Grand Island, NY, USA), respectively. All magnetic bead-conjugated mAbs were purchased from Miltenyi Biotech (Sunnyvale, CA, USA).

Leishmania major *infection*

Leishmania major (MHOM/SU/73/5-ASKH) was maintained in BALB/c mice. Before experiments, parasites were obtained from the infection site on a left hind footpad and promastigotes were propagated at 26°C in Schneider's *Drosophila* medium (GIBCO BRL) containing 15% heat-inactivated FCS. Mice were subcutaneously injected with 5×10^6 of stationary-phase promastigotes at left hind footpads. In some experiments, mice were injected with 1×10^7 of promastigote-parasitized splenocytes from Rag-$2^{-/-}$ mice.

The severity of the disease was evaluated by the footpad swelling and parasite burdens in the infected footpads and, in some experiments, popliteal lymph nodes. The footpad thickness was measured with a vernier caliper and the swelling caused by infection was determined by subtracting the thickness of the uninfected right hind footpad from that of the infected left hind footpad. To determine parasite burdens, infected footpads and/or popliteal lymph nodes were homogenized with steel mesh and 5-fold serially diluted with 15% FCS-containing Schneider's *Drosophila* medium and incubated at 26°C for 14 days. Emerged promastigotes were monitored and the parasite burden was calculated by the last dilution of promastigotes emerged (15).

Cell preparation

To obtain bone marrow (BM) cells, cells were prepared aseptically from femora. After removing RBCs by treatment with ammonium chloride solution, BM cells (2×10^7 cells ml^{-1}) were washed and suspended in PBS. To obtain splenocytes, spleen was first digested by injection of collagenase D solution (Boehringer Mannheim, Indianapolis, IN, USA) with a 26-G needle. After 10-min incubation at 37°C, spleen was minced with forceps. After pipetting, cell suspension was filtrated with nylon mesh to remove connective tissues. To obtain naive $CD4^+$ T cells from splenocytes, B and $CD8^+$ T cells were initially removed by using anti-CD8 and anti-B220 magnetic bead-conjugated mAbs. $CD4^+$ T cells were then positively collected with anti-CD4-conjugated magnetic beads and AutoMACS (Miltenyi Biotech) in accordance with the manufacturer's protocols. Purity of $CD4^+$ T cells was >95% in all experiments. Splenic DCs were obtained by magnetic separation of $CD11c^+$ cells from Rag-$2^{-/-}$ splenocytes. To obtain bone marrow-derived dendritic cells (BMDCs), BM cells from Rag-$2^{-/-}$ mice were cultured in RPMI 1640 medium supplemented with 10% FCS, penicillin and streptomycin, 10 mM HEPES and 200 U ml^{-1} of granulocyte macrophage colony-stimulating factor (PeproTech, London, UK). Ten days after culture, non-adherent cells were collected and $CD11c^+$ DCs were positively collected by magnetic sorting with AutoMACS. Purity of $CD11c^+$ cells was >90%.

In vitro *parasitization*

To prepare parasitized cells, Rag-$2^{-/-}$ splenocytes or BMDCs were mixed with *L. major* promastigotes (cells:promastigotes = 3:1) and incubated for 2 h at 37°C with rotation, washed three times to exclude extracellular promastigotes and suspended in PBS. To prepare DC-removed parasitized Rag-$2^{-/-}$ splenocytes, parasitized cells were mixed with anti-CD11c magnetic beads and $CD11c^+$ DCs were removed with VarioMACS (Miltenyi Biotech) in accordance with the manufacturer's protocols. Over 95% of $CD11c^+$ cells were removed from parasitized Rag-$2^{-/-}$ splenocytes. In some experiments, DC-removed parasitized Rag-$2^{-/-}$ splenocytes were mixed with parasitized $CD11c^+$ DCs at a ratio of 4:1 (determining by the percentage of $CD11c^+$ cells in Rag-$2^{-/-}$ splenocytes). To confirm the rate of parasitization, amastigotes in parasitized cells were directly observed on thin-blood smear specimens by Giemsa staining 3 days after parasitization. The infection rates of BALB/c and B10.D2 cells were nearly the same through all the experiments (data not shown).

T_h1/T_h2 cytokine assay

To determine the T_h1/T_h2 balance in mice, 4.0×10^5 splenocytes (2.0×10^6 ml^{-1}) were stimulated with freeze-thaw killed leishmanial antigens. Two days after stimulation, culture supernatants were harvested and stored at −80°C until performing ELISA. All ELISAs were performed with OptEIA™ ELISA sets (BD PharMingen) in accordance with the manufacturer's protocols.

Statistics

Student's *t*-test was applied to the results of footpad swelling and Mann–Whitney's *U*-test was applied to the results of parasite burdens.

Results

Genetic background of BM cells determines the outcome of L. major *infection*

To examine whether the outcome of *Leishmania* infection is determined by hematopoietic or non-hematopoietic cells, we

employed BM chimeras using Rag-$2^{-/-}$ mice on *L. major*-susceptible BALB/c and -resistant B10.D2 backgrounds. Rag-$2^{-/-}$ mice were lethally irradiated and transplanted with BM cells from BALB/c or B10.D2 WT mice. Two months after the transplantation of BM cells when the proportion of peripheral leukocytes was nearly the same as WT mice, BM chimeras were infected with *L. major*. As shown in Fig. 1, the outcome of infection such as footpad swelling, parasite burden and T_h1/T_h2 balance were reflected on the genetic background of BM donor. B10.D2-Rag-$2^{-/-}$ mice transplanted with BALB/c BM cells were unable to control *L. major* infection as revealed by progressive footpad swelling and high parasite burden in infected footpads as compared with the mice transplanted with B10.D2 BM cells (Fig. 1A and B). Moreover, these chimeras exhibited a T_h2 response as shown by the production of a small amount of IFN-γ and a large amount of IL-4 from splenocytes by re-stimulation with *L. major* antigens (Fig. 1C). In contrast, the BM chimeras based on BALB/c-Rag-$2^{-/-}$ mice transplanted with B10.D2 BM cells efficiently controlled the infection (Fig. 1D and E) and exhibited a characteristic T_h1 response (Fig. 1F) as compared with the mice transplanted with BALB/c BM cells. These results indicate that BM cells carry genetic factors controlling *L. major* infection.

Genetic background of CD4⁺ T cells is minimally involved with the outcome

Next, to examine whether the outcome of *L. major* infection is determined by the genetic background of $CD4^+$ T cells (6), Rag-$2^{-/-}$ mice were reconstituted with $CD4^+$ T cells from naive B10.D2 or BALB/c WT mice and infected with *L. major*. B10.D2-Rag-$2^{-/-}$ mice reconstituted with $CD4^+$ T cells from either B10.D2 or BALB/c mice exhibited milder symptoms as compared with BALB/c-Rag-$2^{-/-}$ mice reconstituted with BALB/c $CD4^+$ T cells (Fig. 2). The ratios of IFN-γ and IL-4 produced by splenocytes were similar in B10.D2-Rag-$2^{-/-}$ mice reconstituted with B10.D2 and BALB/c $CD4^+$ T cells. On the other hand, such ratio was lower in BALB/c-Rag-$2^{-/-}$ mice reconstituted with BALB/c $CD4^+$ T cells. These results indicate that the outcome of disease is largely dependent upon the genetic background of recipient Rag-$2^{-/-}$ mice and, more specifically, non-T and -B cells among BM-derived cells.

Cells that first encounter parasites determine the outcome of infection

It has been suggested that the outcome of the disease upon infection with *L. major* is determined at an early phase of infection (7, 8). For example, the administration of cytokines or neutralizing antibodies against cytokines during the early phase of infection can drastically alter the disease outcome (7, 8). We therefore hypothesized that cells that initially encounter parasites determine the outcome of *L. major* infection. To examine the hypothesis, Rag-$2^{-/-}$ splenocytes containing macrophages and DCs were mixed with *L. major* promastigotes *in vitro* and parasitized cells were subcutaneously inoculated into the footpad of WT mice. Since macrophages are the major reservoir of *Leishmania* (2), leishmaniasis is induced by the inoculation of parasitized Rag-$2^{-/-}$ splenocytes.

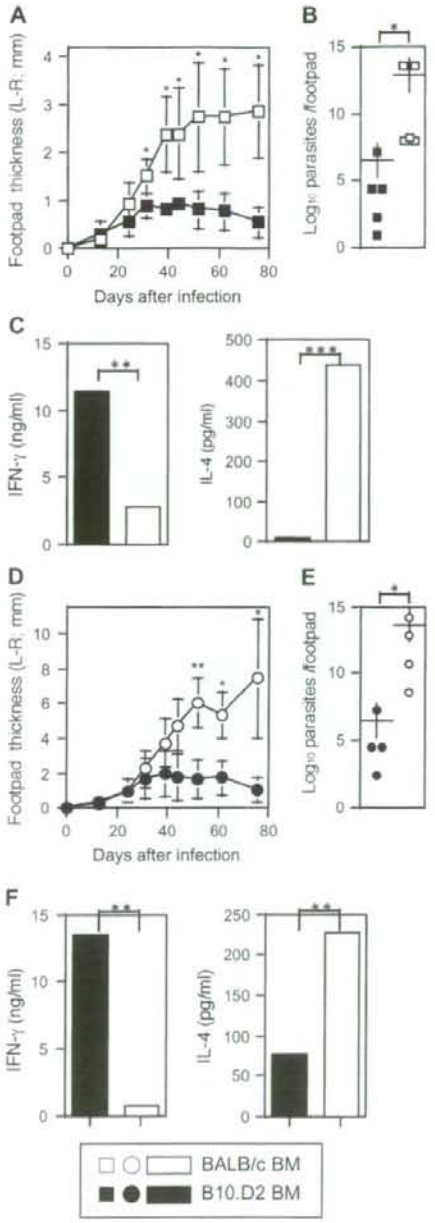

Fig. 1. The outcome of *Leishmania major* infection on BM chimeras is determined by the genetic background of BM cell donor. B10.D2-Rag-$2^{-/-}$ (A–C) and BALB/c-Rag-$2^{-/-}$ (D–F) mice were irradiated at 9 and 6 Gy, respectively, and reconstituted with 10^7 B10.D2-WT (closed symbols and bars) or BALB/c-WT BM (open symbols and bars) cells. Two months after transplantation, mice were administered with *L. major* promastigotes at left hind footpads. (A and D) Footpad swelling caused by *L. major* infection. (B and E) Parasite burdens in infected footpads. +, mean of each group. (C and F) IFN-γ and IL-4 production by splenocytes induced by parasite antigens. Statistical significance: $^*P < 0.01$, $^{**}P < 0.001$ and $^{***}P < 0.0001$.

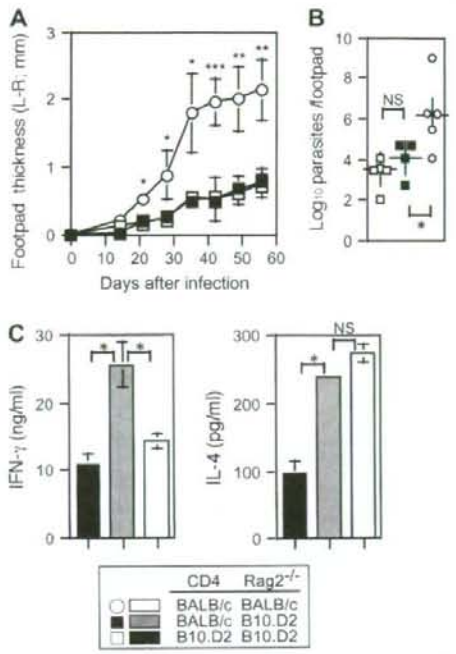

Fig. 2. The outcome of *Leishmania major* infection on Rag-$2^{-/-}$ mice reconstituted with $CD4^+$ T cells is determined by the genetic background of Rag-$2^{-/-}$ recipients. B10.D2-Rag-$2^{-/-}$ mice were reconstituted with 10^7 naive $CD4^+$ T cells collected from either B10.D2 (open squares and closed bars) or BALB/c (closed squares and gray bars). BALB/c-Rag-$2^{-/-}$ mice were reconstituted with BALB/c $CD4^+$ T cells (open circles and open bars). Three days after reconstitution, mice were administered with *L. major* promastigotes. (A) Footpad swelling. Statistical significance was observed in closed squares versus open circles. (B) Parasite burdens in infected footpads. +, mean of each group. (C) IFN-γ and IL-4 production from splenocytes of *L. major*-infected mice upon stimulation with parasite antigens. Although not shown, no $CD8^+$ T cells or B cells were detected in the spleens of Rag-$2^{-/-}$ mice reconstituted with $CD4^+$ T cells. Statistical significance: NS, not significant; $^*P < 0.01$, $^{**}P < 0.001$ and $^{***}P < 0.0001$.

As shown in Fig. 3(A–C), the outcome of disease was largely dependent on the genetic background of parasitized cells. Upon inoculation with parasitized B10.D2-Rag-$2^{-/-}$ splenocytes, both B10.D2 and BALB/c WT mice exhibited a milder symptom (Fig. 3A and B) and a T_h1-biased response (Fig. 3C) as compared with BALB/c WT mice inoculated with parasitized BALB/c-Rag-$2^{-/-}$ splenocytes. These results indicate that BM-derived non-T and -B cells in Rag-$2^{-/-}$ splenocytes that initially encounter

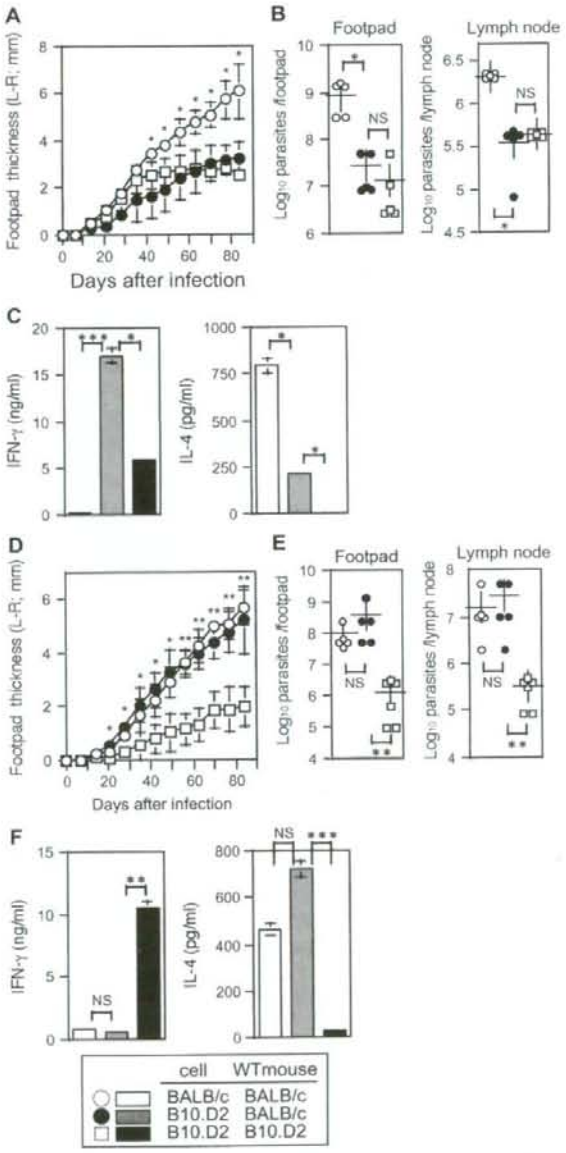

Fig. 3. (A–C) The genetic background of *Leishmania major*-parasitized Rag-$2^{-/-}$ splenocytes determines the outcome of *L. major* infection on WT BALB/c mice. BALB/c WT mice were inoculated with 10^7 parasitized BALB/c-Rag-$2^{-/-}$ (open circles and open bars) or B10.D2-Rag-$2^{-/-}$ (closed circles and gray bars) splenocytes. B10.D2 WT mice were inoculated with 10^7 parasitized B10.D2-Rag-$2^{-/-}$ splenocytes (open squares and closed bars). (D–F) The depletion of $CD11c^+$ DCs from *L. major*-parasitized Rag-$2^{-/-}$ splenocytes impairs the ability to determine the outcome of *L. major* infection. BALB/c WT mice were inoculated with 10^7 DC-depleted parasitized BALB/c-Rag-$2^{-/-}$ splenocytes (open circles and open bars) or DC-depleted parasitized B10.D2-Rag-$2^{-/-}$ splenocytes (closed circles and gray bars). B10.D2 WT mice were inoculated with 10^7 DC-depleted parasitized B10.D2-Rag-$2^{-/-}$ splenocytes (open squares and closed bars). (A and D) Footpad swelling caused by *L. major* infection. (B and E) Parasite burdens in footpads and popliteal lymph nodes of infected legs. +, mean of each group. (C and F) IFN-γ and IL-4 production from *L. major*-infected mouse splenocytes upon stimulation with parasite antigens. Statistical significance: NS, not significant; $^*P < 0.01$, $^{**}P < 0.001$ and $^{***}P < 0.0001$. Asterisks indicated in (A) are open circles versus closed circles and in (D) are closed circles versus open squares.