Natural Killer Cells Protect against Mucosal and Systemic Infection with the Enteric Pathogen *Citrobacter rodentium*

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Natural killer (NK) cells are traditionally considered in the context of tumor surveillance and viral defense, but their role in bacterial infections, particularly those caused by enteric pathogens, is less clear. C57BL/6 mice were orally gavaged with *Citrobacter rodentium*, a murine pathogen related to human diarrheagenic *Escherichia coli*. We used polyclonal anti-asialo GM1 antibody to actively deplete NK cells *in vivo*. Bioluminescent imaging and direct counts were used to follow infection. Flow cytometry and immunofluorescence microscopy were used to analyze immune responses. During *C. rodentium* infection, NK cells were recruited to mucosal tissues, where they expressed a diversity of immune-modulatory factors. Depletion of NK cells led to higher bacterial loads but less severe colonic inflammation, associated with reduced immune cell recruitment and lower cytokine levels. NK cell-depleted mice also developed disseminated systemic infection, unlike control infected mice. NK cells were also cytotoxic to *C. rodentium* *in vitro*.

Natural killer (NK) cells are a subset of lymphocytes with a critical function in innate immune surveillance against the development of tumors and viral infection. NK cell-mediated cytotoxicity is controlled by complex interactions of inhibitory and activating receptors which trigger specialized downstream effector signaling pathways (1). NK cells also produce a wide range of cytokines and immune modulators, with tumor necrosis factor alpha (TNF- α) and gamma interferon (IFN- γ) being the most prominent cytokines (2). During inflammation, bidirectional cross talk with accessory cells, such as dendritic cells (DCs) or macrophages, is required for the full activation and antimicrobial activity of NK cells. Specifically, during systemic bacterial infection, NK cell-derived IFN- γ can enhance phagocytosis of extracellular bacteria or infected host cells by macrophages (3). In addition, NK cells can also modulate both the DC and macrophage antigen presentation function, promoting the generation of the subsequent adaptive immune response (4–6).

The attaching-and-effacing (A/E) pathogens enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) attach to and colonize the host epithelium, causing diarrhea, colon inflammation, and epithelial cell hyperplasia (7). Both of these enteric pathogens are major contributors to the global burden of infectious disease associated with substantial annual mortality, particularly at the extremes of life (8). As EPEC and EHEC are human specific, a related mouse enteric pathogen, *Citrobacter rodentium*, is widely used as an *in vivo* model of A/E bacterial infection (9). Employing similar pathogenic strategies as noninvasive EPEC and EHEC, *C. rodentium* contains the 35-kb pathogenicity island called the locus of enterocyte effacement, which encodes ~41 essential virulence factors required for the formation of A/E lesions (7). *C. rodentium* infection leads to acute colitis, mucosal hyperplasia, and diarrhea, which resolves in C57BL/6 mice by 2 to 3 weeks postinfection (10). Adaptive immune responses, both T_H1 and T_H17, are required for clearance of this pathogen (11–15). While the role of the adaptive immune system in the host response to *C. rodentium* infection is well-known, the contribution of the innate response, in particular, NK cells, remains unclear.

To determine the contribution of NK cells to the overall im-

mune response to A/E bacterial infection, we studied the response of NK cell-depleted mice after oral infection with bioluminescent *C. rodentium*. Our results demonstrate that NK cells play an important integrating role during the immune response to enteric bacterial infection by promoting *C. rodentium* clearance. NK cells are directly cytotoxic to *C. rodentium*, provide important signals required for the recruitment and activation of other innate and adaptive immune populations, and crucially, also prevent bacterial dissemination to extracolonic tissues.

MATERIALS AND METHODS

Mice and NK cell depletion. Female C57BL/6J OlaHsD mice (age, 8 to 10 weeks) from Harlan UK were used. Animal husbandry and experimental procedures were approved by the University College Cork Animal Experimentation Ethics Committee. Mice received intraperitoneal (i.p.) injections (every 3 to 4 days) of 50 μ g anti-asialo GM1 (anti-AGM1) to deplete NK cells or an appropriate rabbit IgG isotype control (6). Less than 1% splenic NK cells were consistently observed in NK cell-depleted animals throughout the study, with no significant reductions in other cellular populations (data not shown).

***C. rodentium* infection and bioluminescent imaging of mice.** The bioluminescent *C. rodentium* derivative strain ICC180 expresses the *lux-CDABE* operon from the entomopathogenic nematode symbiont *Photobacterium luminescens* (10). *C. rodentium* was grown in LB medium at 37°C with nalidixic acid at 50 μ g/ml and kanamycin at 100 μ g/ml. Groups of 6 to 8 mice were inoculated orally by a 200- μ l gavage with approximately 1×10^9 CFU *C. rodentium* at 1 day after antibody injection. Fecal samples

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were recovered aseptically at various time points after inoculation, and the number of viable bacteria per mg of feces was determined by plating onto LB agar containing the appropriate antibiotic. At the end of the study, mice were culled and their small intestine, cecum, colon, kidneys, spleens, and livers were aseptically removed, homogenized, and plated onto LB agar with nalidixic acid at 50 µg/ml and kanamycin at 100 µg/ml. Colonies were enumerated after overnight incubation at 37°C. On days 7 and 14 postinfection, *in vivo* bioluminescence imaging was performed as previously described (15) with an IVIS 100 charge-coupled-device imaging system (Xenogen, Alameda, CA). Emission images of whole bodies were collected with 5-min integration times, and organs were washed with sterile phosphate-buffered saline (PBS) and imaged for 5 min. Bioluminescent signals were quantified by the creation of regions of interest (ROIs). To standardize the data, light emission from the same surface area (ROI) was quantified for each organ type. In addition, background light emission, taken from ROIs created on organs of uninfected control animals, was subtracted from test organs. Imaging data were analyzed and quantified with Living Image software (version 2.50; Xenogen) and expressed as the numbers of photons/second/cm².

Histology and immunofluorescent staining. Six-micrometer distal colon sections were stained with hematoxylin-eosin (H&E) or Alcian blue (counterstained with hematoxylin) according to standard histological procedures or stained with primary monoclonal antibody (MAb), as specified in Table S1 in the supplemental material. Where required, purified MAbs were revealed using the appropriate Alexa Fluor 488- or 568-conjugated anti-Ig antibodies, with Hoechst (Invitrogen) used as a nuclear counterstain. Colon sections were evaluated and in a blinded fashion were assigned scores for evidence of inflammatory damage, such as goblet cell loss, crypt elongation, mucosal thickening, and epithelial injury, including hyperplasia and enterocyte shedding into the gut lumen. Scores were determined on a scale of 0 to 3 (0, none; 1, mild; 2, moderate; 3, severe). A mean inflammatory score was then assigned per mouse distal colon (3 to 4 mice per group) (15). For goblet cell enumeration, the average number of Alcian blue-positive cells per mm² was based on 6 mice per time point, with the numbers of cells in 5 to 10 fields being measured.

Flow cytometry. Single-cell suspensions from spleens and mesenteric lymph nodes (MLNs) of individual mice were prepared as described previously (6). For colonic lamina propria (cLP) cell isolation, colons were removed, cut longitudinally, and washed in PBS to remove debris. Colons were cut into 1-cm pieces and incubated at 37°C with gentle shaking in digestion buffer (Hanks balanced salt solution [HBSS], 1 mM EDTA) for 15 min. Samples were then washed with HBSS and incubated in RPMI medium plus 0.2 mg/ml collagenase type IV and 0.04 mg/ml DNase I at 37°C with gentle shaking for 1.5 h. Cells were suspended in 44% Percoll underlaid with 70% Percoll and centrifuged for 20 min, collected at the interface, and washed twice with cold PBS. Cells were added at a concentration of 2×10^5 cells/well (96-well plate) in blocking buffer (1× PBS, 1% bovine serum albumin, 0.05% sodium azide, 1% rat, hamster, and mouse serum). To this, 50 µl of each MAb dye mix plus 5 µl of amine-reactive viability UV dye (Invitrogen) was added, and the mixture was incubated in the dark at 4°C for 30 min to determine dead cells (16). The MAbs used for flow cytometry are listed in Table S1 in the supplemental material. Cells were washed and resuspended in 200 µl 3% formalin. To perform flow cytometric analyses, a FACSLSR II 5 laser (UV/violet/blue/yellow-green/red) cytometer and BD Diva software (Becton, Dickinson) were used. For each sample, 50,000 to 200,000 events were recorded. Background staining was controlled by the use of labeled isotype controls and fluorescence minus one. The results represent the percentage of positively stained cells in the total cell population with a signal exceeding the background staining signal. For determination of intracellular cytokine production by leukocytes, cells were incubated for 6 h at 37°C with BD activation cocktail plus GolgiPlug (phorbol myristate acetate [PMA], ionomycin, and brefeldin A [BD Biosciences]); unstimulated controls were also set up for each cytokine study. Cells were then washed with staining buffer and stained at 4°C for 30 min with the appropriate surface

MAbs. Controls were stained with the appropriate isotype-matched control MAbs. Cells were then fixed, permeabilized with saponin (Perm/Fix solution; BD Biosciences), and incubated with the MAbs listed in Table S1 in the supplemental material or isotype control MAbs. After 30 min, cells were twice washed in permeabilization buffer (BD Biosciences) and then analyzed by flow cytometry as described above. NK cells were identified as NK1.1⁺/CD3⁻, neutrophils were defined as Ly6G⁺, DCs were defined as CD11c⁺, macrophages were defined as F4/80⁺, B cells were defined as CD19⁺, and T cells were defined as CD3⁺.

Oxidative burst analysis. To analyze respiratory burst activity in neutrophils, intracellular reactive oxygen species (ROS) production was assessed with dihydrorhodamine 123 (DHR; Invitrogen) by flow cytometry. This primarily nonfluorescent dye becomes fluorescent upon oxidation to rhodamine by ROS produced during the respiratory burst. DHR (10 µM) was added to *ex vivo* cultures, this mixture was incubated at 37°C for 5 min, and then cells were incubated for a further 25 min with or without 100 nM PMA before quenching on ice. Cells were then prepped for flow cytometry as described above.

Analysis of total cytokine levels. Colonic homogenates were analyzed using T_h1/T_h2 9-plex kits (IFN-γ, TNF-α, interleukin-10 [IL-10], IL-1β, IL-12 total, IL-2, IL-4, IL-5, and keratinocyte chemoattractant [KC]; Meso Scale Discovery) and enzyme-linked immunosorbent assays (IL-22 and IL-17; BenderMedSystems). All assays were performed per the manufacturers' instructions. Cytokine levels are expressed as pg cytokine/mg colonic tissue (sensitivities of assays, >0.5 to 11 pg/ml).

NK cell-*C. rodentium* killing assay. NK cells were isolated from spleens and lymph nodes of naïve mice, pooled, and sorted by magnetic cell sorting (MACS) with a mouse NK cell isolation kit (Miltenyi Biotec). The purity of sorted populations ranged from 86% to 92%. Sorted non-NK cells were also retained for studies to compare against NK-specific function. *C. rodentium* bacteria were cultured overnight in LB broth and serially diluted in PBS. Bacteria ($\sim 1 \times 10^6$ CFU) were added to 1×10^5 NK cells or non-NK cells in RPMI medium without antibiotics. After 1, 2, or 3 h, the bacterium-cell culture was diluted 1:10 in water for 10 min to lyse the cells, and duplicate serial dilutions were plated on LB agar.

Statistical analysis. Experimental results were plotted and analyzed for statistical significance with Prism4 software (GraphPad Software Inc., CA). A *P* value of <0.05 was considered significant.

RESULTS

NK cells are recruited to the colon and express immune-modulatory factors after *C. rodentium* infection. To determine if NK cells play a role during *C. rodentium* infection, we first examined their characteristics over a number of infection time points (Fig. 1; see Fig. S1 in the supplemental material). Kinetic analysis of the NK cell population revealed significant increases (*P* < 0.05) in the percentage and total number of NK cells within the MLN and spleen at day 7 and day 14 postinfection compared to uninfected control mice (Fig. 1A; see Fig. S1 in the supplemental material). We also observed significant (*P* < 0.001) accumulation of NK cells from day 7 onwards in the colons of these mice (Fig. 1B and C). Functional aspects of the NK cell response to *C. rodentium* infection were addressed by flow cytometric analysis of intracellular cytokine and cytotoxic molecules in colonic and MLN NK cells from *C. rodentium*-infected mice (Fig. 1D and E; see Fig. S1 in the supplemental material). On day 7 and day 14 postinfection, we observed a significant increase (*P* < 0.05) in cLP (Fig. 1D and E) and MLN (see Fig. S1 in the supplemental material) NK cells positive for IFN-γ, IL-17, IL-10, and CD107a (degranulation marker) in infected compared to uninfected mice. Perforin (pf)-positive MLN NK cells were significantly elevated (*P* < 0.01) on day 7, and the number of granzyme B (gzmB)-positive MLN NK cells was significantly higher (*P* < 0.01) at the end of the study (day 14).

FIG 1 NK cells are recruited and express immune-modulatory factors after *C. rodentium* infection. Cells were isolated from the MLNs and spleens of *C. rodentium*-infected or uninfected control C57BL/6 mice at various time points and analyzed by flow cytometry. (A) Mean NK cell percentage $\pm$ SD. (B) Frozen colonic sections (6 μ m) were stained with anti-NKp46 (green) and nuclei (blue) from uninfected control and infected mice at days 7 and 14. A representative picture for each group is shown. Bars, 200 μ m. (C) Total number of positive cells per mm². Values are based on 6 mice/time point, with values in 5 to 10 fields being measured. Arrows highlight the locations of the NK cells. cLP cells were stimulated for 6 h with BD leukocyte activation cocktail plus GolgiPlug and stained with NK1.1 and CD3, permeabilized, and stained with an anti-cytokine fluorochrome-labeled MAb. Data represent the percentage of cytokine-positive NK cells out of the total NK cell population on day 7 (D) and day 14 (E). Data are means $\pm$ SDs ($n = 7$; 2 independent experiments). *, $P < 0.05$ versus uninfected control mice; **, $P < 0.01$ versus uninfected control mice; ***, $P < 0.001$ versus uninfected control mice; NS, not significant; d7, day 7; d14, day 14.

These data show that the numbers of NK cells are persistently increased in gut mucosal tissues and that they express a wide range of cytokine and cytotoxic factors during *C. rodentium* infection.

NK cells reduce *C. rodentium* burden in colon and cecum. To

determine the impact of these changes within the NK cell population on the course of *C. rodentium* infection in mice, polyclonal anti-AGM1 antibody was used to actively deplete the NK cell population from murine tissues (6). We observed that NK cell-depleted mice had significantly higher ($P < 0.05$) numbers of *C. rodentium* organisms than *C. rodentium*-infected control mice by day 7 and until the study end in feces and by day 14 in the colon and cecum (Fig. 2A and B). Bioluminescence imaging analysis confirmed these observations. NK cell-depleted mice infected with *C. rodentium* had a significantly higher ($P < 0.01$) *C. rodentium* bioluminescence signal from the lower abdomen on days 7 and 14 than control infected mice (Fig. 2C and D). When organs were excised and imaged, NK cell-depleted infected mice had significantly higher bioluminescence signals ($P < 0.05$) in the cecum and MLN on days 7 and 14 and the colon on day 14 than control mice infected with *C. rodentium*. Notably, at the end of the study (day 14), NK cell-depleted mice demonstrated delayed *C. rodentium* clearance (Fig. 2). Furthermore, colonic staining for the A/E virulence factor intimin was observed in control and NK cell-depleted mice on day 7 but only NK cell-depleted mice on day 14 (Fig. 2E). Taken together, these data indicate that NK cells are required to reduce the *C. rodentium* burden.

NK cells influence colonic pathology after *C. rodentium* infection. *C. rodentium* infection is associated with colonic crypt hyperplasia, goblet cell depletion, mucosal inflammation, and erosion. Epithelial hyperplasia can be indirectly assessed by changes in distal colonic weights. Infected mice had significantly heavier ($P < 0.05$) colons on days 7 and 14 postinfection than uninfected mice (Fig. 3A). Notably, distal colons from NK cell-depleted mice infected with *C. rodentium* were significantly heavier ($P < 0.05$) on day 14 than colons from control infected mice, suggesting increased hyperplasia during infection (Fig. 3A). However, histological analysis and scoring revealed that NK cell-depleted infected mice had significantly less ($P < 0.01$) inflammation, as exemplified by shorter colonic crypts and reduced leukocyte infiltration, especially at day 14, than control *C. rodentium*-infected mice (Fig. 3B and C). Interestingly, NK cell-depleted mice had larger and significantly greater ($P < 0.001$) numbers of goblet cells at day 14 postinfection, in contrast to the characteristic goblet cell depletion in control infected mice (Fig. 3D to F). Consistently, analysis of cytokine levels in colon homogenates revealed significantly reduced ($P < 0.05$) levels of IFN- γ , IL-10, murine KC (mKC), and IL-17 in NK cell-depleted mice at both 7 and 14 days postinfection than control mice (Fig. 3G and H). TNF- α was significantly reduced ($P < 0.01$) on day 7 but not day 14 in NK cell-depleted mice compared to the levels in control infected mice. Increased levels of IL-12 were found in NK cell-depleted mice at 7 days postinfection but not on day 14 compared to the levels in control *C. rodentium*-infected mice (Fig. 3G and H). Significantly lower levels ($P < 0.001$) of IL-1 β were detected in NK cell-depleted mice on day 14 after *C. rodentium* infection but not on day 7 compared to the levels in control infected mice (Fig. 3G and H). No major changes in colonic IL-2, IL-4, IL-5, and IL-22 were found between the different groups at the different time points analyzed (Fig. 3G and H and data not shown). Thus, the absence of NK cells appears to result in less inflammation and enhanced epithelial function, suggesting that NK cells contribute to the pathology (possibly via recruitment and/or activation of other cell types) associated with infection.

FIG 2 NK cells contribute to *C. rodentium* clearance. C57BL/6 mice were treated with anti-AGM1 or control IgG every 3 to 4 days and infected with *C. rodentium*. (A) The numbers of fecal CFU were determined every 3 to 4 days, and data represent the $\log_{10}$ number of CFU/g feces ($\pm$ SD). (B) Organs (colon and caecum) were also removed on days 7 and 14 to determine the numbers of CFU. (C) Bioluminescent images from the gastrointestinal region (*in vivo* whole-body imaging) are displayed as pseudocolor images, with variations in color representing the light intensity at a given location. Red represents the most intense light emission, while blue corresponds to the weakest signal. (D) Bioluminescent signals were quantified from the whole body and *ex vivo* organs, i.e., colon, caecum, and MLN, and are expressed as means ($\pm$ SDs). Significance was determined using the Mann-Whitney U test: *, $P < 0.05$; **, $P < 0.01$ ($n = 7$ to 10 mice/group). (E) Frozen colonic sections (6 μ m) were stained with anti-intimin (green), F-actin (red), and nuclei (blue). A representative picture for each group is shown. Bars, 200 μ m.

NK cells modulate infiltration and cytokine profiles of MLN immune cells during infection. To explore the influence of NK cells on other immune populations, we analyzed immune cell number and cytokine profiles on days 7 and 14 postinfection in MLNs from NK cell-depleted and control mice, as these lymph nodes drain the murine colon (Fig. 4; see Fig. S2 and S3 in the supplemental material). The percentages of F4/80⁺ macrophages, Ly6G⁺ neutrophils, and CD11c⁺ DCs were significantly reduced

($P < 0.05$) on day 7 postinfection in NK cell-depleted mice compared to control and uninfected animals (Fig. 4A). By day 14, the percentages of B cells and DCs were significantly reduced ($P < 0.05$) in NK cell-depleted infected mice compared to control infected animals (Fig. 4B). Further, the percentages of Ly6G⁺ neutrophils were significantly lower ($P < 0.05$) in NK cell-depleted infected mice than control infected and uninfected controls at both day 7 and day 14 postinfection (Fig. 4A and B). Notably,

FIG 4 NK cells modulate infiltration and cytokine profiles of MLN immune cells during infection. Cells were isolated from MLNs of C57BL/6 mice at 7 days (A) and 14 days (B) after *C. rodentium* infection, stained with fluorochrome-labeled MAb, and analyzed by flow cytometry. Columns represent the mean percentage $\pm$ SD of seven mice per group from two independent experiments. MLN cells were incubated with DHR (a marker for ROS) before addition of PMA. Cells were then analyzed by flow cytometry to determine the percentage of DHR/ROS-positive cells in the Ly6G⁺ and F4/80⁺ populations on day 7 (C) and day 14 (D). Data represent the percentage of ROS-positive cells out of the total specific cell population $\pm$ SD. *P* values were determined using one-way analysis of variance, followed by Bonferroni's multiple-comparison test: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

when total cell numbers were examined, we observed significantly reduced levels of all immune populations analyzed in NK cell-depleted mice compared to control infected animals (see Fig. S2 in the supplemental material).

We also analyzed cytokine profiles from MLN immune cells during *C. rodentium* infection from both NK cell-depleted and control mice (see Fig. S3 in the supplemental material). We observed significantly lower (*P* < 0.001) percentages of T cells expressing IL-17 in NK cell-depleted mice than both control and uninfected mice on days 7 and 14 postinfection and IFN- γ -positive T cells on day 14 (*P* < 0.05). IFN- γ - and TNF- α -positive neutrophils, DCs, and macrophages were also significantly reduced (*P* < 0.05) in NK cell-depleted mice compared to control and uninfected mice at both infection time points. At day 7, IL-10-positive macrophages were significantly lower (*P* < 0.01) in NK cell-depleted infected mice than uninfected and control infected mice. As a marker of antimicrobial activity, ROS production from phagocytes was assessed (Fig. 4C and D). On days 7 and 14 postinfection, NK cell-depleted mice had significantly lower percentages (*P* < 0.05) of macrophages and neutrophils producing ROS than control or uninfected mice. These data indicate that NK cells modulate the activation and function of other immune cell populations during *C. rodentium* infection.

NK cells recruit colonic effector cells during infection. *C. rodentium* induces a localized inflammatory response in the colon, characterized by infiltration of immune cells such as neutrophils and lymphocytes. At day 7 postinfection, NK cell-depleted mice had significantly lower (*P* < 0.001) counts of Ly6G⁺ neutrophils than control mice (Fig. 5A and B). Notably, by day 14, all immune populations examined, including CD3⁺ T cells, CD19⁺ B cells, CD11c⁺ DCs, F4/80⁺ macrophages, and neutrophils, were significantly reduced (*P* < 0.001) in the colons of NK cell-depleted mice compared to the colons of control *C. rodentium*-infected mice (Fig. 5C and D). These data suggest that NK cells are crucial for the regulated recruitment of other immune cell populations to the inflamed colon during infection.

NK cells limit extracolonic dissemination of *C. rodentium*. *C. rodentium* infection is restricted to the cecum and colon of C57BL/6 mice. To determine if NK cells had a role in preventing disseminated infection, we measured the bacterial loads in various systemic tissues (liver, kidney, and spleen) following infection. NK cell-depleted mice had high numbers of bacterial CFU in all systemic organs on days 7 and 14 postinfection, whereas no such increment was detected in control infected mice (Fig. 6A). These observations suggest that the presence of NK cells prevents dissemination of *C. rodentium* from the colon to peripheral tissues.

NK cells can directly kill *C. rodentium* in vitro. Our observation that bacterial loads in NK cell-depleted mice were greater than those in control animals suggested the possibility that NK cells might have a direct cytotoxic effect on *C. rodentium*. To test whether NK cells directly kill *C. rodentium*, live bacteria were mixed with cultured NK cells and bacterial survival was quantitated. NK cells exhibited significant efficiency (*P* < 0.05) in killing *C. rodentium* after 2 h of coculture compared to medium alone (Fig. 6B). Notably, we did not observe any significant killing in

FIG 3 NK cells impact colonic pathology after *C. rodentium* infection. Anti-AGM1- and IgG-treated control mice had colons removed at days 7 and 14 postinfection. (A) Mean colon weight (mg/cm $\pm$ SD). (B) Representative histology of H&E-stained colon. Bars, 200 μ m. (C) Mean inflammatory scores of IgG control or anti-AGM1-treated mice on days 7 and 14 postinfection determined by histological analysis of distal colonic sections. (D) Sections of H&E-stained colonic tissue on day 14, with differences in goblet cells indicated by arrows. Bars, 100 μ m. (E) Alcian blue, counterstained with hematoxylin, indicating (in blue) mucus-producing goblet cells. Bars, 100 μ m. (F) Means $\pm$ SDs of the total number of goblet cell per mm² at day 14 postinfection, based on 6 mice/time point (5 to 10 fields). (G and H) Mean cytokine levels in whole colonic homogenates $\pm$ SD on day 7 (G) and day 14 (H). Data are for 7 mice per group and are representative of two experiments. *P* values were determined using the Mann-Whitney U test: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; NS, not significant.

FIG 5 NK cells induce recruitment of immune populations to the colons of infected mice. Anti-AGM1- and IgG-treated control mice had colons removed at days 7 and 14 postinfection for immunofluorescent staining of cellular populations. (A) Neutrophils (Ly6G, green) and nuclei (blue); (B) mean of the total number of positive cells per $\text{mm}^2 \pm \text{SD}$ at day 7 postinfection; (C) neutrophils (Ly6G, green), T cells (CD3, red), B cells (CD19, red), DCs (CD11c, red), macrophages (F4/80, red), and nuclei (blue); (D) mean of the total number of positive cells per $\text{mm}^2 \pm \text{SD}$ at day 14 postinfection. A representative picture for each group ($n = 7$) is shown. Bars, 200 μm . P values were determined using one-way analysis of variance, followed by Bonferroni's multiple-comparison test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

FIG 6 NK cells limit systemic spread of *C. rodentium* and have a direct antimicrobial effect *in vitro*. C57BL/6 mice were treated with anti-AGM1 or control IgG every 3 to 4 days and infected with *C. rodentium*. (A) The bacterial load (numbers of CFU) in systemic organs was determined on days 7 and 14, and data represent the log₁₀ number of CFU/g tissue ($\pm$ SD; $n = 6$ /time point). ND, not detectable. (B) NK cells or non-NK cells (both at 1×10^5 cells) or medium alone was coincubated with *C. rodentium* (1×10^6 CFU of bacteria) for 1, 2, and 3 h of culture (multiplicity of infection, 10:1). Data are the mean numbers of CFU at each time point and are representative of two independent experiments. *P* values were determined using the Kruskal-Wallis test, followed by Dunn's multiple-comparison test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

non-NK cell-*C. rodentium* cultures compared to medium alone over 3 h. These data indicate that NK cells are directly cytotoxic to *C. rodentium*.

DISCUSSION

Enteroadherent pathogens such as EPEC, EHEC, and *C. rodentium* cause disease characterized by colonic inflammation and diarrhea. While previous studies have elucidated the pathogenic strategies employed by these pathogens, the role of the host immune system, particularly the innate arm of the immune response, is still poorly understood. We provide evidence that NK cells protect the host from prolonged mucosal and systemic infection by generating direct cytokine and antimicrobial cytotoxic factors and by providing signals regulating infiltration and increased activation of other immune cell populations. Therefore, while NK cells contribute to colonic inflammation, this response is also crucial for reducing the bacterial burden.

After oral infection with *C. rodentium*, the distal colon is preferentially colonized by the bacterium through intimate attachment to the gut epithelium. Breaches in the intestinal barrier after A/E lesion formation lead to acute inflammation, peaking at 1 week postinfection in C57BL/6 mice (9). During *C. rodentium* infection, we observed NK cell recruitment, in tandem with strong expression of cytokine and cytotoxic factors, into mucosal and systemic tissues, suggesting that NK cells play an important role during *C. rodentium* infection. Data from bioluminescence whole-body and organ-specific imaging and viable bacterial counts revealed that NK cell-depleted mice had delayed bacterial clearance. In addition to a higher bacterial burden, reduced immune cell infiltration and diminished cytokine levels within the

MLNs and colons of infected mice were observed in NK cell-depleted mice.

Previous studies have highlighted the importance of neutrophils in defense against *C. rodentium* (17). IL-17 coordinates early recruitment and activation of neutrophils to infection sites through chemokine expression, including the induction of mKC (18). It is noteworthy that within the colons of NK cell-depleted infected mice we observed lower levels of IL-17 and mKC and, correspondingly, diminished neutrophil infiltration, potentially due to the absence of NK cell and/or NK cell-activated T cell-derived IL-17. While only neutrophil infiltration was impaired on day 7 postinfection in NK cell-depleted mice, by day 14 all other immune populations examined were markedly reduced within NK cell-depleted colons. IFN- γ is readily produced by NK cells after activation, which we also observed during *C. rodentium* infection. IFN- γ is known to regulate chemokine production and, with regard to bacterial infection, has been shown to determine the outcome of *Staphylococcus aureus* infection through chemokine-induced immune cell recruitment (19). Furthermore, NK cell-derived cytokine production, particularly IFN- γ production, is required for maturation and activation of other immune cell populations for clearance of infection (3). These data support the evidence for the role of NK cell-derived signals in recruitment of immune cells to resolve infection with *C. rodentium*. Several studies have identified a new and distinct population of IL-22-producing, NKp46-positive (NKp46⁺) transcription factor ROR γ t-positive (ROR γ t⁺) gut-associated mucosal cells (20, 21). These cells have been shown to play an IL-22-protective role in host defense against gut pathogens (22). Initially, these cells were thought to be a new subset of classical NK cells, as they express the activation receptor NKp46. However, these cells do not express other well-characterized NK cell markers, such as NK1.1 or DX5, are not cytotoxic, and do not produce IFN- γ . Importantly, we did not detect any significant differences between infected groups for IL-22, suggesting that IL-22 production from the resident NKp46⁺ ROR γ t⁺ subset population and/or the presence of activated T cells cannot explain why NK cell-deficient mice have increased enteric infection in our model (23, 24).

DCs and macrophages require help from NK cells for maturation, proper antigen presentation function, and priming of T cell responses (25). This bidirectional cross talk during the early phases of immunity influences the following type and magnitude of adaptive immune response (2). It is noteworthy that in our study, mucosal infiltration and cytokine expression of both T and B cells were significantly impaired in NK cell-depleted mice. Previous work in RAG1-knockout mice has highlighted the importance of T and B cells for the clearance of *C. rodentium* (11). Interestingly, these mice also had transient, inflammatory, and crypt hyperplastic responses that subsided after 2 weeks, similar to our observations in NK cell-depleted mice during infection (11).

C. rodentium growth is restricted to the colon, with little bacterial translocation except in highly susceptible mouse strains, such as C3H (26). In NK cell-depleted mice, we observed significant dissemination of the bacterium to systemic organs, including the spleen, kidneys, and liver, in stark contrast to control mice, which exhibited no extracolonic spread. Previous work has also shown a role for NK cells in preventing systemic bacterial spread from initial mucosal colonization of the lungs during *Mycobacterium tuberculosis* infection (27). Our data reveal a mechanism by which NK cells may protect the host from systemic bacterial

spread. ROS are involved in host defense to bacterial pathogens and are induced in response to proinflammatory cytokines such as IFN- γ (28). We observed diminished ROS production from both neutrophils and macrophages in NK cell-depleted mice, suggesting that NK cell-derived cytokines may stimulate ROS production by granulocytes, thereby increasing bacterial killing both within the colon and also within distal sites. Another mechanism by which NK cells may protect the host from systemic spread of bacteria is to kill *C. rodentium* directly. NK cells may mediate antibacterial effects through the indirect action of secreted mediators or, in the case of infected host cells, by direct cytolytic activity. We observed that NK cells also have significant antibacterial activity against extracellular *C. rodentium*. Indeed, NK cells may kill bacteria in the colon as well as systemically, where they are present in larger numbers, and thereby prevent dissemination of bacteria to systemic organs.

The signals which activate NK cells to mediate direct damage of free bacteria are poorly understood. Many NK cell receptors are known to bind several viral as well as host cell proteins, and recent studies have shown that NKP44 directly binds ligands on the surface of *M. tuberculosis* and *Pseudomonas aeruginosa* (29). Undefined ligands from *C. rodentium* might be recognized by NK cell receptors, although studies examining NKP46 suggest that this particular activating receptor is dispensable for defense against *C. rodentium* (30). Secreted NK-derived factors, other than cytokines, could also play a role in bacterial clearance. Defensins are antimicrobial peptides, expressed mainly by epithelial cells and immune cells, that disrupt the bacterial membrane, leading to pathogen death (31). Previous studies have shown that NK cells can directly recognize pathogen-associated molecular patterns on the surface of bacteria and respond by production of α -defensins (32). This may also represent a direct cytotoxic pathway involved in NK cell-mediated protection against *C. rodentium*.

Goblet cells are highly specialized secretory cells present in the intestinal tract that produce mucins which form a dynamic defensive mucus barrier against enteric pathogens (33). Previous work has shown that infection by several enteric pathogens, including *C. rodentium*, leads to a marked reduction in the number of goblet cells, and this may represent a mechanism for subversion of this host defense (34). In contrast to infected murine controls, where we observed goblet cell depletion, NK cell-depleted mice had a significant increase in the number and size of goblet cells. Previous studies have demonstrated that while colonic goblet cells can be subject to direct infection and potential subversion by A/E pathogens *in vivo*, it is the host immune system that primarily modulates the function of these cells during infection (35). Concurrent with this idea, the reduced immune cell infiltration observed in NK cell-depleted mice during *C. rodentium* infection may account for the absence of characteristic goblet cell depletion in our study. Indeed, goblet cell hyperplasia may represent a compensatory mechanism in the absence of normal immune cell infiltration and may also account for the increased colonic mass observed within NK cell-depleted mice.

In summary, we show the complex nature of NK cell involvement in protection against the A/E enteric pathogen *C. rodentium*. NK cells are necessary for reducing pathogen burden. They mediate their effects through cytokine production and the subsequent regulation of innate immune populations, leading to the generation of robust adaptive immunity. NK cells are also able to directly kill *C. rodentium* while regulating the antimicrobial activity of

granulocytes and protect the host from bacterial dissemination. Indeed, this comprehensive anti-infection NK cell response may represent a promising therapeutic focus, particularly for approaches against enteric bacterial infections.

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REFERENCES

1. Pegram HJ, Andrews DM, Smyth MJ, Darcy PK, Kershaw MH. 2011. Activating and inhibitory receptors of natural killer cells. *Immunol. Cell Biol.* 89:216–224.
2. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM, Ugolini S. 2011. Innate or adaptive immunity? The example of natural killer cells. *Science* 331:44–49.
3. Horowitz A, Stegmann KA, Riley EM. 2011. Activation of natural killer cells during microbial infections. *Front. Immunol.* 2:88. doi:10.3389/fimmu.2011.00088.
4. Degli-Esposti MA, Smyth MJ. 2005. Close encounters of different kinds: dendritic cells and NK cells take centre stage. *Nat. Rev.* 5:112–124.
5. Nedvetzki S, Sowinski S, Eagle RA, Harris J, Vely F, Pende D, Trowsdale J, Vivier E, Gordon S, Davis DM. 2007. Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses. *Blood* 109:3776–3785.
6. Hall LJ, Clare S, Dougan G. 2010. NK cells influence both innate and adaptive immune responses after mucosal immunization with antigen and mucosal adjuvant. *J. Immunol.* 184:4327–4337.
7. Croxen MA, Finlay BB. 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. *Nat. Rev. Microbiol.* 8:26–38.
8. Nataro JP, Kaper JB. 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* 11:142–201.
9. Mundy R, MacDonald TT, Dougan G, Frankel G, Wiles S. 2005. *Citrobacter rodentium* of mice and man. *Cell. Microbiol.* 7:1697–1706.
10. Wiles S, Clare S, Harker J, Huett A, Young D, Dougan G, Frankel G. 2004. Organ specificity, colonization and clearance dynamics *in vivo* following oral challenges with the murine pathogen *Citrobacter rodentium*. *Cell. Microbiol.* 6:963–972.
11. Vallance BA, Deng W, Knodler LA, Finlay BB. 2002. Mice lacking T and B lymphocytes develop transient colitis and crypt hyperplasia yet suffer impaired bacterial clearance during *Citrobacter rodentium* infection. *Infect. Immun.* 70:2070–2081.
12. Bry L, Brigl M, Brenner MB. 2006. CD4+ T-cell effector functions and costimulatory requirements essential for surviving mucosal infection with *Citrobacter rodentium*. *Infect. Immun.* 74:673–681.
13. Simmons CP, Clare S, Ghaem-Maghami M, Uren TK, Rankin J, Huett A, Goldin R, Lewis DJ, MacDonald TT, Strugnell RA, Frankel G, Dougan G. 2003. Central role for B lymphocytes and CD4+ T cells in immunity to infection by the attaching and effacing pathogen *Citrobacter rodentium*. *Infect. Immun.* 71:5077–5086.
14. Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, Fujikado N, Tanahashi Y, Akitsu A, Kotaki H, Sudo K, Nakae S, Sasakawa C, Iwakura Y. 2009. Differential roles of interleukin-17A and -17F in host defense against mucocutaneous bacterial infection and allergic responses. *Immunity* 30:108–119.
15. Murphy CT, Hall LJ, Hurley G, Quinlan A, Macsharry J, Shanahan F, Nally K, Melgar S. 2012. The sphingosine-1-phosphate analogue FTY720 impairs mucosal immunity and clearance of the enteric pathogen *Citrobacter rodentium*. *Infect. Immun.* 80:2712–2723.
16. Perfetto SP, Chattopadhyay PK, Lamoreaux L, Nguyen R, Ambrozak D, Koup RA, Roederer M. 2006. Amine reactive dyes: an effective tool to discriminate live and dead cells in polychromatic flow cytometry. *J. Immunol. Methods* 313:199–208.
17. Lebeis SL, Bommaris B, Parkos CA, Sherman MA, Kalman D. 2007. TLR signaling mediated by MyD88 is required for a protective innate immune response by neutrophils to *Citrobacter rodentium*. *J. Immunol.* 179:566–577.
18. Hartupée J, Liu C, Novotny M, Li X, Hamilton T. 2007. IL-17 enhances chemokine gene expression through mRNA stabilization. *J. Immunol.* 179:4135–4141.

19. McLoughlin RM, Lee JC, Kasper DL, Tzianabos AO. 2008. IFN-gamma regulated chemokine production determines the outcome of *Staphylococcus aureus* infection. *J. Immunol.* 181:1323–1332.
20. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, Doherty JM, Mills JC, Colonna M. 2009. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* 457:722–725.
21. Sanos SL, Bui VL, Mortha A, Oberle K, Heners C, Johner C, Diefenbach A. 2009. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat. Immunol.* 10:83–91.
22. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, Abbas AR, Modrusan Z, Ghilardi N, de Sauvage FJ, Ouyang W. 2008. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat. Med.* 14:282–289.
23. Colonna M. 2009. Interleukin-22-producing natural killer cells and lymphoid tissue inducer-like cells in mucosal immunity. *Immunity* 31:15–23.
24. Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, Basham B, Smith K, Chen T, Morel F, Lecron JC, Kastelein RA, Cua DJ, McClanahan TK, Bowman EP, de Waal Malefyt R. 2007. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat. Immunol.* 8:950–957.
25. Vitale M, Della Chiesa M, Carlomagno S, Pende D, Arico M, Moretta L, Moretta A. 2005. NK-dependent DC maturation is mediated by TNF α and IFN γ released upon engagement of the NKp30 triggering receptor. *Blood* 106:566–571.
26. Vallance BA, Deng W, Jacobson K, Finlay BB. 2003. Host susceptibility to the attaching and effacing bacterial pathogen *Citrobacter rodentium*. *Infect. Immun.* 71:3443–3453.
27. Feng CG, Kaviratne M, Rothfuchs AG, Cheever A, Hieny S, Young HA, Wynn TA, Sher A. 2006. NK cell-derived IFN-gamma differentially regulates innate resistance and neutrophil response in T cell-deficient hosts infected with *Mycobacterium tuberculosis*. *J. Immunol.* 177:7086–7093.
28. Fang FC. 2011. Antimicrobial actions of reactive oxygen species. *mBio* 2:e00141–11. doi:10.1128/mBio.00141-11.
29. Esin S, Batoni G, Counoupas C, Stringaro A, Brancatisano FL, Colone M, Maisetta G, Florio W, Arancia G, Campa M. 2008. Direct binding of human NK cell natural cytotoxicity receptor NKp44 to the surfaces of mycobacteria and other bacteria. *Infect. Immun.* 76:1719–1727.
30. Satoh-Takayama N, Dumoutier L, Lesjean-Pottier S, Ribeiro VS, Mandelboim O, Renauld JC, Vossenrich CA, Di Santo JP. 2009. The natural cytotoxicity receptor NKp46 is dispensable for IL-22-mediated innate intestinal immune defense against *Citrobacter rodentium*. *J. Immunol.* 183:6579–6587.
31. Doss M, White MR, Tecle T, Hartshorn KL. 2010. Human defensins and LL-37 in mucosal immunity. *J. Leukoc. Biol.* 87:79–92.
32. Chalifour A, Jeannin P, Gauchat JF, Blaecke A, Malissard M, N'Guyen T, Thieblemont N, Delneste Y. 2004. Direct bacterial protein PAMP recognition by human NK cells involves TLRs and triggers alpha-defensin production. *Blood* 104:1778–1783.
33. Kim YS, Ho SB. 2010. Intestinal goblet cells and mucins in health and disease: recent insights and progress. *Curr. Gastroenterol. Rep.* 12:319–330.
34. McGuckin MA, Linden SK, Sutton P, Florin TH. 2011. Mucin dynamics and enteric pathogens. *Nat. Rev. Microbiol.* 9:265–278.
35. Bergstrom KS, Guttman JA, Rumi M, MA C, Bouzari S, Khan MA, Gibson DL, Vogl AW, Vallance BA. 2008. Modulation of intestinal goblet cell function during infection by an attaching and effacing bacterial pathogen. *Infect. Immun.* 76:796–811.