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IL-17 Activates the Canonical NF-κB Signaling Pathway In Autoimmune B Cells of BXD2 Mice to Upregulate the Expression of Regulators of G-Protein Signaling 16

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Abstract

We previously identified that autoreactive B cells from BXD2 mice can be targeted by IL-17, leading to upregulation of the expression of regulators of G-protein signaling (*Rgs*) genes that facilitated the development of spontaneous germinal centers. Little is known about the signaling pathway used by IL-17 to upregulate RGS. In the current study, we found that IL-17 rapidly activates the canonical NF-κB signaling pathway and that BXD2 B cells exhibit higher basal and activated phosphorylated p65 levels than B6 or BXD2-*Il17ra*$^{-/-}$ B cells. Inhibition of p65 phosphorylation down-regulated RGS16 expression and abrogated the IL-17-induced chemotactic arrest of B cells in response to CXCL12. Knockdown of TNFR-associated factor 6 or NF-κB activator 1 in 70Z/3 pre-B cells led to decreased *Rgs16* expression, indicating that both of these two genes are involved in IL-17-mediated activation of NF-κB signaling in B cells. These findings identify the signaling pathway regulated by IL-17 to contribute to the development of spontaneous germinal centers in autoimmune BXD2 mice.

Introduction

Interleukin-17 has been shown to play a major role, especially in enhancing chemotaxis as related to the innate immune response. Chemokines upregulated by IL-17 include CXCL1, CXCL2 (1), CXCL5(2), CXCL8 (IL-8) (3), CXCL9, CXCL10 (4), CCL2, and CCL20 (5). IL-17 upregulates additional pro-inflammatory mediators, including IL-6, TNF-α, G-CSF, GM-CSF, C-reactive protein, matrix metalloproteases, and anti-microbial proteins, and is especially potent in expansion and recruitment of neutrophils (6).

The importance of IL-17 regulation on B cells has been shown recently to be associated with the development of spontaneous germinal centers (GCs) in autoimmune BXD2 mice (7). The BXD2 recombinant inbred mouse strain was generated by inbreeding the intercross progeny of C57BL/6J and DBA/2J mice for more than 20 generations (8,9). These mice develop high titers of pathogenic autoantibodies and a spontaneous erosive arthritis that progresses as the

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mice age. BXD2 mice also exhibit spontaneous development of GCs in the spleen, and we have established that this formation of GCs plays a critical role in the production of pathogenic autoantibodies (7).

IL-17 signaling has been shown to be mediated through NF-κB activator 1 (ACT1) nd TNFR-associated factor 6 (TRAF6) (10,11). The IL-17R family cytoplasmic tails have been shown to have homology with IL-1/TLR/IL-1R domain now referred to as the SEFIR. The SEFIR domain in IL-17RA is essential for IL-17 signaling (10,12,13). The IL-1/TLR domain contains a protein-protein interaction motif found in TLRs (IL-1Rs) and also is found in ACT1, an activator of NF-κB that had been linked to the B cell activation factor from the TNF family (BAFF) and CD40L signaling (11,14). ACT1 also contains a TRAF6 binding motif and IL-17 activation of the NF-κB and MAPK pathways requires TRAF6 to induce IL-6 (10).

The chemokine receptors CXCR4 and CXCR5 and their respective ligands CXCL12 and CXCL13 facilitate recruitment of lymphocytes in lymphoid follicles to create GCs (15). The high levels of IL-17 that are characteristic of the BXD2 mice (7) are associated with the upregulation in the expression of *the regulators of G-protein signaling (Rgs) 16* and *Rgs13* genes in B cells. This increased expression of the *Rgs16* and *Rgs13* genes reduces the B cell chemotactic responses to the CXCL12 and CXCL13 chemokines, thereby promoting the retention of the B cells within the GCs and providing an optimal microenvironment for the generation of pathogenic autoantibodies (7). The associated reduction in the B cell chemotactic responses are absent in BXD2-*Il17ra*$^{-/-}$ mice, suggesting that one important IL-17/IL-17RA signaling function in B cells is to mediate the upregulation of *Rgs* genes.

It has been shown that NF-κB is involved in the mediation of IL-17 downstream signaling in various mammalian cell types, such as myofibroblasts (16), intestinal epithelia cells (17), and articular chondrocytes (18). IL-17 has been shown to use the NF-κB pathway to promote the survival and differentiation of B cells from human lupus patients (19). It is not known whether the IL-17-mediated RGS response also is dependent on the NF-κB signaling pathway. In this study, we show that in autoimmune B cells of BXD2 mice, the IL-17-mediated upregulation in RGS16 expression was associated with rapid phosphorylation and degradation of IκB*α* as well as phosphorylation of p65 (P-p65) and its translocation to the nucleus. Inhibition of phosphorylation of Ser^{276} on p65 with a specific membrane-permeable peptide inhibitor blocked IL-17-induced upregulation of RGS16 expression, and thus the IL-17-induced inhibition of chemotaxis of B cells in responses to CXCL12. In 70Z/3 pre-B cells, knockdown of *Traf6* or *TNFR-associated factor 3 interacting protein 2* (*Traf3ip2*), the gene encoding ACT1, by small interfering RNA (siRNA) also blocked the increase in P-p65 and *Rgs16* expression levels by IL-17 signaling. Together, this finding extends our previously described model in which IL-17 can inhibit B cell chemotactic responses to CXCL12 (7) via its activation of the SEFIR and NF-κB signaling pathway, leading to upregulation of RGS16.

Materials and Methods

Mice

C57BL/6 and BXD2 recombinant inbred mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6-*Il17ra*$^{-/-}$ mice, obtained from Amgen (Thousand Oaks, CA), were backcrossed with the BXD2 mice for seven generations (7). All of the mice were cared for according to the Institutional Animal Care and Use Committee guidelines.

Mouse spleen B cell isolation

B cells were enriched from single-cell suspensions of spleens from 8- to 12-wk-old mice using magnetic anti-CD19 microbeads and an AutoMACS magnetic cell sorter (Miltenyi Biotech, Auburn, CA). The B cell preparations were 98% pure as confirmed by FACS.

Cell line and reagents

The 70Z/3 cell line was obtained from the American Type Culture Collection (Manassas, VA). Abs against p65 (C22B4), P-p65 (93H1), transcription factor RelB (C1E4), IκBα (44D4), phospho-IκBα (14D4), phospho-p38 MAPK (28B10), phospho-p44/42 MAPK (Erk1/2) (Thr^{202}/Tyr^{204}) (197G2) rabbit mAb, phospho-C/EBPβ (Thr^{235}) (Cell Signaling Technology, Beverly, MA), p50 (H119), p52 (K27), SP1 transcription factor (1C6), C/EBPβ (C-19), C/EBPδ (C-22) (Santa Cruz Biotechnology, Santa Cruz, CA), RGS16 (Prosci, Poway, CA), and GAPDH (R&D Systems, Minneapolis, MN) were used to detect their respective Ags.

Quantitative real-time PCR

RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA) and converted to cDNA by a First Strand cDNA Synthesis kit (Fermentas, Burlington, Ontario, Canada). Real-time PCR reactions were performed in triplicate using the SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA) in an iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad) with the following primers: *Il17ra*, GGTGGCGGTTTTCCTTCAG (F), GTGGTTTGGGTCCCCATCA (R); *Il17rc*, GCGGTATTTGACTGTTTCG (F), TGCTCCTCAGAGACATCC (R); *Rgs16*, GGTACTTGCTACTCGCTTTTCC (F), CAGCCGCGTCTTGAACTCT (R); *Rgs13*, TGCAGAGATGAATCTAAGAGGCT (F), GGTTTCACATGCCATCCAGAAT (R); *Rgs1*, TTTTCTGCTAGCCCAAAGGA (F), TGGTTGGCAAGGAGTTTTTC (R); *Traf6*, GCGAGAGATTCTTTCCCTGAC (F), TGTATTAACCTGGCACTTCTGG (R); *Traf3ip*, TTTAGAAGGCACCCTGAAC (F), CCGTAAGTGTGAACCGATG (R); *Gapdh*, AGGTCGGTGTGAACGGATTTG (F), TGTAGACCATGTAGTTGAGGTCA (R).

Western blot analysis

The cytoplasmic and nuclear extracts from B cells were prepared with NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). Equal amounts of protein extracts were electrophoresed on 8–10% SDS polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes. Anti-rabbit HRP-conjugated Ab (Cell Signaling Technology) was used at a 1:3000 dilution, and anti-mouse HRP-conjugated Ab (Santa Cruz Biotechnology) was used at a 1:5000 dilution. HRP Abs were detected using the chemiluminescence reagent (Pierce). The scanned figures were visualized and quantified using ImageJ software.

Immunofluoresence staining

Cytospin preparations of splenic B cells were fixed and permeabilized with BD Cytofix/Cytoperm™ solution (BD Biosciences, San Jose, CA), then sequentially stained with 1:100 dilution of P-p65 rabbit mAb (93H1, Cell Signaling Technology) and 1:200 dilution of FITC-conjugated donkey anti-rabbit Ab (BD Biosciences). The slides were viewed with a DM IRBE inverted Nomarski/epifluorescence microscope (Leica Microsystems, Deerfield, IL) outfitted with TCS NT laser confocal optics (Leica Microsystems).

Flow cytometry analysis

Whole spleen cells from B6, BXD2 and BXD2-*Il17ra*$^{-/-}$ mice were stained with biotin-peanut agglutinin (PNA) (Vector Laboratory, Burlingame, CA), CD19-Alexa Fluor 700 (eBioscience, San Diego, CA) and IL17RA-PE (eBioscience). PNA^{+} cells were revealed by a secondary

allophycocyanin-streptavidin conjugate. The purified splenic B cells from B6 and BXD2 mice were stained in the cold with biotin-PNA (Vector Laboratory) and PE-anti-Fas (Biolegend, San Diego, CA). The B cells were quickly warmed to 37°C and stimulated with IL-17 (30 ng/ml) for 5 min. The B cells then were cooled quickly, fixed and permeabilized with BD Cytofix/Cytoperm™ solution (BD Biosciences), then sequentially stained with 1:100 dilution of P-p65 rabbit mAb (93H1, Cell Signaling Technology) and 1:200 dilution of FITC-conjugated donkey anti-rabbit Ab (BD Biosciences). An isotype control rabbit IgG was used for gating of P-p65-positive cells. The stained whole spleen cells or purified B cells were analyzed on a LSRII FACS analyzer (BD Biosciences).

Specific Inhibitory Peptide

The NF-κB p65 (Ser^{276}) inhibitory peptide (DRQIKIWFQNRRMKWK-KQLRRP**S**DRELSE) contains a protein transduction (PTD) sequence (DRQIKIWFQNRRMKWKK) derived from antennapedia and a Ser^{276} site (boldface) that is phosphorylated during NF-κB activation thereby blocking p65-Ser^{276} phosphorylation (IMGENEX, San Diego, CA). The control peptide consists of the PTD sequence (IMGENEX).

Cell migration assay

The purified spleen B cells from BXD2 mice or 70Z/3 pre-B cells were loaded into the upper well insert of a Transwell system (Costar, Cambridge, MA), and the chemokine CXCL12 (100 ng/ml) was added to the bottom chamber. After incubation for 2 h, the cells that remained in the inserts or that had migrated to the lower chamber were counted. The chemotaxis index was calculated by dividing the number of cells that migrated in response to the chemokine by the number of cells that migrated in the absence of chemokine (7).

Knockdown of Traf3ip2 and Traf6

siRNAs for *Traf3ip2* (AAAGCAUUAGGUAAACUUGGGUCUG, Invitrogen), *Traf6* (AAAGUUCACAAAUUUCACCACCUCC, Invitrogen) and control scrambled siRNAs (Invitrogen) were transfected into 70Z/3 cells at a final concentration of 100 nM using BLOCK-iT™ Transfection Kit (Invitrogen). The transfection efficiency in 70Z/3 is >80%, as determined by the BLOCK-iT™ Fluorescent Oligo. After transfection for 24 h, cells were stimulated with IL-17 (30 ng/ml) for 4h, and harvested for quantitative real-time PCR or for cell migration analysis as described earlier.

Results

Splenic B cells from autoimmune BXD2 mice are highly sensitive to IL-17-induced enhancement of P-p65

NF-κB is the best known mediator of IL-17-associated cellular responses (16–18). It is constitutively activated in many autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus and type1 diabetes (20). We found that the B cells from the spleens of autoimmune BXD2 mice exhibited significantly higher levels of basal P-p65 as compared with those of the B cells from the spleens of the parental B6 mice (Fig. 1*A*, Supplemental Fig 1), and that the levels of P-p65 in the B cells from the spleens of these mice were enhanced by stimulation with IL-17 (30 ng/ml, Fig. 1*A*, Supplemental Fig 1). The levels of P-p65 in the spleens of the BXD2-*Il17ra*$^{-/-}$ mice were markedly lower and were not responsive to IL-17 treatment (Fig. 1*A*, Supplemental Fig 1).

BXD2 mice display high levels of serum IL-17 and IL-17 expression in the splenic CD4 T cells (7), which may account for higher levels of IL-17 signaling. To determine whether B cells of B6 mice could exhibit an equivalent response to IL-17 at higher levels of IL-17 or at longer

incubation times, B cells from BXD2 and B6 mice were analyzed after incubation with IL-17 (150 ng/ml) for 5 min or after 15 and 30 min with 30 ng/ml IL17 (Fig. 1B). Higher levels of IL-17 were not able to significantly elevate P-p65 in B cells of B6 mice compared with those of the control with no IL-17. There was a markedly lower P-p65 induction by IL-17 in B6 B cells at 15 and 30 min with 30 ng/ml IL17 compared with that of BXD2 B cells (Fig. 1*B*).

There is an ~5-fold increase in the *Il17ra* mRNA in total spleen B cells of BXD2 mice compared with those of B6 mice (7). B cells from BXD2 contain a dramatically increased population of Fas^+PNA^+ GC B cells compared with those of B cells from B6 mice (21). We therefore asked whether increased IL-17R and IL-17 stimulation of P-p65 in B cells from BXD2 mice was limited to GC B cells, or all of the B cells exhibited higher responses to IL-17. Responses of B cell subpopulations were analyzed by intracellular staining and FACS analysis of IL-17-stimulated B cells (Fig. *1C*, *1D*). There were increased P-p65 levels in response to IL-17 in both GC and non-GC B cells of BXD2 mice compared with those of the corresponding B cells from B6 mice. Consistent with this result, both non-GC (Fas^-PNA^-) and GC (Fas^+PNA^+) B cells exhibited higher levels of *Il17ra* expression (Fig. 1*E*). Increased expression of IL-17RA on the surfaces of B cells from BXD2 mice also was confirmed by FACS analysis (Fig. 1*F*). B cells from BXD2 mice also exhibited higher levels of *Il-17rc* (Supplemental Fig 2). There are also higher levels of *Il17rc* in BXD2-*Il17ra*$^{-/-}$ mice. Together, these results suggest that higher levels of *Il17ra* expression and higher IL-17-stimulated P-p65 are intrinsic properties of B cells from BXD2 mice.

IL-17 stimulation leads to nuclear translocation of P-p65 in B cells

The addition of IL-17 not only upregulated the levels of P-p65 in the B cells from the spleens of BXD2 mice, but also promoted its translocation to the nucleus. In the absence of IL-17 stimulation, the levels of P-p65 in the cytoplasm were considerably higher in BXD2 B cells than in B6 B cells (Fig. 2, *upper panels*). Within 5 minutes after addition of IL-17, the levels of P-p65 in the cytoplasm of BXD2 B cells were increased in most of these cells (Fig. 2*A*, *middle panels*), compared those of similarly treated B cells from the B6 control mice (Fig. 2*B*, *middle panels*). There was a dramatic reduction of the levels of P-p65 in the nuclei of B cells from BXD2 mice at 15 minutes after the addition of the IL-17, but high levels of P-p65 persisted in the cytoplasm (Fig. 2*A*, *lower panels*). The upregulation of P-p65 observed in almost all B cells of BXD2 mice further supports that this increased response to IL-17 is not due to an increased population of GC B cells compared with that of normal B6 mice.

Increased cytoplasmic and nuclear levels of P-p65 following IL-17 stimulation

Consistent with the results described above, within 5 min after addition of IL-17, the levels of P-p65 in total and cytoplasmic extracts of BXD2 B cells increased (Fig. 3*A*–3*D*). There was also a dramatic increase in the levels of P-p65 in nuclear extract (Fig. 3*E*, 3*F*). The levels of P-p65 in the B cell nuclear extract from BXD2 mice returned to baseline levels at 15 min after the addition of IL-17 (Fig. 3*E*, 3*F*), but high levels of P-p65 persisted in the cytoplasmic extract (Fig. 3*C*, 3*D*).

In most cell types, NF-κB is retained usually in the cytoplasm of the unstimulated cells by IκB family proteins. Upon stimulation, the IκB kinase (IKK) complex is activated, resulting in the phosphorylation of IκBs (22,23). The phosphorylated IκBs are ubiquitinated and subsequently degraded, which will release transcription factor NF-κB (22,23). In this study, we also found that IL-17 stimulation was associated with a significant increase in the levels of phosphorylated IκBα in the cytoplasm at the 5 min time point. Consistent with this, the total levels of IκBα in the cytoplasm decreased at the 5 min time point (Fig 3*C*, 3*D*). The levels of p50, a product of p105, were not elevated in the nuclear extracts of the BXD2 B cells with IL-17 stimulation (Fig. 3*A*, 3*E*).

Activation of p38 MAP kinase (MAPK), p44/42 MAPK (Erk1/2), and C/EBPβ also has been shown as a downstream signaling pathway of IL-17 (10,12,24). There was significantly increased induction of phospho-p38 MAPK and phospho-p44/42 MAPK (Erk1/2) in BXD2 B cells up to 30 min after IL-17 stimulation (Fig. 3*A*, 3*B*), but we did not find the increased induction of phospho-C/EBPβ (Fig. 3*A*, 3*B*) or increased nuclear levels of C/EBPβ or C/EBPδ (Supplemental Fig. 3). We also did not find any increase in the formation of p52, the noncanonical NF-κB subunit, or translocation of p52 or transcription factor RelB to the nucleus (Supplemental Fig. 3). Taken together, IL-17 strongly activates the canonical NF-κB signaling pathway in autoimmune BXD2 B cells.

Upregulation of Rgs16 and Rgs13 following P-p65 activation by IL-17 in BXD2 B cells

We have found previously that after 4 h of culture with IL-17, there was equivalent upregulation of *Rgs16* and *Rgs13* in B cells from the spleens of BXD2 and B6 mice (7). Consistent with these previously published data, we found that treatment of the B cells from BXD2 mice with IL-17 resulted in a significant increase in the expression of *Rgs16* mRNA, as determined by quantitative real-time PCR, within 2 h of IL-17 treatment ($p < 0.01$) (Fig. 4*A*). Similarly, treatment of B6 B cells with IL-17 enhanced the levels of *Rgs16* mRNA, but the magnitude of the response was lower and the response was delayed in that it peaked at 4 h after addition of IL-17 (Fig. 4*A*, left panel). It has been reported that *Rgs1* (25) and *Rgs13* (26,27) are highly expressed in GC B cells and some B cell lines. Although we found that *Rgs13* expression was upregulated significantly in the IL-17-treated B cells ($p < 0.05$) (Fig. 4*B*), the relative magnitude of the response was lower than that observed for *Rgs16*. However, consistent with the results of *Rgs16*, the peak response occurred more rapidly in BXD2 B cells than in B6 B cells. *Rgs1* was not upregulated in B cells after IL-17 stimulation (Fig. 4*C*), which is consistent with the previously published data (7).

Canonical NF-κB pathway is required for upregulation of RGS16 in BXD2 B cells

To determine whether IL-17 signaling through the canonical NF-κB pathway is required for upregulation of RGS16 at the protein level, the BXD2 B cells were preincubated with a membrane-permeable inhibitory peptide that specifically blocks the phosphorylation of Ser^{276} of p65 during NF-κB activation (28). We found that pretreatment with this inhibitor blocked the IL-17-induced RGS16 in the B cells of BXD2 mice (Fig. 5*A*, 5*B*). To further determine whether the down-regulation of RGS16 induced by this peptide inhibitor affected the IL-17-induced migration toward the CXCL12 chemokine, the BXD2 B cells were pretreated with the NF-κB p65-Ser^{276} inhibitory peptide (inhibitor) or control peptide PTD prior to culture for 4 hours in the presence or absence of IL-17. Analysis of the migration toward CXCL12 using a chemotaxis chamber confirmed our previous finding that IL-17 treatment resulted in a significant reduction in the migration activity of BXD2 B cells in response to CXCL12 ($p < 0.05$) (Fig. 5*C*) (7). Furthermore, pre-incubation of the BXD2 B cells with the NF-κB p65-Ser^{276} inhibitory peptide prevented the inhibitory effect of IL-17 on the chemotactic response of the BXD2 splenic B cells to CXCL12 ($p < 0.01$) (Fig. 5*C*). Our data indicate that in the B cells of autoimmune BXD2 mice, IL-17 activates the canonical NF-κB signaling pathway, and that the activation of the canonical NF-κB signaling pathway is associated with the upregulation of RGS16 expression and the physiologically important response of these B cells to the CXCL12 chemokine.

IL-17-mediated upregulation of RGS16 expression in B cells requires TRAF6 and ACT1

TRAF6 and ACT1 have been reported to be involved in upstream signaling of IL-17 in many cell types (13,29). Both of them are expressed at equivalent levels in B cells from BXD2 mice compared with those in B6 and BXD2 *Il17ra*$^{-/-}$ B cells (Supplemental Fig 4). To determine whether TRAF6 or ACT1 was required for IL-17R signaling in B cells, we used siRNA for

Traf6 or *Traf3ip2*, the gene encoding ACT1, to knock down these genes in the 70Z/3 pre-B cell line. Comparison between the 70Z/3 pre-B cell line and B cells from the spleens of BXD2 mice indicated that 70Z/3 cells would be an appropriate model for BXD2 B cells. 70Z/3 cells exhibited comparable expression levels of *Il17ra* (data not shown).

Treatment of 70Z/3 cells with IL-17 resulted in increased levels of P-p65 from 5–60 min after IL-17 stimulation, although the peak time of induction differed somewhat from that for BXD2 B cells (Fig. 6*A*, *6B*). Consistent with the results from BXD2 B cells, treatment of 70Z/3 B cells with IL-17 also led to significantly increased expression of RGS16 from 1–10 h following stimulation (Fig. 6*C*, 6*D*). We also confirmed that upregulation of RGS16 in the 70Z/3 cells was dependent upon P-p65 as determined by pretreatment with the NF-κB p65-Ser^{276} inhibitory peptide (Fig. 6*E*, *6F*). These results are consistent with the report by Li et al. (30) that after LPS stimulation, the expression of *Rgs16* is dependent on the canonical NF-κB pathway in the 70Z/3 pre-B cell line.

Treatment of 70Z/3 cells with siRNA for *Traf6* or *Traf3ip2* resulted in ~67% and 66% reductions in the respective mRNA levels at 24 h after transfection compared with those for the scrambled siRNA results, respectively (Fig. 7*A*). Treatment of 70Z/3 cells with siRNA for *Traf6* or *Traf3ip2* also resulted in a significant decrease in the P-p65 activation level (Fig. 7*B*, 7*C*).

On treatment of the 70Z/3 cells with IL-17 (30 ng/ml) for 4 h after siRNA transfection, we found that pre-treatment with siRNA for *Traf6* resulted in ~57% reduction in expression of *Rgs16* ($p < 0.05$) and that pre-treatment with siRNA for *Traf3ip2* resulted in an ~60% reduction in expression of *Rgs16* ($p < 0.05$) (Fig. 7*D*), compared with that for the scrambled siRNA. That is to say, a block in expression of either of these two genes resulted in a dramatic reduction in the IL-17-stimulated expression of *Rgs16*. Chemotaxis of B cells in response to CXCL12 was reduced significantly in 70Z/3 cells pretreated with scrambled siRNA after culture with IL-17. This decreased chemotaxis was strongly reversed by treatment with *Traf6* siRNA (Fig. 7*E*) and was restored partially by treatment with *Traf3ip2* siRNA (Fig. 7*E*). These results indicate that IL-17/IL-17RA signaling in B cells requires both TRAF6 and ACT1 and signals through the canonical NF-κB pathway to upregulate RGS16 expression, which inhibits chemotaxis in response to CXCL12.

Discussion

An understanding of the signaling pathways associated with stimulation of IL-17R in B cells should shed light on the circumstances that promote B cell retention in the GCs and the subsequent development of pathogenic autoantibodies. We previously showed that IL-17 (20 ng/ml) stimulation of purified B cells from B6 or BXD2 mice resulted in ~10-fold upregulation of *Rgs16* and a lesser increase in *Rgs13* levels at 4 h after stimulation. There was equivalent upregulation of *Rgs16* levels in BXD2 compared with those in B6 mice (7). In the present experiment, we found that *Rgs16* is the major RGS that is upregulated in BXD2 B cells compared with those in B6 B cells. We also found that the kinetics of upregulation *Rgs* mRNA is faster in BXD2 B cells compared with that in B6 B cells. Upregulation of *Rgs16* levels in BXD2 B cells peaks at 2 h and is higher than the upregulation of *Rgs16* levels in B6 B cells, which peaks at 4 h. IL-17-induced upregulation of *Rgs13* also peaked at 2 h in BXD2 B cells and 4 h in B6 B cells. The present study therefore clearly showed that there was more rapid and greater upregulation of both *Rgs13* and *Rgs16* levels in BXD2 compared with those in B6 B cells.

A question is whether IL-17 acts on a small population of B cells, such as GC B cells in BXD2 mice, or on a larger population of B cells in B6 and BXD2 mice. The present experiments show

that *Il17ra* expression is greatly increased on all B cells of BXD2 mice compared with that of B6 B cells. The level of stimulation indicated by P-p65 is upregulated significantly on all B cells of BXD2 mice compared with that off B cells from B6 mice. There is no significant greater increase in P-p65 signaling in GC B cells in BXD2 mice compared with that of non-GC B cells. These results indicate that IL-17RA expression and signaling, although higher on B cells of BXD2 mice, are features of all of the B cells and are not restricted to a subpopulation of B cells. However, because the majority of IL-17 producing CD4 T cells locate in the vicinity of a GC (7), this may be one factor that promotes the migration arrest of B cells in the GC area in the spleen of BXD2 mice *in vivo*.

IL-17R signaling occurs through homodimers or heterodimers of IL-17A and IL-17F, which interact through heterodimers of IL-17RA and IL-17RC, usually at a 2:1 ratio. Downstream signaling pathways have been characterized to use the NF-κB, MAPK and C/EBP pathways. IL-17 was shown to induce NF-κB signaling in mouse fibroblasts and human macrophages (31,32). IL-17 also was shown to activate the NF-κB subunits p50 and p65 in intestinal epithelial cells (17). This activation appears to require TRAF6 and IκBα kinase, IKKα. IL-17 induced signal transduction resulted in regulation of three different families of MAPKs in IEC-6 cells (17). These can be linked to the NF-κB activation pathways. TRAF proteins also can activate JNK and NF-κB. Therefore, activation of ERK, JNK/stress-activated protein kinase, and p38 MAPKs by IL-17 in IEC-6 cells may be linked to the NF-κB activation pathway. Because the NF-κB pathway appeared to be most critical, it was studied in B cells in the current study.

Our results show that there is rapid upregulation of the P-p65 canonical pathway in B cells of B6 and BXD2 mice. Upregulation of P-p65 is increased greatly in B cells of BXD2 mice compared with those of B6 mice, which may be in part due to increased expression of *Il17ra* on the surfaces of both GC and non-GC B cells of BXD2 mice. Cytoplasmic and nuclear staining also demonstrates that almost all off the B cells in BXD2 mice exhibit upregulation of P-p65 first in the cytoplasm and then in the nucleus at 5 min after IL-17 stimulation. There was nearly complete downregulation of P-p65 and NF-κB in the nuclei of BXD2 B cells at 15 min, but P-p65 levels remained high in the cytoplasm and in cytoplasmic extracts from BXD2 mice. B cells from B6 mice exhibited a lesser degree of upregulation of P-p65 in both the cytoplasm and the nucleus in response to IL-17 stimulation. Also, at 5 min after IL-17 stimulation, P-IκBα levels increased in the cytoplasm. Activation of P-p65 was associated with induction of p38 MAPK and p44/42 MAPK (Erk1/2) as has been reported previously in other cells (17), but C/EBPβ was not upregulated in B cells. Similar to cytoplasmic levels of P-p65, which persists for 1 h after IL-17 stimulation, P-p38 MAPK and P-p44/42 MAPK (Erk1/2) remained high in total lysate for 30–60 min after IL-17 stimulation. However, levels of P-p65 in the nucleus were rapidly regulated at 5 min and were down-regulated at 15 min as confirmed by confocal microscopy and analysis of nuclear extract. These results suggest that NF-κB target genes are quickly modulated after IL-17 activation in B cells of BXD2 mice.

NF-κB is essential for upregulation of RGS16. B cells incubated with a specific decoy peptide inhibitor of P-p65 that contained a PTD and could permeate into primary B cells of BXD2 mice resulted in nearly complete block of upregulation of RGS16 levels after IL-17 treatment. Treatment of primary B cells from BXD2 mice with IL-17 exhibited a decreased response to CXCL12. Treatment of primary B cells with the P-p65 decoy peptide was highly effective in blocking this IL-17-induced inhibition of chemotaxis to CXCL12. Together, these results indicate that IL-17 specifically signals through the classical NF-κB P-p65 pathway to induce expression of RGS16.

To determine whether TRAF6 and ACT1 are required for IL-17R signaling in B cells, we used a 70Z/3 pre-B cell line. This cell line expresses IL-17R and also signals upregulation of RGS16

to the canonical NF-κB pathway, because pretreatment with the P-p65 peptide inhibitor blocked IL-17 upregulation of RGS16. 70Z/3 B cells transfected with siRNA for *Traf6* or *Traf3ip2* exhibited significant inhibition of P-p65 signaling. siRNA for *Traf6* or *Traf3ip2* also significantly blocked IL-17 induction of RGS16. Correspondingly, there was normalization of the chemotactic response to CXCL12 after IL-17 treatment in B cells transfected with siRNA for either *Traf6* or *Traf3ip2*. These results indicate that IL-17 signaling in B cells is similar to mechanisms in previously described in non-B cells and require TRAF6 and ACT1 to signal NF-κB (13,29).

Finally, the results show that IL-17 induces a relatively larger increase in *Rgs16* level (10 fold) compared with that for *Rgs13* (2- to 3- fold). This is consistent with previous findings that NF-κB is a known transcription factor for RGS16 (30). However, RGS13 levels are constitutively higher in B cells and IL-17 leads to a larger absolute increase in the RGS13 level compared with that in untreated B cells. We propose that although RGS13 and RGS16 both regulate B cell chemotaxis and GC formation in response to IL-17, RGS16 upregulation, compared with that for RGS13, may provide a more finely tuned response to IL-17 to regulate the spontaneous GC response in BXD2 mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

ACT1	NF-κB activator 1
GC	germinal center
PNA	peanut agglutinin
P-p65	phospho-p65
PTD	protein transduction domain
RGS	regulators of G-protein signaling
siRNA	small interfering RNA
Traf3ip2	TNFR-associated factor 3 interacting protein 2
TRAF6	TNFR-associated factor 6

References

1. Fossiez F, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, Maat C, Pin JJ, Garrone P, Garcia E, Saeland S, Blanchard D, Gaillard C, Das Mahapatra B, Rouvier E, Golstein P, Banchereau J, Lebecque S. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. J Exp Med 1996;183:2593–2603. [PubMed: 8676080]
2. Ruddy MJ, Shen F, Smith JB, Sharma A, Gaffen SL. Interleukin-17 regulates expression of the CXC chemokine LIX/CXCL5 in osteoblasts: implications for inflammation and neutrophil recruitment. J Leukoc Biol 2004;76:135–144. [PubMed: 15107456]
3. Laan M, Cui ZH, Hoshino H, Lotvall J, Sjostrand M, Gruenert DC, Skoogh BE, Linden A. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. J Immunol 1999;162:2347–2352. [PubMed: 9973514]
4. Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, Cilley GE, Shen F, Eaton SM, Gaffen SL, Swain SL, Locksley RM, Haynes L, Randall TD, Cooper AM. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. Nat Immunol 2007;8:369–377. [PubMed: 17351619]
5. Van Kooten C, Boonstra JG, Paape ME, Fossiez F, Banchereau J, Lebecque S, Bruijn JA, De Fijter JW, Van Es LA, Daha MR. Interleukin-17 activates human renal epithelial cells in vitro and is expressed during renal allograft rejection. J Am Soc Nephrol 1998;9:1526–1534. [PubMed: 9697677]
6. Gaffen SL. An overview of IL-17 function and signaling. Cytokine 2008;43:402–407. [PubMed: 18701318]
7. Hsu HC, Yang P, Wang J, Wu Q, Myers R, Chen J, Yi J, Guentert T, Tousson A, Stanus AL, Le TV, Lorenz RG, Xu H, Kolls JK, Carter RH, Chaplin DD, Williams RW, Mountz JD. Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. Nat Immunol 2008;9:166–175. [PubMed: 18157131]
8. Hsu HC, Zhou T, Kim H, Barnes S, Yang P, Wu Q, Zhou J, Freeman BA, Luo M, Mountz JD. Production of a novel class of polyreactive pathogenic autoantibodies in BXD2 mice causes glomerulonephritis and arthritis. Arthritis Rheum 2006;54:343–355. [PubMed: 16385526]
9. Mountz JD, Yang P, Wu Q, Zhou J, Tousson A, Fitzgerald A, Allen J, Wang X, Cartner S, Grizzle WE, Yi N, Lu L, Williams RW, Hsu HC. Genetic segregation of spontaneous erosive arthritis and generalized autoimmune disease in the BXD2 recombinant inbred strain of mice. Scand J Immunol 2005;61:128–138. [PubMed: 15683449]
10. Chang SH, Park H, Dong C. Act1 adaptor protein is an immediate and essential signaling component of interleukin-17 receptor. J Biol Chem 2006;281:35603–35607. [PubMed: 17035243]
11. Li X. Act1 modulates autoimmunity through its dual functions in CD40L/BAFF and IL-17 signaling. Cytokine 2008;41:105–113. [PubMed: 18061473]
12. Maitra A, Shen F, Hanel W, Mossman K, Tocker J, Swart D, Gaffen SL. Distinct functional motifs within the IL-17 receptor regulate signal transduction and target gene expression. Proc Natl Acad Sci U S A 2007;104:7506–7511. [PubMed: 17456598]
13. Qian Y, Liu C, Hartupee J, Altuntas CZ, Gulen MF, Jane-Wit D, Xiao J, Lu Y, Giltiay N, Liu J, Kordula T, Zhang QW, Vallance B, Swaidani S, Aronica M, Tuohy VK, Hamilton T, Li X. The adaptor Act1 is required for interleukin 17-dependent signaling associated with autoimmune and inflammatory disease. Nat Immunol 2007;8:247–256. [PubMed: 17277779]
14. Novatchkova M, Leibbrandt A, Werzowa J, Neubuser A, Eisenhaber F. The STIR-domain superfamily in signal transduction, development and immunity. Trends Biochem Sci 2003;28:226–229. [PubMed: 12765832]
15. Allen CD, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, Cyster JG. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. Nat Immunol 2004;5:943–952. [PubMed: 15300245]
16. Hata K, Andoh A, Shimada M, Fujino S, Bamba S, Araki Y, Okuno T, Fujiyama Y, Bamba T. IL-17 stimulates inflammatory responses via NF-kappaB and MAP kinase pathways in human colonic myofibroblasts. Am J Physiol Gastrointest Liver Physiol 2002;282:G1035–1044. [PubMed: 12016129]

17. Awane M, Andres PG, Li DJ, Reinecker HC. NF-kappa B-inducing kinase is a common mediator of IL-17-, TNF-alpha-, and IL-1 beta-induced chemokine promoter activation in intestinal epithelial cells. J Immunol 1999;162:5337–5344. [PubMed: 10228009]

18. Shalom-Barak T, Quach J, Lotz M. Interleukin-17-induced gene expression in articular chondrocytes is associated with activation of mitogen-activated protein kinases and NF-kappaB. J Biol Chem 1998;273:27467–27473. [PubMed: 9765276]

19. Doreau A, Belot A, Bastid J, Riche B, Trescol-Biemont MC, Ranchin B, Fabien N, Cochat P, Pouteil-Noble C, Trolliet P, Durieu I, Tebib J, Kassai B, Ansieau S, Puisieux A, Eliaou JF, Bonnefoy-Berard N. Interleukin 17 acts in synergy with B cell-activating factor to influence B cell biology and the pathophysiology of systemic lupus erythematosus. Nat Immunol 2009;10:778–785. [PubMed: 19483719]

20. Brown KD, Claudio E, Siebenlist U. The roles of the classical and alternative nuclear factor-kappaB pathways: potential implications for autoimmunity and rheumatoid arthritis. Arthritis Res Ther 2008;10:212. [PubMed: 18771589]

21. Hsu HC, Wu Y, Yang P, Wu Q, Job G, Chen J, Wang J, Accavitti-Loper MA, Grizzle WE, Carter RH, Mountz JD. Overexpression of activation-induced cytidine deaminase in B cells is associated with production of highly pathogenic autoantibodies. J Immunol 2007;178:5357–5365. [PubMed: 17404321]

22. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. Cell 2008;132:344–362. [PubMed: 18267068]

23. Sun SC, Ley SC. New insights into NF-kappaB regulation and function. Trends Immunol 2008;29:469–478. [PubMed: 18775672]

24. Shen F, Li N, Gade P, Kalvakolanu DV, Weibley T, Doble B, Woodgett JR, Wood TD, Gaffen SL. IL-17 receptor signaling inhibits C/EBPbeta by sequential phosphorylation of the regulatory 2 domain. Sci Signal 2009;2:ra8. [PubMed: 19244213]

25. Reif K, Cyster JG. RGS molecule expression in murine B lymphocytes and ability to down-regulate chemotaxis to lymphoid chemokines. J Immunol 2000;164:4720–4729. [PubMed: 10779778]

26. Druey KM. Regulation of G-protein-coupled signaling pathways in allergic inflammation. Immunol Res 2009;43:62–76. [PubMed: 18810336]

27. Shi GX, Harrison K, Wilson GL, Moratz C, Kehrl JH. RGS13 regulates germinal center B lymphocytes responsiveness to CXC chemokine ligand (CXCL)12 and CXCL13. J Immunol 2002;169:2507–2515. [PubMed: 12193720]

28. Takada Y, Singh S, Aggarwal BB. Identification of a p65 peptide that selectively inhibits NF-kappa B activation induced by various inflammatory stimuli and its role in down-regulation of NF-kappaB-mediated gene expression and up-regulation of apoptosis. J Biol Chem 2004;279:15096–15104. [PubMed: 14711835]

29. Huang F, Kao CY, Wachi S, Thai P, Ryu J, Wu R. Requirement for both JAK-mediated PI3K signaling and ACT1/TRAF6/TAK1-dependent NF-kappaB activation by IL-17A in enhancing cytokine expression in human airway epithelial cells. J Immunol 2007;179:6504–6513. [PubMed: 17982039]

30. Li J, Peet GW, Balzarano D, Li X, Massa P, Barton RW, Marcu KB. Novel NEMO/IkappaB kinase and NF-kappa B target genes at the pre-B to immature B cell transition. J Biol Chem 2001;276:18579–18590. [PubMed: 11279141]

31. Yao Z, Fanslow WC, Seldin MF, Rousseau AM, Painter SL, Comeau MR, Cohen JI, Spriggs MK. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. Immunity 1995;3:811–821. [PubMed: 8777726]

32. Yao Z, Spriggs MK, Derry JM, Strockbine L, Park LS, VandenBos T, Zappone JD, Painter SL, Armitage RJ. Molecular characterization of the human interleukin (IL)-17 receptor. Cytokine 1997;9:794–800. [PubMed: 9367539]

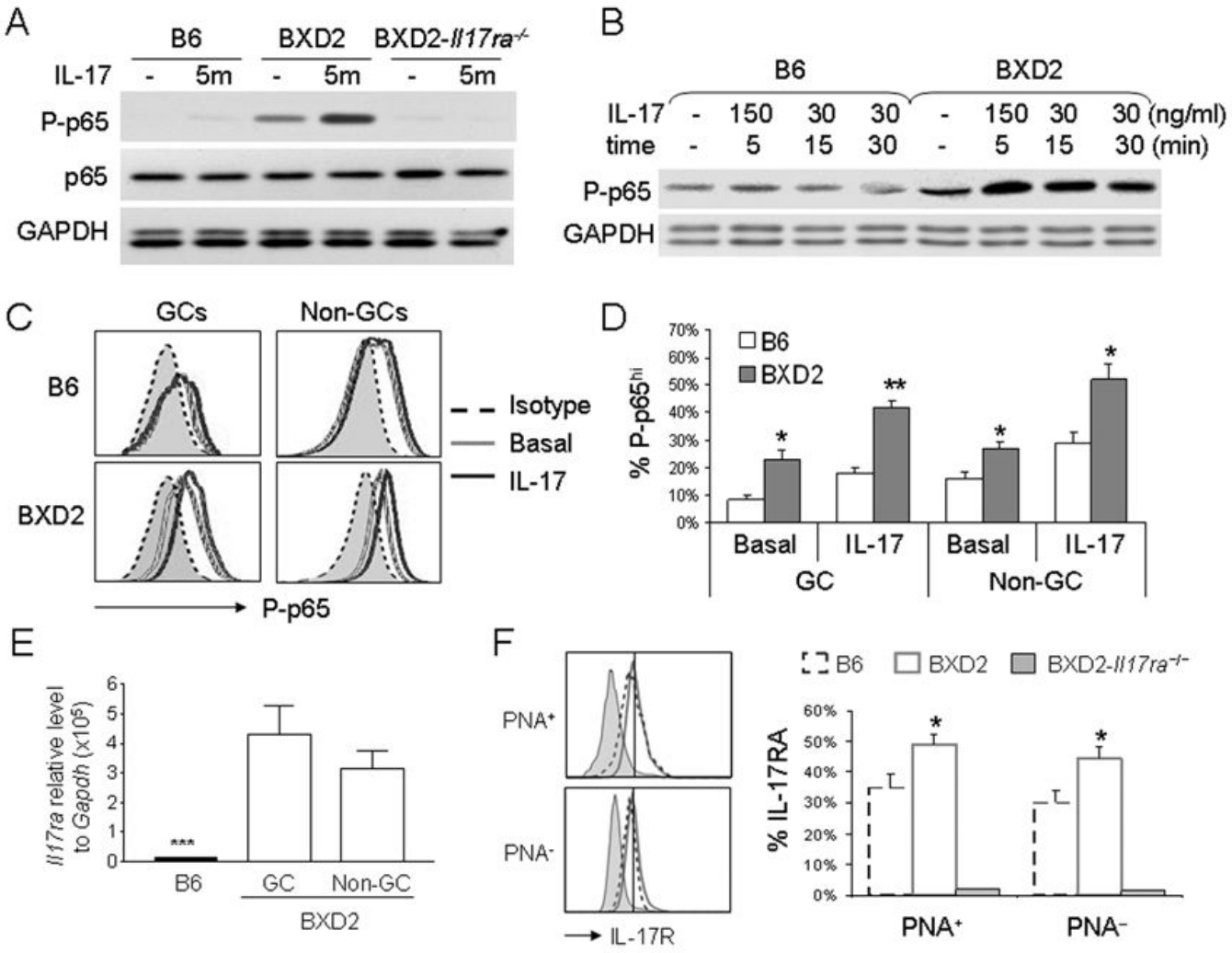

FIGURE 1.

High basal and activated P-p65 by IL-17 in autoimmune B cells of BXD2 mice. MACS-purified $CD19^+$ B cells from B6, BXD2 or BXD2-*Il17ra*$^{-/-}$ mice were treated with or without IL-17 (30 ng/ml) for 5 min. *A*, Whole cell lysates were prepared and analyzed by Western blot analysis with P-p65 and p65 Abs to detect their respective proteins (30 μg in each lane). GAPDH was used as a loading control. The results are representative of three different experiments. *B*, Western blot analysis of the levels of P-p65 and GAPDH in purified B cells from B6 and BXD2 mice after stimulation with IL-17 by the indicated doses and time points. *C* and *D*, Flow cytometry analysis of the levels of P-p65 in GC (Fas^+PNA^+) and non-GC (Fas^-PNA^-) B cells from the indicated mouse strains. Cells were either unstimulated or stimulated with IL-17 (30 ng/ml) for 5 min. *C*, Representative staining for P-p65 in cells gated on GC or non-GC B cells. *D*, the percentages of P-$p65^+$ GC and non-GC B cells are graphed as the mean ± SEM. The data in *C and D* are representative of three independent experiments (*$p < 0.05$; **$p < 0.01$; between B6 and BXD2 B cells). *E*, Real-time quantitative PCR analysis of the levels of *Il17ra* in purified B cells from B6 mice, non-GC (Fas^-PNA^-) and GC (Fas^+PNA^+) B cells from BXD2 mice. The results represent the mean ± SEM. N≥5 from each group. ***$p < 0.005$, between B6 and BXD2 B cells. *F*, Flow cytometry analysis of the expression of IL17RA in B cells from the indicated mouse strains. The percentages of IL17RA in PNA^+ and PNA^- B cells

are graphed as the mean ± SEM. The data in *F* are representative of three independent experiments. *$p < 0.05$, between B6 and BXD2 B cells.

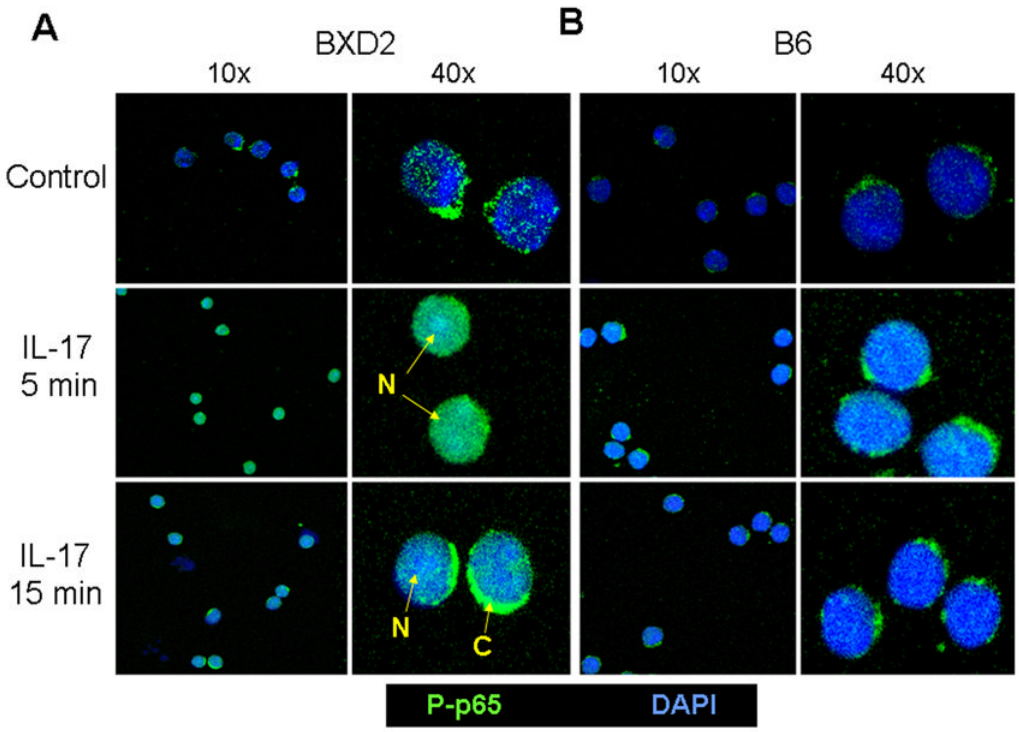

FIGURE 2.

IL-17 rapidly activates nuclear translocation of P-p65 in BXD2 B cells. *A* and *B*, MACS-purified $CD19^+$ B cells from BXD2 (*A*) or B6 (*B*) mice were treated with or without IL-17 (30 ng/ml) for the indicated time points. The cells were fixed and permeablized, and stained with primary P-p65 Ab followed by a FITC-conjugated secondary Ab (Green). The nuclei were stained with DAPI (Blue). The magnification of the objective lens is shown on the top.

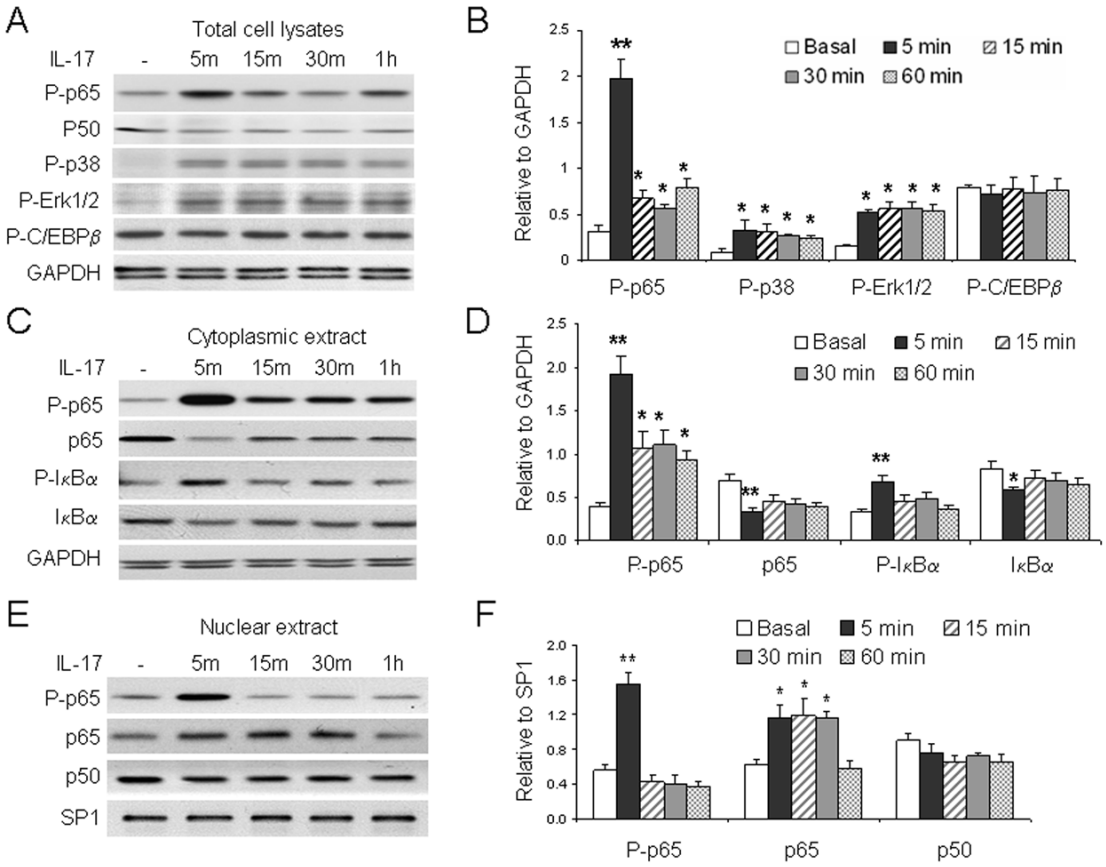

FIGURE 3.

IL-17 rapidly activates the canonical NF-κB signaling pathway in BXD2 B cells. Purified splenic B cells from BXD2 mice were treated with IL-17 (30 ng/ml). Cells were harvested at the indicated time points. *A–F*, The levels of the indicated protein in total cell lysates (*A*), cytoplasmic extract (*C*), and nuclear extract (*E*) were analyzed by Western blot analysis with the respective Abs. Quantitation of the indicated protein after normalization to the levels of GAPDH or SP1 transcription factor is graphed as the mean ± SEM for total cell lysates (*B*), cytoplasmic extract (*D*), and nuclear extract (*F*). The results are representative of three independent experiments. $*p < 0.05$; $**p < 0.01$ compared with unstimulated control.

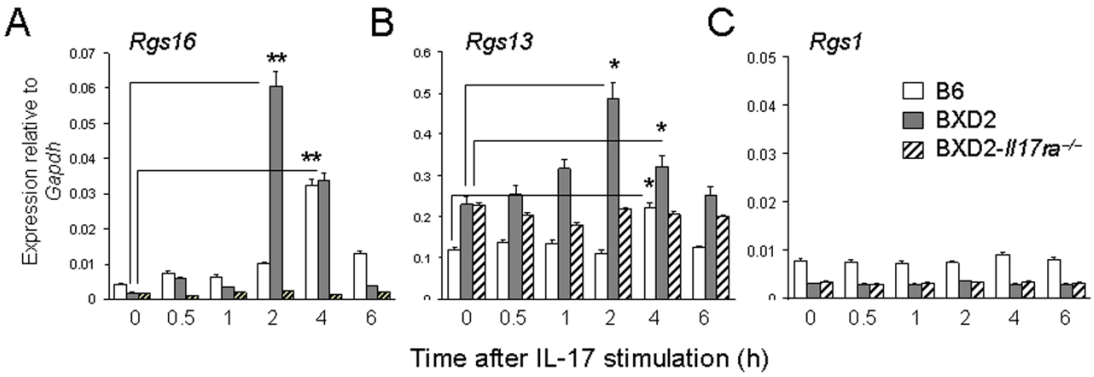

FIGURE 4.

IL-17 upregulated the levels of *Rgs16* and *Rgs13* in B cells. *A–C*, Total RNA was isolated from the purified spleen B cells of BXD2, B6 or BXD2-*Il17ra*$^{-/-}$ mice at different time points after treatment with IL-17 (30 ng/ml). The mRNA levels of *Rgs16* (*A*), *Rgs13* (*B*), and *Rgs1* (*C*) were measured by quantitative real-time PCR and were normalized to *Gapdh*. Significant increases relative to the untreated control are shown. *$p < 0.05$; **$p < 0.01$ compared with unstimulated control. The results are mean ± SEM from three different experiments.

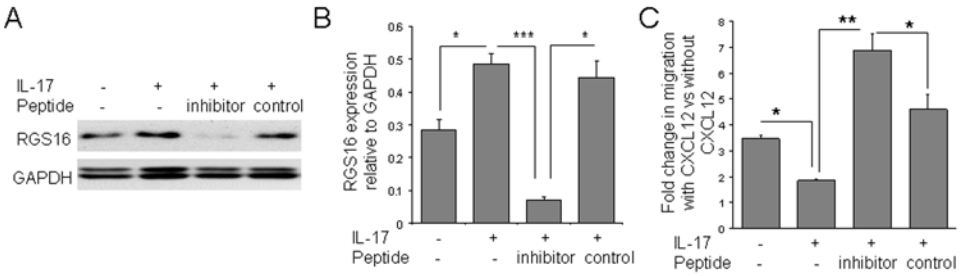

FIGURE 5.

NF-κB peptide inhibitor released the migration arrest of BXD2 B cells by IL-17. *A*, BXD2 B cells were preincubated with peptide inhibitor for 1 h and followed by stimulation with IL-17 (30 ng/ml) for 6 h before being harvested for Western blot analysis of RGS16. The peptide inhibitor is NF-κB p65-Ser^{276} inhibitory peptide, Peptide control is a nonfunctional peptide that corresponds to the PTD sequence of the inhibitor peptide. *B*, Quantitation of the expression of RGS16 after normalization to the levels of GAPDH is graphed as the mean ± SEM. *$p < 0.05$; ***$p < 0.005$ between the indicated comparisons. *C*, BXD2 B cells, after treatment with peptide inhibitor (1 h) and IL-17 (4 h) sequentially, were subjected to a transwell chamber to determine the CXCL12-mediated (100 ng/ml) chemotactic response. The chemotaxis index was calculated by dividing the number of cells that migrated in response to chemokine by the number of cells that migrated in the absence of CXCL12. *$p < 0.05$; **$p < 0.01$ between the indicated comparisons. The results are mean ± SEM from three different experiments.

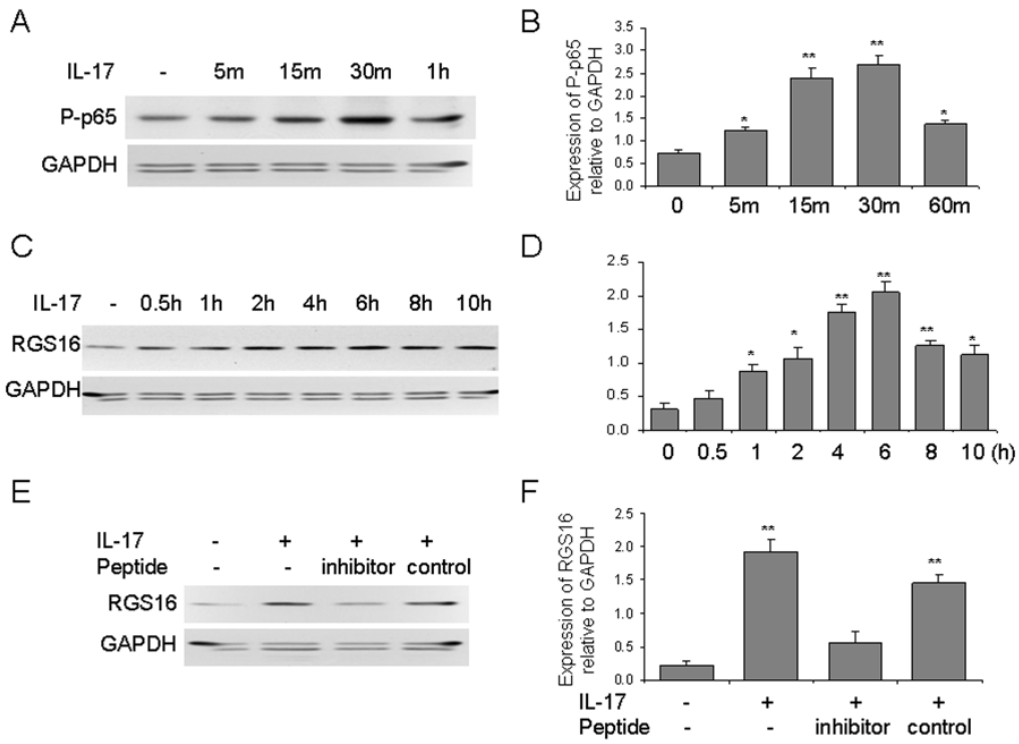

FIGURE 6.

IL-17 upregulated RGS16 via the P-p65 pathway in 70Z/3 pre-B cells. *A* and *B*, 70Z/3 pre-B cells were treated with IL-17 (30 ng/ml) at the indicated time points. Whole-cell lysates were prepared and analyzed by Western blot analysis with Abs against P-p65. *$p < 0.05$; **$p < 0.01$ denotes significant increase relative to the untreated control and normalized to GAPDH. *C and D*, 70Z/3 pre-B cells were treated with IL-17 (30 ng/ml) for the indicated time points. Whole-cell lysates were prepared and analyzed by Western blot analysis with Abs against RGS16. *$p < 0.05$; **$p < 0.01$ denotes significant increase relative to the untreated control. *E and F*, 70Z/3 cells were preincubated with peptide inhibitor for 1 h, and followed by stimulation with IL-17 (30 ng/ml) for 6 h before being harvested for Western blot analysis of RGS16. **$p <$ 0.01 denotes significant decrease relative to the inhibitor control peptide. The results are mean ± SEM from three different experiments.

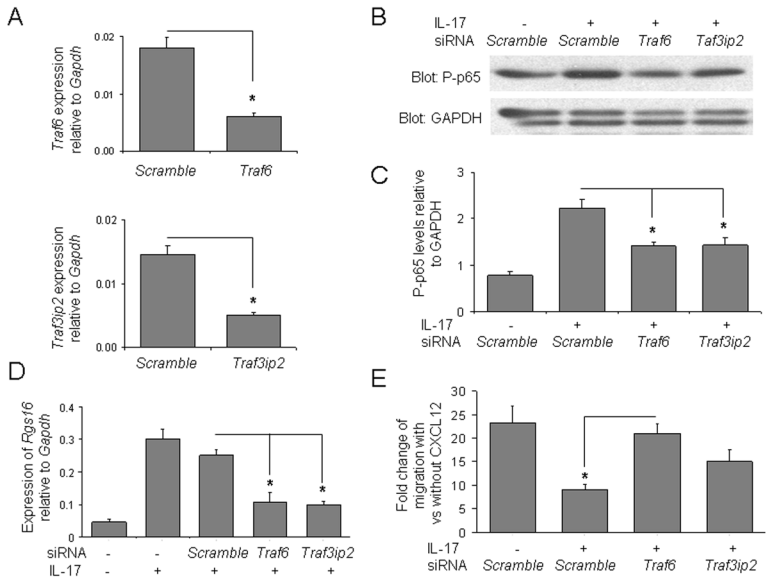

FIGURE 7.

Traf6 and *Traf3ip2* are involved in IL-17-mediated upregulation of *Rgs16* in 70Z/3 pre-B cells. 70Z/3 pre-B cells were transfected with siRNA for *Traf6*, *Traf3ip2*, or scrambled siRNA. After 24 h of siRNA transfection, the cells were treated with IL-17 (30 ng/ml). *A*, The expression of *Traf6* (*upper panel*) and *Traf3ip2* (*lower panel*) at the mRNA levels was measured by quantitative real-time PCR. *B* and *C*, Western blot analysis of the levels of P-p65 in the indicated siRNA-transfected 70Z/3 cells (*B*). Quantitation of P-p65 after normalization with the levels of GAPDH is graphed as the mean ± SEM (*C*). *D* and *E*, siRNA-transfected 70Z/3 cells were stimulated with IL-17 (30 ng/ml) for 4 h, the expression of *Rgs16* was analyzed by real-time PCR (*D*), and the migration response of these cells to CXCL12 was analyzed by a transwell migration chamber (*E*). *$p < 0.05$ between the indicated comparisons. $n = 3$; mean ± SEM for all panels.