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Cutting Edge: Resistance to Bacillus anthracis Infection Mediated by a Lethal Toxin Sensitive Allele of Nalp1b/Nlrp1b

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Cutting Edge

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Cutting Edge: Resistance to *Bacillus anthracis* Infection Mediated by a Lethal Toxin Sensitive Allele of *Nalp1b/Nlrp1b*

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Pathogenesis of *Bacillus anthracis* is associated with the production of lethal toxin (LT), which activates the murine *Nalp1b/Nlrp1b* inflammasome and induces caspase-1–dependent pyroptotic death in macrophages and dendritic cells. In this study, we investigated the effect of allelic variation of *Nlrp1b* on the outcome of LT challenge and infection by *B. anthracis* spores. *Nlrp1b* allelic variation did not alter the kinetics or pathology of end-stage disease induced by purified LT, suggesting that, in contrast to previous reports, macrophage lysis does not contribute directly to LT-mediated pathology. However, animals expressing a LT-sensitive allele of *Nlrp1b* showed an early inflammatory response to LT and increased resistance to infection by *B. anthracis*. Data presented here support a model whereby LT-mediated activation of *Nlrp1b* and subsequent lysis of macrophages is not a mechanism used by *B. anthracis* to promote virulence, but rather a protective host-mediated innate immune response. *The Journal of Immunology*, **2010, 184: 17–20.**

Bacillus anthracis is the pathogenic bacterium responsible for the acute disease anthrax. Virulence of *B. anthracis* is mediated in large part via the production of a protein exotoxin called lethal toxin (LT). Indeed, purified LT induces many symptoms associated with fulminant anthrax including vascular collapse and death (1–3). LT is a bipartite toxin in which the binding subunit, protective Ag (PA), attaches to anthrax toxin receptors and subsequently delivers the catalytic moiety, lethal factor (LF), into the host cell cytosol. Once intracellular, LF functions as a zinc-dependent metalloproteinase, cleaving the *N* termini of MAPK kinases and thereby disrupting cell signaling through the ERK1/2, JNK, and p38 pathways (3). As a result, LT cripples the host innate immune system by blocking cytokine production from numerous cell types, inhibiting chemotaxis of neutrophils, and inducing apoptosis in activated macrophages (3). At high concentrations, similar to those found late in infection, LT induces cytokine-independent shock and death in animals that is associated with vascular collapse (1, 2, 4).

Interestingly, LT induces rapid cell lysis in macrophages and dendritic cells derived from a subset of inbred mouse and rat strains (3, 5). This finding led to the model that the cytokine burst resulting from LT-induced macrophage lysis contributes to pathology associated with this toxin (6, 7). Such a model is attractive, as rapid release of proinflammatory cytokines concomitant with macrophage lysis could, in theory, exacerbate the vascular damage associated with anthrax and LT-mediated pathology (3). Furthermore, macrophages play an important role in limiting *B. anthracis* infection (8–10), and their rapid destruction by LT would be predicted to result in increased bacterial fitness. However, this model is at odds with the observations that animals resistant to purified LT are sensitive to challenge by *B. anthracis* spores and vice versa (11). A similar inverse relationship exists in inbred mouse strains whereby many strains whose macrophages lyse in response to LT display increased resistance to infection by *B. anthracis* (12). Therefore, contrary to one model, LT-mediated lysis of macrophages appears to be associated with protection against infection by *B. anthracis*.

A single gene, *Nlrp1b,* controls macrophage and dendritic cell sensitivity to LT (3, 13), and when heterologously expressed with caspase-1 in human fibroblasts, confers susceptibility to LT in these cells (14). *Nlrp1b* is a member of the nucleotide-binding domain–leucine rich repeat family of proteins found in plants, called R proteins, and animals, termed NLR proteins (6, 13). Plant R proteins function in host immunity by recognizing pathogens and/or danger signals and initiating a hypersensitive response that can function locally through induction of cell

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Abbreviations used in this paper: ERP, early response phenotype; IL-1β, β form of pro-IL-1; LF, lethal factor; LT, lethal toxin; LT^R, lethal toxin-resistant; LT^S, lethal toxin-sensitive; PA, protective Ag; PMN, polymorphonuclear neutrophil.

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death or distally through production and release of antimicrobial products and signaling molecules. Localized cell death induced by R proteins represents a mechanism to limit bacterial infection and can be triggered by a number of upstream stimuli including the presence of bacterial proteases in the host cytosol (6, 15). We reasoned that a similar hypersensitive response may also occur in *B. anthracis*-exposed animals and could explain why macrophage susceptibility to LT varies inversely with susceptibility to spore challenge as described above. Therefore, we sought to determine how *Nlrp1b* influences outcome to LT and spore challenge.

Materials and Methods

Mouse maintenance and breeding

All mice were cared for in accordance with the University of California Animal Research Committee and the U.S. Army Medical Research Institute of Infectious Diseases Animal Care and Use Committee. C57BL/6J (B6) mice were purchased from the The Jackson Laboratory (Bar Harbor, ME). Transgenic mice expressing a 129S1/SvImJ(129S1)-derived lethal toxin-sensitive (LT^S) allele of *Nlrp1b* on a LT-resistant (LT^R) B6 background ($B6^{Nlrp1b(129S1)}$), backcrossed to B6 for seven generations, were obtained from Drs. E. Boyden and W. Dietrich (Harvard Medical School, Boston, MA). Heterozygous $B6^{Nlrp1b(129S1)}$ were intercrossed or crossed with B6, and transgene-positive offspring were identified by PCR genotyping as previously described (13).

Toxin preparation and challenge

PA was expressed in *Escherichia coli* and purified as previously described (16), followed by Sephacryl S-200 (GE Healthcare, Piscataway, NJ) size exclusion chromatography. LF was obtained from Dr. J. Mogridge (University of Toronto, Toronto, Ontario, Canada). A dose of 5 μg PA and 2.5 μg LF, diluted in pharmaceutical grade saline, per g body weight was injected i.p. Alternatively, PA and LF were purified from *B. anthracis* strain BH450 (17). LF produced from strain BH450 displayed 3-fold lower activity (18), and consequently a dose of 15 μg PA and 7.5 μg LF per g body weight was used to achieve a similar mortality rate. Endotoxin was removed from all toxin preparations as described (16). Walking ataxia was scored as follows: mild: reduced exploratory behavior or rearing on hind limbs, a slower and/or less steady gait, but free ambulation throughout the cage; moderate: preferred sedentary state, but the mouse was able to generate a slow, unsteady (e.g., wobbly) gait usually for <7 s before resting; and severe: typically in a stationary state, but on stimulation the mouse could generate a few unstable steps (e.g., severe wobble and/or tremor) before stopping. Body temperatures were measured following LT injection using a rectal thermometer. Baseline temperatures were determined prior to LT injection and no differences were observed between animal groups (not shown). For cytokine analysis, blood was collected via cardiac puncture and allowed to coagulate. Sera was collected and stored at −80°C. Cytokines were detected using the Millipore Milliplex MAP Mouse Cytokine Kit per the manufacturer's instructions (Millipore, Billerica, MA).

Spore challenge and cellular analysis

$B6^{Nlrp1b(129S1)}$ and nontransgenic littermate/cagemate mice were injected i.p. with $\sim 2.5 \times 10^7$ unencapsulated, toxigenic Sterne strain (7702) or 4×10^2 Ames strain spores per mouse and monitored daily for 14 d. For cellular analysis, mice were infected i.p. with $\sim 1.6 \times 10^7$ Sterne spores and euthanized at 4, 28, 52, 76, and 135 h postinfection. Uninfected mice were used to determine baseline cell populations in the peritoneal cavity of each strain. Peritoneal exudates were harvested by injecting 7 ml of sterile HBSS and 3 ml of air into the peritoneal cavity, followed by extraction. Samples were stained with fluorescently conjugated Abs to surface markers Mac1/Cd11b (Invitrogen, Carlsbad, CA) and Ly6G (BD Pharmingen, San Diego, CA) and analyzed by flow cytometry. Due to the cross-reactivity of the anti-Mac1 Ab, polymorphonuclear neutrophils (PMNs) were defined as $Ly6G^+$ and $Ly6G^+/Mac1^+$. Monocytes were defined as $Mac1^+/Ly6G^-$. The average percentage of each cell type per mouse was then converted to total cell number by multiplying with the mean hemocytometer count for each mouse group. Similar values were obtained by histochemical and microscopic analyses (data not shown).

Results

Nlrp1b-*mediated response to LT*

To determine whether the presence of a LT^S allele of *Nlrp1b* controls whole animal susceptibility to purified LT, we challenged $B6^{Nlrp1b(129S1)}$ mice with LT via i.p. injection (13). Surprisingly, $B6^{Nlrp1b(129S1)}$ mice displayed a time to a moribund state similar to nontransgenic littermate controls following LT challenge (Fig. 1*A*), indicating that the expression of a LT^S allele of *Nlrp1b* does not contribute to whole-animal susceptibility to LT. Histopathological analysis also revealed no differences at the end stage of disease (data not shown), consistent with earlier reports (1). However, a previously undescribed rapid and transitory response was observed following LT challenge, which was characterized by ataxia (Fig. 1*B*), bloat, dilated vessels on pinnea, loose/watery feces, labored abdominal breathing, and/or mild hypothermia (Fig. 1*C*). This distinctive response was designated as the early response phenotype (ERP) as some animals presented as early as 30 min after LT administration, and the remaining animals typically presented by 1 to 2 h. Wild-type B6 and littermate control (not shown) animals displayed no significant ERP following LT challenge (Fig. 1*B*, 1*C*). Surprisingly, $B6^{Nlrp1b(129S1)}$ mice recovered to seemingly normal behavior following the ERP before succumbing to LT in a manner similar to control animals (Fig. 1*B*).

The pathology, timing, and clinical presentations associated with the ERP are consistent with an inflammatory response, the rate of macrophage lysis ex vivo, and the previously reported cytokine response in LT^S strains of mice (1, 2). We therefore tested whether expression of a LT^S allele of *Nlrp1b* is sufficient to induce a proinflammatory cytokine response to LT. Activation of Nlrp1b results in formation of a caspase-1–containing inflammasome and subsequent proteolytic maturation of the β form of pro-IL-1 (IL-1β) (13, 19). As expected, IL-1β increased rapidly after LT administration (Fig. 1*D*). In addition, several proinflammatory cytokines not directly activated by caspase-1 also increased (Fig. 1*D*) (1, 2). In contrast to previous findings with LT^S strains of mice (1, 2), there was a mild increase in TNF-α in $B6^{Nlrp1b(129S1)}$ mice (Fig. 1*D*). No changes were observed in either IL-1α or IFN-γ. Endotoxin contamination of PA or LF was not responsible for cytokine induction as no response was detected following injection of a 2× dose of individual toxin components (data not shown). Further, B6 animals showed no ERP or cytokine response to LT (Fig. 1*D*), indicating that these responses are a result of Nlrp1b detection of LF activity rather than LPS contamination. Therefore, expression of a LT^S allele of *Nlrp1b* in LT^R B6 mice is sufficient to induce a proinflammatory cytokine response to LT in mice.

LT^S Nlrp1b *alleles provide protection against* B. anthracis *infection*

To test the role of *Nlrp1b* in an infection model, $B6^{Nlrp1b(129S1)}$ mice and transgene-negative littermate control animals were challenged with the unencapsulated, toxigenic *B. anthracis* Sterne strain. Within 6 d, eight of nine control animals succumbed to infection, whereas all $B6^{Nlrp1b(129S1)}$ mice survived for the duration of the experiment (Fig. 2*A*). To test the role of a LT^S *Nlrp1b* allele in response to a fully virulent *B. anthracis* infection, $B6^{Nlrp1b(129S1)}$ mice were challenged with *B. anthracis* Ames strain. Although $B6^{Nlrp1b(129S1)}$ mice displayed a trend toward protection, the data were not statistically significant (Fig. 2*B*). The latter finding is not surprising given that virulence associated with the Ames strain is governed primarily by the presence of a poly-*D*-glutamic acid capsule rather than LT in the mouse model (20).

To determine the cellular mediators contributing to Nlrp1b-mediated resistance to infection, peritoneal exudates were collected and analyzed at various time points following spore

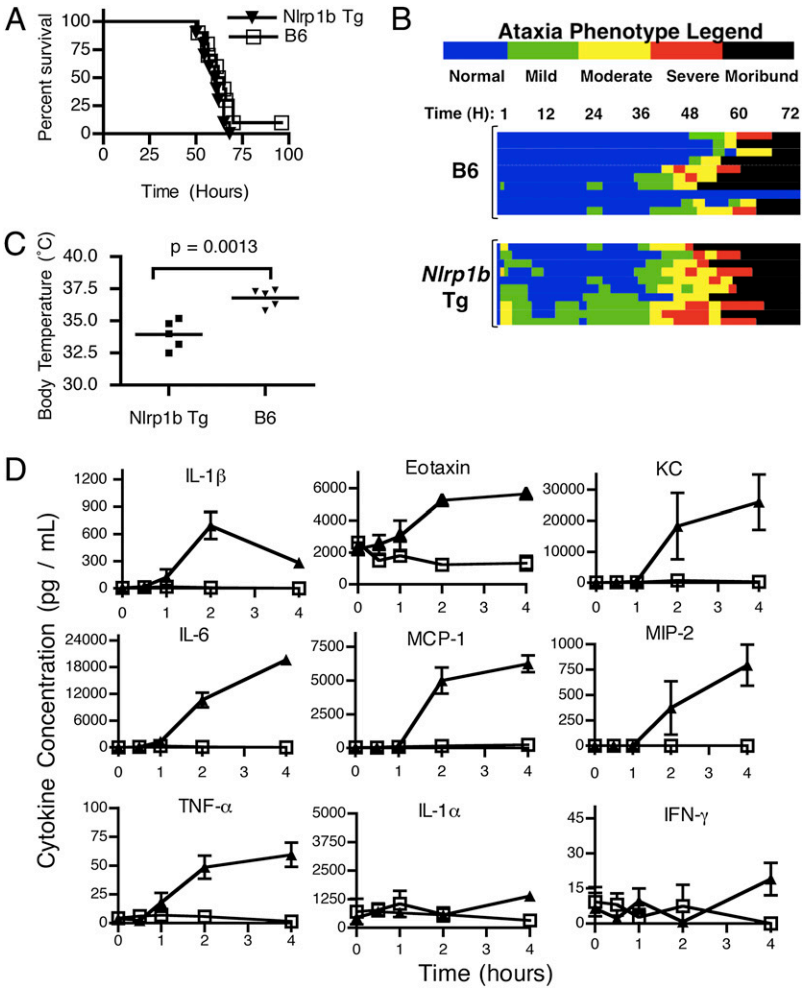

FIGURE 1. Influence of *Nlrp1b* on the response in mice to LT. *A*, B6$^{Nlrp1b(129S1)}$ transgenic mice (Nlrp1b Tg) (n = 10) expressing a LT^S allele of *Nlrp1b* or transgene-negative control animals (B6) (n = 10) were challenged with 5 μg PA + 2.5 μg LF per g body weight via i.p. injection. Animals were closely monitored for the first 4–6 h following LT injection and then every 3 h for 5 d and euthanized upon reaching a moribund state. *B*, Heat map representing ataxia severity of the animals shown in *A*. Each horizontal line represents an individual animal from time of LT injection (*left*) until the end of the experiment (*right*). Ataxia severity is indicated by color. Data are representative of seven independent experiments. *C*, Body temperature of B6$^{Nlrp1b(129S1)}$ (n = 5) or nontransgenic littermate control mice (n = 5) was measured following i.p. injection of 15 μg PA + 7.5 μg LF per g body weight. Temperature was monitored hourly and lowest temperature observed during first 5 h posttoxin injection is plotted. *D*, B6$^{Nlrp1b(129S1)}$ transgenic mice (closed triangles) or transgene-negative control animals (open squares) were challenged with LT as in *C*. Uninjected animals served as t = 0 controls. Animals were sacrificed at 0.5, 1, 2, and 4 h posttoxin injection, and serum cytokines levels were measured. Data represent the average values of five animals ± SD.

challenge. Both strains responded with an increase in the number of Ly6G$^+$ PMNs (Fig. 2*C*). However, the levels of PMNs were higher in B6$^{Nlrp1b(129S1)}$ mice at early time points following spore challenge compared with nontransgenic littermate control animals. This influx of PMNs was followed by more Ly6G$^-$/Mac1$^+$ monocytes in both strains (Fig. 2*D*) that were maintained in B6$^{Nlrp1b(129S1)}$ but not control mice.

Discussion

Based on LT and spore-challenge data from different animal species, Lincoln et al. (11) hypothesized that animals resistant to infection by *B. anthracis* were susceptible to challenge by its toxin and that the inverse was true for infection-susceptible species. Using inbred and recombinant strains of mice, Welkos and colleagues (12, 21, 22) substantiated this proposed inverse correlation between the sensitivity of animals to challenge with purified LT and with *B. anthracis* spores and explored the genetic basis for this phenomenon. Specifically, mice whose macrophages rapidly lyse in response to LT were more resistant to spore challenge than mice whose macrophages were LT^R (12, 13, 23). Further, mice resistant to spore challenge had increased rates of PMN infiltration at early time points and sustained higher monocyte numbers at the site of *B. anthracis* infections (22). Here we report that allelic variation at *Nlrp1b* accounts for these previously observed phenomena, thereby providing molecular insight into host defense against anthrax.

B. anthracis triggers activation of TLRs and NOD2 in human and mouse macrophages, resulting in production of TNF-α through a MAPK signaling pathway (24). However, the presence of LT blocks this response by cleaving and inactivating MAPK kinase proteins (24). LT^S alleles of *Nlrp1b* counteract this immunosuppressive effect by triggering a rapid proinflammatory programmed cell death. Interestingly, IL-1β is released upon LT-mediated macrophage lysis (19). IL-1β is a proinflammatory cytokine that recruits PMNs and monocytes, cell types that are predicted to resolve infection (9, 10, 25). Although Nlrp1b inflammasome activation in response to LT is detrimental to the toxin-exposed macrophage, our data demonstrate that Nlrp1b activation is ultimately beneficial for the host by inducing inflammation (e.g., enhanced cytokine production and PMN infiltration) at the site of LT production. Of note, a similar mechanism has been described in plants where R proteins recognize bacterial virulence factors in the host cell cytosol and induce localized cell death to limit infection. Importantly, the finding that the *Nlrp1b*-mediated inflammatory response is protective against *B. anthracis* infection is consistent with previous data that mice deficient in caspase-1, IL-1β, or IL-1R display increased sensitivity to anthrax (25, 26). Therefore, we propose that *Nlrp1b*-mediated cell death provides a selective advantage to the host rather than pathogen.

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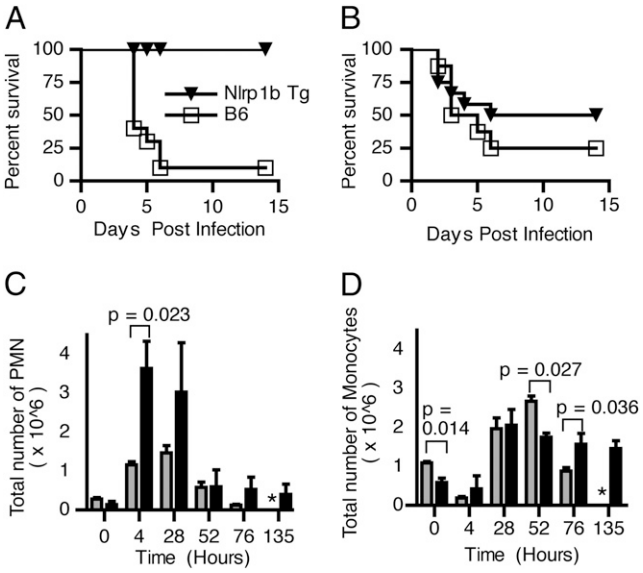

FIGURE 2. LT^S allele of *Nlrp1b* provides protection from *B. anthracis* spore challenge. *A*, $B6^{Nlrp1b(129S1)}$ transgenic mice ($n = 11$) or transgene negative control animals ($n = 9$) were challenged i.p. with 2.5×10^7 spores of *B. anthracis* Sterne strain 7702 ($p < 0.0001$, log-rank test of Kaplan-Meier survival curves). *B*, $B6^{Nlrp1b(129S1)}$ transgenic mice ($n = 12$) or transgene negative control animals ($n = 8$) were challenged i.p. with 4×10^2 spores of *B. anthracis* Ames strain ($p = 0.3847$, log-rank survival curve). *C* and *D*, $B6^{Nlrp1b(129S1)}$ transgenic mice (black bars) or transgene-negative control animals (gray bars) were challenged i.p. with 1.6×10^7 spores of *B. anthracis* Sterne strain 7702. Animals were euthanized at the indicated time points and the number of PMNs (*C*) and monocytes (*D*) in the peritoneal cavity were determined as described in the *Materials and Methods* section. Data represent mean values ($n = 2$ at 135-h time point and $n = 3$ at all other time points) ± SD. Asterisk indicates no B6 survivors at the 135-h time point.

Disclosures

The authors have no financial conflicts of interest.

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