Entamoeba histolytica is a protozoan parasite that causes amebic colitis, an invasive disease responsible for as many as 100,000 deaths per year globally (14). A well-appreciated feature of E. histolytica infection is that it progresses to invasive disease only a minority of colonized individuals (5, 9, 13). Host factors likely contribute to this diversity in clinical outcome, but the identity of these factors is not entirely clear.

We have utilized a murine model of intestinal amebiasis that reveals mouse strain-dependent susceptibility to establishment of infection. After intracanal inoculation of strain CBA mice with animal-passaged E. histolytica, most individuals develop persistent infection with colitis. Other mouse strains, such as strains 129 and FVB, are also reasonably susceptible. In contrast, the vast majority of C57BL/6 mice are resistant to infection by a mechanism that occurs within the first few hours or days. We hypothesize that epithelial cells govern the initial susceptibility to infection, since bone marrow chimera experiments showed that this phenotype segregates with the nonhemopoietic compartment (11). We also have found that interleukin-10 (IL-10) contributes to the resistance of C57BL/6 mice, and again we postulate that this action is associated with preservation of the epithelial barrier function.

Given the rapidity with which clearance occurs compared with successful infection, it is plausible that a rapid response, such as nuclear factor-κB (NF-κB) activity, may occur. NF-κB is a pleiotropic transcription factor that regulates diverse cellular functions ranging from the inflammatory response to cell proliferation, and it directs gene expression in response to pathogens, including E. histolytica (17, 18, 23, 24). NF-κB is a dimeric transcription factor consisting of 5 possible subunits, p50, p52, p65, RelB, and c-Rel, and p50-p65 is the canonical heterodimer that typically leads to proinflammatory gene expression in immune cells through carefully orchestrated transcription from the cytoplasm to the nucleus (19). A key enzyme in the canonical NF-κB activation pathway is IKKβ (IκB kinase β), which serine phosphorylates IκB, targets it for ubiquitination, and thus liberates p50-p65 to translocate into the nucleus to bind κB cis-acting elements of proinflammatory genes (19, 20). Most research on NF-κB in infectious diseases such as the disease caused by Entamoeba has focused on this canonical p50-p65 heterodimer. In a SCID xenograft intestinal amebiasis model p65 participates in the proinflammatory response associated with recruitment of neutrophils and increased epithelial permeability (24). In other work, treatment of cell lines with amebic antigen led to expression of chemokine genes through a mechanism that involves posttranslational modification of p65 (18).

However, NF-κB activation in response to amebae can be more nuanced; for instance, amebic antigens can also suppress NF-κB activation in epithelial cells via heat shock protein action on IκB kinase activity (17). Furthermore, the gut is a unique site, where NF-κB activity often results in a restorative response, not a proinflammatory response (20). In particular, studies with Helicobacter hepaticus and Campylobacter jejuni models have suggested an important role for NF-κB p50 in resolving intestinal inflammation (8, 26, 27). Since previously there has not been an examination of any NF-κB subunits in the mouse model of intestinal amebiasis, here we identified which NF-κB subunits were most abundant during intestinal E. histolytica infection in this model, found that p50a was the predominant subunit in the gut, and then examined the role of this subunit with available mice.

Materials and Methods

Mice. CBA/J, C57BL/6, C57BL/6 Cdcs1btm1Bal, and C57BL/6.SJL-Tg(Vil-cre)997Gum/J mice were purchased from The Jackson Laboratory. C57BL/6 IL-10−/− and C57BL/6 IL-10−/− mice recombinantly inbred for the C3H/HeJ/Bir Cdx1 locus (BC-R1) were obtained from the research colony of E. H. Leiter at The Jackson Laboratory (3). For mouse strain 129 experiments, NF-κB p105-
The data are from 3 separate experiments. Mice were sacrificed at 7 to 10 days postinoculation for evaluation of the culture-positive rate and inflammation score. Data for the cecal antigen and the histologic ameba score were not obtained in these experiments.

\[ P < 0.05 \] for a comparison of the C57BL/6 IL-10−/− mice and the other groups.

<table>
<thead>
<tr>
<th>Background strain IL-10 genotype</th>
<th>IL-10 locus</th>
<th>n</th>
<th>Culture-positive rate (%)</th>
<th>Inflammation score</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 +/+</td>
<td>C57BL/6</td>
<td>17</td>
<td>6 0.62 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>C57BL/6 −/−</td>
<td>C57BL/6</td>
<td>14</td>
<td>64b 2.68 ± 0.54b</td>
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<tr>
<td>C57BL/6 −/−</td>
<td>C3H/HeJ</td>
<td>21</td>
<td>5 0.71 ± 0.25</td>
<td></td>
</tr>
</tbody>
</table>

a The data are from 3 separate experiments. Mice were sacrificed at 7 to 10 days postinoculation for evaluation of the culture-positive rate and inflammation score. Data for the cecal antigen and the histologic ameba score were not obtained in these experiments.

b \( P < 0.05 \) for a comparison of the C57BL/6 IL-10−/− mice and the other groups.

RESULTS

Rationale for examining the NF-kB system in mice. We examined the NF-kB system in gut underwent different patterns of activation in different mouse strains. We were interested in the NF-kB system because previous work with C57BL/6 IL-10 knockout (KO) mice, which we have previously shown to exhibit increased susceptibility to intestinal amebic infection (11), revealed a dependence on the chromosome 3 locus, which contains the NF-kB p50 subunit encoding gene Nfkbia (1). Specifically, the C6esl locus from C3H/HeJ mice significantly reduced the susceptibility of C57BL/6 IL-10 KO mice, as measured by using positive cultures after sacrifice and the histologic inflammation score (\( P < 0.05 \) (Table 1)). Other workers have reported that this C3H/HeJ locus may confer a high level of p50 expression (3); therefore, we speculated that p50 activity may contribute to protection.

Nuclear translocation of the NF-kB p50 subunit after intracecal inoculation. We started by broadly characterizing the NF-kB subunit profile in the mouse cecum early after inoculation of  E. histolytica  into the intestine. We examined cecal tissue 2 days after inoculation, since this is the time frame when resistant mice are clear or have cleared infections (2). In the ceca of C57BL/6 and CBA mice, as well as in the ceca of 129 mice (which have intermediate susceptibility to  E. histolytica  [10]), there was an increase in NF-kB activity after inoculation of  E. histolytica , as indicated by increased binding of the radiolabeled NF-kB probe in nuclear extracts of whole cecal tissue (Fig. 1A). We performed supershift experiments that showed that p50 was the predominant subunit in all of the mouse strains, and p65 was more apparent in the intermediatesusceptible and susceptible strains. To examine the pattern in epithelial cells specifically, we isolated crypts from C57BL/6 and 129 mice (both naive mice and mice 2 days after inoculation of amebae), isolated nuclear extracts, and performed an ELISA for p50 and p65. The ELISA results corroborated the finding that there was abundant p50 in the epithelium (Fig. 1B).

p50 is protective during amebic infection in vivo. To examine the role of the p50 subunit in this model, we utilized p50 KO
FIG. 1. Nuclear translocation of the NF-κB p50 subunit predominates in C57BL/6 ceca after inoculation of *E. histolytica*. (A) Nuclear extracts of cecal tissue from mice with C57BL/6 (upper panels), 129S6/SvEvTac (middle panels), and CBA (lower panels) backgrounds at 2 days after intracecal inoculation were incubated with a radiolabeled NF-κB consensus sequence oligonucleotide probe and resolved on nondenaturing polyacrylamide gels. To identify NF-κB subunits, supershift experiments were performed with mouse-specific anti-p50, -p65, -p52, -cRel, and -RelB (right panels). The arrows indicate the locations of p50 and p65 subunits if they shifted. ss, supershift. (B) Cecal epithelial crypts were isolated from C57BL/6 and 129 mice at 2 days after inoculation of *E. histolytica*, nuclear extracts were prepared, and quantities of p50 (open bars) and p65 (filled bars) were assayed by oligonucleotide capture ELISA. Optical densities (O.D.) were multiplied by the specific NF-κB oligonucleotide binding fraction according to the manufacturer’s instructions. The data are the means and standard errors of the means for 8 mice per group. *, $P < 0.05$ for a comparison of p50 of naive mice and p50 of inoculated mice for each strain.
mice, which were available with the 129 and C57BL/6 backgrounds. Mice were inoculated intracceally with *E. histolytica* and sacrificed for evaluation of infection. For both strains the p50 KO mice exhibited higher infection indices. The 129 p50 KO mice were more susceptible by all measures, including the histologic ameba score, the inflammation score, the cecal ELISA results, and the positive culture results (Fig. 2A). C57BL/6 mice were statistically more susceptible only when the histologic ameba and inflammation scores were used; this result was not surprising since in our hands these measures are most useful at early time points because waning antigen and culturable amebae persist. The histological examination of the p50 KO ceca revealed amebae and inflammation in the mucosa and submucosa, while the wild-type (WT) ceca were normal (Fig. 2A). The NF-κB translocation pattern in the C57BL/6 KO mice postinoculation was examined after sacrifice, which revealed a significant reduction in the amount of NF-κB binding proteins in the nucleus without clear banding (Fig. 2B), suggesting that p50 was important for NF-κB translocation in these mice.

**NF-κB p50 prevents a decrease in epithelial resistance during *E. histolytica* infection.** We then examined whether we could attribute a protective role to p50 on the epithelium itself. To do this, we developed a transwell assay in which we placed 25 mm² of mouse cecal tissue in transwell plates, added *E. histolytica* to the luminal surface, and measured the transepithelial electrical resistance over time. Using WT and KO tissues, the decline in the TEER mirrored the *in vivo* susceptibility data; ceca from strain 129 p50 KO mice exhibited massive loss of epithelial resistance during *E. histolytica* infection, and ceca from strain 129 WT mice exhibited a more modest but statistically significant decline compared with uninfected explants (Fig. 3A). Likewise, ceca from C57BL/6 WT mice with amebae did not exhibit a decrease in the TEER, but ceca from C57BL/6 p50 KO mice did exhibit a statistically significant decrement (Fig. 3B).

**Epithelial IKKβ is not required for resistance in vivo.** We also examined the role of p65 in amebic infection; however, it is well known that p65 KO mice are embryonic lethal (4). As an alternate method, since the canonical pathway by which the p50-p65 heterodimer accesses the nucleus is via IKKβ phosphorylation of IκB, we obtained mice in which IKKβ was specifically deleted in epithelial cells (32). These mice had the C57BL/6 background yet after inoculation exhibited resistance to amebiasis similar to that exhibited by WT mice by all measures (Fig. 4).

**DISCUSSION**

This study is the first work to characterize the pattern of NF-κB activation in intestinal amebiasis in the mouse model that we used. We found that p50 was predominant in the gut during infection, as has been observed previously with other systems (16, 25). Most importantly, this p50 contributed to resistance to infection in at least two mouse strains, C57BL/6 and 129S6/SvEvTac.

NF-κB activation is a response mechanism for the host cell to rapidly drive gene expression upon external stimulation, such as stimulation with pathogen-associated molecular patterns, and *E. histolytica* expresses at least two of these patterns, Toll-like receptor 2 (TLR2) and TLR4 ligands (23). The canonical NF-κB pathway is typically proinflammatory, and a cascade of phosphorylation events culminates in translocation of the NF-κB p50-p65 heterodimer to the nucleus to drive expression of inflammatory cytokines, such as IL-1β, tumor necrosis factor alpha (TNF-α), IL-12p40, and monocyte che-

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**FIG. 2.** NF-κB p50 is protective in C57BL/6 and 129 mice. NF-κB p50 KO mice were obtained with both the intermediate susceptible 129S6/SvEvTac resistant C57BL/6 backgrounds, inoculated intracceally with *E. histolytica*, and sacrificed after 8 and 2 days, respectively, to evaluate the outcome of infection. Histologic ameba scores and inflammation scores were determined as indicated in Materials and Methods, and cecal luminal contents were assayed for infection by ELISA and culture methods. The data are from two experiments per strain with 12 mice per group for 129 mice and 11 mice per group for C57BL/6 mice and are means and standard errors of the means. *P* < 0.05; NS, not significant. Representative cecal histology is shown for both KO and WT mice, and amebae are indicated by arrows. OD, optical density. (B) Nuclear extracts of cecal tissue from C57BL/6 WT mice (mice 3 to 6) and C57BL/6 p50 KO mice (mice 7 to 10) were prepared 2 days after inoculation of *E. histolytica*. Extracts were incubated with the radiolabeled NF-κB consensus sequence oligonucleotide probe and resolved on a nondenaturing polyacrylamide gel. The arrows indicate NF-κB binding proteins mainly in WT ceca; little specific binding was seen in KO ceca.
mooattractant protein 1 (MCP-1) (19). Indeed, each of these cytokines has been shown to be expressed after *E. histolytica* stimulation (18, 24).

One might predict that NF-κB activation, through recruitment of the inflammatory response, should protect against the parasite and thus that resistant C57BL/6 mice could exhibit robust p50-p65 activation. However, previous work has shown that resistance is not associated with inflammation; histologically, C57BL/6 mice exhibit modest inflammation after inoculation compared with susceptible strains, and indeed inflammation-prone C57BL/6 IL-10 KO mice are more susceptible, not less susceptible, to infection (2, 11). Rather, we hypothesized that a different, noninflammatory NF-κB response may operate in the gut of C57BL/6 mice, which our results indicate is characterized by p50 predominance.

The finding that NF-κB p50 activity in C57BL/6 and 129 mice promoted resistance to infection and inflammation in amebiasis has a precedent in other intestinal models. Genetic deficiencies of a range of NF-κB subunits in mice have demonstrated the role of these subunits in dampening, not promoting, intestinal inflammation due to various stimuli (20). One explanation for this is that the behavior of NF-κB in the intestine is attenuated by a number of regulatory factors given the abundant TLR triggers from the commensal flora (29). p50 KO results similar to our results have been obtained for murine intestinal infections with *Trichuris, Helicobacter, Campylobacter,* and *Citrobacter,* where the KO mice have generally exhibited worse infection parameters and/or worse pathology (1, 6–8). In these infection models the abnormal phenotype has been observed 10 to 60 days postinfection, which is far later than it was observed in our amebiasis model. In the *Trichuris* model a defect in the p50 KO mice could be traced to the T-lymphocyte compartment, while in the *Helicobacter* model a role for p50 in macrophages appears to be likely (27).

In contrast, we feel that in our *E. histolytica* model the role of p50 is associated with the epithelial cell itself, based on the TEER findings.

The next question is how p50 protects the C57BL/6 gut epithelium. The simplest explanation is that p50 homodimers repress activation of pathogenic NF-κB-regulated genes directly (since p50 contains DNA binding domains but not transcriptional activation domains [28, 30]), and we are now examining the gene expression profile of p50-deficient epithelial cells responding to *E. histolytica*. However, the p50 function in the nucleus may be more complex than simple competition for p50-p65 binding; p50 and p65 can exhibit a preference for distinct DNA sequence elements (21), and p50 can be selective in terms of which genes it suppresses (28). Another possibility is that p50 acts through the action of p105, the precursor of p50 which has homology to the IkB family of proteins that inhibit NF-κB in the cytoplasm. We were somewhat surprised that the epithelial cell-deficient IKKβ mice remained resistant, since in most systems IKKβ is a major enzyme in the cascade that leads to p50-p65 translocation into the nucleus. Traditionally, IKKβ has been closely linked with p65 activation (19); for instance, the embryo lethality and pathology of the IKKβ KO mice are very similar to those of the p65 KO mice, and therefore perhaps the resistance of the Cre-villin IKKβ mice reflects a lack of contribution of p65 to C57BL/6 resistance.

This work raises some questions. First, the C57BL/6/p50 KO mice were not 100% susceptible, indicating that there are reasons for their resistance other than their NF-κB response. Second, the p50 KO substantially reduced NF-κB activity in cecal tissue, yet infection and inflammation occurred, indicating that there are unknown NF-κB-independent pathways that can mediate inflammation in amebic colitis. Third, as observed for the CBA mice, based on this work we can make no conclusions about the role of NF-κB in susceptibility. We need proper tools to study p50 and other subunits with this susceptible background; however, since the mice also exhibited a predominance of p50 in the ceca after challenge, it is clear that p50 is not absolutely or uniformly protective.

This work shows a role for NF-κB p50 in amebiasis for the first time. Given the predominance of p50 in our cecal tissues, future studies of the role of NF-κB in amebic colitis that focus on p65 should examine p50. Understanding the control of the NF-κB program and how it resists *E. histolytica* infection should shed light on why some hosts exhibit inflammation, while others remain in an asymptomatic colonized state.

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