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IkBNS Regulates Murine Th17 Differentiation during Gut Inflammation and Infection

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IL-17–producing Th17 cells mediate immune responses against a variety of fungal and bacterial infections. Signaling via NF-κB has been linked to the development and maintenance of Th17 cells. We analyzed the role of the unusual inhibitor of NF-κB, IkBNS, in the proliferation and effector cytokine production of murine Th17 cells. Our study demonstrates that nuclear IkBNS is crucial for murine Th17 cell generation. IkBNS is highly expressed in Th17 cells; in the absence of IkBNS, the frequencies of IL-17A–producing cells are drastically reduced. This was measured in vitro under Th17-polarizing conditions and confirmed in two colitis models. Mechanistically, murine IkBNS−/− Th17 cells were less proliferative and expressed markedly reduced levels of IL-2, IL-10, MIP-1α, and GM-CSF. Citrobacter rodentium was used as a Th17-inducing infection model, in which IkBNS−/− mice displayed an increased bacterial burden and diminished tissue damage. These results demonstrate the important function of Th17 cells in pathogen clearance, as well as in inflammation-associated pathology. We identified IkBNS to be crucial for the generation and function of murine Th17 cells upon inflammation and infection. Our findings may have implications for the therapy of autoimmune diseases, such as inflammatory bowel disease, and for the treatment of gut-tropic infections. The Journal of Immunology, 2015, 194: 2888–2898.

The identification of the proinflammatory Th17 cell subset marked a significant breakthrough in our understanding of the Th cell compartment (1, 2), expanding the established Th1/Th2 paradigm (3). Previously, only the regulation of cellular immune responses against viruses by Th1 cells and humoral responses by Th2 cells in response to extracellular pathogens were known (3). Today, we know that Th17 cells are essential for host protection against a variety of extracellular bacteria and fungi located predominantly in the gastrointestinal tract, skin, and lungs. Consequently, most Th17 cells are found in these mucosal tissues upon infection; however, some reside in the lamina propria (LP) under steady-state conditions (4, 5). Th17 cells depend on the transcription factor RORγt (6) and primarily produce the cytokine IL-17A, which is a potent inducer of other proinflammatory cytokines, such as IL-6, IL-1β, and TNF-α, in a variety of IL-17R–expressing cell types, including endothelial cells, epithelial cells, and macrophages (7, 8). IL-17A is also involved in the recruitment and activation of neutrophils to sites of infection (7). Moreover, Th17 cells secrete GM-CSF, which controls granulocyte and macrophage maturation. Expression of GM-CSF was reported to be responsible for the pathogenicity of Th17 cells during experimental autoimmune encephalomyelitis (EAEd) (9, 10), the murine disease model for multiple sclerosis. Of note, IL-17–producing T cells can be generated in vitro by treating naive CD4+ cells with TGF-β and IL-6 (11, 12) because both cytokines induce the expression of RORγt (6).

NF-κB is an inducible transcription factor and is crucial for lymphocyte development and function (13). In T cells, activation of NF-κB signaling is governed by the CARMA1, MALTI1, and BCL10 complex that activates the inhibitor of κB kinase (IKK) complex (14). The IKK complex phosphorylates IkBα, which binds to NF-κB transcription factors and causes cytoplasmic retention of NF-κB by blocking the nuclear-localization signal (15). After phosphorylation by IKKβ, IkBα becomes polyubiquitinated and, finally, undergoes proteasomal degradation (13). Consequently, NF-κB is released, enters the nucleus, and regulates gene expression via binding to κB sites. NF-κB transcription factors are dimers composed of two members of the Rel-protein family, comprising p50, p52, p65/RelA, c-Rel, and RelB (13). In the nucleus, these transcription factors are regulated via atypical IkB proteins, which include IkBNS (encoded by the gene Nkbnd1), IkBζ, BCL-3, and IkBη (16).

We reported recently that the atypical NF-κB inhibitor IkBNS regulates regulatory T cell (Treg) generation (17). Although IkBNS−/− mice display a severe reduction in Treg numbers, they do not develop signs of spontaneous autoimmunity (17). Thus, we speculated that IkBNS might be important for the activation,
proliferation, or cytokine production of proinflammatory effector T cells. Indeed, we observed strong expression of Il27 in CD4+ cells and strongly impaired Th17 responses in vitro and in vivo. Our results demonstrate that Il27 is required for Th17 differentiation and function.

Materials and Methods

Mice strains

B6.129 SV-NFkb2tm1Clay (C57BL/6 background, later referred to as Il27null/mice, were described previously (18). Il27null mice were crossed to Foxp3DTR-EGFP mice, as previously described, later referred as Il27null/ Foxp3DTR-EGFP reporter mice (17). Il27null/ Foxp3DTR-EGFP, and B6.PL-Thy1+ mice (Thy1.1 mice) were bred under specific pathogen–free conditions in the animal facility of the Helmholtz Centre for Infection Research. B6.129S7-Rag2tm1Mom (RAG1−/−) mice (19) were bred at the animal facility of the Charité – Universitätsmedizin Berlin. All animal experiments and breeding were performed in accordance with the guidelines of national and local authorities.

Chronic dextran sodium sulfate–induced colitis

To induce colitis, mice were fed in four cycles, each comprising 7 d, with drinking water containing 3% (w/v) dextran sodium sulfate (DSS) as an initial dose and 2% (w/v) DSS for all further treatments, followed by 14 d of feeding with DSS-free water. Inflammation of the colon was assessed at the end of the last cycle. Body weight was measured during the progression of colitis. Consistency of the stool and rectal bleeding were scored as described previously (17). At the end of the experiment, the length of the colon was measured from the cecum to the anus. To analyze histological changes in the colon, colon samples were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 2-μm sections. The colon sections were deparaffinized and stained with H&E. Fixed colon samples were scored in a blinded manner, as described earlier (17). The histological score of DSS colitis is the sum of the individual scores for inflammatory cell infiltrations and tissue damage.

Adoptive-transfer colitis

For adoptive-transfer colitis, spleen and lymph nodes were used to isolate CD4+CD25− T cells from wild-type or Il27null mice or CD4+GFPP Tregs from wild-type or Il27null/ Foxp3DTR-EGFP reporter mice. The cells were sorted via flow cytometry (FACSAria II; BD Biosciences) or by using MACS separation kits (CD4 T Cell Isolation Kit, CD25-PE Kit; Miltenyi Biotec), as described in the supplier’s manual. A total of 200 μl 1× PBS containing 5 × 106 CD4+CD25− T cells, alone or together with 5 × 106 CD4+GFPP Tregs, were injected i.p. into RAG1−/− mice. Body weight was monitored during colitis progression. Consistency of the stool and rectal bleeding were scored as described earlier (17). The length of the colon was measured from the cecum to the anus, and colon samples were scored in a blinded manner, as described previously (17).

Citrobacter rodentium infection

Mice were infected with C. rodentium strain ICC180 expressing the luxoperon (courtesy of S. Wiles, Auckland, New Zealand). C. rodentium was cultured in Lennox broth medium (Roth) at 37°C overnight. The next day, the bacteria were adjusted to 1 × 1011 CFU in 1 ml 1× PBS. A total of 1 × 1010 C. rodentium was administered to mice orally. Body weight was monitored during the progression of infection. Bacteria-derived biofilm nesence during infection was measured in vivo by bioluminescence imaging using a Berthold LB 9507 microimager. At day 10, the length of the colon measured from the cecum to the anus. The stool was collected in 1 ml Lennox broth, weighed, homogenized, and diluted serially. To determine the bacterial load in organs, the liver and spleen were homogenized and diluted serially. Serial dilutions were added to MacConkey Agar (Roth) and cultured at 37°C for 24 h. CFU were counted and, for feces, bacterial burden was normalized to stool weight. For histology of C. rodentium–infected mice, whole-colon samples were placed in a shape of a “Swiss roll” and fixed in 4% Roti-Histofix (Roti), embedded in paraffin, cut into 8-μm sections, deparaffinized, stained with H&E, and scored in a blinded manner. The histological sections were scored for epithelial hyperplasia (score based on percentage above the height of the control where 0 = no change, 1 = 1–50%, 2 = 51–100%, and 3 = > 100%). Epithelial integrity was scored as follows: 0, no change; 1, mild epithelial ulceration and cryptic destruction; 2, moderate epithelial ulceration and cryptic destruction; and 3, severe epithelial ulceration and cryptic destruction. Tissue sections were scored for mononuclear cell infiltration as follows: 0, no change; 1, mild; 2, moderate; and 3, severe.

Isolation of LP mononuclear cells

To isolate cells from LP, colon samples were placed into 15 ml ice-chilled 0.5 mM EDTA for 30 min (on ice), followed by successive rinsing with 1× PBS to remove residual epithelium. Then the tissue was cut into fine pieces and digested in DMEM (Life Technologies) supplemented with 1 mg/ml Collagenase D (Roche) and 0.1 mg/ml DNase I (Roche) three times for 30 min at 37°C. After each round of incubation, cells were suspended by passing them through a 100-μm mesh. After centrifugation, the cell pellet was resuspended in a 40% isotonic Percoll solution (GE Healthcare) and underlay with an 80% isotonic Percoll solution. After centrifugation (900 × g, room temperature for 20 min), LP lymphocytes (LPLs) were yielded from the interface cell ring of the 40–80% Percoll gradient. Cells were washed with PBS containing 2% FCS and used for further studies.

In vitro generation of Th cell subsets

CD4+CD25+B220+CD25− naive T cells were sorted via flow cytometry (FACS Aria II; BD Biosciences or MoFlo; Beckman and Coulter) and resuspended in primary T cell medium (IMDM containing 10% FCS, 50 μg/ml penicillin/streptomycin, 25 mM HEpes, 0.05 mM 2- ME, 1 nonessential amino acids, 1 mM sodium pyruvate). Two × 10^6 cells were seeded per well (96-well plate) and were activated with plate-bound anti–CD3 (both from BD Biotech) in the presence of priming cytokines and inhibitory Abs, according to the respective Th subset: Th0- 2 μg/ml anti–CD3 (145-2C11; BioLegend), 2 μg/ml anti–CD28 (37.51; BioLegend), 10 μg/ml anti–IL-4 (11B11; made in-house), and 10 μg/ml anti–IFN-γ (XMG1.2; made in-house); Th1- 2 μg/ml anti–CD3 (145-2C11; BioLegend), 2 μg/ml anti–CD28 (37.51; BioLegend), 10 μg/ml anti–IL-4 (11B11; made in-house), and 10 ng/ml IL-12 (R&D System); Th17– 3 μg/ml anti–CD3 (145-2C11; BioLegend), 5 μg/ml anti–CD28 (37.51; BioLegend), 10 μg/ml anti–IL-2 (JES6-1A12; BioLegend), 10 μg/ml anti–IFN-γ (XMG1.2; made in-house), 2 mg/ml porcine TGF-β (R&D Systems), 30 ng/ml IL-6 (R&D Systems), 10 ng/ml IL-1β (R&D Systems), and 20 ng/ml TNF-α (PeproTech). Cells were analyzed on day 4.

In vitro expansion of CD4+ CD25− T cells

For expansion of CD4+CD25− T cells, 4 × 10^6 cells were expanded in RPMI 1640 supplemented with 10% FCS, 1 mM sodium pyruvate, 1× nonessential amino acids, 50 ng/ml penicillin/streptomycin, and 50 μM 2-ME. For expansion, cells were stimulated in a six-well plate with 1 μg/ml plate-bound anti–CD3 and 2 μg/ml soluble anti–CD28 in the presence of 10 ng/ml murine IL-2. On day 3, cells were transferred into a 10-cm dish in a total volume of 15 ml fresh RPMI 1640 with supplements. On day 6, 5 × 10^6 to 10 × 10^6 cells were seeded on a 12-well plate and centrifuged for 2 min at 800 rpm at room temperature. Cells were stimulated with coated anti–CD3 (10 μg/ml) plus anti–CD28 (5 μg/ml) for 15 or 30 min or for 1, 2, or 4 h; stimulated with PMA (10 ng/ml) plus ionomycin (1 μM) for 2 h; or left untreated. Subsequently, cells were analyzed by Western blotting.

Proliferation analysis by CFSE staining

For proliferation analysis, sorted naive T cells were stained with CFSE (eBioscience). Briefly, T cells were washed two times with 1× PBS and resuspended in 1× PBS to a concentration of 2 × 10^6/ml. CFSE was added to a final concentration of 5 μM. Cells were incubated for 10 min (CFSE) at 37°C in the dark and cultured under Th0-, Th1-, or Th17-polarizing conditions. On day 4, cells were stained for surface markers and intracellular proteins and analyzed by flow cytometry.

Enrichment of Th17 cells via mouse IL-17 Secretion Assay

Naive T cells were isolated and cultured under Th17-polarizing conditions, as described above. On day 4, cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μM; both from Sigma-Aldrich) for 3 h at 37°C. A mouse IL-17 Secretion Assay (Miltenyi Biotec) was used for enrichment of IL-17+ cells. The cells were incubated and enriched according to the supplier’s instruction manual, and isolated cells were used to analyze cytokine secretion.

Analyses of cytokine expression

To analyze the cytokine secretion of enriched IL-17–secreting cells by Proteome Profiler Array, 0.8 × 10^6 cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μM; both from Sigma-Aldrich). After 4 h, the supernatant was taken and used undiluted or diluted 1:10 or 1:50 for cytokine analysis using the Proteome Profiler Array (R&D Systems). The array membrane was activated and thereafter incubated with the Th17 supernatant, as described in...
controls. (17A+ isolated, and the expression of surface markers and intracellular cytokines was analyzed by flow cytometry. Adoptive transfer of freshly isolated CD4+CD25 T cells from IκBNS−/− (n = 35) or IκBNS−/− (n = 35) mice. Mice injected with PBS (n = 17) served as controls. (A) The histological score is the sum of individual scores of inflammatory cell infiltration and tissue damage. (B–E) Lymphocytes of the colon were analyzed by flow cytometry, as described above, and an Fc-block step (10 min, on ice) was included before cell surface staining and the fixation/permeabilization buffers (eBioscience) were used.

**Cell lysis and determination of protein concentration**

Total-cell lysates were obtained by lysing in TPNE lysis buffer (1X PBS, 300 mM NaCl, 2 mM EDTA, 1% v/v Triton X-100) supplemented with 1 mM PMSF, 1 µg/ml protease inhibitor mix (aprotinin, leupeptin, Pepstatin A, chymostatin), and 0.4 mM sodium orthovanadate. Incubation for 20 min on ice was followed by centrifugation (14,000 rpm, 4°C, 15 min). The protein concentration of the lysate was determined by a BCA Protein Assay (Thermo Scientific), following the manufacturer’s instructions.

**Western blot analysis**

For protein separation via SDS-PAGE, 20–40 µg the protein lysate was loaded onto a gel. Proteins were separated on a 12% polyacrylamide gel. For further analysis, the proteins were blotted onto a polyvinylidene difluoride membrane (GE Healthcare) and detected with the following Abs: β-actin (AC-74; Sigma-Aldrich), c-Rel (2851; R&D Systems), ERK (9102; Cell Signaling Technology), IκB (rabbit; made in-house), IκBα (C-21; Santa Cruz), p38 (9212; Cell Signaling Technology), p50/105 (E381; Epitomics), p52/100 (4882; Cell Signaling Technology), p65 (C-20; Santa Cruz Biotechnology), p-Akt (4060; Cell Signaling Technology), p-ERK (4370; Cell Signaling Technology), p-IκBα (2859; Cell Signaling Technology), p-p38 (9211; Cell Signaling Technology), p-SAPK-JNK (9251; Cell Signaling Technology), p-Tyrosine (4610; kind gift of Prof. B. Schravan, Otto-von-Guericke University), and RelB (C-19; Santa Cruz Biotechnology).

**Chromatin immunoprecipitation**

For chromatin immunoprecipitation (ChIP) analyses, Th17 cells were generated and stimulated with PMA/ionomycin for 4 h, as described above. ChIP was performed with a ChIP-IT Express Enzymatic kit (Active Motif), using a modified procedure to achieve ChIP without cross-linking. Hence, procedural steps to remove paraformaldehyde and
to reverse cross-linking were not performed. Rabbit polyclonal IκBNS Ab was used; after elution of the precipitated DNA, IL-10 was amplified by real-time PCR.

**Real-time PCR**

cDNA was used as a template for real-time PCR using SYBR Green (Roche). Ubiquitin C (UBC) was used as a housekeeping gene for normalization. Measurements were run in duplicates in the LightCycler 480 (Roche). Ubiquitin C (UBC) was used as a housekeeping gene for normalization. Measurements were run in duplicates in the LightCycler 480 (Roche).

**Statistics**

GraphPad Prism software (GraphPad) was used for all statistical analyses. To determine statistical significance, the two-tailed Mann-Whitney U test or Student t test was used; error bars represent the SEM.

**Results**

**IkBNS triggers Th17 development during gut inflammation**

In response to intestinal infections and inflammation, Th17 cells accumulate in the gut and surrounding lymphoid tissues (20, 21). To unveil whether IkBNS deficiency affects Th cell differentiation, we induced chronic colitis that is promoted by Th1 and Th17, as well as B cells (22), in IkBNS−/− and wild-type mice by repeated DSS feeding. Although DSS-fed IkBNS−/− and wild-type mice did not display marked histological differences, there was inherent colon damage in the nontreated IkBNS−/− mice that was not present in wild-type mice (Supplemental Fig. 1A–D). Most importantly, the frequency of IL-17A+ T cells was decreased in LPLs of IkBNS−/− mice compared with wild-type mice (Supplemental Fig. 1E, 1F). Interestingly, Th17 frequencies already were reduced in nontreated, IkBNS−/− animals compared with respective wild-type mice (Supplemental Fig. 1E, 1F).

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**FIGURE 2.** Impaired Th1 and Th17 differentiation in the absence of IkBNS. CD4+CD25−CD62L− naïve T cells were isolated by flow cytometry and cultured under Th1-polarizing, Th17-polarizing, or nonpolarizing (Th0) conditions. Cells were analyzed with or without PMA/ionomycin (P/I) (A, B, E, and F) or anti-CD3/anti-CD28 stimulation (4 h) (C and D). (A and C) The induction of IkBNS/Nfkbid mRNA was analyzed by real-time PCR. The relative expression of Nfkbid was calculated by normalization to unstimulated T cells. (B and D) Western blot analyses of IkBNS and β-actin protein expression. (D) The single blot (right panel) represents the protein expression of IkBNS-deficient Th17 cells. (E) The expression of IFN-γ and IL-17A was analyzed by flow cytometry in wild-type and IkBNS−/− T cells upon Th1 and Th17 polarization. Representative dot plots are shown. (F) Statistical analysis of the frequencies of in vitro–differentiated IL-17A IFN-γ+ (Th1) and IL-17A IFN-γ− (Th17) cells. Data shown are representative of at least three (A–D) or four (E) independent experiments. Error bars show SEM. *p < 0.05, **p < 0.01, two-tailed Mann-Whitney U test.
Next, we investigated whether Th17 differentiation also is impaired in T cell–based transfer colitis. Rag1\(^{-/-}\) mice receiving I\(\kappa\)BNS\(^{-/-}\) T cells exhibited significantly more colon damage than did wild-type controls (Fig. 1A). Importantly, the induction of both IL-17A+IFN-\(\gamma\) (Fig. 1B, 1C) and IL-17A+IFN-\(\gamma\)^+ (Fig. 1D) T cells was completely blunted in I\(\kappa\)BNS\(^{-/-}\) mice. In contrast, the frequency of IL-17A+IFN-\(\gamma\) T cells (Fig. 1E) was slightly, but significantly, increased in I\(\kappa\)BNS\(^{-/-}\) mice, demonstrating the importance of I\(\kappa\)BNS for the formation of Th17 cells in the inflamed gut. Previously, we reported a reduced number of Tregs in I\(\kappa\)BNS\(^{-/-}\) mice (17). To analyze whether the increased frequency of Th1 cells in I\(\kappa\)BNS-deficient animals arose from reduced numbers of Tregs, Tregs from I\(\kappa\)BNS\(^{-/-}\)Foxp3\(^{DTR-eGFP}\) or I\(\kappa\)BNS\(^{+/+}\)Foxp3\(^{DTR-eGFP}\) reporter mice were cotransferred (Fig. 1F). The cotransfer of Tregs had no effect on the increased Th1 cell frequency in I\(\kappa\)BNS\(^{-/-}\) mice, regardless of whether the Tregs were I\(\kappa\)BNS deficient or proficient.

In summary, both colitis models revealed a requirement for I\(\kappa\)BNS in Th17 cell differentiation during gut inflammation.

I\(\kappa\)BNS drives Th17 and Th1 development in vitro

To determine whether I\(\kappa\)BNS is differentially expressed in Th cell subsets, we analyzed I\(\kappa\)BNS (Nfkbid) expression at the mRNA level by real-time PCR (Fig. 2). We detected comparable Nfkbid mRNA expression in unprimed T cells (Th0) and in Th1- and Th17-polarized cells upon restimulation with PMA and ionomycin (Fig. 2A). In addition, high I\(\kappa\)BNS expression was observed at the protein level in reactivated T cells (Fig. 2B). Similar findings were obtained when T cells were restimulated with anti-CD3 and anti-CD28 Abs (Fig. 2C, 2D). Th1 cells exhibited a higher I\(\kappa\)BNS protein expression than did Th17 cells. Notably, the nonactivated Th17 cells displayed higher I\(\kappa\)BNS expression compared with the other nonactivated cells (Fig. 2B, 2D), substantiating a pivotal role for I\(\kappa\)BNS specifically in this T cell subset. Indeed, during in vitro priming we monitored severely reduced frequencies of both IL-17A+ Th17 cells and IFN-\(\gamma\)^+ Th1 cells in the absence of I\(\kappa\)BNS (Fig. 2E, 2F). We conclude that I\(\kappa\)BNS expression is not restricted to a single Th cell subset but forces the differentiation of Th17 and Th1 cells in vitro.

Proliferation defect in I\(\kappa\)BNS-deficient Th17 cells

To identify the reason for the reduced frequency of Th17 and Th1 cells induced by I\(\kappa\)BNS deficiency, we first analyzed Th cell proliferation in vitro. Although all T cells proliferated equally during the first 2 d of culture, I\(\kappa\)BNS\(^{-/-}\) T cells primed for both Th17 and Th1 differentiation proliferated much less compared with the wild-type cells in the following days (Supplemental Fig. 2). To determine whether wild-type cells could compensate for the proliferation defect of I\(\kappa\)BNS\(^{-/-}\) cells when cocultured, we dif-
differentiated \( \text{IkB}_{\text{NS}}^{-/-} \) (CD90.2\(^+\)) T cells in the presence of congenic wild-type (CD90.1\(^+\)) cells, both stained with CFSE. Again, the frequencies of \( \text{IkB}_{\text{NS}}^{-/-} \) Th0, Th1, and Th17 cells showed a 50% reduction compared with wild-type cells, suggesting a cell-intrinsic effect of \( \text{IkB}_{\text{NS}} \) on T cell differentiation (Fig. 3A). The CFSE profile and mean number of divisions/cell (division index) revealed enhanced proliferation of wild-type Th0, Th1, and Th17 cells compared with \( \text{IkB}_{\text{NS}}^{-/-} \) cells (Fig. 3B, 3C). Remarkably, the proliferation defect caused by \( \text{IkB}_{\text{NS}} \) deficiency was most pronounced in Th17-differentiated cells. Of note, the proliferation defect was not due to impaired proximal TCR signaling because tyrosine phosphorylation; the activation of the kinases Akt, ERK, and p38; expression of the NF-\( \kappa \)B family members c-Rel, NF-\( \kappa \)B1, NF-\( \kappa \)B2, p65, and RelB; and the expression and phosphorylation status of \( \text{IkB}_{\alpha} \) were unaltered (Supplemental Fig. 3).

\( \text{IkB}_{\text{NS}} \) regulates Th17 cytokine expression

Because ROR\( \gamma \)t is an essential transcription factor for Th17 differentiation and function (6), we analyzed the expression of Rorc (encoding ROR\( \gamma \)t) in \( \text{IkB}_{\text{NS}}^{-/-} \) and wild-type Th1 and Th17 cells by real-time PCR. Despite defective Th17 differentiation of \( \text{IkB}_{\text{NS}}^{-/-} \) cells, Rorc expression was comparable in wild-type and \( \text{IkB}_{\text{NS}}^{-/-} \) Th17 cells (Fig. 4A). Next, we analyzed whether \( \text{IkB}_{\text{NS}}^{-/-} \) Th17 cells display an altered cytokine expression profile. Coculturing wild-type and \( \text{IkB}_{\text{NS}}^{-/-} \) T cells under Th17-polarizing conditions, we observed, in addition to the proliferation defect (Fig. 3), a significantly decreased mean fluorescence intensity (MFI) of IL-17A in \( \text{IkB}_{\text{NS}}^{-/-} \) cells (Fig. 4B, 4C), indicating a role for \( \text{IkB}_{\text{NS}} \) in Th17 cell function. To determine whether \( \text{IkB}_{\text{NS}} \) also impairs the expression of other cytokines, a cytokine array membrane was performed. We enriched in vitro-generated wild-type and \( \text{IkB}_{\text{NS}}^{-/-} \) Th17 cells to obtain comparable numbers of IL-17A–secreting cells. The results revealed reduced secretion of IL-2, IL-10, GM-CSF (Csf2), and MIP-1\( \alpha \) (Ccl3) by \( \text{IkB}_{\text{NS}}^{-/-} \) Th17 cells (Fig. 4D, Supplemental Fig. 4). Using a bead-based assay and flow cytometry, the reduced secretion of the cytokines IL-2, IL-10, and MIP-1\( \alpha \) in \( \text{IkB}_{\text{NS}}^{-/-} \) Th17 cells was verified (Fig. 4E). Nevertheless, secretion of the

![Image](http://www.jimmunol.org/)

**FIGURE 4.** Expression of Th17-specific cytokines is impaired by \( \text{IkB}_{\text{NS}} \) deficiency. CD4\(^+\)CD25\(^-\)CD62L\(^+\) naive T cells were isolated by flow cytometry and cultured under Th1-polarizing, Th17-polarizing, or nonpolarizing (Th0) conditions. (A) T cells were stimulated with PMA/ionomycin (P/I) for 4 h or were left untreated. Subsequently, the expression of Rorc was measured by real-time PCR, and the fold induction was calculated by normalization to UBC. (B) CFSE-labeled CD4\(^+\)CD25\(^-\)CD62L\(^+\) naive \( \text{IkB}_{\text{NS}}^{+/+} \) (CD90.1\(^+\)) and \( \text{IkB}_{\text{NS}}^{-/-} \) (CD90.2\(^+\)) T cells were cultured at a 1:1 ratio under Th17-polarizing conditions. On day 4, IL-17A expression was analyzed by flow cytometry. MFI of IL-17A for every proliferation cycle was analyzed. Representative dot plots showing different MFI of IL-17A within five proliferation steps (G1–G5). (C) The fold reduction in the MFI of IL-17A in \( \text{IkB}_{\text{NS}}^{-/-} \) cells compared with \( \text{IkB}_{\text{NS}}^{+/+} \) cells was determined within the five proliferation steps shown in (B). (D) Isolated naive T cells were cultured under Th17-priming conditions. IL-17A–expressing cells were enriched via a cytokine-secretion assay. Relative levels of cytokines in the supernatant of stimulated \( \text{IkB}_{\text{NS}}^{+/+} \) and \( \text{IkB}_{\text{NS}}^{-/-} \) Th17 cells were assessed using a cytokine array membrane. The expression of IL-2, IL-10, MIP-1\( \alpha \), and GM-CSF in \( \text{IkB}_{\text{NS}}^{-/-} \) cells relative to wild-type Th17 cells is shown. (E) Supernatants were prepared as in (D), and cytokines were measured by a bead-based flow cytometry assay. (F) Isolated naive T cells were cultured under Th17-priming conditions. Subsequently, RNA was prepared for analyses by real-time PCR. The fold induction of Il2, Il10, Il17a, Il17f, Il22, Ccl3, and Csf2 in Th17 cells was calculated relative to nonstimulated Th17 cells after normalization to UBC. Error bars represent the SEM of two (D), three (A), or four (C and E) independent experiments or the mean of seven differentiations in five independent experiments (F). * \( p < 0.05 \), ** \( p < 0.01 \), two-tailed Mann–Whitney \( U \) test.
Th17 cytokines IL-17A, IL-17F, and IL-22 was not affected by IkBNS deficiency. Furthermore, we analyzed the effect of IkBNS on mRNA expression of the respective cytokines in stimulated Th17 cells by real-time PCR. In accordance with the protein data, we detected reduced mRNA expression of Il10 and Csf2 in IkBNS\(^{-/-}\) Th17 cells, whereas Il17a and Il17f expression was not affected (Fig. 4F). Interestingly, in contrast to IL-22 cytokine secretion, IkBNS\(^{-/-}\) Th17 cells showed an increased expression of IL-22 mRNA. We conclude that IkBNS regulates the expression of multiple key Th17 cytokines.

IkBNS\(^{-/-}\) mice display impaired Th17 development and high susceptibility to C. rodentium infection

C. rodentium is a noninvasive pathogen that establishes acute infections in the murine large intestine (23). The adaptive immune response plays an important role for the host response to C. rodentium because mice lacking B and T cells are unable to clear the infection (24). C. rodentium induces a strong Th17 response (25, 26). Therefore, we examined whether IkBNS is required for an appropriate host response against C. rodentium.

The bacterial burden in feces of IkBNS\(^{-/-}\) mice at day 10 postinfection was significantly increased compared with wild-type mice (Fig. 5A). A closer investigation of colonization using live in vivo imaging indicated a similar infection kinetic in mice of both genotypes until day 5 (Fig. 5B, 5C). However, although the bioluminescent signal from the gut of wild-type mice started to increase between days 7 and 10, the signal intensity increased in IkBNS\(^{-/-}\) mice, confirming impaired C. rodentium clearance in these animals (Fig. 5B, 5C). In line with this observation, IkBNS\(^{-/-}\) mice also suffered from an elevated systemic infection, because dissemination of the pathogen to the liver was significantly enhanced in IkBNS\(^{-/-}\) mice compared with wild-type mice (Fig. 5D). These findings indicate that IkBNS is required for restricting C. rodentium infections to the gut.

We next studied the impact of IkBNS deficiency on the host T cell response. We found that the frequencies of Th17 cells were reduced by \(\sim 50\%\) in the colonic LP and in the spleen (Fig. 6A, 6B). Interestingly, the frequency of IL-17A/IFN-\(\gamma\) double-positive cells showed a similar reduction in both organs (Fig. 6A, 6D). In addition, we examined LP cell–derived mRNA levels of the cytokines IFN-\(\gamma\), IL-10, IL-17A, IL-17F, and IL-22 by real-time PCR. Consistent with the previous data, IkBNS deficiency resulted in decreased mRNA expression of Ifng, Il17a, Il17f, and Il10 in the spleen (Fig. 6E). In contrast with the in vitro data, but consistent with the other cytokines in vivo, the expression of Il22 mRNA was slightly down-regulated in IkBNS\(^{-/-}\) mice (Fig. 6E).

To assess whether IkBNS deficiency also affects pathology of the colon, we investigated cellular infiltration and the level of tissue damage in the colon of infected mice. Despite the increased bacterial burden, IkBNS\(^{-/-}\) mice exhibited significantly less immunopathology in the large intestine compared with wild-type mice (Fig. 6F), suggesting that the defective Th17 immune response in these mice results in less inflammation-induced tissue destruction. Altogether, our data demonstrate that IkBNS promotes Th17 differentiation and cytokine expression to fight intestinal infections.

IkBNS binds to the Il10 gene locus

The expression of several cytokines is regulated by the proteins of the IkB family (16). For instance, it was described that IkB\(\zeta\) and IkBNS regulate the expression of IL-6 by binding to its promoter (27, 28). In this study, we consistently found that expression of IL-10 is affected by IkBNS deficiency in various in vitro and in vivo experiments. To investigate whether IkBNS regulates the expression of IL-10 directly by binding to its gene locus, we performed

FIGURE 5. IkBNS\(^{-/-}\) mice are more susceptible to C. rodentium infection. IkBNS\(^{+/+}\) or IkBNS\(^{-/-}\) mice were infected orally with \(1 \times 10^{10}\) C. rodentium bacteria expressing the lux operon. (A) Analysis of C. rodentium burden in stool of IkBNS\(^{+/+}\) (n = 14) or IkBNS\(^{-/-}\) (n = 15) mice. CFU/g of stool are plotted on a logarithmic scale. (B) The bacterial load in IkBNS\(^{+/+}\) (n = 3) or IkBNS\(^{-/-}\) (n = 3) mice infected with bioluminescent C. rodentium was measured in vivo by bioluminescence imaging (IVIS Spectrum CT). Representative bioluminescence images are shown for the day of infection (day 0) and for days 5 and 10 postinfection. (C) Statistical analyses of the total flux of bioluminescence of C. rodentium during the course of the experiment. (D) The bacterial load in liver (IkBNS\(^{+/+}\) n = 6, IkBNS\(^{-/-}\) n = 8) and spleen (IkBNS\(^{+/+}\) n = 4, IkBNS\(^{-/-}\) n = 9) was analyzed 10 d postinfection. Horizontal lines in (A), (C), and (D) represent the mean, and error bars show the SEM. Data were pooled from at least three independent experiments with at least three mice/group (A and D) or are representative of one experiment with three mice/group (B and C). \(*p < 0.05, **p < 0.01, \text{two-tailed Mann–Whitney } U\text{ test (A and D) or Student } t\text{ test (C).}
native ChIP experiments. We detected increased binding of IκBNS to the Il10 gene locus in stimulated Th17 cells compared with unstimulated Th17 cells and IκBNS-deficient controls (Fig. 7). This suggests that IL-10 is a direct IκBNS target gene and that IκBNS regulates Th17 differentiation, at least in part, by direct control of cytokine expression.

**Discussion**

We recently reported that IκBNS regulates the induction of Foxp3 expression in Tregs (17). Although IκBNS−/− mice displayed a severe reduction in immunosuppressive Tregs, we found no signs of autoimmune disease. Thus, we speculated that IκBNS promotes the development or function of proinflammatory T cells. In this study, we show that IκBNS additionally controls the proliferation and cytokine expression of differentiated CD4+ effector T cells. Although impaired in vitro proliferation of IκBNS−/− thymic and peripheral CD4+ and CD8+ cells upon anti-CD3 and anti-CD28 stimulation was reported (18), our data indicate that Th17 cells are particularly affected by IκBNS deficiency. We further unveiled that the proliferation defect of IκBNS−/− T cells primed for Th17 differentiation could not be overcome by the presence of wild-type cells, indicating that IκBNS’s function is T cell intrinsic.

Remarkably, we also detected expression of IκBNS in acutely stimulated Th1 and Th0 cells. Overall, IκBNS expression in Th1 and Th0 cells was comparable to the expression measured in Th17 cells. Differences between mRNA and protein expression might be due to posttranscriptional control of IκBNS expression by Roquin, which was described previously in cell lines (29). Of note, fewer Th1 cells were generated in vitro from IκBNS−/− naive cells as a result of impaired proliferation. Importantly, IκBNS does not affect proximal TCR signaling. Thus, IκBNS forces proliferation further downstream in the signaling cascade. Noteworthy, in vivo proliferation of IκBNS−/− Tregs is not altered (17). Therefore, IκBNS acts differentially in Tregs and effector T cells. For instance, IκBNS is required for development but not function of Tregs (17), whereas in differentiated CD4+ Th cells, it regulates proliferation and effector cytokine production. Thus, our findings
provide an explanation for our previous observation that Treg reduction does not cause an autoimmune phenotype in IkBNS−/− mice (17).

We (17) and other investigators (30) previously described severe pathological changes in the colon in IkBNS−/− mice as a result of DSS-induced and transfer colitis, respectively. In the current study, chronic DSS colitis led to a reduced number of IL-17A+ Th17 cells in IkBNS−/− mice compared with wild-type mice. Furthermore, using a T cell–dependent transfer colitis model, we demonstrated that almost no Th17 cells or IL-17A+ IFN-γ+ T cells developed from transferred IkBNS−/− T cells. Hence, IkBNS is not only an important regulator of Th17 priming in vitro, it is also essential for Th17 and IL-17A+IFN-γ+ T cell development in mouse models of gut inflammation. Furthermore, tissue damage in the gut was increased in recipients of IkBNS−/− T cells as a result of the reduced numbers of immunosuppressive Tregs, as previously shown, error bars display the SEM.

In addition to proinflammatory or pathogenic Th17 cells, protective or suppressive Th17 cells have been described (21, 41). For instance, IL-10–expressing Th17 cells that were primed with TGF-β and IL-6 were not pathogenic (41). Therefore, IL-10 coexpression by Th17 cells is presumably a self-regulatory mechanism to reduce the inflammatory damage of Th17 cells. Although it was initially believed that Th17 cells produce IL-10 early on in an infection (41), it was later shown that Th17 cells migrate from the spleen to the gut in a CCR6-dependent manner and acquire suppressive function and IL-10 expression there (21). We found decreased IL-10 expression in IkBNS−/− Th17 cells both when generated in vitro and in mice infected with C. rodentium. Therefore, IkBNS is crucial for the differentiation of proinflammatory, as well as suppressive, Th17 cells.

We showed previously that IkBNS in Tregs is essential to induce the expression of the transcription factor Foxp3 (17). IkBNS binds together with p50 and c-Rel to CNS3 and the promoter of Foxp3. Because of the extended expression of IL-10 in vivo and in vitro, we analyzed the influence of IkBNS in IL-10 expression by ChIP, which revealed binding of IkBNS to the IL-10 gene locus (42, 43). Thus, we suggest that IkBNS binding to the IL-10 gene locus is mediated by p50 and/or c-Rel, because IkBNS itself has no DNA-binding domain. Although IL-10 appears to be directly regulated by IkBNS, IL-17A, IL-17F, and IL-22 do not contain canonical NF-κB–binding sites. Consequently, we conclude that IkBNS does not regulate these genes directly; further experiments are needed to clarify how IkBNS regulates the expression of cytokines in an indirect manner.

Several components of the NF-κB signaling pathway are important for Th17 development. Most upstream in the signaling complex, CARMA1 deficiency results in defective Th17 cell differentiation (44). MALT1 is highly expressed in Th17 cells generated in vitro, and MALT1−/− Th17 cells produce less IL-17 and GM-CSF (45). Loss of p65/RelA or c-Rel results in impaired Th17 induction in vitro, and c-Rel was shown to interact directly with the Rorc and Il21 promoters (46, 47). Moreover, the atypical nuclear IκB protein IκBζ, another nuclear IκB protein with high sequence similarity to IkBNS, regulates Th17 proliferation in a cell-intrinsic way (48). IκBζ interacts with RORα and RORγt to...


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Supplementary Figures

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Supplementary Figure 1: IκBNS deficiency results in impaired Th17 development in chronic DSS-induced colitis. DSS colitis was induced in IκBNS+/+ (n=6) or IκBNS−/− (n=9) mice by four cycles of DSS feeding. Control mice (n=3) received non-supplemented water. (A) The normalized weight during the disease course is shown. (B) Representative hematoxylin and eosin staining of colon sections are shown (scale bar represents 200 µm). (C) The histological score plotted is the sum of individual scores of inflammatory cell infiltration and tissue damage. (D) The length of the colon was measured. (E) LPL cells were stained and analyzed by flow cytometric measurement. Representative dot blots pre-gated to CD4+ cells are shown. (F) Frequencies of CD4+ IL-17+ T cells among LPL cells. Cells were analyzed as in (E) from untreated IκBNS+/+ (n=3) and IκBNS−/− (n=5) mice as well as DSS-treated IκBNS+/+ (n=6) and IκBNS−/− (n=9) mice. Error bars display the standard error of the mean. Statistical analyses were performed by two-tailed Mann-Whitney U test, (*) p<0.05
Supplementary Figure 2: IκBNS−/− T cells display a proliferation defect during Th-cell differentiation in vitro. CD4+CD25−CD62L+ naïve T cells were isolated by flow cytometry and cultured under non-polarizing (Th0, upper panel), Th1- (middle panel) and Th17- (lower panel) conditions. The number of IκBNS+/+ (closed symbols) and IκBNS−/− cells (open symbols) during T cell differentiation from day 1 to 6 is shown.
Supplementary Figure 3: IκBNS does not affect the activation of CD4⁺ T cells. *In vitro* expanded CD4⁺CD25⁻ T cells from IκBNS⁺/+ and IκBNS⁻/− mice were left untreated or stimulated with anti-CD3 plus anti-CD28 for the indicated time points. Control cells were stimulated with PMA/ ionomycin (P/I) for 2 hours. (A) TCR-proximal T cell activation was determined by Western Blot analysis using the anti-phospho-tyrosine antibody 4G10. (B) Expression and phosphorylation status of the kinases Akt, Erk and p38 was determined by Western blotting. (C) The expression of IκBNS as well as the expression and phosphorylation status of IκBα was analyzed by Western Blot analysis. (D and E) The expression of the five NF-κB family members c-Rel, RelB, p65, NF-κB1 and NF-κB2 was analyzed by Western Blot using the indicated antibodies. Analysis of β-actin expression served as a loading control. All Western blot analyses shown are representative for 3 independent experiments.
Supplementary Figure 4: Cytokine localisation on the cytokine array membrane. (A)
The location of cytokines, positive (pos.) and negative (neg.) controls on the array membrane shown in Figure 6D are indicated. (B,C) Isolated naïve T cells were cultured under Th17-priming conditions. IL-17A expressing cells were enriched via cytokine secretion assay. Relative levels of cytokines in the supernatant of stimulated IkB$_{NS}$$^{+/+}$ (B) and IkB$_{NS}$$^{-/-}$ (C) Th17 cells were profiled with a cytokine array membrane. Representative membranes of 2 independent experiments are shown.