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NOD2 Regulates CXCR3-Dependent CD8+ T Cell Accumulation in Intestinal Tissues with Acute Injury

Xingxin Wu,* Amit Lahiri,* G. Kenneth Haines, III, † Richard A. Flavell,‡,§ and Clara Abraham*‡

Polymorphisms in NOD2 confer risk for Crohn’s disease, characterized by intestinal inflammation. How NOD2 regulates both inflammatory and regulatory intestinal T cells, which are critical to intestinal immune homeostasis, is not well understood. Anti-CD3 mAb administration is used as therapy in human autoimmune diseases, as well as a model of transient intestinal injury. The stages of T cell activation, intestinal injury, and subsequent T tolerance are dependent on migration of T cells into the small intestinal (SI) lamina propria. Upon anti-CD3 mAb treatment of mice, we found that NOD2 was required for optimal small intestinal IL-10 production, in particular from CD8+ T cells. This requirement was associated with a critical role for NOD2 in SI CD8+ T cell accumulation and induction of the CXCR3 ligands CXCL9 and CXCL10, which regulate T cell migration. NOD2 was required in both the hematopoietic and nonhematopoietic compartments for optimal expression of CXCR3 ligands in intestinal tissues. NOD2 synergized with IFN-γ to induce CXCL9 and CXCL10 secretion in dendritic cells, macrophages, and intestinal stromal cells in vitro. Consistent with the in vitro studies, during anti-CD3 mAb treatment in vivo, CXCR3 blockade, CD8+ T cell depletion, or IFN-γ neutralization each inhibited SI CD8+ T cell recruitment, and reduced chemokine expression and IL-10 expression. Thus, NOD2 synergizes with IFN-γ to promote CXCL9 and CXCL10 expression, thereby amplifying CXCR3-dependent SI CD8+ T cell migration during T cell activation, which, in turn, contributes to induction of both inflammatory and regulatory T cell outcomes in the intestinal environment.


Inflammatory bowel diseases (IBDs), including Crohn’s disease (CD) and ulcerative colitis, are characterized by leukocyte accumulation in intestinal tissues (1). Of the common genetic variants identified to date, loss-of-function polymorphisms in NOD2, an intracellular sensor of the bacterial cell-wall component peptidoglycan, confer the greatest susceptibility for development of CD (1). The mechanisms through which NOD2 regulates intestinal immune homeostasis are incompletely understood, although multiple mechanisms are likely involved, including through NOD2 regulation of intestinal epithelial cells, myeloid-derived cells, and T cell differentiation (1–4). Interestingly, although loss-of-function in NOD2 confers risk for CD (1) and intestinal inflammation in specific experimental mouse models (5), there is increasing evidence that loss of NOD2 function may be beneficial in certain situations, such as with infectious challenges (2, 6). For example, mice expressing the CD-associated L1007fsinsC NOD2 polymorphism demonstrate decreased inflammation and lethality postinfection with Enterococcus faecalis (6), and T cell–intrinsic NOD2 deficiency protects mice from Toxoplasma gondii–associated colitis (2). Further supporting this beneficial effect is that human carriers of NOD2 polymorphisms that result in decreased NOD2 expression (7) are less likely to have chronic disease from Mycobacterium leprae (8). This beneficial effect may help explain the relatively frequent presence of loss-of-function NOD2 polymorphisms in the population. Therefore, the inflammation associated with certain infectious exposures or acute injury appears to be attenuated with decreased NOD2 expression or function.

Anti-CD3 mAb treatment is being studied in ongoing trials for various human immune-mediated diseases, including IBD, type 1 diabetes mellitus (T1DM), psoriatic arthritis, and graft-versus-host disease (GVHD) (9). This treatment results in T cell activation (10), transient intestinal injury (11), and induction of regulatory T cell populations (e.g., IL-10–producing T cells; Foxp3+ Tregs) in the small intestine (SI) (12–15), thereby highlighting the regulation of critical stages of intestinal T cell differentiation. Both the intestinal inflammation and induction of intestinal regulatory T cells are dependent on T cell recruitment into the intestinal lamina propria (LP) (13, 14, 16). Importantly, the regulatory T cells generated upon anti-CD3 mAb treatment can mediate protection of systemic immune-mediated diseases, including GVHD (17), skin graft rejection (18), T1DM (19), and autoimmune encephalomyelitis (20). Furthermore, the systemic protection under these conditions is dependent on the generation of regulatory T cells within the intestinal LP (13). Loss-of-function Leu1007insC NOD2 CD patients were found to have decreased Foxp3+ Tregs in colonic tissue compared with wild-type (WT) NOD2 CD patients (21), pointing to the possibility of dysregulation in the generation of intestinal-derived regulatory T cell populations in the absence of NOD2 function or expression.
To dissect the role of NOD2 in mediating intestinal T cell responses in vivo, we selected the clinically relevant anti-CD3 mAb treatment model. We found that NOD2 was critical for the induction of IL-10–producing CD8+ T cells in the SI LP; this was due to a NOD2 requirement for intestinal CD8+ T cell accumulation during anti-CD3 mAb treatment. The T cell trafficking CXCR3 ligands CXCL9 and CXCL10 were dramatically decreased in NOD2+/− mice after anti-CD3 mAb treatment. Consistently, CXCR3 blockade inhibited CD8+ T cell recruitment to the SI with anti-CD3 mAb injection, which led to attenuation of small intestinal chemokines and cytokines (e.g., IL-10). NOD2 expression in the hematopoietic and nonhematopoietic cell compartments was necessary for optimal CXCL9 and CXCL10 production in intestinal tissues upon anti-CD3 mAb injection. Interestingly, NOD2 synergized with IFN-γ to significantly enhance CXCL9 and CXCL10 expression in bone marrow–derived dendritic cells (BMDCs), bone marrow–derived macrophages (BMMs), and intestinal stromal cells in vitro. T cells are a significant source of IFN-γ upon anti-CD3 activation; consistently, depletion of CD8+ T cells or neutralization of IFN-γ reduced intestinal expression of chemokines and ultimately IL-10 during anti-CD3 mAb injection. NOD2 deficiency similarly attenuated chemokine induction and T cell infiltration in a separate CXCR3-dependent acute intestinal injury model, the piroxicam–induced colitis model in IL-10−/− mice. Taken together, NOD2 is critical for the increased injury–induced chemokine expression in intestinal tissues, in particular, CXCL9 and CXCL10, which, in turn, mediates amplification of CXCR3-dependent T cell recruitment to the intestinal LP. This recruitment, in turn, regulates both the inflammatory and regulatory T cell outcomes within the intestinal LP.

Materials and Methods

Mice

NOD2−/− mice (Jackson Laboratory, Bar Harbor, ME) were crossed with IL-10−/−GFP reporter mice (12) or C57BL/6 Thy1.1+/+ mice (Jackson Laboratory). Mice were maintained in a specific pathogen-free facility and used between 2 and 5 mo of age. Experiments were performed in agreement with the Yale University Institutional Animal Care and Use Committee and according to National Institutes of Health guidelines for animal use.

Abs and staining reagents

The following Abs were used on an LSR II (BD Biosciences, San Jose, CA): allophycocyanin-Cy7– and allophycocyanin-labeled anti-CD4, eFlour 650NC– and FITC-labeled anti-CD8, eFlour450–labeled anti-CD3, eFlour650NC–labeled anti–MHC class II (anti-MHCII), PE and PE-Cy7–labeled Thy1.2, PerCP-Cy5.5 and PerCP-Cy5.5 and allophycocyanin-labeled anti-CXCR3 (eBioscience, San Diego, CA), CXCR3-173 (anti-CXCR3 neutralizing Ab; Biologend, San Diego, CA), 145-2C11 (anti-CD3), 2.43 (anti-CD8), and XMGL1.2 (anti–IFN-γ; Bio X Cell, West Lebanon, NH) were used in vivo. The following were assessed by ELISA: IL-10, IL-17A, IFN-γ (BD Biosciences), CXCL9 (R&D Systems, Minneapolis, MN), and CXCL10 (eBioscience).

Anti-CD3 mAb treatment model

Mice were injected i.p. with 15 μg anti-CD3 mAb or isotype control hamster IgG at 0 and 48 h. Mice were analyzed 4 h after the final injection. Mice were given 100 μg anti-CXCR3 mAb (or Armenian hamster IgG isotype control) i.p. 2 h before each anti-CD3 mAb injection to block CXCR3 signaling. To deplete CD8+ T cells, mice were given 250 μg anti-CD8 mAb (or rat IgG2b isotype control) i.p. 48 h before the first anti-CD3 mAb injection, and CD8+ T cell depletion was confirmed by flow cytometry. Mice were given 500 μg anti–IFN-γ (or rat IgG1 isotype control) i.p. 24 h before each anti-CD3 mAb injection to block IFN-γ. In some cases, mice were first primed with a 4-wk antibiotic regimen in drinking water consisting of vancomycin hydrochloride (500 mg/l; Hospira, Lake Forest, IL), ampicillin (1 g/l; DAVCA Pharmaceuticals, Fort Lee, NJ), metronidazole (1 g/l; Teva Pharmaceuticals, Sellersville, PA), and neomycin sulfate (1 g/l; MP Biomedicals, Solon, OH) as per Rakoff-Nahoum et al. (22).

FITC-dextran permeability assay

Mice were orally gavaged with FITC-dextran (40 mg/100 g body weight) 4 h before sacrifice. Serum concentration of FITC-dextran was measured by fluorometer at 488 nm.

Intestinal LP cell isolation

Proximal small intestinal (first 8 cm of SI) LP cells were isolated as previously described (23). In brief, the proximal SI was cut longitudinally and then into ~1-mm pieces. SI pieces were washed thoroughly (ice-cold PBS, 5% FCS) and then digested (PBS, 5 nM EDTA, 5% FCS) at 37°C in a rotating incubator to remove epithelial cells. The supernatants containing epithelial cells and intraepithelial lymphocytes (IELs) were discarded, and intestines were washed twice in ice-cold PBS to remove residual EDTA. The remaining tissue was then incubated for 1 h at 37°C in a rotating incubator in collagenase buffer consisting of RPMI 1640, 10% FCS, 200 U/ml collagenase VIII (Sigma-Aldrich), 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, 50 μM gentamicin, and 50 U/ml penicillin-50 μg/ml streptomycin. The cells were then filtered through a 40-μm filter (BD Biosciences) and washed twice in RPMI 1640 media. For intestinal stromal cells, intestinal LP cells were plated overnight. The next day, nonadherent cells were removed and the adherent fibroblast-like cells were cultured for 7 d. The intestinal stromal cells expressed α-smooth muscle actin, vimentin, P-HA1, desmin, and Myh10 consistent with a myofibroblast phenotype. In contrast, they were negative for hematopoietic markers (e.g., CD45, CD11b, CD11c). They did not contain T cell or B cell contamination as assessed by CD3 and CD19, respectively.

Generation and culture of BMDCs and BMMs

BM single-cell suspensions were cultured in complete RPMI 1640 media containing 20 mg/ml GM-CSF (for BMDCs; Peprotech, Rocky Hill, NJ) and 10% L929-conditioned medium (for BMMs). Cultures were fed fresh medium every 3 d and used at 6–8 d. Purity was >98% as assessed by flow cytometry. BMDCs were CD11c+ and F4/80+, whereas BMMs were CD11b+, F4/80+, and CD11c+. IFN-γ (Peprotech), muramyl dipeptide (MDP; Bachem, King of Prussia, PA), and lipid A (Peptides International, Louisville, KY) were used in vitro.

Tissue mRNA expression and protein analysis

Total RNA (TRizol; Life Technologies, Carlsbad, CA) from cells or homogenized organ tissue was isolated, reverse transcribed, and quantitative PCR was performed as previously described (23). Each sample was run in duplicate and normalized to GAPDH. Primers sequences are shown in Supplemental Table I. For protein analysis, tissue was suspended in a Triton lysis buffer and homogenized (VWR International, Radnor, PA), and ELISA was performed.

Bone marrow chimera

Donor BM cells (1 × 106) were adoptively transferred into lethally irradiated recipients (1100 cGy total body irradiation) between 8 and 12 wk of age. Mice were analyzed 6 wk later. We confirmed engraftment in the blood (90.1 ± 1.2%) and intestinal LP (71.2 ± 2.3%). The presence of radioresistant Thy1+ cells in intestinal tissues was observed by others in such sites as isolated lymphoid follicles (24).

Statistical analyses

Statistical comparisons were assessed using a two-tailed Student t test. Bonferroni correction was applied for multiple comparisons. The p values <0.05 were considered significant.

Results

NOD2 is required for accumulation of IL-10–producing cells in the SI of mice during anti-CD3 mAb treatment

Anti-CD3 mAb treatment induces an immunoregulatory environment marked by induction of IL-10–producing cells mainly in the SI (12). To determine the role of NOD2 in generating intestinal regulatory T cell populations, we crossed NOD2−/− mice with IL-10–GFP reporter mice, and compared NOD2−/− IL-10–GFP and NOD2+/− IL-10–GFP littermate controls. As expected, the per-
the percentage of IL-10–producing cells significantly increased in the SI LP of NOD2−/− mice after anti-CD3 mAb treatment (Fig. 1A, 1B). In contrast, the accumulation of IL-10–producing cells in the SI of NOD2−/− mice was significantly decreased relative to littermate control mice (Fig. 1A, 1B). This was associated with a decreased number of IL-10–producing cells in the SI of NOD2−/− mice (Fig. 1C). Consistent with the pattern of IL-10–producing cells in the SI, induction in SI IL-10 mRNA (Fig. 1D) and serum IL-10 (Fig. 1E) after anti-CD3 mAb treatment was decreased in NOD2−/− mice relative to littermate controls. Of note is that Foxp3+ Tregs can also be induced in the SI with anti-CD3 mAb injection (12, 13). Furthermore, a study in human loss-of-function Leu1007insC NOD2 CD patients identified decreased colonic Foxp3+ CD4+ Tregs in patients with active CD (21). However, we did not observe differences in the percentage of Foxp3+ Tregs in CD4+ T cells at baseline or after anti-CD3 mAb treatment in NOD2−/− compared with NOD2+/− mice (data not shown). In summary, NOD2 is required for the induction of IL-10–producing cells in the SI of mice upon anti-CD3 mAb treatment.

**CD8+ T cells are a major source of IL-10–producing cells in the SI of mice during anti-CD3 mAb treatment**

We next sought to dissect which SI LP cells were producing IL-10 upon anti-CD3 mAb injection, and which of these IL-10–producing cell subsets was decreased in NOD2−/− mice. We found that upon anti-CD3 mAb injection, ~90% of IL-10+ cells were T cells (Thy1.2+ cells); the majority of these IL-10–producing T cells were CD8+ T cells (Fig. 2A, 2B). Relative to CD4+ T cells, regulation of intestinal CD8+ T cells during anti-CD3 mAb treatment has not been well studied. However, treatment of T1DM patients with anti-CD3 mAb leads to increased circulating regulatory CD8+ T cell populations (25), highlighting the importance of CD8+ T cells in therapy for human disease. We found the percentage (Fig. 2C) and number (Fig. 2D) of IL-10–producing CD8+ T cells in the SI of NOD2−/− mice was increased with anti-CD3 mAb treatment; this increase was significantly reduced in NOD2−/− mice (mean + SEM; n = 3/group). Data are representative of at least five independent experiments. **p < 0.01.

**NOD2 is required for CD8+ T cell accumulation in the SI during anti-CD3 mAb treatment**

Prior studies showed that T cells accumulate in the proximal SI a few hours after anti-CD3 mAb treatment (13, 14). The decreased number of IL-10–producing CD8+ T cells in NOD2−/− mice after anti-CD3 mAb treatment NOD2 may be because of a defect in IL-10 induction in the CD8+ T cells within the SI or because of an overall decrease in accumulation of CD8+ T cells in the SI LP. To dissect these two possibilities, we first gated on CD8+ T cells isolated from the SI LP after anti-CD3 mAb treatment and found that the percentage of IL-10–producing cells within the CD8+ T cells that were present in the SI of NOD2−/− and NOD2+/− mice was equivalent (Fig. 3A, 3B). In contrast, the dramatic increase in the percentage (Fig. 3C) and number (Fig. 3D) of CD8+ T cells observed within the SI of NOD2+/− mice after anti-CD3 mAb treatment was significantly diminished in NOD2−/− mice, high-

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**FIGURE 1.** NOD2 is required for optimal accumulation of IL-10–producing cells in the SI of mice during anti-CD3 mAb treatment. NOD2−/− IL-10–GFP and NOD2−/− IL-10–GFP mice were treated with 15 μg anti-CD3 mAb or IgG isotype control at 0 and 48 h. Four hours after the last injection, animals were sacrificed. (A) Representative flow cytometry plots of IL-10–producing cells in the SI LP (gated on live cells). (B) Percentage of IL-10–producing cells in the SI. (C) Number of IL-10–producing cells in the SI (mean + SEM; n = 3/group; representative of five independent experiments). (D) IL-10 mRNA expression in the SI. (E) Serum IL-10. (D and E) Mean + SEM; n = 6/group from two independent experiments. Comparisons are between anti-CD3 mAb and IgG treatment in the same genotype or as indicated. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 2.** CD8+ T cells are the major source of IL-10–producing cells in the SI of mice during anti-CD3 mAb treatment. Mice were treated with anti-CD3 mAb as in Fig 1. (A) Representative flow cytometry gated on IL-10–producing cells in the SI LP. Surface CD3 is internalized with anti-CD3 mAb treatment, whereas Thy1.2 expression remains intact. Cells were stained for Thy1.2, CD4, and CD8 expression. (B) Pie chart of the proportion of IL-10–producing cell types in the SI after anti-CD3 mAb injection (mean + SEM; n = 3). (C) Percentage and (D) number of IL-10–producing CD8+ T cells (CD4+Thy1.2+) in the SI of NOD2−/− and NOD2+/− mice (mean + SEM; n = 3/group). Data are representative of at least five independent experiments. **p < 0.01.
lighting a role for NOD2 in optimal CD8+ T cell accumulation in the SI. Of note is that, as expected, the percentage of CD8+ T cells in the LP at baseline is low; the increase in CD8+ T cells is observed specifically upon anti-CD3 mAb treatment. We ensured that the increase in LP CD8+ T cells upon anti-CD3 mAb treatment was not due to contamination by IELs through staining for characteristic IEL populations (TCRγδ and TCRαβCD8α T cells, data not shown). The CD8+ T cells accumulating in the LP express CD45 and Thy1.2, and are MHCII+ (Supplemental Fig. 1). For experiments that ensue, we gate on live CD8+CD4+Thy1.2+ (MHCII+) cells to examine CD8+ T cells (Supplemental Fig. 1). Given the decreased CD8+ T cells in the SI, we examined other T cell–derived cytokines, and observed that IFN-γ and IL-17A mRNA expression in the SI were also decreased in NOD2−/− mice after anti-CD3 mAb treatment (Fig. 3E). Therefore, upon anti-CD3 mAb treatment, NOD2 is required for the significant increase in CD8+ T cells in the SI LP, and for the subsequent increase in cytokines and IL-10–producing CD8+ T cells that occurs.

Optimal anti-CD3–mediated increases in IL-10–producing cells and CD8+ T cell accumulation in the SI LP depends on intestinal microbiota

The dependency on NOD2 for optimal accumulation of IL-10–producing CD8+ T cells in the SI LP upon anti-CD3 mAb treatment highlighted a likely role for intestinal microbiota in mediating these outcomes. Consistent with prior reports (26), we observe increased intestinal permeability after anti-CD3 treatment (Fig. 3F).

**FIGURE 3.** NOD2 is required for CD8+ T cell migration to and cytokine production in the SI upon anti-CD3 mAb treatment. Mice were treated with anti-CD3 mAb as in Fig 1. (A) Representative flow cytometry plots of cells from the SI gated on CD8+ T cells (CD4+Thy1.2+). (B) Percentage of IL-10–GFP+ cells in CD8+ T cells in the SI of NOD2+/+ and NOD2−/− mice after anti-CD3 mAb treatment. (C) Percentage and (D) number of CD8+ T cells in the SI LP (mean + SEM; n = 3/group; representative of five independent experiments). (E) IFN-γ and IL-17A mRNA expression in the SI (mean + SEM; n = 6–9/group from three independent experiments). Comparisons are between anti-CD3 mAb and IgG treatment in the same genotype or as indicated. (F) Oral FITC-dextran was administered 4 h before sacrifice (coincident with the 48-h anti-CD3 mAb treatment time point) and measured in the serum at the time of sacrifice. (G–I) NOD2−/− IL-10–GFP mice were administered an antibiotic (ABX) regimen consisting of vancomycin, metronidazole, ampicillin, and neomycin for 4 wk in the drinking water. Antibiotic-treated or -untreated (specific pathogen-free [SPF]) mice were then treated with anti-CD3 mAb as in Fig. 1 and examined for (G) percentage of IL-10–GFP+ cells (gated on live cells), (H) percentage of IL-10–GFP+ T cells, and (I) percentage of CD8+ T cells in the SI LP. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 4.** NOD2 is required for optimal CXCR3 ligand induction in the SI upon anti-CD3 mAb treatment. (A–D) NOD2+/+ and NOD2−/− mice were treated with anti-CD3 mAb as in Fig. 1. CXCL9 and CXCL10 mRNA levels in (A) SI and (B) MLN (mean + SEM; n = 6/group; representative of two independent experiments). (C and D) Percentage of CD8+ T cells expressing CXCR3 in SI, MLN, and spleen was assessed. (C) Representative flow cytometry plots for CXCR3 expression on CD8+ T cells (solid black line). Low CXCR3–expressing MHCII+ cells are shown for comparison (shaded gray histogram). (D) Summary graph for CXCR3–expressing cells within CD8+ T cells (mean + SEM; n = 3/group; representative of two independent experiments). *p < 0.05, **p < 0.01, ***p < 0.001.
(Fig. 3F), thereby increasing exposure of LP cells to intestinal microbiota. To directly assess the role of intestinal bacteria in the anti-CD3 treatment–mediated outcomes observed, we administered mice a commonly used oral antibiotic regimen consisting of vancomycin, metronidazole, ampicillin, and neomycin for 4 wk in the drinking water (22). The antibiotic treatment resulted in a 96.6 ± 1.3% reduction in bacterial 16S rRNA levels in the feces. Upon anti-CD3 treatment, mice treated with antibiotics demonstrated significantly decreased accumulation of SI LP IL-10–producing cells (Fig. 3G), IL-10–producing CD8+ T cells (Fig. 3H), and total CD8+ T cells (Fig. 3I) compared to nonantibiotic-treated mice. Taken together, intestinal bacteria contribute to the increased IL-10–producing CD8+ T cells in the SI upon anti-CD3 treatment. 

**NOD2 is required for optimal CXCL9 and CXCL10 expression in the SI after anti-CD3 mAb treatment**

To define the mechanism through which NOD2 mediates the increased CD8+ T cell accumulation in the SI after anti-CD3 mAb treatment, we evaluated pertinent trafficking molecules, including integrins and chemokines. CD18, CD11a, CD49d, and β1 play an important role in T cell trafficking to intestinal tissues (27). In some cases, the expression of these integrins increased with anti-CD3 mAb (Supplemental Fig. 2). However, the expression of the assessed integrins on CD8+ T cells did not differ between NOD2+/− and littermate controls at baseline or with anti-CD3 mAb treatment in the SI, or in lymphoid organs from which they might be recruited such as mesenteric lymph nodes (MLNs) or spleen (Supplemental Fig. 2).

We next considered the regulation of chemokines in NOD2+/− mice during anti-CD3 mAb treatment. CXCR3 regulates T cell migration to inflammatory sites (28). The CXCR3 receptor has three ligands, CXCL9, CXCL10, and CXCL11. Both CXCL9 and CXCL10 were significantly induced in the SI and MLN of NOD2+/− mice after anti-CD3 mAb treatment; this dramatic increase was not observed in NOD2−/− mice (Fig. 4A, 4B). CXCL11 was induced to an equivalent degree in the SI of NOD2+/− and NOD2−/− mice after anti-CD3 mAb treatment (data not shown). In contrast, with the decreased CXCL9 and CXCL10 in NOD2−/− mice upon anti-CD3 mAb treatment, CXCR3 expression on CD8+ T cells did not differ between NOD2+/− mice and littermate controls (Fig. 4C, 4D). Therefore, NOD2 is critical for the significant induction of CXCR3 ligands in intestinal lymphoid tissues observed with anti-CD3 mAb treatment.

**CD8+ T cell accumulation in the SI and subsequent outcomes upon anti-CD3 mAb treatment are CXCR3 dependent**

To establish that CXCR3 is playing a definitive role in CD8+ T cell accumulation in the SI during anti-CD3 mAb treatment, and in the induction of cytokines, including IL-10, associated with this accumulation, we injected CXCR3 blocking Ab before anti-CD3 mAb treatment. CXCR3 blockade significantly attenuated the accumulation of CD8+ T cells in the SI (Fig. 5A, 5B). Anti-CXCR3 mAb administration in the absence of anti-CD3 mAb treatment (with Armenian hamster IgG isotype control) did not alter the percentage or number of CD8+ T cells in the SI (data not shown). Importantly, CXCR3 blockade also resulted in decreased intestinal CXCL9 and CXCL10 mRNA expression (Fig. 5C), and decreased IFN-γ, IL-17A, and IL-10 expression in the SI (Fig. 5D) and serum (Fig. 5E) during anti-CD3 mAb treatment. In contrast, we did not observe differences in LP CD8+ T cell apoptosis with anti-CD3 mAb treatment between NOD2+/− and NOD2−/− mice (data not shown). Moreover, whereas LP CD8+ T cell proliferation (assessed by Ki67 expression) increased significantly upon anti-CD3 mAb treatment, there was no difference between NOD2+/− and NOD2−/− mice in this induced proliferation (data not shown). Thus, CXCR3 is critical for the T cell migration into the SI LP, amplification of its own chemokine ligands, and the ultimate induction of IL-10 that occurs upon anti-CD3 mAb treatment.

**NOD2 in hematopoietic and nonhematopoietic compartments is required for optimal CD8+ T cell accumulation in the SI upon anti-CD3 mAb treatment**

Because NOD2 is expressed in both hematopoietic and nonhematopoietic cells, we sought to define the contributions of these cell types to the accumulation of CD8+ T cells and IL-10 production in the SI after anti-CD3 mAb treatment. To address this, we generated bone marrow chimeras in which either the donors or recipients were NOD2 deficient. On transfer of NOD2+/− Thy1.1+ into WT Thy1.2+ mice, accumulation of NOD2+/− donor CD8+ T cells in the SI was significantly attenuated upon anti-CD3 mAb treatment.

**FIGURE 5.** CXCR3 is essential for the amplification of SI CD8+ T cell accumulation, chemokines, and subsequent IL-10 induction upon anti-CD3 mAb treatment. To block CXCR3, we treated mice with 100 μg anti-CXCR3 mAb (or Armenian hamster IgG isotype control) i.p. 2 h before each anti-CD3 mAb injection (at 0 and 48 h). Mice were harvested 4 h after the second anti-CD3 mAb injection. (A) Percentage and (B) number of CD8+ T cells in the SI LP (mean + SEM; n = 3/group; representative of three independent experiments). (C) CXCL9 and CXCL10 mRNA expression in the SI. (D) IL-10, IFN-γ, and IL-17A mRNA expression in SI. (E) Serum levels of IL-10, IFN-γ, and IL-17A (mean + SEM; n = 6/group from two independent experiments for (C)–(E)). *p < 0.05, **p < 0.01, ***p < 0.001.
injection relative to donor NOD2−/− Thy1.1+ (Fig. 6A). Induction of CXCL9 and CXCL10 expression was reduced in NOD2−/− donors compared with NOD2+/− donors upon anti-CD3 mAb injection (Fig. 6B), consistent with the decreased CD8+ T cell accumulation in these mice. Moreover, IL-10, IFN-γ, and IL-17A intestinal mRNA expression (Fig. 6C) and serum levels (Fig. 6D) were decreased in NOD2−/− compared with NOD2+/− donors. In the reciprocal transfer of WT Thy1.2+ into NOD2−/− Thy1.1+ cells. Serum cytokine levels. Data are mean + SEM; n = 3/group, and representative of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 6. NOD2 in hematopoietic and nonhematopoietic cells is required for optimal CD8+ T cell migration into the SI LP upon anti-CD3 mAb treatment. Chimeric mice were generated and treated with anti-CD3 mAb as in Fig 1. (A–D) Transfer of NOD2+/− or NOD2−/−Thy1.1+ BM cells into irradiated Thy1.2+ WT mice. (E–H) Transfer of WT Thy1.2+ BM cells into NOD2+/− or NOD2−/− Thy1.1+ mice. (A and E) Percentage of CD8+ T cells donor cells in the SI LP. (B and F) Chemokine mRNA expression in SI. (C and G) Cytokine mRNA expression in SI. (D and H)
mice, similar reductions were observed relative to transfer of WT Thy1.2+ into NOD2−/− Thy1.1+ mice (Fig. 6E–H). Therefore, NOD2 expression is required in the hematopoietic and non-hematopoietic cell compartments for the CD8+ T cell accumulation, and induced chemokine and cytokine expression observed with anti-CD3 mAb injection.

NOD2 stimulation synergistically enhances IFN-γ-induced CXCL9 and CXCL10 expression in BMMs, BMDCs, and intestinal stromal cells in vitro

Given the dependence on NOD2 in both hematopoietic and non-hematopoietic cells for chemokine production and CD8+ T cell accumulation in the SI during anti-CD3 mAb treatment, we evaluated the ability of NOD2 stimulation to induce CXCL9 and CXCL10 in both hematopoietic (e.g., BMMs, BMDCs) and non-hematopoietic (e.g., intestinal stromal) cells. NOD2 stimulation alone with MDP induced a low level of expression of both these chemokines in BMMs and BMDCs (Fig. 7A, 7B), although not in intestinal stromal cells (Fig. 7C). NOD2 can synergize with other molecules for certain downstream outcomes (29). CD8+ T cells significantly increase in the SI upon anti-CD3 activation (Fig. 3C, 3D), and CXCR3-dependent blockade of CD8+ T cells led to decreased intestinal CXCL9 and CXCL10 (Fig. 5C). IFN-γ can induce CXCL9 and CXCL10 (28), and activated CD8+ T cells are an important source of IFN-γ. We therefore evaluated interactions between MDP and IFN-γ, and observed a clear synergy between MDP and IFN-γ in inducing CXCL9 and CXCL10 secretion from BMMs (Fig. 7A), BMDCs (Fig. 7B), and intestinal stromal cells (Fig. 7C). This synergy was absent in BMMs, BMDCs, and intestinal stromal cells from NOD2−/− mice (Fig. 7). In contrast, synergy between NOD2 and TLR4 (Fig. 7) and other TLRs (data not shown) was not observed for CXCL9 or CXCL10 secretion. Therefore, NOD2 synergizes with IFN-γ, a cytokine secreted particularly by activated T cells, to produce chemokines from myeloid-derived cells and intestinal stromal cells that can then further enhance T cell recruitment into intestinal tissues.

Anti-CD3 mAb treatment-dependent intestinal outcomes require CD8+ T cells and IFN-γ

We next sought to clearly establish in vivo that CD8+ T cells and IFN-γ are each required for the intestinal outcomes observed with anti-CD3 mAb treatment. We therefore injected mice with anti-CD8 mAb to deplete CD8+ T cells before anti-CD3 mAb treatment; CD8+ T cells were effectively depleted in the SI (Fig. 8A), MLN, and spleen (data not shown). CD8+ T cell depletion significantly decreased the induction of CXCL9 and CXCL10 mRNA (Fig. 8B) and protein (Fig. 8C, 8G) in the SI, and of IL-10, IFN-γ, and IL-17A expression in the SI (Fig. 8D) upon anti-CD3 mAb treatment. IFN-γ blockade also significantly attenuated the accumulation of CD8+ T cells in the SI (Fig. 8E), induction of CXCL9 and CXCL10 (Fig. 8F, 8H), and IL-10, IFN-γ, and IL-17A in the SI (Fig. 8H) during anti-CD3 mAb treatment. Thus, CD8+ T cells and IFN-γ are critical for the optimal induction of intestinal chemokines and cytokines, including of IL-10, upon anti-CD3 mAb treatment in vivo, highlighting the ability of activated CD8+ T cells to amplify their own recruitment to the SI through modulation of chemokine induction (Fig. 9).

NOD2 promotes T cell accumulation in the colon in CXCR3-dependent acute piroxicam-induced colitis.

To determine whether...
NOD2 regulation of T cell accumulation and T cell–mediated injury extends to additional intestinal injury models, we selected a colitis model known to be dependent on CXCR3–CXCL10 interactions. In IL-10−/− mice, CXCL10 is highly expressed at sites of colitis, and CXCL10 neutralization can attenuate colitis severity (30). Short-term exposure of IL-10−/− mice to the nonsteroidal anti-inflammatory drug piroxicam simulates an environmental trigger that can play a role in human IBD, and results in a synchronous, rapid induction of colitis (31) that is similarly CXCL10 dependent (32). Piroxicam-fed IL-10−/− mice demonstrated rapid weight loss (Supplemental Fig. 3A), colon shortening (Supplemental Fig. 3B), and moderate-to-severe colitis (Supplemental Fig. 3C, 3D); such changes were markedly attenuated in NOD2−/− IL-10−/− mice. Consistent with these findings, T cell infiltration into the colon (Supplemental Fig. 3E) and MLN (Supplemental Fig. 3F) of NOD2−/− IL-10−/− mice was significantly decreased compared with IL-10−/− mice. Consistent with the decreased T cell recruitment, colonic CXCL9 and CXCL10 expression was significantly decreased in NOD2−/− IL-10−/− mice compared with IL-10−/− mice (Supplemental Fig. 3G), as was colonic TNF-α, IFN-γ, and IL-17A mRNA expression (Supplemental Fig. 3H). These results indicate that similar to the NOD2 requirement for T cell recruitment and chemokine and cytokine production in the SI during anti-CD3 mAb injection, NOD2 is required for T cell accumulation and chemokine and cytokine production in the colon in acute piroxicam–induced colitis in IL-10−/− mice.

**Discussion**

In this study, we demonstrate a novel role for NOD2 in CD8+ T cell accumulation in the intestinal LP during acute intestinal injury, which contributes to consequences with respect to inflammatory and regulatory T cell outcomes. NOD2 is required for optimal CD8+ T cell migration into the SI LP during anti-CD3 mAb treatment, which then enhances CXCL9 and CXCL10 induction, thereby amplifying CXCR3-dependent CD8+ T cell recruitment. The NOD2 ligand MDP synergizes with IFN-γ (produced by activated T cells), but not lipid A, to induce CXCL9 and CXCL10 in BMMs, BMDCs, and intestinal stromal cells. We show that these interactions between CD8+ T cells, IFN-γ, NOD2, intestinal bacteria, and CXCR3 in vivo are critical in driving the CD8+ T cell recruitment amplification loop (Fig. 9). Therefore, we find that through its contributions in multiple cell subsets to the regulation of T cell trafficking, NOD2 is required for both the inflammatory and regulatory T cell outcomes observed in the intestinal environment.

NOD2 can play a complex role in intestinal immune outcomes in vivo, with varying contributions in different cell subsets. On the one hand, NOD2 confers protection in certain situations, such as GVHD (33) and Helicobacter hepatitis–driven inflammation (34). On the other hand, NOD2 contributes to the adverse outcomes in other situations, such as in E. faecalis infection (6), in T. gondii–associated colitis (2), and in the colitis observed in older IL-10−/− mice (associated with contributions to cytokine secretion in macrophages) (35). We now define contributions of NOD2 to both inflammatory and regulatory T cell outcomes through its regulation of T cell–recruiting chemokines, which, in turn, initiates a self-amplifying loop of T cell migration into the intestinal LP, thereby providing insight into at least one mechanism for these dual effects.

The ability of NOD2 to regulate chemokines, and thereby cell recruitment into the intestinal LP, has been previously described in the context of C. rodentium infection; stromal cell–derived CCL2 induction and monocyte recruitment is NOD2 dependent (36). The CXCR3-CXCL9/10 axis is important in T cell trafficking and effector T cell generation (28). Accordingly, we now find a clear role for NOD2 in the hematopoietic and nonhematopoietic compartment in vivo and in myeloid-derived cells and intestinal stromal cells in vitro for induction of chemokines critical for intestinal recruitment of T cells. Interestingly, NOD2 in stromal cells can also directly contribute to T cell differentiation (37). Of note is that our studies demonstrate that CXCR3 is required for optimal induction of its own ligands within intestinal tissues during anti-CD3 mAb treatment, consistent with the amplification loop that occurs during this acute form of injury (Fig. 9). Blockade of CXCR3-CXCL10 can protect mice from colitis (30, 32, 38); we now identify a clear role for these interactions in clinically relevant anti-CD3 mAb therapy. Moreover, a fully humanized anti-CXCL10 Ab is in development for treating IBD (39). Our studies demonstrate that although blockade of the CXCR3-CXCL9/10 axis can downregulate proinflammatory cytokines, it can also impact on the generation of IL-10–producing T cells that occurs in the intestinal environment, which may ultimately have significant consequences on protective mechanisms given the important role of the intestine for broad regulatory T cell outcomes.

In contrast with IELs, which consist of predominantly CD8+ T cells, LP lymphocytes are predominantly CD4+ T cells. However, under anti-CD3 treatment conditions, we observed a significant increase in the percentage of LP CD8+ T cells. This increase has been previously described with anti-CD3 treatment (14). Moreover, a dramatic increase in LP CD8+ T cells has been observed during viral infections, such as with vesicular stomatitis virus (40), highlighting that CD8+ T cells can accumulate in the intestinal LP under select conditions of T cell activation. Although we find that optimal intestinal LP CD8+ T cell accumulation is dependent on CXCR3-mediated migration (Fig. 5), there are likely additional factors that contribute to both CD8+ T cell accumulation and induction of cytokines and chemokines during anti-CD3 mAb treatment. Other chemokine receptors (e.g., CCR6) can contribute to T cell migration into the SI during injury (14), as can various adhesion molecules, a subset of which we found to be upregulated upon anti-CD3 mAb treatment (Supplemental Fig. 2).
We did not observe differences between cell death (Annexin V+) or proliferation (Ki67+) in SI LP CD8+ T cells between NOD2−/− and NOD2+/+ mice during anti-CD3 mAb treatment (data not shown). Therefore, it appears that at least at the time point examined, the majority of CD8+ T cell accumulation is attributable to trafficking into the SI LP. However, it remains possible that differences in cell death, proliferation, or T cell egress into the intestinal lumen (14) may be detected through alternative approaches or may exist at later time points, which could then contribute to differences between T cell accumulation, and cytokine and chemokine induction between NOD2−/− and NOD2+/+ mice.

Anti-CD3 mAb treatment has been undergoing therapeutic trials for a number of human immune-mediated diseases, with T1DM being the most well investigated (9, 19). Patients receiving anti-CD3 therapy demonstrate an increase in circulating regulatory CD8+ T cells (25). Interestingly, studies in humanized mouse models have shown that the anti-CD3 mAb–mediated protection is dependent on T cell migration into the SI, where it acquires a regulatory phenotype; this intestinal T cell migration is required for the anti-CD3 mAb–mediated regulatory T cell protection in systemic diseases (13). The anti-CD3 mAb injection model in mice has also been used to simulate viral infection (14); that anti-CD3 mAb results in an acute transient inflammation and subsequent intestinal-dependent induction of T cell tolerance highlights the unique and important role of the local intestinal environment for the proper differentiation of protective T cells. Therefore, identifying those factors that are required for T cell recruitment into the SI LP during acute T cell activation is essential to understanding the ability of anti-CD3 mAb therapy and other acute intestinal T cell activation conditions to mediate their beneficial effects. In this study, we have found that CD8+ T cells are the main source of IL-10–producing cells within the intestinal LP and that NOD2 is required for the CXCR3-dependent T cell migration into the SI that ultimately leads to these IL-10–producing CD8+ T cells; the function and regulation of CD8+ T cells in intestinal injury have been investigated much less than have CD4+ T cells. Taken together, NOD2 promotes CXCL9 and CXCL10 expression in the SI, thereby regulating of CD8+ T cells in intestinal injury have been investigated much less than have CD4+ T cells. Taken together, NOD2 promotes CXCL9 and CXCL10 expression in the SI, thereby promoting CXCL9 and CXCL10 expression in the SI, thereby promoting interleukin-17 production in human memory T cells. 565.

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Disclosures
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