IκBNS Regulates Murine Th17 Differentiation during Gut Inflammation and Infection

Michaela Annemann, Zuobai Wang, Carlos Plaza-Sirvent, Rainer Glauben, Marc Schuster, Frida Ewald Sander, Panagiota Mamareli, Anja A. Kühl, Britta Siegmund, Matthias Lochner and Ingo Schmitz

_J Immunol_ 2015; 194:2888-2898; Prepublished online 18 February 2015; doi: 10.4049/jimmunol.1401964

http://www.jimmunol.org/content/194/6/2888

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2015/02/18/jimmunol.1401964.DCSupplemental

**References**

This article cites 52 articles, 11 of which you can access for free at: http://www.jimmunol.org/content/194/6/2888.full#ref-list-1

**Subscription**

Information about subscribing to _The Journal of Immunology_ is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
IkBNS Regulates Murine Th17 Differentiation during Gut Inflammation and Infection

Michaela Annemann,†‡ Zuobai Wang,§ Carlos Plaza-Sirvent,*,† Rainer Glauben,§
Marc Schuster,*,† Frida Ewald Sander,*,† Panagiota Mamareli,§ Anja A. Kühl,§
Britta Siegmund,§ Matthias Lochner,‡ and Ingo Schmitz*,†

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 conditions, 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 (EAE) (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, MALT1, 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 IkBNS in CD4+ cells and strongly impaired Th17 responses in vitro and in vivo. Our results demonstrate that IkBNS is required for Th17 differentiation and function.

Materials and Methods

Mouse strains

B6.129SV-NFkb1tm1Clel (Clel) mice, later referred to as IkBNS−/− mice, were described previously (18). IkBNS−/− mice were crossed to Foxp3DTR-eGFP mice, as previously described. later referred as IkBNS−/−, Foxp3DTR-eGFP reporter mice (17). IkBNS−/−, C57BL/6-Typl.1 (Thy1+) mice (Thy1.1 mice) were bred under specific pathogen–free conditions in the animal facility of the Helmholtz Centre for Infection Research. Cy1J mice (Thy1.1 mice) were bred under specific pathogen–free conditions at the animal facility of the Helmholtz Centre for Infection Research. B6.129S7-Rag1tm1Mom (RAG1−/−) mice (19) were bred at the animal facility of the National Cancer Institute. 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% (v/v) dextran sodium sulfate (DSS) as an initial dose and 2% (v/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, deparaffinized, stained with H&E, and 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 IkBNS−/− mice or CD4+GFP+ Tregs, were injected i.p. into RAG1−/− mice. Body weight was measured from the cecum to the anus, and colon samples were scored in a blinded manner, as described previously (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 ICL180 expressing the lux operon (courtesy of S. Wiles, Auckland, New Zealand). C. rodentium were 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 bioluminescence during infection was measured in vivo by bioluminescence imaging using IVIS Imaging CT. 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 were placed into 15 ml ice-chilled 0.5 mM EDTA for 30 min (on ice), followed by sufficient 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 underlain with an 80% isotonic Percoll solution. After centrifugation (900 × g, room temperature for 20 min), LP lymphocytes (LPLs) were isolated from the cell yield 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+CD45R+B220− naive T cells were sorted via flow cytometry (FACS-Aria II; BD Biosciences or MoFlo; Beckman and Coulter) and resuspended in primary Th cell medium (IMDM containing 10% FCS, 50 μg/ml penicillin/streptomycin, 25 mM HEPES, 0.5 mM 2-ME, 1 μM nonessential amino acids, 1 mM sodium pyruvate). Two × 106 cells were seeded per well (96-well plate) and were activated with plate-bound anti–CD3 (1 μg/ml) and anti–CD28 (2 μg/ml) 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 Systems); 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

After sorting of CD4+CD25− T cells, four × 106 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 × 106 to 10 × 106 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 × 106/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

Naïve 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 × 106 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 supemant, as described in...
the user manual. Finally, the chemiluminescence of the membrane was measured using the Fusion FX-7 camera (Vilber Lourmat), and the volume of bands was quantified with the program BIO-ID (Peqlab).

To analyze the cytokine secretion of Th17 cells by flow cytometry, 0.8 × 10^6 enriched IL-17–secreting cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μM; both from Sigma-Aldrich). After 4 h, the supernatant was used for cytokine analysis with a FlowCytomix kit (eBioscience). The supernatant was used undiluted or was diluted 1:10 or 1:50. The standard curves and samples were prepared according to the manufacturer’s instructions and measured by flow cytometry in the BD LSR II Flow Cytometer System or the BD LSRFortessa (BD Biosciences).

Flow cytometric analyses

For intracellular cytokine staining, cells were stimulated for 4 h with PMA (10 ng/ml) and ionomycin (1 μM; both from Sigma-Aldrich). Brefeldin A (10 μg/ml; Sigma-Aldrich) was added during the last 2 h of stimulation. Live/dead staining was performed in 100 μl 1× PBS (Invitrogen) containing one of the LIVE/DEAD dyes from Life Technologies (30 min, 4°C, in the dark). Surface markers were incubated in FACS buffer (1× PBS, 2% v/v BSA, 0.01% v/v NaN₃) at 4°C for 15 to 20 min in the dark, using the following fluorochrome-conjugated Abs: CD3–allophycocyanin–eFluor 780 (17A2; eBioscience), CD3–FITC (145-2C11; BD), CD3–PECy7 (145-2C11; eBioscience), CD4–Alexa Fluor 488 (RM4-5; BD), CD4–allophycocyanin (RM4-5; eBioscience), CD4–FITC (RM4-5; eBioscience), CD4–PerCP–Cy5.5 (RM4-5; eBioscience), CD8–allophycocyanin (RM4-5; eBioscience), CD8–Pacific Blue (RM4-5; BioLegend), CD8–PE (RM4-5; BD), CD8–PECy7 (XMG1.2), and CD62L–PerCPCy5.5 (MEL-14; eBioscience). Subsequently, the cells were fixed and permeabilized using the FoxP3 Staining Buffer Set (Miltenyi Biotec) for 30 min (4°C, in the dark). Intracellular cytokine staining was performed in 100 μl 1× permeabilization buffer using IFN-γ–FITC (XMG1.2), IFN-γ–PE (XMG1.2), IFN-γ–PECy7 (XMG1.2), and IL-17A–allophycocyanin (eBiol7B7; all from eBioscience) Abs (4°C, for 30 min in the dark). Labeled cells were analyzed with a BD LSR II Flow Cytometer System or BD LSRFortessa (BD Biosciences).

For flow cytometric analysis upon C. rodentium infection, cells from the spleen and mesenteric lymph nodes, as well as LPLs, were restimulated for 6 h in medium containing IL-23 (20 ng/ml) with PMA (10 ng/ml) and ionomycin (1 μg/ml; both from Sigma-Aldrich). Brefeldin A (10 μg/ml; Sigma-Aldrich) was added for the last 3 h. Cells were processed for 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 (1× 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 (2890; Cell Signaling Technology), ERK (9102; Cell Signaling Technology); IkBα (rabbit; made in-house); IκBα (C-21; Santa Cruz), p38 (9212; Cell Signaling Technology), p50 (p50105 (E381; Epitomics), p52/100 (4882; Cell Signaling Technology), p65 (C-20; Santa Cruz Biotechnology), p–Akt (4066; Cell Signaling Technology), p–ERK (4370; Cell Signaling Technology), p–IkBα (2859; Cell Signaling Technology), p–p38 (9211; Cell Signaling Technology), p–SAPK–JNK (9251; Cell Signaling Technology), p–Tyrosine (4910; kind gift of Prof. B. Schraven, 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 the user manual. Finally, the chemiluminescence of the membrane was measured using the Fusion FX-7 camera (Vilber Lourmat), and the volume of bands was quantified with the program BIO-ID (Peqlab).

To analyze the cytokine secretion of Th17 cells by flow cytometry, 0.8 × 10^6 enriched IL-17–secreting cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μM; both from Sigma-Aldrich). Brefeldin A (10 μg/ml; Sigma-Aldrich) was added for the last 3 h. Cells were processed for 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 (1× 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 (2890; Cell Signaling Technology), ERK (9102; Cell Signaling Technology); IkBα (rabbit; made in-house); IκBα (C-21; Santa Cruz), p38 (9212; Cell Signaling Technology), p50 (p50105 (E381; Epitomics), p52/100 (4882; Cell Signaling Technology), p65 (C-20; Santa Cruz Biotechnology), p–Akt (4066; Cell Signaling Technology), p–ERK (4370; Cell Signaling Technology), p–IkBα (2859; Cell Signaling Technology), p–p38 (9211; Cell Signaling Technology), p–SAPK–JNK (9251; Cell Signaling Technology), p–Tyrosine (4910; kind gift of Prof. B. Schraven, 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
The single blot (dependent experiments. Error bars show SEM. *p < 0.05, **p < 0.01, two-tailed Mann-Whitney U test or Student t test was used; error bars represent the SEM.

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 system using the following primers: GM-CSF forward (fwd), 5'-CAGGCTGTCTTGCCATCAAGA-3' and GM-CSF reverse (rev), 5'-AATTGAATGATCCACACATCC-3'; IFN-γ fwd, 5'-ATGAGTCATTGCCATTTCCTTC-3' and IFN-γ rev, 5'-GGGTTTCTTCTCCATCTTCT-3'; IL-17A fwd, 5'-GGGACACAATCCAGCTTGTC-3' and IL-17A rev, 5'-CGGGTCTGGAAATG-3'; IL-10 fwd, 5'-GGGACACAATCCAGCTTGTC-3' and IL-10 rev, 5'-CCAGAGCAATCTCAGTCC-3'; IL-21 fwd, 5'-TGCCAAAGCTCAGAATG-3'.

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
IκBNS 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 IκBNS deficiency affects Th cell differentiation, we induced chronic colitis that is promoted by Th1 and Th17, as well as B cells (22), in IκBNS−/− and wild-type mice by repeated DSS feeding. Although DSS-fed IκBNS−/− and wild-type mice did not display marked histological differences, there was inherent colon damage in the nontreated IκBNS−/− 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 IκBNS−/− mice compared with wild-type mice (Supplemental Fig. 1E, 1F). Interestingly, Th17 frequencies already were reduced in nontreated, IκBNS−/− animals compared with respective wild-type mice (Supplemental Fig. 1E, 1F).

![Figure 2](http://www.jimmunol.org/) Impaired Th1 and Th17 differentiation in the absence of IκBNS. 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 IκBNS/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 IκBNS and β-actin protein expression. (D) The single blot (right panel) represents the protein expression of IκBNS-deficient Th17 cells. (E) The expression of IFN-γ and IL-17A was analyzed by flow cytometry in wild-type and IκBNS−/− 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 (F) 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<sup>−/−</sup> mice receiving IκB<sub>NS</sub>−/− T cells exhibited significantly more colon damage than did wild-type controls (Fig. 1A). Importantly, the induction of both IL-17A<sup>+</sup>IFN-γ<sup>+</sup> (Fig. 1B, 1C) and IL-17A<sup>+</sup>IFN-γ<sup>−</sup> (Fig. 1D) T cells was completely blunted in IκB<sub>NS</sub>−/− mice. In contrast, the frequency of IL-17A<sup>+</sup>IFN-γ<sup>+</sup> T cells (Fig. 1E) was slightly, but significantly, increased in IκB<sub>NS</sub>−/− mice, demonstrating the importance of IκB<sub>NS</sub> for the formation of Th17 cells in the inflamed gut. Previously, we reported a reduced number of Tregs in IκB<sub>NS</sub>−/− mice (17). To analyze whether the increased frequency of Th1 cells in IκB<sub>NS</sub>-deficient animals arose from reduced numbers of Tregs, Tregs from IκB<sub>NS</sub>−/−Foxp3<sup>DTR-eGFP</sup> or IκB<sub>NS</sub><sup>+</sup>/+Foxp3<sup>DTR-eGFP</sup> reporter mice were cotransferred (Fig. 1F). The cotransfer of Tregs had no effect on the increased Th1 cell frequency in IκB<sub>NS</sub>−/− mice, regardless of whether the Tregs were IκB<sub>NS</sub> deficient or proficient.

In summary, both colitis models revealed a requirement for IκB<sub>NS</sub> in Th17 cell differentiation during gut inflammation.

IκB<sub>NS</sub> drives Th17 and Th1 development in vitro

To determine whether IκB<sub>NS</sub> is differentially expressed in Th cell subsets, we analyzed IκB<sub>NS</sub> (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 IκB<sub>NS</sub> 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 IκB<sub>NS</sub> protein expression than did Th17 cells. Notably, the nonactivated Th17 cells displayed higher IκB<sub>NS</sub> expression compared with the other nonactivated cells (Fig. 2B, 2D), substantiating a pivotal role for IκB<sub>NS</sub> specifically in this T cell subset. Indeed, during in vitro priming we monitored severely reduced frequencies of both IL-17A<sup>+</sup> Th17 cells and IFN-γ<sup>+</sup> Th1 cells in the absence of IκB<sub>NS</sub> (Fig. 2E, 2F). We conclude that IκB<sub>NS</sub> expression is not restricted to a single Th cell subset but forces the differentiation of Th17 and Th1 cells in vitro.

Proliferation defect in IκB<sub>NS</sub>-deficient Th17 cells

To identify the reason for the reduced frequency of Th17 and Th1 cells induced by IκB<sub>NS</sub> deficiency, we first analyzed Th cell proliferation in vitro. Although all T cells proliferated equally during the first 2 d of culture, IκB<sub>NS</sub>−/− 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 IκB<sub>NS</sub>−/− cells when cocultured, we dif-

**FIGURE 3.** IκB<sub>NS</sub>-deficient T cells display a proliferation defect during Th cell differentiation in vitro. CD4<sup>+</sup>CD25<sup>−</sup>CD62L<sup>+</sup> naive T cells were isolated by flow cytometry and cultured under Th1-polarizing, Th17-polarizing, or nonpolarizing (Th0) conditions. Cells were analyzed after PMA/ionomycin (P/I, 4 h) stimulation. Naive IκB<sub>NS</sub><sup>+</sup>/+ (CD90.1<sup>+</sup>) and IκB<sub>NS</sub>−/− (CD90.2<sup>+</sup>) T cells were mixed at a ratio of 1:1, labeled with CFSE, and cultured under Th1-polarizing, Th17-polarizing, or nonpolarizing (Th0) conditions. Proliferation of each genotype was analyzed by flow cytometry. (A) Representative dot plots of viable IκB<sub>NS</sub><sup>+</sup>/+ (CD90.1<sup>+</sup>) and IκB<sub>NS</sub>−/− (CD90.2<sup>+</sup>) cells. (B) Representative graphs of CSFE intensity of IFN-γ<sup>+</sup> or IL-17A<sup>+</sup> cells (pregated to CD90.1<sup>+</sup> or CD90.2<sup>+</sup> cells). (C) Statistical analysis of the division index of wild-type and IκB<sub>NS</sub>−/− T cells. Error bars show the SEM of four independent experiments. *p < 0.05, two-tailed Mann–Whitney U test.
ferentiated IκBNS−/− (CD90.2+) T cells in the presence of con-
genetic wild-type (CD90.1+) cells, both stained with CFSE. Again, the frequencies of IκBNS−/− Th0, Th1, and Th17 cells showed a 50% reduction compared with wild-type cells, suggesting a cell-
intrinsic effect of IκBNS 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 IκBNS−/− cells (Fig. 3B, 3C). Remarkably, the proliferation defect caused by IκBNS 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 NFκB family members c-Rel, NFκB1, NFκB2, p65, and RelB; and the expression and phosphorylation status of IκBα were unaltered (Supplemental Fig. 3).

IκBNS regulates Th17 cytokine expression

Because RORγt is an essential transcription factor for Th17 differ-
entiation and function (6), we analyzed the expression of Rorc
(encoding RORγt) in IκBNS−/− and wild-type Th1 and Th17 cells

FIGURE 4. Expression of Th17-specific cytokines is impaired by IκBNS 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 IκBNS+/+ (CD90.1+) and IκBNS−/− (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 IκBNS−/− cells compared with IκBNS+/+ 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 IκBNS+/+ and IκBNS−/− Th17 cells were assessed using a cytokine array membrane. The expression of IL-2, IL-10, MIP-1α, and GM-CSF in IκBNS−/− 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.

Because RORγt is an essential transcription factor for Th17 differ-
entiation and function (6), we analyzed the expression of Rorc
(encoding RORγt) in IκBNS−/− and wild-type Th1 and Th17 cells

By guest on April 4, 2017 http://www.jimmunol.org/ Downloaded from
Th17 cytokines IL-17A, IL-17F, and IL-22 was not affected by IκBNS deficiency. Furthermore, we analyzed the effect of IκBNS 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 IκBNS−/− Th17 cells, whereas Il17a and Il17f expression was not affected (Fig. 4F). Interestingly, in contrast to IL-22 cytokine secretion, IκBNS−/− Th17 cells showed an increased expression of IL-22 mRNA. We conclude that IκBNS regulates the expression of multiple key Th17 cytokines.

IκBNS−/− 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 against 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 IκBNS is required for an appropriate host response against C. rodentium.

The bacterial burden in feces of IκBNS−/− 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 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. (B) The bacterial load in IκBNS+/

We next studied the impact of IκBNS deficiency on the host cell response. We found that the frequencies of Th17 cells were reduced by ∼50% in the colonic LP and in the spleen (Fig. 6A, 6B). Interestingly, the frequency of IL-17A/IFN-γ double-positive cells showed a similar reduction in both organs (Fig. 6A, 6C), whereas the frequency of IFN-γ Th1 cells was slightly reduced in the spleen of IκBNS−/− mice but not in the other organs investigated (Fig. 6A, 6D). In addition, we examined LP cell-derived mRNA levels of the cytokines IFN-γ, IL-10, IL-17A, IL-17F, and IL-22 by real-time PCR. Consistent with the previous data, IκBNS deficiency resulted in decreased mRNA expression of Ifng, Il17a, Il17f, and Il10 by LPILs of the colon (Fig. 6E). In contrast with the in vitro data, but consistent with the other cytokines in vivo, the expression of Il22 mRNA was slightly downregulated in IκBNS−/− mice (Fig. 6E).

To assess whether IκBNS 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, IκBNS−/− 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 IκBNS promotes Th17 differentiation and cytokine expression to fight intestinal infections.

IκBNS binds to the Il10 gene locus

The expression of several cytokines is regulated by the proteins of the IκB family (16). For instance, it was described that IκBζ and IκBNS 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 IκBNS deficiency in various in vitro and in vivo experiments. To investigate whether IκBNS regulates the expression of IL-10 directly by binding to its gene locus, we performed
native ChIP experiments. We detected increased binding of IkBNS to the Il10 gene locus in stimulated Th17 cells compared with unstimulated Th17 cells and IkBNS−/− controls (Fig. 7). This suggests that IL-10 is a direct IkBNS target gene and that IkBNS regulates Th17 differentiation, at least in part, by direct control of cytokine expression.

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

Remarkably, we also detected expression of IkBNS in acutely stimulated Th1 and Th0 cells. Overall, IkBNS 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 IkBNS expression by Roquin, which was described previously in cell lines (29). Of note, fewer Th1 cells were generated in vitro from IkBNS−/− naive cells as a result of impaired proliferation. Importantly, IkBNS does not affect proximal TCR signaling. Thus, IkBNS forces proliferation further downstream in the signaling cascade. Noteworthy, in vivo proliferation of IkBNS−/− Tregs is not altered (17). Therefore, IkBNS acts differentially in Tregs and effector T cells. For instance, IkBNS 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

FIGURE 6. Fewer Th17 cells develop in IkBNS−/− mice upon C. rodentium infection. A total of 1 × 10^10 bioluminescent C. rodentium bacteria was administered orally to IkBNS+/+ or IkBNS−/− mice. Animals were analyzed 10 d postinfection. (A) Cells from the colon were isolated, stained with Abs directed against CD3, CD4, IFN-γ, and IL-17A, and measured by flow cytometry. Representative dot plots are shown. (B–D) Cells from colon, spleen, and mesenteric lymph nodes (mLN) were isolated, stained, and measured by flow cytometry as in (A). Frequency of IL-17A+ (B), IL-17A+/IFN-γ+ (C), and IFN-γ+ cells (D) within CD4+CD3+ T cells. (E) The relative expression of Ifng, II10, II17a, II17f, and II22 in LPLs was measured by real-time PCR and normalized to UBC. (F) Representative H&E staining of colon section. Scale bar, 200 μm (upper panels). The histological score was determined from colon sections of IkBNS+/+ (n = 6) or IkBNS−/− (n = 4) mice on day 10 postinfection (lower panel). Data are representative of two experiments with at least three mice/group (in total IkBNS+/+ n = 9, IkBNS−/− n = 11 [E]) or were pooled from three independent experiments with two to four mice/group (B–D) or two independent experiments with at least two mice/group (F). Horizontal lines represent the mean, and error bars show the SEM. *p < 0.05, **p < 0.01, two-tailed Mann–Whitney U test.
provide an explanation for our previous observation that Treg reduction does not cause an autoimmune phenotype in IκBNS−/− mice (17).

We (17) and other investigators (30) previously described severe pathological changes in the colon in IκBNS−/− mice as a result of DDS-induced and transfer colitis, respectively. In the current study, chronic DDS colitis led to a reduced number of IL-17A+ Th17 cells in IκBNS−/− 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 IκBNS−/− T cells. Hence, IκBNS 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 IκBNS−/− T cells as a result of the reduced numbers of immunosuppressive Tregs, as previously reported (17). In addition to a lower Treg frequency, the elevated frequency of IFN-γ+ Th1 cells is likely to be responsible for the enhanced pathological damage. Although both IκBNS−/− Th1 and Th17 cells showed a proliferation defect in vitro, TH1 development was not affected during colon inflammation in vivo. Because co-transfer of Tregs did not affect the enhanced TH1 differentiation, this might indicate the presence of additional signals that promote TH1 development in vivo.

In addition to the autoimmune models of colitis, we addressed the question of whether IκBNS is instrumental for TH17 responses during infection. To this end, we used C. rodentium because this murine pathogen induces strong TH17 responses (25, 26, 31). C. rodentium shares virulence factors with enteropathogenic Escherichia coli and enterohemorrhagic E. coli and, thus, is a commonly used murine model to study immune responses against these gut-tropic pathogens (32). Similar to the colitis models, IκBNS deficiency resulted in impaired TH17 and IL-17A+IFN-γ+ T cell generation in infected mice. Because IFN-γ, as well as the p80 and the p19 subunit of IL-23, which is known to stabilize the TH17 phenotype (33), are required for C. rodentium clearance (25, 34, 35), it is conceivable that the lack of IL-17A+ and IFN-γ+ T cells in IκBNS−/− mice upon C. rodentium infection is responsible for the increased bacterial burden in these mice. Interestingly, NF-κB1/p50−/− mice are also defective in C. rodentium clearance (36), which is in line with the observation that IκBNS preferentially interacts with this NF-κB family member (17, 37). Furthermore, because of the normal IL-17A and IL-17F expression in vitro, we speculate that the reduced mRNA expression in vivo arises from the decreased frequency of TH17 and IL-17A+IFN-γ+ cells.

In addition to IL-17A and IL-17F, which were involved in late host defense against C. rodentium, the IL-10 cytokine family member IL-22 is essential in early host protection (26, 38). IL-22−/− mice exhibit increased damage to the intestinal epithelium, systemic bacterial burden, and mortality (39). In this study, we observed an increased mRNA, but unaltered protein, expression of IL-22 by IκBNS−/− cells in vitro. Of note, Il22 expression was reduced in vivo upon C. rodentium infection. The reduced IL-22 levels might be sufficient to suppress protection of the intestinal epithelium; however, the defects in the expression of IL-22 and other cytokines were insufficient to reduce bacterial burden.

Why do we see decreased histological damage in C. rodentium–infected IκBNS−/− mice despite the increase in bacterial burden? Interestingly, IL-23 also was reported to induce GM-CSF expression in TH17 cells, which causes pathogenicity in EAE, the murine model for multiple sclerosis (9, 10). Thus, the reduction in GM-CSF production by IκBNS−/− TH17 cells may account for the reduced tissue damage in the gut. Alternatively, impaired bacterial clearance in conjunction with reduced tissue damage was reported recently (40). In this infection model, TH17 differentiation depended on the presence of Tregs and the consumption of IL-2. Thus, it appears that TH17 cells during C. rodentium infection are not only involved in pathogen clearance (i.e., beneficial), they also cause tissue damage and, therefore, can be pathogenic.

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 IκBNS−/− TH17 cells both when generated in vitro and in mice infected with C. rodentium. Therefore, IκBNS is crucial for the differentiation of proinflammatory, as well as suppressive, TH17 cells.

We showed previously that IκBNS in Tregs is essential to induce the expression of the transcription factor Foxp3 (17). IκBNS 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 IκBNS in IL-10 expression by CHIP, which revealed binding of IκBNS to the IL-10 gene locus. Previous studies identified the binding of NF-κB to the IL-10 gene locus (42, 43). Thus, we suggest that IκBNS binding to the IL-10 gene locus is mediated by p50 and/or c-Rel, because IκBNS itself has no DNA-binding domain. Although IL-10 appears to be directly regulated by IκBNS, IL-17A, IL-17F, and IL-22 do not contain canonical NF-κB–binding sites. Consequently, we conclude that IκBNS does not regulate these genes directly; further experiments are needed to clarify how IκBNS 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). MAL1 is highly expressed in TH17 cells generated in vitro, and MAL1−/− 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 IκBNS, regulates TH17 proliferation in a cell-intrinsic way (48). IκBζ interacts with RORα and RORγt to
El-Behi, M., B. Ciric, H. Dai, Y. Yan, M. Cullimore, F. Safavi, G. X. Zhang, a crucial regulator of Th17 differentiation. Furthermore, Heissmeyer and colleagues (55) demonstrated that paired Th17 differentiation. Taken together, I infiltration in spinal cord (54). Consistent with our data, the gene Th17 frequencies in draining lymph nodes and a lack of lymphocyte pharmacological target in future drug development. While this manuscript was in revision, it was reported that IkBNS is necessary for murine Th17 differentiation during inflammation and infection, and it belongs to the group of NF-kB-sIGNALING proteins that commonly regulate Treg and Th17 cell differentiation. Hence, IkBNS exhibits diverse T cell proliferation and cytokine secretion functions and may represent a specific pharmacological target in future drug development.

Acknowledgments
We thank Sabrina Schumann for technical assistance, Alisha Broniatzki and Dr. Yvonne Neumann for critical reading of the manuscript, David Dettbarn and colleagues at the Helmholtz Centre for Infection Research (HZI) Animal Facility for excellent mouse stock breeding, and Dr. Marina Pils (HZI) and Elena Wiebe (Medical School Hannover) for help with the histology. We are grateful to the FACs facilities, especially Dr. Gerd Groothuis (HZI) and Dr. Desiree Kunkel (Berlin-Brandenburg Centre for Regenerative Therapies–Flow Cytometry Laboratory, Berlin, Germany), for help with cell sorting. Rag1–/– mice were kindly provided by Dr. Thomas Blankenstein (Charité – Universitätsmedizin Berlin).

Disclosures
The authors have no financial conflicts of interests.

References


