(anti-IL-4 mAb)-coupled beads as previously described (57). IL-5 was purified according to previously described procedures (36,56). PI-3 kinase inhibitor, LY 294002 (Calbiochem, San Diego, CA) and PKC inhibitors, Gö6976 and Gö6983 (Calbiochem), were dissolved in DMSO and further diluted with cell culture medium before use.

B cell culture

Splenic B cells were prepared using MACS column CS (Myltenyi, Stuttgart, Germany) by negatively selecting with anti-CD3–biotin, anti-Mac-1–biotin, and streptavidin–magnetic beads as previously described. This protocol yielded >95% pure population of B cells that were B220$^+$ CD3$^-$, as determined by flow cytometry. The B cells were cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY), supplemented with 8% FCS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, penicillin (50 U/ml) and streptomycin (50 μg/ml) in 96-well flat-bottom microtiter plates (Coaster, Pittsburgh, PA) at a concentration of 1×10^5 cells/well in a volume of 200 μl of medium with or without stimulants. To determine proliferative responses, cells were cultured in triplicate for 3 days and pulse-labeled with [^{3}H]thymidine (0.2 μCi/well; 1 μCi = 37 GBq, Amersham Life Science, Little Chalfont, UK) for the last 6 h of the culture. Incorporation of [^{3}H]thymidine was measured according to procedures previously described (38). Results were expressed as the arithmetic mean c.p.m. ± SD of triplicate cultures. To determine Ig secretion, splenic B cells were cultured at a density of 1×10^5 cells/well in 200 μl of media for 7 days as previously described (37). Anti-CD38 (1.0 μg/ml), anti-CD40 (1.0 μg/ml), IL-5 (100 U/ml), IL-4 (50 ng/ml) or a selected combination of those agents was added at the time the cells were plated. Cultures were set up in triplicate. The amounts of total IgM and IgG1 present in the culture supernatants were determined by ELISA. In some cases, cells were pretreated with LY294002 (10 μM), Gö6976 (300 nM) or Gö6983 (500 nM) for 1 h before adding anti-CD38 mAb (final concentration of DMSO did not exceed 0.1%). Each experiment was repeated at least 3 times, and one of the representative results is shown. For FACS analysis and preparation of RNA, B cells were cultured in a six-well plate at a density of 1×10^6 cells/ml.

Flow cytometry

For single-color flow cytometric analysis, cells were stained with FITC- or PE-conjugated mAb on ice for 30 min, and washed with PBS containing 2% FCS and 0.5% NaN_3. Stained cells were analyzed on a FACSCalibur instrument (Becton Dickinson, Mountain View, CA) equipped with CellQuest software. 7-Aminoactinomycin D (2 μg/ml) (Sigma) was used to exclude dead cells from the analysis. For each sample, at least 1×10^4 cells were collected and analyzed.

Nuclear protein extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts were prepared according to described procedures (58–60). In brief, Cells were disrupted for 10 min on ice by hypotonic lysis buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl and 1.5 mM $MgCl_2$. To extract their nuclear proteins, cells were suspended for 20 min on ice in extraction buffer (20 mM HEPES, pH 7.9, 420 mM KCl, 1.5 mM $MgCl_2$ and 25% glycerol) containing leupeptin (2 μg/ml), pepstatin (2 μg/ml), aprotinin (2 μg/ml), PMSF (0.5 mM), sodium orthovanadate (1 mM) and DTT (1 mM). After incubation, samples were centrifuged, and supernatants were collected as nuclear protein extracts and stored at –70°C. Protein concentration was determined by the Bradford method (Biorad, Hercules, CA) (61).

EMSA was carried out as previously described (58,59) in the following buffer: 10 mM HEPES pH 7.9, 50 mM sodium chloride, 1.5 mM EDTA, 5% glycerol and 0.1% NP-40. Each reaction mixture (25 μl) also contained 3 μg/ml of poly(dI:dC) (Amersham Pharmacia Biotech) and 4×10^4 c.p.m./2 μl of ^{32}P-end-labeled probe. The probe was the following: sense, 5′-AGCTTCAGAGGGGACTTTCCGAGAGG-3′; anti-sense, 5′-TCGACCTCTCGGAAAGTCCCCTCTGA-3′. For EMSA supershift experiments or for antibody inhibition experiments, optimal amounts of antibodies specific for each component of the NF-κB/Rel family of proteins were incubated with a mixture of nuclear protein extracts and poly(dI:dC) in DNA binding buffer at 4°C for 60 min before the addition of ^{32}P-labeled probe. Reaction mixtures were incubated at room temperature for another 30 min and the DNA–protein complexes were resolved on a native 4% polyacrylamide gel in 0.25× Tris borate–EDTA buffer for electrophoretic analysis at 150 V. Gels were dried and subjected to image analyzer (Fuji Photo Film, Tokyo, Japan). Antibodies for supershift or inhibition experiments against c-Rel, p50, p52, p65, RelB and normal rabbit serum IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Semi-quantitative RT-PCR analysis of germline γ1 transcripts

Total RNA was extracted from splenic B cells before or after culture (2 day culture for germline γ1 transcript assay) using TRIzol (Gibco/BRL, Gaithersburg, MD) according to the manufacturer's instructions. cDNA synthesis was carried out in 20-μl aliquots of reaction mixture containing 5 μg total RNA and oligo(dT) primer and Superscript II RNase H$^-$ reverse transcriptase (Gibco/BRL) as described previously (38). For semi-quantitation, serial dilutions of the cDNA templates were subjected to PCR amplification using the following primers: $I_\gamma 1$ and $C_\gamma 1R$ for the germline γ1 transcript and HPRT S1 and HPRT AS1, as described previously (38). PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

Western blot analysis

Cellular extracts of B cells were prepared as previously described (36) and subjected to SDS–PAGE (7.5% gel). For Akt immunoblotting, equal amounts of whole-cell lysates (equivalent to 4×10^5 cells/sample) were loaded onto each lane of a 7.5% SDS–PAGE gel. Proteins were transferred on a PVDF membrane (Amersham) that was blocked with 5% non-fat dry milk in TBST buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.05% Tween 20) for 1 h at room temperature. The membranes were further incubated with optimal concentrations of anti-phospho-Akt antibodies (1:1000) (New England Biolabs, Mississauga, Ontario, Canada). The membranes were then washed 3 times with TBST and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit IgG (PharMingen, San Diego, CA) in

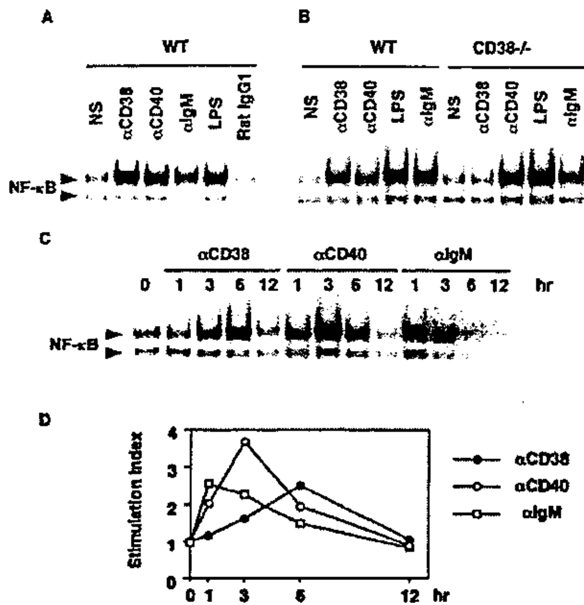

Fig. 1. Activation of NF-κB in splenic B cells by CD38 ligation. Splenic B cells (10×10^6 cells/stimuli) from C57BL/6 or BALB/c (A, C and D) and CD38$^{-/-}$ (B) mice were stimulated with either anti-CD38 (1.0 μg/ml), anti-CD40 (1.0 μg/ml), anti-IgM (10 μg/ml) or LPS (10 μg/ml) for 6 h and then lysed. Nuclear proteins (0.5 μg) prepared from each of the treatments were subjected to EMSA using a γ-^{32}P-radiolabeled DNA probe containing the NF-κB-binding site. (C and D) Kinetic analysis for NF-κB activation in anti-CD38-stimulated B cells. Arrows indicate probe–NF-κB complexes.

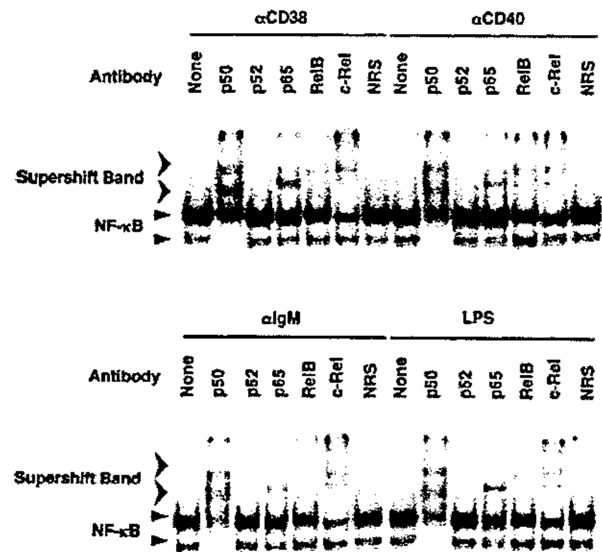

Fig. 2. NF-κB family members in B cells activated by CD38 ligation differ from those in CD40-activated B cells. Nuclear proteins (0.5 μg) prepared from each of the treatments were subjected to EMSA using a γ-^{32}P-radiolabeled DNA probe containing the NF-κB-binding site. Rabbit antibody (1 μg) against p50, p52, p65, RelB or c-Rel was added to a separate set of reactions for 1 h prior to EMSA. As a control, normal rabbit serum (NRS) was added. Smaller arrows indicate probe–NF-κB complexes and larger arrows indicate supershift complexes with anti-NF-κB antibodies.

TBST plus 5% BSA. The membrane was washed with TBST 3 times and immunoreactive bands were visualized by ECL detection (Amersham Pharmacia Biotech). The same membrane was re-probed with anti-Akt antibody (New England Biolabs) after removing anti-phospho-Akt.

Results

Activation of NF-κB upon stimulation of naive B cells with agonistic anti-CD38 antibody

It has been reported that treatment of murine splenic B cells with various mitogenic stimuli induces activation of NF-κB. Cross-linking of CD38 on the murine B cell surface by agonistic anti-CD38 (CS/2 clone) generates a proliferation signal and the increased expression of germline γ1 transcripts (35–39,42). However, it still remains unclear whether CD38 ligation of naive B cells activates NF-κB. To examine whether CD38 ligation activates NF-κB, we stimulated purified splenic B cells for various periods of time with anti-CD38. As controls, B cells were stimulated with anti-CD40, anti-IgM or LPS. Nuclear protein extracts of cells from each group of stimulation were prepared and subjected to EMSA using a NF-κB binding DNA probe. As can be seen in Fig. 1(A), anti-CD38 stimulation of wild-type B cells induced NF-κB activation at similar levels to that seen in the anti-CD40, anti-IgM or LPS stimulation. As expected, CD38$^{-/-}$ B cells did not show NF-κB activation upon anti-CD38 stimulation, but showed NF-κB activation similar to that of wild-type B cells in response to anti-CD40, anti-IgM and LPS stimulation (Fig. 1B). Kinetic analysis revealed that the NF-κB activation by anti-CD38 stimulation was observed within 3 h, peaked at 6 h and declined by 12 h. The kinetics of anti-CD38-induced NF-κB activation differed from that of anti-IgM and anti-CD40, which peaked at 1 and 3 h respectively (Fig. 1C and D). These results indicate that CD38 ligation on splenic B cells induces the NF-κB activation differently from that of CD40 and BCR stimulation.

To address which NF-κB/Rel proteins are activated by CD38 ligation, we carried out EMSA by using antibodies specific for each NF-κB/Rel family member. Nuclear extracts isolated from splenic B cells incubated with anti-CD38, anti-CD40, anti-IgM or LPS for 6 h were used for EMSA. Nuclear complexes induced by anti-CD38 were supershifted with antibodies specific for p50, p65 and c-Rel, but not p52 or RelB (Fig. 2). These supershifted patterns were similarly to those observed upon anti-IgM or LPS stimulation, indicating that CD38 ligation of splenic B cells induces p50, p65 and c-Rel activation like BCR cross-linking and LPS stimulation. Interestingly, anti-CD40 stimulation activated RelB in addition to p50, p65 and c-Rel (Fig. 2).

Requirement of Btk and downstream signaling molecules for CD38-induced activation of NF-κB

We have reported that the activation of Btk, Lyn and Fyn is involved in CD38-induced B cell activation (43,44). Prior studies have demonstrated that BCR-induced NF-κB activation in murine B cells is profoundly impaired in Btk-deficient (Btk$^{-/-}$) mice (15,16). However, a biochemical link between Btk

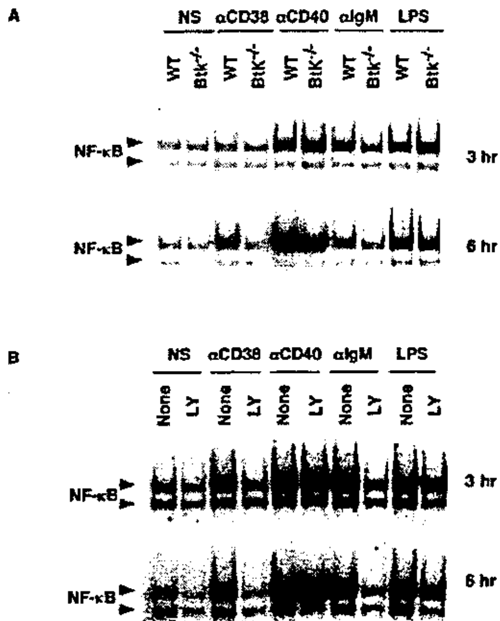

Fig. 3. (A) Impaired NF-κB activation of Btk$^{-/-}$ B cells in response to CD38 ligation. The γ-^{32}P-labeled probe containing an NF-κB-binding site was incubated with 0.5 μg of splenic B cell nuclear extract (10 × 10^6 cells/stimuli) from Btk$^{-/-}$ B cells (10 × 10^6 cells/stimuli) stimulated with anti-CD38 (1.0 μg/ml), anti-CD40 (1.0 μg/ml), anti-IgM (10 μg/ml) or LPS (10 μg/ml) for 3 and 6 h. EMSA was performed as above. The arrows indicate DNA-binding complexes. (B) Effect of PI-3 kinase inhibitor on anti-CD38-induced NF-κB activation. Splenic B cells (10 × 10^6 cells/stimuli) were pre-treated with PI-3 kinase inhibitor, LY294002 (10 μM) for 60 min at 37°C before addition of anti-CD38 (1.0 μg/ml), anti-CD40 (1.0 μg/ml), anti-IgM (10 μg/ml) or LPS (10 μg/ml). The B cells were incubated for another 3 or 6 h. EMSA was performed as above.

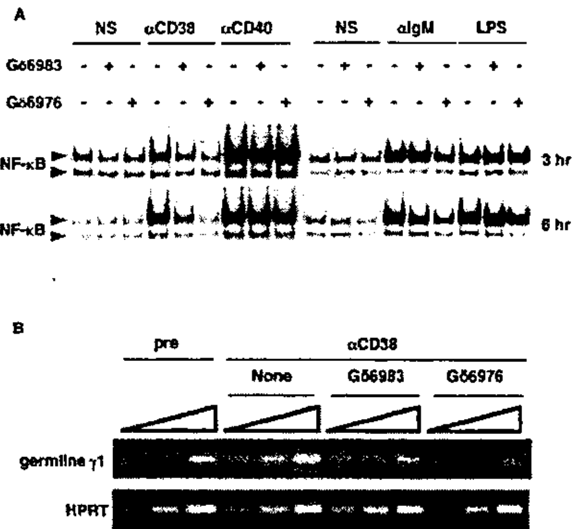

Fig. 4. Effect of PKC inhibitor on CD38-induced NF-κB activation. (A) Splenic B cells were pre-treated with PKC inhibitor Gö6976 or Gö6983 for 60 min at 37°C, and stimulated with anti-CD38 (1.0 μg/ml), anti-CD40 (1.0 μg/ml), anti-IgM (10 μg/ml) or LPS (10 μg/ml) for 3 and 6 h. EMSA was performed as above. (B) Semi-quantitative RT-PCR analysis of germline γ1 transcript expression in B cells activated by CD38 ligation. Splenic B cells (5 × 10^6 in a 5 ml culture) were pre-treated with PKC inhibitors as Fig. 4(A) and cultured in the presence of anti-CD38 (1.0 μg/ml) for 48 h. Total RNA was prepared from both pre-cultured and cultured cells, and cDNA was prepared. Serial dilutions (4-fold) of the cDNA templates were subjected to PCR analysis using a set of primers amplifying germline γ1 transcript. The HPRT gene was amplified in order to calibrate quantities of cDNA in each sample.

and NF-κB has not been well established. Splenic B cells from either wild-type or Btk$^{-/-}$ mice were exposed to various B cell stimuli that induce NF-κB activation. Nuclear extracts were prepared and analyzed for NF-κB DNA-binding activity by EMSA. As shown in Fig. 3(A), anti-CD38, anti-CD40, anti-IgM or LPS stimulation of splenic B cells led to a marked increase in NF-κB activity (Fig. 3A, *cf.* lanes 1 and 3, 5, 7 and 9). However, anti-CD38- and anti-IgM-mediated induction of NF-κB was negligible in Btk$^{-/-}$ B cells (Fig. 3A, lanes 4 and 8). Interestingly, anti-CD40 and LPS stimulation led to the activation of NF-κB (Fig. 3A, *cf.* lanes 5 versus 6 and 9 versus 10).

One of the critical downstream events following activation of Btk is the recruitment of PI-3 kinase to the plasma membrane. Activated Btk, together with Syk, phosphorylates and activates PLC-γ2 (10,11), resulting hydrolysis of phosphatidylinositol 4,5-bisphosphate and production of the second messengers inositol 1,4,5-triphosphate and diacylglycerol (12). These second messengers stimulate the activity of PKC and increase intracellular calcium levels, resulting in activation of downstream transcription factors (13,14). When splenic B cells were stimulated with anti-CD38, tyrosine phosphorylation of Btk was clearly evident (36). However, tyrosine phosphorylation of BLNK, PLC-γ2 or Vav was not observed in anti-CD38-stimulated B cells (data not shown). This was in sharp contrast to anti-IgM stimulation in which significant tyrosine phosphorylation of Vav, BLNK and PLC-γ2 was induced within 2 min (data not shown).

To investigate the relationship between PI-3 kinase and NF-κB in anti-CD38-induced B cell activation, we stimulated splenic B cells with anti-CD38 in the presence of the well-characterized PI-3 kinase-specific inhibitor LY294002 and monitored NF-κB activation. LY294002 abrogated the NF-κB activation induced by anti-CD38 and anti-IgM (Fig. 3B). This was not due to an increased rate of cell death because anti-CD38 stimulation maintained cell viability following treatment with LY294002. Interestingly, LY294002 showed little inhibition, if any, of NF-κB activation upon stimulation with anti-CD40 and LPS.

Another important protein kinase thought to be involved in NF-κB activation is PKC. To address whether PKC activation is involved in NF-κB activation, we added PKC inhibitors Gö6983 and Gö6976 to the B cell culture with various stimuli. Gö6983 and Gö6976 can inhibit the activity of PKC isoforms of α, β, γ, δ and ζ, and α, β and μ respectively, all of which are expressed in B cells. Our results revealed that both Gö6983 and Gö6976 inhibited NF-κB activation induced by anti-CD38, but not by anti-CD40 or LPS (Fig. 4). To elucidate the role of PKC on germline γ1 expression of splenic B cells activated by anti-CD38, we performed RT-PCR using B cells pre-treated with PKC inhibitors. Both Gö6983 and Gö6976 inhibited germline

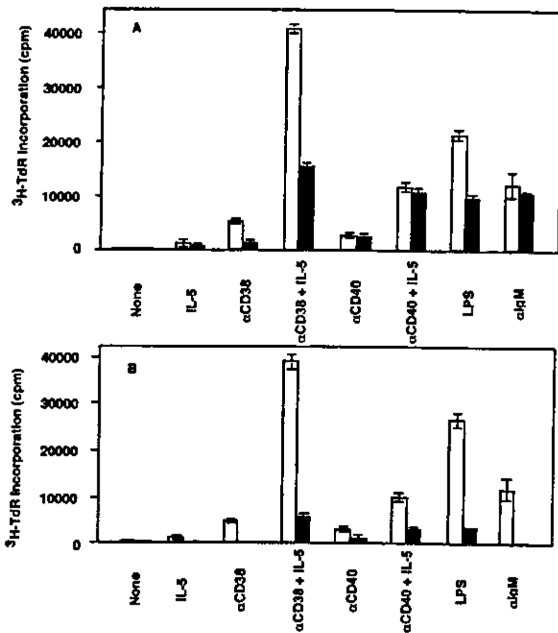

Fig. 5. Proliferative response of splenic B cells from p50$^{-/-}$ and c-Rel$^{-/-}$ mice in response to CD38 ligation. Splenic B cells from either 8-week-old wild-type littermate, p50$^{-/-}$ mice (A) and c-Rel$^{-/-}$ mice (B) were cultured (1 × 10^5 cells in a 200 μl culture) for 3 days with anti-CD38 (1.0 μg/ml), anti-CD40 (1.0 μg/ml), anti-IgM (10 μg/ml) or LPS (10 μg/ml). Cells were pulse-labeled with 0.2 μCi of [^{3}H]thymidine for the last 6 h of the culture. The results represent mean c.p.m. + SD of triplicate cultures. We tested four mice for each group.

γ1 transcript expression in response to anti-CD38 (Fig. 4B). These data indicate that PKC and NF-κB activation is involved in CD38-mediated germline γ1 transcript expression.

Proliferation in p50$^{-/-}$ and c-Rel$^{-/-}$ B cells in response to CD38 activation

There is numerous evidence that NF-κB/Rel family proteins play distinctive roles in cell growth and differentiation. To address whether NF-κB family proteins play a role in CD38-induced B cell activation, we stimulated splenic B cells from p50$^{-/-}$ and c-Rel$^{-/-}$ mice with various individual and combinations of stimuli including anti-CD38, LPS, IL-5 and anti-IgM for 72 h. B cell proliferation was monitored by [^{3}H]thymidine incorporation. As a control, splenic B cells of wild-type littermate mice were cultured separately. The proliferative response of p50$^{-/-}$ B cells to anti-CD38 and anti-CD38 plus IL-5 and LPS were significantly lower than that of wild-type B cells (Fig. 5A). The proliferation induced by anti-CD40 and anti-CD40. plus IL-5 was similar to that of wild-type B cells. In contrast, proliferative responses of c-Rel$^{-/-}$ B cells to various stimuli examined were all severely impaired to the same extent (Fig. 5B). These results indicate that c-Rel activation is indispensable for B cell proliferation in response to anti-CD38, anti-CD40, LPS and anti-IgM. In contrast, p50 activation is required for inducing maximum levels of B cell proliferation induced by anti-CD38 and LPS.

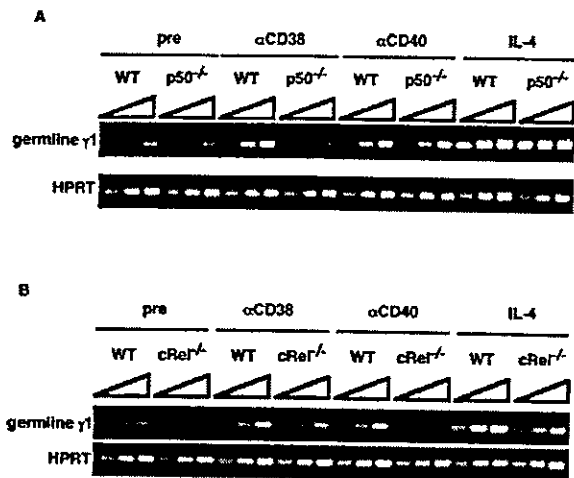

Fig. 6. Semi-quantitative RT-PCR analysis of germline γ1 transcript expression in B cells activated by CD38 ligation. Splenic B cells (5 × 10^6 in a 5 ml culture) from either wild-type littermate, p50$^{-/-}$ mice (A) or c-Rel$^{-/-}$ mice (B) were cultured in the presence of anti-CD38 (1.0 μg/ml), anti-CD40 (1.0 μg/ml) or IL-5 (50 ng/ml). Total RNA was prepared from both pre-cultured and cultured cells 48 h after plating, and cDNA was prepared. Serial dilutions (4-fold) of the cDNA templates were subjected to PCR analysis using sets of primers amplifying germline γ1 transcript. The HPRT gene was amplified in order to calibrate quantities of cDNA in each sample.

Role of NF-κB activation in the expression of germline γ1 transcripts

To elucidate a role of NF-κB activation in the expression of germline γ1 transcripts, we stimulated splenic B cells from wild-type, p50$^{-/-}$ or c-Rel$^{-/-}$ mice with anti-CD38, anti-CD40 and IL-4 for 2-days, and the expression of germline γ1 transcript was monitored by RT-PCR. In wild-type B cells, anti-CD38 stimulation induced a significant expression of the germline γ1 transcripts (Fig. 6A and B). However, in p50$^{-/-}$ B cells, the anti-CD38-induced expression of germline γ1 transcripts was significantly decreased (Fig. 6A). The stimulation of p50$^{-/-}$ B cells with anti-CD40 or IL-4 induced the expression of germline γ1 transcripts to a similar extent to that of wild-type B cells. In contrast, c-Rel$^{-/-}$ B cells showed markedly reduced expression of germline γ1 transcripts in response to various stimuli including anti-CD38 (Fig. 6B). In particular, anti-CD40-induced expression of γ1 transcripts was not induced in c-Rel$^{-/-}$ B cells. These results imply that activation of both p50 and c-Rel is indispensable for anti-CD38-induced germline γ1 transcript expression. Furthermore, c-Rel plays a more important role than p50/NF-κB in CD40-induced germline γ1 RNA expression.

IgM and IgG1 production by p50$^{-/-}$ and c-Rel$^{-/-}$ B cells stimulated with anti-CD38 and IL-5

We cultured splenic B cells from wild-type, p50$^{-/-}$ and c-Rel$^{-/-}$ mice with anti-CD38, anti-CD40, IL-4, IL-5 or combinations of these stimuli for 7 days after which the concentration of IgM and IgG1 in the culture supernatants were then measured. Stimulation of splenic B cells from wild-type mice with IL-5,

Table 1. IgM and IgG1 production by wild-type and p50$^{-/-}$ B cells in response to various B cell mitogen and cytokine[a]

Stimulant	IgM[b]		IgG1[b]	
	Wild-type	p50$^{-/-}$	Wild-type	p50$^{-/-}$
Medium	24 ± 3	42 ± 14	9 ± 3	14 ± 4
Anti-CD38	119 ± 13	129 ± 19	44 ± 27	38 ± 24
Anti-CD38 + IL-4	153 ± 20	157 ± 32	55 ± 14	56 ± 15
Anti-CD38 + IL-5	3855 ± 342	971 ± 219	1127 ± 392	128 ± 74
Anti-CD40	80 ± 10	28 ± 18	22 ± 7	17 ± 9
Anti-CD40 + IL-4	892 ± 92	253 ± 149	849 ± 204	58 ± 12
Anti-CD40 + IL-5	4347 ± 541	485 ± 68	1162 ± 185	89 ± 47

[a]Splenic B cells from either wild-type mice or p50$^{-/-}$ mice were cultured for 7 days with anti-CD38 (1.0 μg/ml), anti-CD40 (1.0 μg/ml) in conjunction with IL-5 (100 U/ml) or IL-4 (50 ng/ml). After the culture, IgM and IgG1 antibody secreted in cultured supernatants were titrated by ELISA.
[b]Results were expressed mean concentrations of IgM and IgG1 (ng/ml) ± SD. Representative results of a series of two different experiments are shown.

Table 2. IgM and IgG1 production by wild-type and c-Rel$^{-/-}$ B cells in response to various B cell mitogen and cytokine[a]

Stimulant	IgM[b]		IgG1[b]	
	Wild-type	c-Rel$^{-/-}$	Wild-type	c-Rel$^{-/-}$
Medium	20 ± 6	9 ± 6	26 ± 9	5 ± 3
Anti-CD38	105 ± 13	51 ± 23	58 ± 32	23 ± 4
Anti-CD38 + IL-4	147 ± 14	69 ± 24	85 ± 18	28 ± 7
Anti-CD38 + IL-5	4383 ± 120	193 ± 50	1035 ± 39	139 ± 68
Anti-CD40	127 ± 30	31 ± 2	77 ± 45	13 ± 6
Anti-CD40 + IL-4	1540 ± 201	612 ± 182	791 ± 88	84 ± 25
Anti-CD40 + IL-5	3087 ± 338	570 ± 58	1078 ± 167	34 ± 7

[a]Splenic B cells from either wild-type mice or c-Rel$^{-/-}$ mice were cultured for 7 days with anti-CD38 (1.0 μg/ml), anti-CD40 (1.0 μg/ml) in conjunction with IL-5 (100 U/ml) or IL-4 (50 ng/ml). After the culture, IgM and IgG1 antibody secreted in cultured supernatants were titrated by ELISA.
[b]Results were expressed mean concentrations of IgM and IgG1 (ng/ml) ± SD. Representative results of a series of two different experiments are shown.

anti-CD38 plus IL-5 and anti-CD40 plus IL-5 induced significant IgM production, while p50$^{-/-}$ and c-Rel$^{-/-}$ B cells showed impaired IgM production (p50$^{-/-}$ B cells in Table 1 and c-Rel$^{-/-}$ B cells in Table 2). Stimulation of the wild-type B cells with anti-CD38 plus IL-5 and anti-CD40 plus IL-5 induced significant IgG1 production (Tables 1 and 2).

Neither anti-CD38 plus IL-5 nor anti-CD40 plus IL-5 induced IgG1 production in splenic B cells from p50$^{-/-}$ mice (Table 1) and c-Rel$^{-/-}$ mice (Table 2). Stimulation of p50$^{-/-}$ and c-Rel$^{-/-}$ B cells with anti-CD40 plus IL-4 did not induce significant IgG1 production (data not shown). These results indicate that both p50 and c-Rel are indispensable for IgG1 production by B cells stimulated with either anti-CD38 or anti-CD40 in conjunction with IL-5 or IL-4.

Discussion

As we reported, anti-CD38 and IL-5 can induce a high level of IgM and IgG1 production in addition to promoting switching from IgM to IgG1 in naive (sIgD+) splenic B cells (37). In this system, ligation of splenic B cells with anti-CD38 induces the expression of germline γ1 transcripts and enhances the expression of the IL-5Rα (36,37). Furthermore, IL-5 stimulation of the anti-CD38-activated B cells induces μ–γ1 class switch recombination and IgM and IgG1 production (37–39). Accumulating data suggest that activation of NF-κB family proteins is indispensable for CSR. Thus, we examined the activation of NF-κB by anti-CD38 stimulation in murine B cells. The data presented in this paper demonstrated that stimulation of naive B cells with anti-CD38 alone activates NF-κB family proteins, resulting in the expression of germline γ1 transcripts.

CD38 ligation of splenic B cells activates NF-κB/Rel

Our studies with murine splenic B cells indicated that CD38 ligation induced the NF-κB activation (Figs 1 and 2). Analysis of B cells from mice deficient for CD38 or individual NF-κB family members demonstrated that like CD40- and BCR-mediated B cell activation and proliferation (62,63), NF-κB is important in CD38-mediated B cell activation and proliferation (Figs 1, 6 and 7). Both p50 and c-Rel together with p65 were activated in B cells by anti-CD38 ligation (Fig. 2). Kinetic analysis revealed that the time-course of p50 and c-Rel activation induced by anti-CD38 differed from anti-CD40 stimulation (Fig. 1), suggesting that anti-CD38 stimulation activates NF-κB differently from anti-CD40 stimulation. IL-5 by itself did not activate NF-κB family proteins (data not shown). While this is the first report which has highlighted a link between NF-κB activation and CD38 ligation of B cells, the underlying molecular mechanisms by which they are linked are still obscure. It would be important in future to examine whether the NF-κB activation is primary response or separate response requiring *de novo* protein synthesis.

Btk is essential for CD38-induced NF-κB activation

Btk is critical in early B cell development as well as in mature B cell activation and survival. Genetic defects in Btk cause human X-linked agammaglobulinemia, which is characterized by reduced numbers of peripheral B lymphocytes, low concentrations of serum Ig and varying degrees of bacterial infections. Likewise, *xid* mice, as well as Btk$^{-/-}$ mice, show impaired B cell development and function. NF-κB activation is one of the major downstream effects of Btk.

The NF-κB/Rel transcription factors play an important role in the expression of genes involved in B cell development, differentiation and function. Nuclear NF-κB is induced in B cells by engagement of either the BCR, CD40 cross-linking or stimulation with LPS. Two groups independently reported diminished IgM-, but not CD40-mediated NF-κB/Rel nuclear translocation and DNA binding in B cells from *xid* as well as Btk$^{-/-}$ mice (15,16). As shown in Fig. 3(A), NF-κB activation was severely impaired in Btk$^{-/-}$ B cells upon anti-CD38 or anti-IgM stimulation. Interestingly, in the absence of Btk, NF-κB was activated by CD40 ligation and LPS. These results are in agreement with Bajpai's report (15).

PI-3 kinase and PKC are involved in CD38-mediated NF-κB activation

The pleckstrin homology (PH) domain is the best characterized domain of Btk. Molecules binding to the PH domain include phosphoinositides, several isoforms of PKC, TFII-I transcription factor (also known as BAP-135), F-actin, STAT3 and Fas (64). Mutation of the conserved arginine residue at position 28 (R28) in the PH domain of Btk in X-linked agammaglobulinemia patients markedly reduces the affinity between Btk and phosphoinositides, and highlights the importance of this domain in Btk signal transduction.

Despite the crucial role of PI-3 kinase in cell growth and differentiation, the downstream signaling events following PI-3 kinase activation have only recently begun to be addressed. In B cells, the PH domain-containing kinases Btk and Akt are activated in response to BCR cross-linking in a PI-3 kinase-dependent manner (21,22). Using a specific pharmacological inhibitor of PI-3 kinase, LY294002, Bone and Williams (65) first demonstrated the vital role of PI-3 kinase in BCR-induced NF-κB DNA-binding activity. They also showed that PI3-kinase is critical in triggering NF-κB DNA-binding ability following LPS stimulation. Using the PI-3 kinase inhibitor LY294002, we also demonstrated that PI-3 kinase is required for anti-CD38- and anti-IgM-induced NF-κB activation (Fig. 3B). Due to a decrease in cell viability by LY294002, we could not examine its inhibitory effect on CD38-mediated germline γ1 transcript expression. Our findings indicate a critical role of PI-3 kinase in the activation of NF-κB following CD38 ligation and BCR cross-linking. This is the first report which has highlighted a link between PI-3 kinase and NF-κB activation following CD38 ligation of B cells, and may add supporting evidence for the importance of PI-3 kinase activation not only for BCR-stimulated B cells, but also for CD38-activated B cells.

A major downstream effector of PI-3 kinase, Akt, has recently been shown to be involved in NF-κB activation (66). Together with two recent reports showing a direct interaction between Akt and IKK-α in cell lines (24,25), PI-3 kinase was proposed to regulate IκB degradation in splenic B cells via Akt. We observed that Akt phosphorylation was induced by anti-CD38 stimulation and blocked by PI-3 kinase inhibitor LY294002 (data not shown). However, addition of an Akt inhibitor did not affect anti-CD38-induced NF-κB activation (data not shown). Our study, while establishing the signaling pathway responsible for CD38-mediated NF-κB/Rel activation involves PI-3 kinase, indicates it is unlikely that PI-3 kinase-dependent CD38- and BCR-induced activation of NF-κB occurs via an Akt-dependent route.

Among several PKC family members capable of binding to the PH domain of the Btk, Btk phosphorylates PKC-βI upon BCR stimulation. Phosphorylated PKC may, in turn, directly or indirectly down-regulate the kinase activity of Btk, as observed in the case of PKC-μ. PKC is necessary for BCR- but not anti-CD40-induced activation of NF-κB (67). Btk is in turn responsible for the activation of PKC and calcium mobilization, and the activation of NF-κB. We showed in Fig. 4 that specific PKC inhibitors Gö6983 and Gö6976 potently blocked CD38- and BCR-induced NF-κB activation. These results demonstrate a critical role of PKC in that process.

NF-κB activation is essential for the germline γ1 expression

In primary splenic B cells, BCR cross-linking or CD40 ligation leads to nuclear translocation and DNA binding by NF-κB/Rel transcription factors. Phenotypic analysis of mice deficient in individual NF-κB/Rel family members has demonstrated the essential role of these transcription factors in the CD40, LPS and BCR pathways leading to B cell proliferation. NF-κB/Rel proteins are important for initiating CSR to IgG1 in response to T-dependent antigens. CD40 engagement contributes to this preferential isotype production by activating NF-κB/Rel to induce germline γ1 transcripts, which are essential for CSR. While the CD40 signal transduction pathway leading to NF-κB activation has been well-characterized (8,68), there is no information about the molecular signaling events connecting the CD38 to NF-κB activation. It is quite important to clarify a link between NF-κB activation and germline γ1 expression in anti-CD38-stimulated naive B cells.

As we demonstrated in Fig. 2, both anti-CD38 and anti-CD40 stimulation can activate p50 in wild-type B cells. However, RT-PCR analysis of germline γ1 transcripts in $p50^{-/-}$ B cells revealed that anti-CD38-induced expression of germline γ1 transcripts was diminished, while anti-CD40-induced expression of germline γ1 transcripts was in the normal range (Fig. 6A). This may be due to kinetic differences or to the induction of RelB by anti-CD40, but not by anti-CD38. Interestingly, IgG1 production was profoundly impaired in response to anti-CD38 plus IL-5 and anti-CD40 plus IL-5 (Table 1). There are at least two possibilities to account for these observations. First, p50 may play a critical role in IL-5-induced CSR from C_μ to $C_\gamma 1$ in both CD38- and CD40-activated B cells. Alternatively, p50 may play an important role in terminal maturation of sIgG1 B cells to IgG1-producing cells. We do not have concrete evidence at this moment to support either one of the possibilities. RelB, which can be activated in response to anti-CD40 (Fig. 2), may compensate germline γ1 transcript expression in anti-CD40-stimulated $p50^{-/-}$ B cells. In $c\text{-Rel}^{-/-}$ B cells, both anti-CD38- and anti-CD40-induced expression of germline γ1 transcripts was severely impaired (Fig. 6B). We infer from these results that NF-κB activation is indispensable for the expression of germline γ1 transcripts in B cells stimulated with either anti-CD38 or anti-CD40. We recently found that BCR cross-linking and LPS were able to induce the expression of germline γ1 transcripts.

CD38-mediated signaling is supposed to resemble BCR-mediated signaling (30). In our preliminary experiments, anti-CD38 seems not to activate BLNK, PLC-γ2 and Vav, which are activated by anti-IgM under the same conditions (data not shown). These data suggest to us that a part of the CD38 signaling pathway may not be coupled with the BCR-mediated pathway. Therefore understanding the specific control of NF-κB activation could provide clues to the differential physiological outcomes of these B cell activation pathways.

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Abbreviations

Btk	Bruton's tyrosine kinase
CS/2	agonistic anti-CD38 mAb
CSR	class switch recombination
EMSA	electrophoretic mobility shift assay
IL-5Rα	IL-5 receptor α chain
LPS	lipopolysaccharide
PE	phycoerythrin
PH	pleckstrin homology
PI-3 kinase	phosphatidylinositol-3 kinase
PKC	protein kinase C
PLC	phospholipase C

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Development of Colitis in Signal Transducers and Activators of Transcription 6-Deficient T-Cell Receptor α-Deficient Mice

A Potential Role of Signal Transducers and Activators of Transcription 6-Independent Interleukin-4 Signaling for the Generation of Th2-Biased Pathological CD4$^+$ ββT Cells

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Forbidden CD4$^+$ββ T cells, which produce interleukin (IL)-4 predominantly, are a pathological subset in the development of colitis in T-cell receptor α chain (TCRα)-deficient mice. Stimulation of naive CD4$^+$ T cells with IL-4 induces Th2 development via the activation of signal transducers and activators of transcription (STAT) 6. In the present study, we had found that IL-4 enhanced the expression of STAT6 in CD4$^+$ββ T cells isolated from TCRα$^{-/-}$ mice with colitis, suggesting that the IL-4 signal in the CD4$^+$ββ T cells is mediated by STAT6. To further investigate the role of STAT6 in the development of colitis induced by TCRα deficiency, we generated double-deficient mice by crossing TCRα$^{-/-}$ mice and STAT6$^{-/-}$ mice. Surprisingly, STAT6 deficiency did not result in decreased severity of colitis in TCRα$^{-/-}$ mice. STAT6-deficient CD4$^+$ββ T cells produced IL-4 and intraperitoneal injection of anti-IL-4 monoclonal antibody in the nondiseased TCRα$^{-/-}$ and STAT6 double-deficient mice prevented the colitis formation, thus indicating that the cells differentiated into the Th2 phenotype have the ability to mediate the development of the colitis in the absence of STAT6. *(Am J Pathol 2003, 162:263–271)*

Human inflammatory bowel disease (IBD) consists of two distinctive types, ulcerative colitis and Crohn's disease, on the basis of clinical and pathological features.[1–3] Distinct cytokine imbalance is considered important for the induction of these two types of disease.[4–6] Th1 cytokine [interferon-γ, interleukin (IL)-2, and tumor necrosis factor-α] seems to be predominant in Crohn's disease, whereas Th2 cytokine (IL-4, IL-5, and IL-6) tends to be associated with ulcerative colitis.[7–9]

Colitis spontaneously develops in various gene-manipulated murine models: T-cell receptor α chain-deficient (TCRα$^{-/-}$) mice, IL-2-deficient mice, IL-7-transgenic mice, IL-10-deficient mice, macrophage-specific signal transducers and activators of transcription 3 (STAT3)-deficient mice, $G\alpha_{i2}$-deficient mice, and severe combined immunodeficient mice restored with CD45RB^{hi}CD4$^+$ T cells.[3,10–16] Most of these rodent IBD models exhibit disruption of T-cell regulatory networks, with cytokine imbalance of pathogenic CD4$^+$ T cells predominantly shifted to the Th1 type. In contrast, the Th2 pathway is a major causative agent for the development of colitis in TCRα$^{-/-}$ mice,[17–20] hapten-induced murine colitis,[21] and oxazolone-induced colitis.[22]

TCRα$^{-/-}$ mice spontaneously develop chronic colitis under specific pathogen-free conditions at ~10 weeks of age.[18] We have previously shown that CD4$^+$ββ T cells,

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Table 1. Histological Scoring System (Colon)

Histological changes	Score 0	1	2	3
Loss of goblet cells	None	A few	Numerous and focal	Numerous and diffuse
Crypt abscesses	0	1–4	>5	-
Epithelial erosion	None	Focal	Diffuse	-
Hyperemia	None	A few	Numerous and focal	Numerous and diffuse
Cellular infiltration in the lamina propria	None	A few	Numerous and focal	Numerous and diffuse
Thickness of colonic mucosa	<100%	101–140%	141–180%	>181%
Increase of the colonic gland	None	A few	Numerous and focal	Numerous and diffuse

which produce predominantly IL-4, are increased in the colons of TCR$\alpha^{-/-}$ mice with the disease.[17,18,23] Furthermore, administration of a monoclonal antibody (mAb) against IL-4 suppressed the onset of IBD in TCR$\alpha^{-/-}$ mice.[19] It has been reported also that TCR$\alpha^{-/-}$ × IL-4$^{-/-}$ double-mutant mice have a decreased propensity to develop IBD.[20] Thus, Th2-driven, IL-4-producing CD4$^{+}\beta\beta$ T cells seem to be a fundamental pathological element for the induction of colitis in TCR$\alpha^{-/-}$ mice.

STAT6 are members of the STAT family of proteins, which have been cloned and characterized as IL-4-activated transcription factors.[24,25] The binding of IL-4 to its receptor leads to the tyrosine phosphorylation of STAT6 by JAK1 and JAK3.[26] The phosphorylated STAT6 then forms homodimers, translocates to the nucleus, and binds to the promoter regions of IL-4-responsive genes to initiate the gene expression.[26] From analysis of mice lacking the *STAT6* gene it has been concluded that the signal transducer is essential for the biological functions of IL-4, including the development of Th2 cells from naive CD4^{+} T cells, the class switching of B cells to the production of IgE and IgG1, induction of antigen-dependent airway hyperresponsiveness, and IL-4-mediated up-regulation of cell-surface molecules such as MHC class II and CD23.[24,25] These observations indicate that STAT6 is an essential signal transduction molecule for IL-4 signaling in T lymphocytes.[24,25]

To investigate the role of STAT6 in the development of IBD mediated by Th2-type pathological CD4$^{+}\beta\beta$ T cells, we crossed TCR$\alpha^{-/-}$ mice with STAT6$^{-/-}$ mice. Surprisingly, we found that the TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice spontaneously developed IBD, with characteristic clinical and histopathological abnormalities. STAT6-deficient CD4$^{+}\beta\beta$ T cells isolated from the diseased double-knockout mice retained the ability to produce IL-4, suggesting that the cells possessed the typical Th2-type phenotype. In addition, anti-IL-4 antibody (Ab) treatment in double-deficient mice prevented the development of colitis indicating that the STAT6-independent IL-4-signaling pathway is critical for the pathogenesis.

Materials and Methods

Mice

TCR$\alpha^{-/-}$ mice, with a background of C57Bl/6, were obtained from the Jackson Laboratory (Bar Harbor, ME). TCRα and STAT6 double-knockout mice were generated by backcrossing TCR$\alpha^{-/-}$ mice into STAT6$^{-/-}$ mice with a background of C57Bl/6.[25] The animals were maintained in the Experimental Animal Facility at the Research Institute for Microbial Diseases, Osaka University, under specific pathogen-free conditions and given sterilized food and autoclaved distilled water *ad libitum*. To determine the genotype of the mice, polymerase chain reaction (PCR) analysis was performed by using tail DNA.[25,27]

Anti-IL-4 mAb Treatment

In this study, a standard protocol was used for mAb *in vivo* treatment.[18,19] TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice from the beginning of 4 weeks to 10 weeks of age were intraperitoneally injected with rat anti-mouse IL-4 mAb (1 mg/mouse) prepared from hybridoma (11B11; American Type Culture Collection, Manassas, VA) or rat IgG1 (R3-34, 1 mg/mouse; PharMingen, San Diego, CA) as mock Ab in 100 μl of phosphate-buffered saline (PBS) twice a week. These treatments did not induce any signs of serum sickness.

Histopathological Analysis

For the assessment of the severity of IBD, the colons of TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice and TCR$\alpha^{-/-}$ mice were examined histologically. Tissue samples obtained from the proximal, middle, and distal colon were fixed in 4% paraformaldehyde in PBS for 4 hours, embedded in paraffin, and sectioned at a thickness of 6 μm.[19] The tissue sections were stained with hematoxylin and eosin. The histopathological score shown in Table 1 was used for the assessment of the severity of the colitis. It is a modification of the scoring system described previously.[21,28] The scoring was performed blindly by two independent investigators (YO and YK). The thickness of the colonic mucosa was determined to compare with the mucosal layer of the equivalent colonic segment in normal C57Bl/6 mice using a micrometer. The total histological score represents the sum of each item score.

Preparation of Cell Suspensions

Mice anesthetized with ketamine (Sigma Chemical Co., St. Louis, MO) were sacrificed at 10 weeks age. The spleens and mesenteric lymph nodes (MLNs) were aseptically removed, and single-cell suspensions were pre-

pared by a standard mechanical procedure.[18,29,30] Mononuclear cells from the lamina propria (LP) of the colon were dissociated using type IV collagenase (Sigma) to obtain single-cell preparations as described.[18,29,30]

Flow Cytometric Analysis and Cell Sorting

For analysis of the distribution of CD4$^+\beta\beta$ T cells by flow cytometry, single-cell suspensions of the mononuclear cells (10^6/sample) prepared from various tissues were stained with optimal concentrations of phycoerythrin-conjugated anti-CD4 mAb (L3T4) and fluorescein isothiocyanate (FITC)-conjugated anti-TCR-β mAb (H57-597). The samples were then subjected to flow cytometric analysis by using a FACScan (Becton Dickinson, Mountain View, CA). Data were analyzed by using CellQuest software (Becton Dickinson). For the analysis of the cytokine mRNA expression by the CD4$^+\beta\beta$ T cells, the cells were purified by FACS Vantage (Becton Dickinson) as described previously.[19]

Quantitative Reverse-Transcriptase-PCR

To analyze the cytokine-specific mRNA expression by CD4$^+\beta\beta$ T cells isolated from colonic LP of the diseased TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice, a highly sensitive, quantitative RT-PCR was performed.[31–33] Total RNA was isolated from fluorescence-activated cell sorting-purified CD4$^+\beta\beta$ T cells by using TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA was reverse-transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen), RNase inhibitor (Toyobo, Tokyo, Japan), oligo(dT)12-18 primer (Invitrogen), and dNTPs (Amersham Pharmacia Biotech, Arlington Heights, IL). The mixture was incubated at 42°C for 120 minutes and heated to 90°C for 5 minutes.[34] After treatment with RNase H (Toyobo), the synthesized cDNA was extracted by phenol/chloroform. Then, the cytokine-specific cDNA was quantified with LightCycler (Roche Diagnostics, Mannheim, Germany) technology by using LightCycler-DNA Master Hybridization Probes (Roche Diagnostics). For the amplification of cDNA, 20 μl of PCR mix was added to each tube to give a final concentration of 0.05 μmol/L 5′ primer, 0.05 μmol/L 3′ primer, 0.2 μmol/L FITC-labeled probe, 0.2 μmol/L LightCycler Red 640-labeled probe, 2 mmol/L $MgCl_2$, and 1× LightCycler-DNA master hybridization probes mix (Roche Diagnostics). The oligoprimers specific for the IL-4 (sense, 5′-ATGGGTCTCAACCCCCAGCTAGT-3′; anti-sense, 5′-GCTCTTTAGGCTTTCCAGGAAGTC-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense, 5′-TTCACCACCATGGAGAAGGC-3′; anti-sense, 5′-GGCATGGACTGTGGTCATGA −3′) were used.[35,36] For detection of the target molecule, FITC-labeled hybrid probe and LightCycler Red 640 (LCR)-labeled hybrid probe to IL-4 (FITC, 5′-CGTTTGGCACATCCATCTCCGT-3′; LCR, 5′-CATGGCGTCCCTTCTCCTGTG −3′), and GAPDH (FITC, 5′-TGGGTGTGAACCACCAGAAATATGAC-3′; LCR, 5′-ACTCACTCAAGATTGTCAGCAATGCA-3′) were prepared according to instructions provided by the manufacturer. After heating at 94°C for 2 minutes, cDNA were amplified for 40 cycles, each cycle consisting of 95°C for 10 seconds, 55°C for 30 seconds, and 72°C for 30 seconds.[33] Once during the cycle in which the log-linear signal could be distinguished from background, it was possible to compare the target concentrations (external standard) in samples with an internal standard in the same samples.[33] After the PCR had been completed, the LightCycler software (Roche Diagnostics) automatically converted the raw data into copies of target molecules. In this study, the relative quantitative expression of cytokine-specific mRNA in each sample was expressed as the amount of cytokine mRNA divided by the amount of mRNA GAPDH.[37]

Cytokine Enzyme-Linked Immunosorbent Assay

Purified CD4$^+$ T cells were co-cultured with mAb anti-CD28 (2 μg/ml, 37.51; PharMingen) in anti-CD3 mAb (10 μg/ml, 145-2C11; PharMingen) precoated 96-well tissue culture plates (5 × 10^5 cells/well) for 60 hours.[38] Culture supernatants were then harvested for analysis of cytokine production by cytokine enzyme-linked immunosorbent assay kit (Amersham Pharmacia Biotech) for IL-4.

Western Blotting Analysis for the Expression of Phosphorylated STAT6

Purified CD4$^+$ T cells isolated from MLNs of wild-type C57Bl/6 mice and TCR$\alpha^{-/-}$ mice were stimulated *in vitro* with IL-4 (20 ng/ml) for 15 minutes, then lysed in cell lysis buffer (5% sodium dodecyl sulfate, 0.5 mol/L Tris-HCl, pH 6.8, 0.5 mol/L ethylenediaminetetraacetic acid, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethyl sulfonyl fluoride, and 10 μg leupeptin) for 30 minutes at 4°C, and centrifuged at 10,000 rpm for 30 minutes. The supernatants were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with anti-phospho STAT6Ab (Phospho-STAT Antibody Sampler; Cell Signaling, Beverly, MA) and anti-STAT6Ab (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was finally developed by use of an enhanced chemiluminescence kit (ECL plus; Amersham Pharmacia Biotech).

Statistical Analysis

Data were statistically analyzed by Student's two-tailed t-test or Pearson's correlation coefficient test with $P < 0.05$ considered statistically significant.

Results

STAT6 Mediated the IL-4 Signaling in CD4$^+\beta\beta$T Cells

It has been shown that IL-4-mediated differentiation of naive CD4$^+$ T cells into Th2 cells is dependent on STAT6.[24,25] Thus, it was important to examine whether the STAT6-mediated signaling pathway was involved in the activation of IL-4-producing pathological CD4$^+\beta\beta$ T

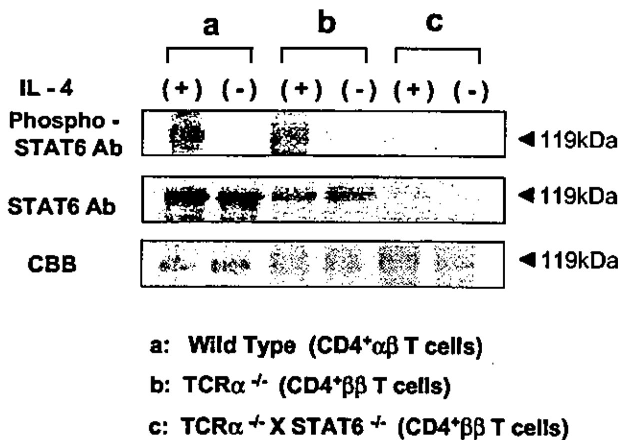

Figure 1. Expression of phosphorylated STAT6 in CD4$^+\beta\beta$ T cells from TCR$\alpha^{-/-}$ mice and CD4$^+\alpha\beta$ T cells from wild-type C57Bl/6 mice. MACS-purified CD4$^+$ T cells from MLNs were stimulated *in vitro* with recombinant IL-4 (20 ng/ml) for 15 minutes, and then phosphorylated STAT6 protein was examined by Western blotting analysis. **Top**, Anti-phospho STAT6 Ab; **middle**, anti-STAT6 Ab; **bottom**, Coomassie brilliant blue staining for the confirmation of that equivalent amounts of cellular protein were present. The data are representative from one of three independent experiments.

cells clonally expanded in the diseased TCR$\alpha^{-/-}$ mice. According to the method described previously,[18,19,23] the CD4$^+\beta\beta$ T cell-enriched fraction was prepared by MACS separation. Western blotting analysis revealed that phosphorylated STAT6 was induced in MLN-derived $\beta\beta$ T cell-enriched CD4$^+$ T cells after stimulation with IL-4, thus indicating that STAT6 was involved in the IL-4 signaling event in the cells (Figure 1).

TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ Mice Developed Colitis

To assess the role of STAT6 in the development of colitis in the TCRα-deficient mice, we generated TCRα- and STAT6-double-mutant (TCR$\alpha^{-/-}$ × STAT6$^{-/-}$) mice. The expression of STAT6 protein was not detected in CD4$^+\beta\beta$ T cells isolated from MLNs of TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice (Figure 1). Unexpectedly, the TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice, like TCR$\alpha^{-/-}$ mice, lost significant body weight from 11 weeks of age when compared with C57Bl/6 wild-type mice and STAT6$^{-/-}$ mice (Figure 2). The average body weight at the end of observation was: wild-type mice, 26.42 ± 0.62 g; STAT6$^{-/-}$ mice, 26.19 ± 0.62 g; TCR$\alpha^{-/-}$ mice, 21.17 ± 0.93 g; and TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice, 20.63 ± 0.73 g. TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice also displayed the physical changes typical of those in TCRα-deficient mice with colitis, ie, hunched posture, anorectal prolapse, and diarrhea. Some TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice had died after 8 to 10 weeks of age, and all were dead by 15 weeks. Histologically, colons of the TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice at age 10 weeks were thickened, eroded, and infiltrated with inflammatory mononuclear cells, just as in TCR$\alpha^{-/-}$ mice with IBD (Figure 3A). In contrast, the colons of STAT6$^{-/-}$ mice and wild-type mice had no evident inflammatory changes. We further compared the degree of histopathological changes present in the colonic tissues of TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice with those in other mice by

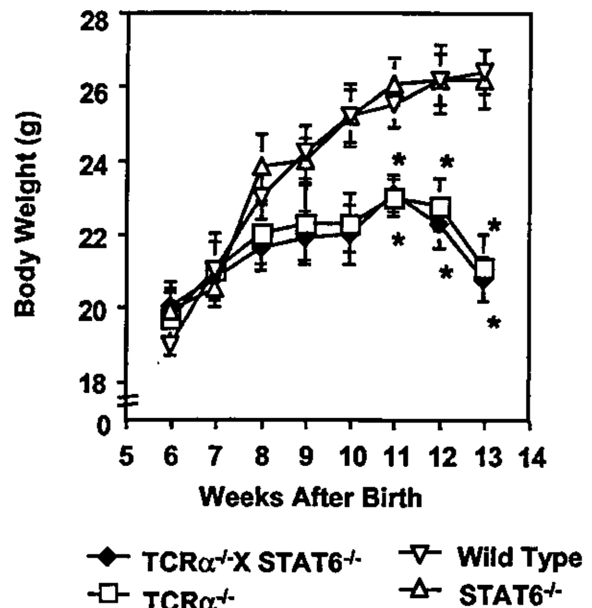

Figure 2. Decreased body weight in TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice and TCR$\alpha^{-/-}$ mice but not in STAT6$^{-/-}$ mice or wild-type C57Bl/6 mice. Body weight of the mice was measured weekly for 14 weeks. All of the mice used in this study have the same inbred C57Bl/6 background. Statistical analysis was performed using Student's two-tailed *t*-tests to compare TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice, TCR$\alpha^{-/-}$ mice, and STAT6$^{-/-}$ mice with C57Bl/6 mice. The results are expressed as mean ± SEM of three independent experiments (10 mice/group). *, $P < 0.05$.

using the histological scoring system as described (Table 1).[21,28] The scores in TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice (8.7 ± 1.1) were comparable to those in TCR$\alpha^{-/-}$ mice with colitis (9.4 ± 0.5) (Figure 3B), whereas the scores in C57Bl/6 wild-type and STAT6$^{-/-}$ mice were both 0.

STAT6-Deficient CD4$^+\beta\beta$T Cells Were Present in TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ Mice with Colitis

Because CD4$^+\beta\beta$ T cells reportedly are crucial for the induction of IBD in TCR$\alpha^{-/-}$ mice,[18,23,39] we next assessed the presence of this unique subset of T cells in TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice with IBD. When mononuclear cells isolated from the colonic LP and MLNs of the TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice with IBD were examined for the subset of CD4$^+\beta\beta$ T cells, increased numbers of the cells were present (MLN, 1.89 ± 0.41%; colonic LP, 19.24 ± 3.90%). The degree of increase was similar to those in TCR$\alpha^{-/-}$ mice with IBD [MLN, 1.67 ± 0.44%; colonic lamina propria lymphocytes (LPLs), 19.86 ± 2.22%] (Figure 4, A and B).

It has been reported also that the severity of IBD in TCR$\alpha^{-/-}$ mice correlates with the increase of colonic CD4$^+\beta\beta$ T cells.[18] We observed a similar and significant correlation in the TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice with IBD when the correlation coefficient between histopathological changes and increase of CD4$^+\beta\beta$ T cells was calculated ($r = 0.85$, $P < 0.05$) (Figure 4C).

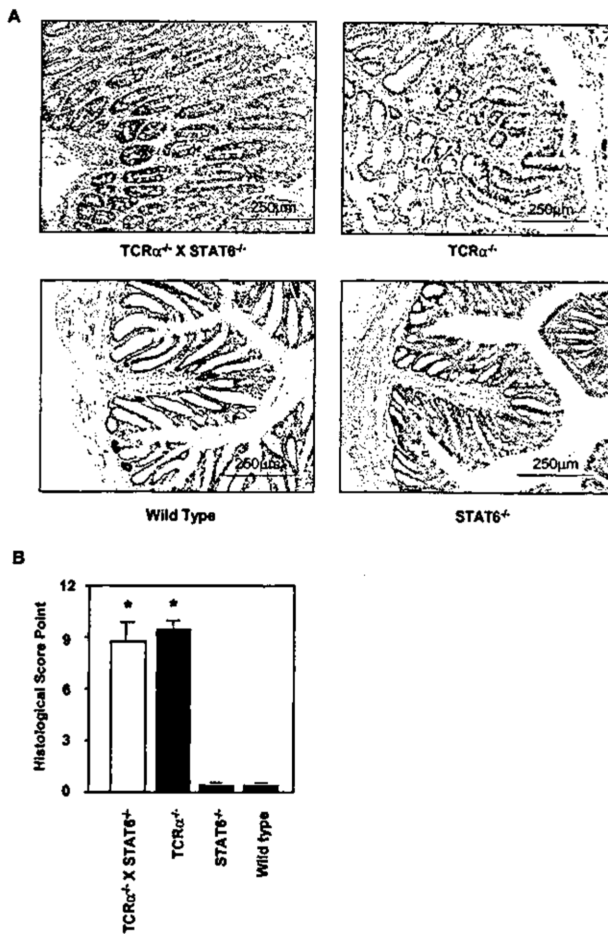

Figure 3. Histological analysis of colons isolated from TCRα$^{-/-}$ × STAT6$^{-/-}$ mice, TCRα$^{-/-}$ mice, STAT6$^{-/-}$ mice, and wild-type C57Bl/6 mice. All of the mice were sacrificed at 10 weeks of age. **A:** The tissue sections prepared from paraffin-embedded fixed colon were stained with H&E. **B:** The severity of the colitis was also examined by using the histological disease scoring system (Table 1) as described previously.[21,28] The histological score shown in each strain of mice (n = 7) was determined according to the diagnosis and grading of the colitis and was expressed as mean ± SD. Statistical analysis was performed for the comparison of TCRα$^{-/-}$ × STAT6$^{-/-}$ mice, TCRα$^{-/-}$ mice, and STAT6$^{-/-}$ mice. *, $P < 0.05$.

STAT6-Deficient Intestinal CD4$^+$ββT Cells Produced IL-4 in the Double-Mutant Mice with Colitis

Th2-type pathological CD4$^+$ββ T cells from TCRα$^{-/-}$ mice produce predominantly IL-4,[17,18] and the administration of mAb against IL-4 reportedly suppressed the onset of IBD in TCRα$^{-/-}$ mice.[19] Because CD4$^+$ββ T cells were increased in the colons of our TCRα$^{-/-}$ × STAT6$^{-/-}$ colitic mice, we next investigated cytokine production by CD4$^+$ββ T cells isolated from the mice. Total RNA isolated from purified pathogenic CD4$^+$ββ T cells of colonic LP and MLNs was subjected to cytokine-specific quantitative RT-PCR. An equivalent or slightly increased amount of IL-4-specific mRNA was found in the cells of these diseased animals as compared with amounts expressed in conventional CD4$^+$ββ T cells isolated from TCRα$^{-/-}$ mice with IBD (Figure 5A). We also examined, by enzyme-linked immunosorbent assay, the levels of the cytokine in the culture supernatant of purified CD4$^+$ T

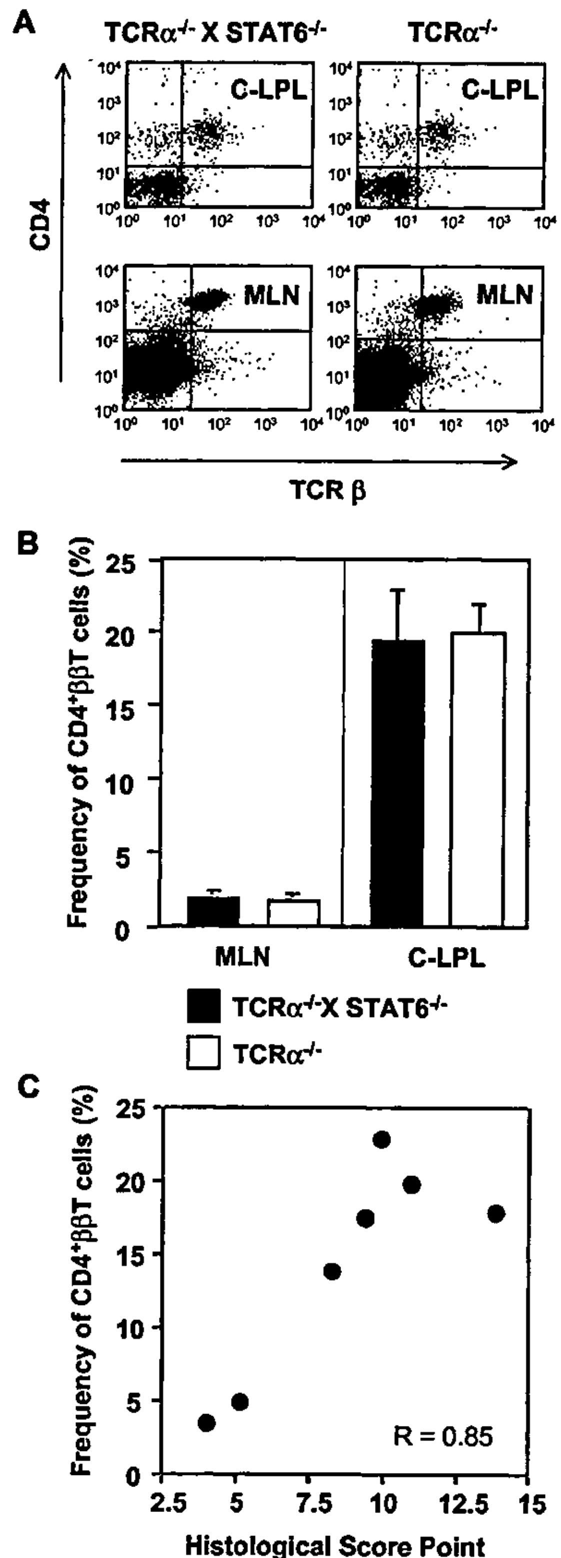

Figure 4. Flow cytometric analysis of CD4$^+$ββ T cells in TCRα$^{-/-}$ × STAT6$^{-/-}$ mice with IBD. **A:** Lymphocytes were isolated from the colonic LP (C-LPLs) and MLNs of the double-mutant and TCRα$^{-/-}$ mice with IBD and co-stained with appropriate fluorescence-conjugated anti-CD4 (L3T4) and anti-TCR-β (H57-597) mAb for fluorescence-activated cell sorting analysis. **B:** The frequency of CD4$^+$ββ T cells in the C-LPLs and MLNs of TCRα$^{-/-}$ × STAT6$^{-/-}$ mice (■) and TCRα$^{-/-}$ mice (□) was calculated. **C:** The increase of CD4$^+$ββ T cells and the disease score in each TCRα$^{-/-}$ × STAT6$^{-/-}$ mouse was correlated (P = 0. 016, r = 0.85, n = 7). The data are representative of three independent experiments.

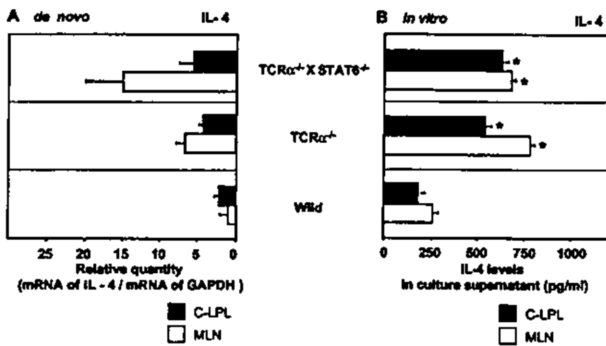

Figure 5. IL-4 production by $CD4^+\beta\beta$ T cells isolated from $TCR\alpha^{-/-}$ × $STAT6^{-/-}$ mice with colitis. **A:** Relative quantity of cytokine-specific mRNA expression by $CD4^+\beta\beta$ T cells in the mucosal compartment of $TCR\alpha^{-/-}$ × $STAT6^{-/-}$ mice and $TCR\alpha^{-/-}$ mice *de novo*. $CD4^+\beta\beta$ T cells in the C-LPLs (■) and MLNs (□) were purified by flow cytometry, and cytokine-specific mRNA expression was analyzed by quantitative RT-PCR. Cytokine-specific mRNA production was expressed as the amounts of relative quantity against GAPDH. (C-LPL: $TCR\alpha^{-/-}$ × $STAT6^{-/-}$, 5.39 ± 2.42; $TCR\alpha^{-/-}$, 4.16 ± 0.81; wild-type, 3.01 ± 1.08; MLN: $TCR\alpha^{-/-}$ × $STAT6^{-/-}$, 14.94 ± 5.72; $TCR\alpha^{-/-}$, 6.58 ± 1.53; wild-type, 0.75 ± 0.13). **B:** Cytokine production by MACS-purified $CD4^+$ T cells isolated from $TCR\alpha^{-/-}$ × $STAT6^{-/-}$ mice and $TCR\alpha^{-/-}$ mice with IBD. The T lymphocytes from the C-LPLs (■) and MLNs (□) were cultured *in vitro* with precoated anti-CD3 mAb in the presence of anti-CD28 mAb for 60 hours. Then culture supernatants were collected and cytokine production was analyzed by cytokine enzyme-linked immunosorbent assay. (C-LPL: $TCR\alpha^{-/-}$ × $STAT6^{-/-}$, 634.2 ± 47.6 pg/ml; $TCR\alpha^{-/-}$, 542.2 ± 51.4 pg/ml; wild-type, 219.5 ± 28.2 pg/ml; MLN: $TCR\alpha^{-/-}$ × $STAT6^{-/-}$, 682.6 ± 35.8 pg/ml; $TCR\alpha^{-/-}$, 784.0 ± 36.3 pg/ml; wild-type, 249.9 ± 16.5 pg/ml). The results are expressed as mean ± SEM of three independent experiments. *, $P < 0.05$.

cells from $TCR\alpha^{-/-}$ × $STAT6^{-/-}$ mice (Figure 5B), and found levels similar to those produced by cells from $TCR\alpha^{-/-}$ mice. Thus, even in the absence of STAT6, $CD4^+\beta\beta$ T cells in the $TCR\alpha^{-/-}$ × $STAT6^{-/-}$ mice with colitis produced much IL-4.

Anti-IL-4 mAb Administration Suppressed the Onset of Colitis in $TCR\alpha^{-/-}$ × $STAT6^{-/-}$ Mice

To clarify that IL-4 plays an essential role for the development of IBD in $TCR\alpha^{-/-}$ × $STAT6^{-/-}$ mice, the double-mutant mice were treated intraperitoneally with anti-IL-4 mAb from the beginning of 4 weeks to 10 weeks of age. The mice treated with mock IgG developed IBD, including anorectal prolapse, diarrhea, hemorrhagic stool, and the weight loss. In contrast, the mice treated with anti-IL-4 mAb showed no sign for the development of IBD. Histological examination of the colon demonstrated anti-IL-4 mAb treatment prevented the formation of colonic inflammation (Figure 6A). We further compared the degree of histopathological changes present in the colonic tissues of $TCR\alpha^{-/-}$ × $STAT6^{-/-}$ mice with and without anti-IL-4 mAb by using the histological scoring system (Table 1). The score in anti-IL-4 mAb treated mice (0.6 ± 0.4) was significantly less than that in mock IgG-treated mice (8.3 ± 1.2) (Figure 6B). We next assessed the alteration of $CD4^+\beta\beta$ T cells in $TCR\alpha^{-/-}$ × $STAT6^{-/-}$ mice with and without the anti-IL-4 mAb treatment. A significant difference was not observed between anti-IL-4-treated (0.91 ± 0.20%) and untreated (or mock IgG treated, 1.78 ± 0.38%) groups in the frequency of $CD4^+\beta\beta$ T cells in MLNs (P = 0.26). However, the frequency of the pathogenic $CD4^+\beta\beta$ T cells was significantly decreased in the colon of double-knockout mice treated with anti-IL-4 mAb (anti-IL-4 mAb, 4.60 ± 0.87%; mock IgG, 18.59 ± 3.76%) (Figure 6C). These findings indicated that IL-4 plays a critical role for the onset of IBD in $TCR\alpha^{-/-}$ × $STAT6^{-/-}$ mice despite in the absence of STAT6.

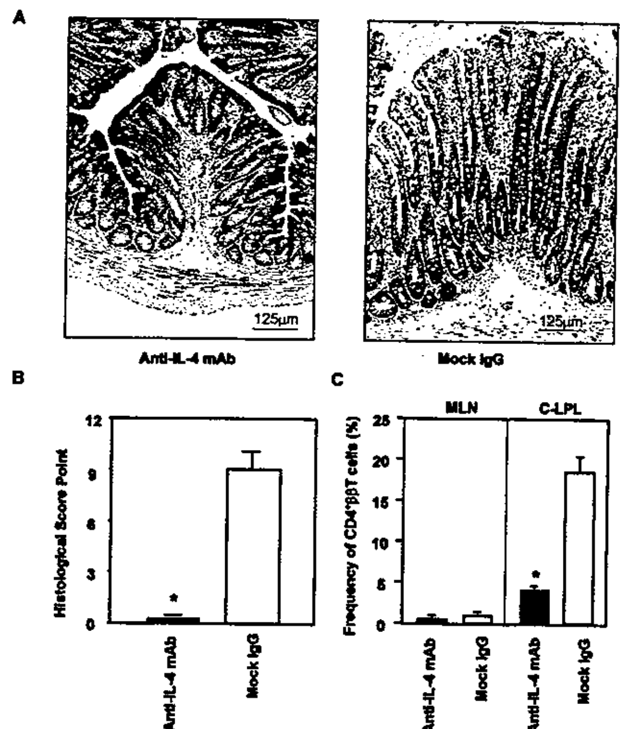

Figure 6. The suppressive effect of anti-IL-4 mAb treatment for the development of colitis in $TCR\alpha^{-/-}$ × $STAT6^{-/-}$ mice. **A:** Histological analysis of the colon in $TCR\alpha^{-/-}$ × $STAT6^{-/-}$ mice treated intraperitoneally with anti-IL-4 mAb or mock Ab (three mice/group). The treatment was initiated at 4 weeks of age and all of the mice were then sacrificed at 10 weeks of age. The tissue sections were prepared from colon and stained with H&E. **B:** The severity of the colitis was also determined in each group of mice (n = 3) by using the histological disease scoring system (Table 1) and was expressed as mean ± SD. The administration of anti-IL-4 Ab ameliorated the severity of the colitis. **C:** Flow cytometric analysis of $CD4^+\beta\beta$ T cells in $TCR\alpha^{-/-}$ × $STAT6^{-/-}$ mice with and without anti-IL-4 mAb. Lymphocytes were isolated from the C-LPLs and MLNs, then co-stained with anti-CD4 (L3T4) and anti-$TCR\beta$ (H57-597) mAbs. The results are expressed as mean ± SEM, *, $P < 0.05$.

Discussion

IL-4 produced by $CD4^+\beta\beta$ T cells is critical for the development of IBD in $TCR\alpha^{-/-}$ mice.[18–20] Previously we reported that the administration of anti-IL-4 antibody changed the pattern of cytokine production in $CD4^+\beta\beta$ T cells from dominant Th2 to Th1 and resulted in reduced ability to induce IBD in $TCR\alpha^{-/-}$ mice.[19] Others have reported that $TCR\alpha^{-/-}$ and $IL\text{-}4^{-/-}$ double-knockout mice, but not $TCR\alpha^{-/-}$ and interferon-$\gamma^{-/-}$ double-knockout mice, have a decreased frequency of IBD when compared to $TCR\alpha^{-/-}$ mice.[20] STAT6 is a transcriptional molecule that is a constitutive ingredient for IL-4 functions;[24,25,40] in the absence of STAT6, IL-4 failed to activate naive T cells to differentiate into Th2 cells or to enhance the proliferation of differentiated

Th2 cells.[24,25,40,41] Further, STAT6 was shown to be an essential transcriptional molecule for IL-4-driven Th2 differentiation and cell expansion in CD4$^+$ T cells.[42] Also, in a murine model of allergy, the loss of STAT6 abrogated antigen-induced respiratory hyperresponsiveness.[43,44] Thus, it was logical to postulate that the removal of the *STAT6*-specific gene from the pathological CD4$^+\beta\beta$ T cells would result in diminished development of colitis in TCRα$^{-/-}$ mice.

Contrary to this postulate, we found that TCRα$^{-/-}$ × STAT6$^{-/-}$ mice developed IBD similar in severity to that seen in TCRα$^{-/-}$ mice (Figures 2 and 3). Because anti-IL-4 mAb treatment suppressed the induction of IBD in TCRα$^{-/-}$ × STAT6$^{-/-}$ mice, a STAT6-independent signaling pathway responsible for the induction of IL-4-producing CD4$^+\beta\beta$ T cells in murine colitis was implied. Such a possibility seems reasonable especially because several additional molecules reportedly are critical for IL-4 signaling in Th2-type cells.[26,45] Ouyang and colleagues[46] reported that Th2 development is dependent on GATA-3 expression independent of IL-4 and STAT6, and activation of GATA-3 is a central player in Th2 differentiation.[46,47] GATA-3 has been shown to express naive T cells,[48] followed by a substantial increase during Th2 development with down-regulation of Th1 development.[49] Thus, GATA-3 can inhibit Th1 development by repressing IL-12Rβ expression.[49,50] Further, GATA-3 provides an instructive signal for the development of Th2 type cells,[47] and GATA-3 generates stability of Th2 commitment via the chromatin remodeling of Th2-specific cytokine loci.[51,52] Our preliminary results indicated that the levels of expression of GATA-3 were enhanced in CD4$^+\beta\beta$ T cells purified from the diseased TCRα$^{-/-}$ × STAT6$^{-/-}$ mice, implying that GATA-3 is at least one alternative component responsible for Th2 development of CD4$^+\beta\beta$ T cells in the double-knockout mice with colitis (data not shown). This point is now carefully addressed in a separate study.

In addition to GATA-3, STAT6-independent IL-4 signaling reportedly is mediated via several other molecules, such as phosphotyrosine-binding domain proteins;[26] insulin receptor substrate;[53] src homology 2 domain-containing α2 collagen-related protein (Shc);[54] IL-4 receptor-interacting protein FRIP;[55] and Th2-related transcription factors such as GATA-3[48,56] c-Maf,[57] NF-AT,[58] and BCL-6. A proto-oncogene, BCL-6, binds to the same DNA-binding motifs of STAT transcription factors as a transcriptional repressor.[59,60] The removal of the *BCL-6* gene resulted in the overproduction of Th2 cells, leading to severe inflammation of the heart and lung[59,61] and creation of double-mutant mice in both BCL-6 and either STAT6 or IL-4 unexpectedly resulted in the development of lethal Th2-type inflammation.[62] These findings further support our present result in which pathological Th2 type CD4$^+\beta\beta$ T cells developed in the absence of STAT6. Thus, there might be multiple redundant pathways for IL-4 production by CD4$^+\beta\beta$ T cell, exhibiting equivalent effects on the differentiation of the unique CD4$^+\beta\beta$ T cells into the Th2-biased phenotype.

STAT6 deficiency did not affect the ability of CD4$^+\beta\beta$ T cells to secrete IL-4 and proliferate. These results imply that STAT6 is not an absolute requirement for Th2 differentiation of CD4$^+\beta\beta$ T cells, although it has been reported that STAT6 is necessary and sufficient to mediate both IL-4-driven Th2 differentiation and cell expansion in naive CD4$^+$ T cells in normal mice.[42] It should be noted that the concept of a primary role of IL-4 and STAT6 signaling in the induction of Th2-type cells has recently been put in doubt: in mice genetically deficient in IL-4, IL-4R, and STAT6, Th2-type cells and those associated immune responses emerged in the absence of the IL-4/IL-4R and STAT6 signaling cascade,[63–66] and classical IL-4- and IL-5-producing Th2-type cells developed in helminth-infected STAT6-deficient mice.[67] Further, repetitive anti-CD3 stimulation of memory type CD4$^+$CD62L^{high} T cells from STAT6-deficient mice resulted in the generation of IL-4-producing Th2-type cells.[68] Moreover, the number of CD4$^+\beta\beta$ T cells was not different in TCRα$^{-/-}$ × IL-4$^{-/-}$ and TCRα$^{-/-}$ mice with IBD.[20] Taken together, the previous and present findings strongly suggest that the STAT6 signaling pathway is not essential for the development of pathological Th2-type CD4$^+\beta\beta$ T cells.

Our previous data also showed that anti-IL-4 treatment did not influence the number of CD4$^+\beta\beta$ T cells in TCRα$^{-/-}$ mice.[19] Attempts to culture CD4$^+\beta\beta$ T cells isolated from TCRα$^{-/-}$ mice failed to replicate these aberrant T cells under *in vitro* Th2-skewing-conditions (data not shown). Because IL-4 did not act as a trophic factor for CD4$^+\beta\beta$ T cells it probably does not directly affect the development of CD4$^+\beta\beta$ T cells. This implies that a unique environment exists *in vivo* that is capable of driving Th2 responses independent of IL-4-STAT6 signaling. Interestingly, CD4$^+\beta\beta$ T cells were not detected in TCRα$^{-/-}$ × MHC class II$^{-/-}$ mice.[30] Therefore, CD4$^+\beta\beta$ T cells may develop and expand in response to unidentified MHC class II-restricted antigens. TCRα$^{-/-}$ mice with IBD showed humoral immune responses to food, self antigens,[18] and intestinal bacteria, such as *Bacteroides vulgatus*.[38] Perhaps then CD4$^+\beta\beta$ T cells exhibit Ag specificities against innocuous luminal antigens for the production of Th2-type cytokines, eventually leading to the development of IBD.

The results of the present study illustrate the complexity of mechanisms involved in the pathogenesis of colitis in TCRα-deficient condition. Further investigation of signaling molecules other than STAT6 involved in IL-4 signaling may clarify the molecular mechanism behind Th2-type colitis. Moreover, the TCRα$^{-/-}$ × STAT6$^{-/-}$ mouse strain is a useful animal model for investigating the mechanism of STAT6-independent Th2 differentiation of the unique pathogenic CD4$^+\beta\beta$ T cells.

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Combined intrarectal/intradermal inoculation of recombinant *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) induces enhanced immune responses against the inserted HIV-1 V3 antigen

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Abstract

The development of a successful recombinant *Mycobacterium bovis* bacillus Calmette–Guérin (rBCG) vector-based vaccine for human immunodeficiency virus type 1 (HIV-1) requires the induction of high levels of HIV-1-specific immunity while at the same time maintaining immunity to tuberculosis. To examine a combined vaccination strategy for enhancement of immune responses specific for HIV-1, guinea pigs were inoculated with either a single or combination intradermal (i.d.), intrarectal (i.r.) and intranasal (i.n.) administration of rBCG–pSOV3J1 which secretes a chimeric protein of HIV-1 V3J1 peptide and α-antigen. Significant level of delayed-type hypersensitivity to both V3J1 peptide and tuberculin was induced in guinea pigs inoculated with human doses of rBCG–pSOV3J1 by a combination of intrarectal and intradermal routes. Guinea pigs inoculated by combined routes also had significantly higher titers of HIV-1-specific serum IgG and IgA compared with those animals immunized only intrarectally, which led to the enhanced neutralization activity against HIV-1_{MN}. In addition, the induction of high levels of IFNγ and interleukin-2 (IL-2) mRNA in PBMC, splenocytes, and intraepithelial lymphocytes from the immunized animals was detected until at least 110 weeks post-inoculation. These results suggest that enhanced immune responses specific for HIV-1 are efficiently induced by combined intrarectal and intradermal immunization with rBCG–HIV, and antigen-specific Th1-type memory cells are maintained for more than 2 years in the immunized animals. Thus, inoculation with rBCG–HIV by combined routes represents an effective vaccination strategy to elicit high levels of HIV-1-specific immune responses.
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Keywords: Mycobacterium bovis; BCG; HIV-1; Recombinant BCG vaccine

1. Introduction

Globally, the incidence of new infections with human immunodeficiency virus type 1 (HIV-1) continues to increase rapidly [1–3]. To control further spread of the virus, a preventive vaccine must be developed with the goal of inducing protective immunity to HIV-1. To be successful, such a vaccine must take into consideration not only protective immunity, but also issues of safety and stability, ease of administration, compatibility with other administered vaccines, and affordability—particularly in developing countries. Recent studies have shown that the correlates of protective immunity may be due to complex interactions of immunological [4–6], viral [7] and genetic factors [8]. Importantly, vigorous cellular and humoral immune responses may be responsible for controlling HIV-1 viral load and limiting the progression of disease [9]. *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) is considered a potential vaccine vector that is safe for use in humans [10–13] and may be capable of inducing high levels of HIV-specific immunity while maintaining protection against tuberculosis. Previously, we reported use of a recombinant BCG (rBCG) containing V3 sequences from the Japanese consensus HIV (rBCG–pSOV3J1) that induced strong delayed-type hypersensitivity (DTH) reactions, cytotoxic T lymphocytes (CTL), and neutralizing antibodies to HIV-1 [13]. In addition, a clade E V3 rBCG

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was also constructed and produced cross-clade neutralizing antibodies against HIV-1 clades E and B′ [14]. The vaccine elicited long-term V3J1 peptide-specific neutralizing immunity in Th1- and Th2-deficient conditions [15]. These findings strongly suggest that rBCG has potential as a candidate vaccine against HIV-1. However, these results were obtained by inoculating rBCG–pSOV3J1 subcutaneously (s.c.) at dosages approximately 100-fold higher than those used in humans for BCG vaccination against tuberculosis. Moreover, subcutaneous vaccination is generally considered inefficient in stimulating mucosal immunity [16,17] which may be a critical component for blocking transmission of HIV-1 at the site of entry. Others have emphasized the mucosal route for administration of rBCG expressing highly immunogenic antigens of lacZ which induced potent cellular and humoral immune responses against β-galactosidase [18]. Furthermore, an HIV-1 V3J1 antigen was reported to induce antibodies at low efficiency in guinea pigs [19]. Here, we used combinations of intradermal, intrarectal (i.r.) and nasal rBCG–pSOV3J1 inoculations, at human doses, and found that combined inoculation by rectal and intradermal routes effectively enhanced the levels of humoral and cellular immune responses against HIV-1 and tuberculin in systemic and mucosal compartments.

Although the guinea pig has been used as an animal model for mycobacterial infection, cytokine gene expression in these animals has not been extensively studied. Previously, Scarozza et al. [20] examined gene expression of several cytokines in a mitogen-stimulated mononuclear cell fraction from peripheral blood and lymphoid organs of non-immunized guinea pigs. However, these studies did not include examination of IFNγ which is suggested to be one of the major factors responsible for protection against tuberculosis and other cell-associated pathogens [21,22]. It is conceivable that Th1 cytokine production may provide resistance to HIV infection and progression to AIDS [23]. Therefore, we evaluated antigen-specific gene expression of two Th1 cytokines, IFNγ and interleukin-2 (IL-2), in guinea pigs immunized with rBCG–pSOV3J1.

2. Materials and methods

2.1. Animals

Hartley guinea pigs (Shizuoka Laboratory Center, Shizuoka, Japan), weighing 300–350 g each, were used in a P2 level animal facility at the National Institute of Infectious Diseases, Tokyo, Japan.

2.2. Construction of the HIV-1 V3J1/α-antigen plasmid

A mycobacterial codon-optimized DNA fragment encoding 19 amino acids of the tip V3 sequence of Japanese consensus HIV-1 (V3J1, NTRKSIHIGPGRAFYATGS) was inserted into the *Xho*I site of the plasmid pSO246 [24]. A recombinant *M. bovis* BCG substrain Tokyo which stably expressed the inserted synthetic DNA fragment (designated rBCG–pSOV3J1) was then selected and used for all mucosal inoculations.

2.3. Mucosal immunization of guinea pigs with rBCG–pSOV3J1

Hartley strain female guinea pigs were deprived of food overnight to remove feces from their intestines prior to i.r. inoculation with rBCG–pSOV3J1. The next day the animals were anesthetized with ketamine hydrochloride (Sankyo Co. Ltd., Tokyo, Japan) and 80 mg of rBCG–pSOV3J1 in 500 μl of saline was gently introduced into the rectum via a feeding tube (Termo Co., Tokyo, Japan) once a week for two, three or four consecutive weeks. For cutaneous inoculation, 1.0 or 0.1 mg of rBCG–pSOV3J1 in 0.1 ml of saline was dermally injected into the same guinea pigs that received rectal inoculation. Another group of guinea pigs was also nasally inoculated with rBCG–pSOV3J1 at a concentration of 10 μg in 50μl of saline four times at weekly intervals for 3 weeks.

2.4. Measurement of the HIV-1 V3J1 chimeric protein

An ELISA was used for detection of the chimeric HIV V3/α protein in culture filtrates of rBCG–pSOV3J1. Ninety-six-well ELISA plates (MaxiSorp, Nunc A/S, Roskilde, Denmark) were coated with C25 monoclonal antibody, which recognizes the GPGR sequence characteristic of the HIV-1 clade B V3 domain [13], at 1 μg/ml. The wells were washed and treated with 2% bovine serum albumin (BSA). Duplicate samples containing either standards or test filtrates at appropriate dilutions were then added and incubated for 1 h at 37 °C. An affinity column-purified HIV-1 V3 chimeric protein was diluted in sample-diluent to concentrations ranging from 5 to 40 μg/ml and used as a standard. The wells were incubated with biotinylated antibody to the *M. kansasii* α-antigen (α-K) [25] followed by addition of an avidin–phosphatase solution (Bethesda Research Laboratory, Rockville, MD). The plates were measured for optical density at 450 nm with a precision microplate reader (Emax; Molecular Devices Co., Sunnyvale, CA). The concentrations of chimeric protein in the culture filtrates were evaluated from a calibration curve drawn with software developed for the reader (SOFTmax; Molecular Devices, Osaka, Japan).

2.5. Induction of delayed-type hypersensitivity (DTH)

To investigate DTH skin reactions, 0.5 μg of purified protein derivative of tuberculin (PPD) or 40 μg of keyhole limpet hemocyanin (KLH)-conjugated HIV-1 V3J1 peptide (KLH–V3J1) per 100 μl of saline were injected intradermally into the rBCG–pSOV3J1 immunized guinea pigs. Saline (100 μl) was used as a negative control. After 24 h, the skin reactions were measured.

2.6. Expression of cytokine-specific mRNA

To investigate expression of cytokine mRNA, PBMC, splenocytes, alveolar lavage cells and intestinal intraepithelial leukocytes (i-IEL) from the immunized guinea pigs were harvested and purified. PBMC were isolated using Lymphosepar 2 according to the manufacturer's instructions (Immuno-Biological Laboratories Co. Ltd., Gunma, Japan). For isolation of splenocytes, alveolar lavage cells and i-IEL, the immunized guinea pigs were sacrificed under anesthesia with ketamine, and the spleen, lungs and large intestine were collected. Splenic cells were prepared by gentle homogenization through a 70 μm nylon cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ), and the preparations were treated with ACK lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA) for 1 min at room temperature. Alveolar lavage cells were isolated by washing the airway of the lung with PBS, followed by purification using ACK lysing buffer. i-IEL were isolated as described previously [26–28]. The purified cells were cultured at a density of 1×10^6 cells/ml in the presence of 100 μg/ml of PPD or V3J1 peptide. After 4 days, the cells were harvested to obtain total RNA using a RNeasy MiNi Kit (QIAGEN, Valencia, CA). For detection of cytokine-specific mRNA (IFNγ, IL-2), a standard RT-PCR analysis was performed using a one-step RT-PCR kit (Takara Shuzo Co. Ltd., Kyoto, Japan). The RT reaction was performed at 45 °C for 60 min and inactivated at 94 °C for 7 min. PCR conditions consisted of 32 cycles of amplification at 94 °C for 30 s; 53 °C for 30 s; and 65 °C for 4 min. PCR products were then electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining (0.2 μg/ml). The remaining PCR products were sequenced using a ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA) to confirm the PCR product as guinea pig IFNγ. Oligonucleotide primers specific for IL-2 (5′-CCACAGAATTGAAACATCTTCAGTG-3′, 5′-CTTTGACAAAAGGTAATCCATCTGTTCAG-3′) and a β-actin internal control (5′-ATGTGCAAGGCCGGCTTCG-3′, 5′-TTAATGTCACGCACGATTTCC-3′) were prepared according to published results [20]. The cDNA of guinea pig IFNγ was kindly supplied by Dr. Yoshimura T. NCI-FCRC, NIH, and amplified using oligonucleotide primers specific for IFNγ (5′-ATTTTGAAGAATTGCCAAGAGG-3′, 5′-AAATTCAAATATTATAGGCAGA-3′).

2.7. Serum anti-PND antibody titration by HIV V3 peptide-based ELISA

Sera were collected from each guinea pig at several time points and stored at −80 °C until further use. Antigen-specific antibody titers were determined by ELISA using a modified method as described previously [29,30]. Maxisorp plates were coated with V3J1-peptide (2 μg/well) at 4 °C overnight. Serially diluted sera were added to the wells for 2 h at 37 °C. After three washes, rabbit anti-guinea pig IgG–horseradish peroxidase (HRP) conjugate (Zymed Laboratories Inc., San Francisco, CA) was added to the wells for 2 h at 37 °C, and the plates were then washed, and visualized by adding TMB substrate (Moss Inc., Pasadena, MD). After 30 min at room temperature, absorbance was read with an ELISA reader at 470 nm. For detection of HIV-PND-specific IgA, 100 μl of rabbit anti-guinea pig IgA (diluted 1:2500) (Bethyl Laboratories Inc., Montgomery, TX) was added to the wells for 2 h at 37 °C. After three washes, the wells were treated with 100 μl of goat anti-rabbit Ig–HRP conjugate (diluted 1:2500) (Nordic Immunological Laboratories, Tilburg, Netherlands) for 1 h at 37 °C. The plates were then washed six times and TMB was added to visualize. Endpoint titers for Ag-specific IgG and IgA were calculated using Microsoft Office Excel, and expressed as the reciprocal $\log_2$ of the last dilution giving an OD_{470} of 0.1 U above samples obtained from pre-immunized animals [31].

2.8. PBMC-based virus neutralization assay

The HIV-1_{MN} (H9/HTLV-III MN, AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, MD) was cultured with phytohemagglutinin (PHA)-activated PBMC in the presence of 40 U/ml of human IL-2 (Shionogi Pharmaceutical Co., Osaka, Japan) for 7 days and the virus stocks were measured for 50% tissue culture infective doses ($TCID_{50}$) using PHA-activated PBMC isolated from healthy individuals. Serum antibodies were purified from pre-immune and rBCG-immunized guinea pig sera by using protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden). In vitro virus neutralization assay using diluted serum antibodies was performed as previously described [14]. The neutralization titer is given as concentration of serum IgG antibody which reduces the p24 antigen production by 50% (IC_{50}) compared to control wells with purified pre-immune IgG from guinea pigs.

2.9. Statistical analysis

Data analysis was carried out using the Statistica program (StatSoft, Tulsa, OK) and $P < 0.05$ were considered significant. One-way and two-way ANOVA for repeated measures were used to evaluate the statistical significance of differences in levels of DTH skin reactions and antibody titers between different routes of immunization.

3. Results

3.1. Construction of rBCG–pSOV3J1 that stably expresses soluble HIV-1 chimeric protein

To obtain stable secretion of a rBCG–HIV-1 chimeric protein, we began with the plasmid pSO246 and inserted 19 amino acids of the V3 sequence of the Japanese Consensus