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Expression of the p60 Autolysin Enhances NK Cell Activation and Is Required for *Listeria monocytogenes* Expansion in IFN-γ-Responsive Mice

Jessica Humann,† Ryan Bjordahl,† Karl Andreasen,* and Laurel L. Lenz2*†

Both peptidoglycan and muropeptides potently modulate inflammatory and innate immune responses. The secreted *Listeria monocytogenes* p60 autolysin digests peptidoglycan and promotes bacterial infection in vivo. Here, we report that p60 contributes to bacterial subversion of NK cell activation and innate IFN-γ production. *L. monocytogenes* deficient for p60 (Δp60) competed well for expansion in mice doubly deficient for IFNAR1 and IFN-γR1 or singly deficient for IFN-γR1, but not in wild-type, IFNAR1−/−, or TLR2−/− mice. The restored competitiveness of p60-deficient bacteria suggested a specific role for p60 in bacterial subversion of IFN-γ-mediated immune responses, since in vivo expansion of three other mutant *L. monocytogenes* strains (ΔActA, ΔNamA, and ΔPlcB) was not complemented in IFN-γR1−/− mice. Bacterial expression of p60 was not required to induce socs1, socs3, and il10 expression in infected mouse bone marrow macrophages but did correlate with enhanced production of IL-6, IL-12p70, and most strikingly IFN-γ. The primary source of p60-dependent innate IFN-γ was NK cells, whereas bacterial p60 expression did not significantly alter innate IFN-γ production by T cells. The mechanism for p60-dependent NK cell stimulation was also indirect, given that treatment with purified p60 protein failed to directly activate NK cells for IFN-γ production. These data suggest that p60 may act on infected cells to indirectly enhance NK cell activation and increase innate IFN-γ production, which presumably promotes early bacterial expansion through its immunoregulatory effects on bystander cells. Thus, the simultaneous induction of IFN-γ production and factors that inhibit IFN-γ signaling may be a common strategy for misdirection of early antibacterial immunity. *The Journal of Immunology*, 2007, 178: 2407–2414.

Innate antimicrobial resistance involves cooperation between neutrophils, macrophages, and NK cells. The discourse between these cells involves both cell surface molecules and cytokines such as TNF-α, IL-1β, IL-12, IFN-γ, IL-6, and type I IFNs (IFN-α/β). Induction of these cytokines coordinates innate resistance to viral replication (1–5) or in some cases exerts deleterious effects on antibacterial immunity. The basis for such deleterious effects is not clear but may involve the induction of immune pathology (6–8) or immunosuppression. For example, IL-6−/− mice show increased inflammatory cytokine production and neutrophil infiltration following aerosol exposure to LPS (9), and IL-6 treatment mimics the immunosuppressive effects of IL-10 under conditions of suppressor of cytokine signaling (SOCS)3 gene deficiency (10). Likewise, high doses of IL-12 and IFN-γ suppress cellular immunity through induction of immune inhibitory factors such as inducible NO synthase and IDO (11, 12). These latter processes have actually been shown to promote infection by *Bordetella pertussis* (13), *Salmonella typhimurium* (7), and *Toxoplasma gondii* (14), although it is not known whether specific microbial factors contribute to subversion of immune regulatory cytokine responses in vivo.

*Listeria monocytogenes* is a Gram-positive intracellular bacterial pathogen that generaly causes acute infections in immunocompetent individuals or animals. However, impairment of adaptive immunity increases the severity and lethality of *L. monocytogenes* infections in pregnant, aged, and immunosuppressed individuals. Several pathogenic factors contributing to intracellular infection by *L. monocytogenes* have been characterized. These include a secreted hemolysin that permits bacterial access to the cytoplasm of infected host cells, two secreted phospholipases that promote vacuolar escape and cell-cell spread (phospholipases A and B; PlcB), and a bacterial surface protein (ActA) that hijacks host proteins to nucleate actin and promote intercellular and intracellular bacterial spread (15, 16). In addition, we have recently shown that a novel secretion system (SecA2) and two autolytic enzymes secreted by this system (NamA and p60) also contribute to *L. monocytogenes* pathogenicity during in vivo infection of mice but not during in vitro infection of cultured macrophages or fibroblasts (17). The data suggest that these autolysins promote in vivo bacterial infection through modulating the release of peptidoglycan (PGN) fragments (muropeptides) detected by host innate receptors such as TLR2, Nod1, Nod2, or others. However, the effects of autolysins or autolysin-dependent bacterial products on host immune responses in vivo have not been previously reported.

Here, we report that p60 is required for bacterial expansion at early times after infection of wild-type (wt) mice, but is largely dispensable during infection of mice that are nonresponsive to

*Abbreviations used in this paper: SOCS, suppressor of cytokine signaling; CI, competitive index; NOD, nucleotide oligomerization domain; CARD, caspase activation and recruitment domain; PlcB, phospholipase B; PGN, peptidoglycan; wt, wild type; Ern, erythromycin; ErnR, Ern resistant; BMM, bone marrow-derived macrophages; NWNA, nylon wool-nonadherent.*

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IFN-γ. In addition, we show that bacterial p60 expression enhanced both NK cell activation and serum IFN-γ concentrations, although purified p60 protein failed to activate NK cells in vitro. Thus, bacterial production of p60 indirectly enhances the ability of Listeria infection to stimulate NK cell activation yet contributes to bacterial growth. Because infected macrophages produced socs1, socs3, and il10, we propose that p60-induced hyperproduction of innate IFN-γ disregulates innate immunity without enhancing activation of infected macrophages.

Materials and Methods

Bacterial strains

The wt L. monocytogenes strain used for these studies was 10403S. Bacterial strains with in-frame deletions of ActA and PlcB were generously provided by Dr. Daniel Portnoy (University of California, Berkeley, CA). Before use for competitive index (CI) assays, each mutant strain was transduced with Tiρ17 from the donor strain DPL-3903 to generate an erythromycin (Erm)-resistant (ErmR) variant, as described (17). The site of Tiρ17 integration in the donor and transductants does not affect in vivo virulence of L. monocytogenes (18). ErmR and ErmR strains with deletions of NamA or p60 and the complemented Δp60 strain were described previously (17). Before animal infections, each strain was passaged through a C57BL/6 mouse, reisolated from infected livers, and grown to log phase. Frozen stocks of these cultures were stored at −80°C.

Mice

TLR2−/− and Tlr4−/− mice backcrossed 10–15 times on BALB/c or C57BL/6 were generously provided by Dr. Daniel Portnoy. 129Sv/J and 129/SvJ, and C57BL/6 mice were gifts from Dr. Sujan Shresta (La Jolla Institute of Allergy and Immunology, La Jolla, CA). C57BL/6, BALB/c, and IFN-γ−/− mice on the C57BL/6 background were obtained from The Jackson Laboratory and bred in our colony. Mice were maintained under pathogen-free conditions in the National Jewish Biological Resource Center. All animal work was approved by the National Jewish animal care and use committee.

Bacterial infections

Mice were infected i.v. through the lateral tail vein. Infections with individual bacterial strains were used to evaluate growth kinetics under non-competitive conditions, to determine serum cytokine concentrations, and before intracellular cytokine staining experiments. The inoculum for these experiments was ~106 CFU/mouse, which is approximately one-half the LD50 for strain 10403S. For CI experiments, mice were inoculated with 105 total CFU per mouse to avoid founder effects, as previously described (17). Briefly, 10403S (wt) and an Erm-resistant test strain were grown separately to log phase in brain–heart infusion broth, washed in PBS to remove excess bacteria, and resuspended in 0.2% Nonidet P-40 containing Erm at 0.2 mg/ml (to induce expression of the p60 autolysin) and stored at −80°C. Before use for competitive index (CI) assays, each mutant strain was transduced with a wt strain and the complemented Δp60 and the complemented Δp60 strain was grown up to log phase in brain–heart infusion broth, washed in PBS to remove excess bacteria, and stored at −80°C.

Intracellular cytokine staining

Spleens were harvested from infected or control mice and dissected into a single-cell suspension using nylon mesh. After lysis of RBC using ACK lysis buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM Na2EDTA, pH 7.4), white blood cells were counted and incubated in RPMI 1640, 10% FCS, and 2 mM L-glutamine plus GolgiStop (BD Pharmingen) for 3 h at 2 × 10^5 cells per well in 96-well plates. Before staining, cells were incubated in anti-CD16/32 (2.4G2 hybridoma supernatant) to block FcRs. Cells were surface stained with PE-conjugated anti-NK1.1 (clone PK136) and PE-Cy5-conjugated CD3 (clone 145-2C11). Cells were then fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen) and stained with FITC-conjugated anti-IFN-γ (clone XMG1.2) to detect intracellular IFN-γ. A FACSCalibur (BD Biosciences) was used to collect 100,000 events, and samples were analyzed with FlowJo software (Treestar).

BMM infections and RT-PCR for SOCS and cytokine gene expression

BMMs were cultured from C57BL/6 mice and plated at 10^5 cells/well in six-well tissue culture plates on day 6 of culture. On day 7, BMMs were infected with log-phase L. monocytogenes at a multiplicity of 5 bacterial cell/macrophage. Macrophages were transiently transfected with plasmid expressing hemagglutinin epitope tagged p60 at 0.5–1.0 μg DNA per well in 24-well plates using Fugene (Roche) and grown to log phase. Total RNA was isolated from triplicate wells of infected macrophages at the indicated times using the Qiagen mini kit (Qiagen) and reverse-transcribed using oligo(dT) primers and the IMPROM II RT system (Promega). cDNAs were used as template for 28–32 cycles of PCR with Taq polymerase (Promega). Reactions using template RNA incubated without reverse transcriptase served to control for genomic DNA contamination and gave no products in the experiments shown. In addition, primers used for semiquantitative analyses were designed to span introns as follows: IL-10 sense (CTGGCTCTAG CACTGCTAT); IL-10 antisense (ATTCTAGGCTTGTAGACAC); GAPDH sense (AAGGACCCCTTATGGAC); GAPDH antisense (TCACG GACACTTACGAC); SOCS1 sense (ACACTACCTTCCGACCTT); SOCS1 antisense (GAAGCCTACCTTCCGACCTAG); SOCS3 sense (TGGC CCATGGTACCCCAAGCAAGT); and SOCS3 antisense (GCTCT TAAATGGGACGATCATCAGTA).

Purification of His-p60 and in vitro assay for NK cell activation

An N-terminally His-tagged recombinant p60 was engineered in the pTrcHis TOPO (Invitrogen), and the recombinant protein was purified from induced Escherichia coli cultures using nickel affinity chromatography and passed over a polyvinyl B column to remove E. coli LPS. The recombinant full-length protein was >95% pure as judged by SDS-PAGE and tested for its ability to digest PGN in renaturation zymography assays as described previously (17). To evaluate the ability of p60 to activate NK cells, splenocytes of naïve C57BL/6 mice were passed over nylon wool columns (Polysciences) to enrich for nonadherent (NWNA) cells. As shown in Fig. 5, NWNA cell populations were enriched for NK and T cells and depleted of B cells and macrophages. To test for activity against the enriched NK cell population, NWNA cells were cultured for 24 h with either PMA plus ionomycin or purified His-p60 protein. Brefeldin A was added during the last 4 h of culture to block cytokine secretion before intracellular cytokine staining.

Results

The p60 autolysin enhances L. monocytogenes infection of wt mice but is not essential in IFN-γ-nonresponsive mice

On the basis of data from coinfection (competitive index) experiments in wt BALB/c mice, we previously reported that bacterial expression of p60 enhanced L. monocytogenes colonization of livers and spleens of wt mice by 48 h postinfection (17). Likewise, the kinetics of bacterial survival/growth (hereafter referred to as expansion) in tissues of wt C57BL/6 mice infected individually with wt or Δp60 strains revealed a reduced recovery of p60-deficient bacteria from infected livers beginning after 24 h of infection (Fig. 1A). Interestingly, p60-deficient bacteria were not cleared from the infected animals but rather persisted in mouse tissues for at least 4 days after infection with little or no net increase in bacterial numbers. Thus, expression of p60 was not required for in vivo survival of bacteria but rather supported the increase in bacterial numbers that is typically seen at early times after in vivo infection of mouse livers and spleens. Considering that Δp60 bacteria readily infect and show efficient cell–cell spread in fibroblasts, BMMs, and epithelial cells in vitro (Ref. 17 and data not shown), we hypothesized that p60 might influence bacterial survival by impairing specific host innate immune responses. To test this hypothesis, we asked whether the requirement for p60 in bacterial resistance was obviated in mice lacking specific immune effector mechanisms. Both type I (αβ) and type II (γ) IFNs are produced.
during *L. monocytogenes* infection, and it was recently shown that type I IFNs promote in vivo replication/expansion of *L. monocytogenes* (19–21). We therefore evaluated the ability of Δp60 *L. monocytogenes* to competitively colonize tissues of mice lacking both IFN-γR and IFNAR1 using the CI assay. Remarkably, the ability of Δp60 bacteria to compete in the livers (Fig. 1B) and spleens (not shown) of these mice was restored to nearly wt levels. To determine whether these effects were due to the loss of response to IFN-γ, IFN-α/β, or both, we also evaluated the CI in mice lacking IFN-γR or IFNAR1 alone (Fig. 1, C and D). Due to the limited availability of the various knockout alleles, these experiments were done using mice on different genetic backgrounds. However, we do not believe our results are affected by this, since we observed a similar magnitude of virulence defect in control (wt) mice in each background. In addition, we observed similar results in a CI experiment using IFNAR1+/− mice (on a BALB/c background) (E). Thus, p60 promotes in vivo bacterial expansion independent of TLR2.

Enhancement of *L. monocytogenes* expansion by p60 is independent of TLR2

The p60 protein is expressed during cytosolic *L. monocytogenes* infection (22), and its C-terminal catalytic domain is required to enhance bacterial expansion in vivo (17). Because TLR2, Nod1, Nod2, and other host receptors involved in innate immunity recognize PGN or muropeptides that might be released during *L. monocytogenes* infection, we previously hypothesized that p60 might modulate PGN or muropeptide recognition by these receptors (17). We have not yet been able to extensively evaluate the requirements for p60 expression in Nod1−/− or Nod2−/− mice. However, CI assays in both TLR2−/− and congenic BALB/c mice revealed similar (~50-fold) defects in expansion of the Δp60 bacterial strain (Fig. 1E). Thus, p60 promotes in vivo bacterial expansion independent of TLR2.

Enhanced competition of bacterial mutants in IFN-γR−/− mice is unique to the p60-deficient strain

A caveat to the above experiments was that IFN-γ is an important component of resistance intracellular pathogens (23–25). It was thus conceivable that any mutant *L. monocytogenes* strain might show wt expansion in this immunodeficient mouse strain. To address this issue, we engineered selectable (ErmR) versions of several other mutant *L. monocytogenes* strains and tested their ability to compete with wt *L. monocytogenes* using the CI assay in IFN-γR−/− mice (Fig. 2A). The mutants tested include a PlcB-deficient strain reported to have a similar virulence defect as Δp60 (ΔPlcB), a strain deleted for the SecA2-dependent NamA autolysin (ΔNamA), and a strain incapable of actin-based motility and cell-cell spread (ΔActA). Only the Δp60 *L. monocytogenes* showed complementation in the IFN-γR−/− mice. Together with the observation that the Δp60 strain is not complemented in several other mutant mouse strains (above), these data strongly suggest that p60 expression enables *L. monocytogenes* to resist or subvert innate IFN-γ-dependent immune effector mechanisms.

Induction of socs1 and socs3 in *L. monocytogenes*-infected BMM

Given the increased virulence of the p60-deficient bacteria in IFN-γR−/− hosts, we hypothesized that p60 expression might contribute to the ability of *L. monocytogenes* to induce expression of host genes that impair the host response to IFN-γ in infected cells. Indeed, when cultured mouse BMMs were infected with *L. monocytogenes*, we detected by semiquantitative RT-PCR an up-regulation of several molecules known to impede or counteract IFN-γ...
signalinng (Fig. 2B). The earliest response was seen with socs3 and il10, both of which were clearly up-regulated within 2 h of infection by wt L. monocytogenes (Fig. 2B). Interestingly, increased socs1 mRNA was not seen until after 4 h of infection. Given that the induction of these genes was also seen during infection with Δp60 L. monocytogenes in this and several other experiments, these data suggest that cytosolic L. monocytogenes infection triggers a p60-independent up-regulation of host genes that impede IFN-γ responses in infected cells. Importantly, we have repeatedly failed to detect IFN-γ production in either uninfected or infected (up to 24 h) BMM cultures (data not shown), arguing against the possibility that IFN-γ stimulation is responsible for the induction of these responses in our experiments.

Bacterial expression of p60 enhances innate IFN-γ production during in vivo infection

Because expression of p60 was not required for induction of socs1 and 3 and il10 by L. monocytogenes-infected BMM, we considered the possibility that p60 expression might instead normally act to impair host IFN-γ production. Thus, we used ELISAs to evaluate serum concentrations of this cytokine in C57BL/6 (B6) mice at 22–26 h after infection with wt or Δp60 bacteria. Strikingly, however, IFN-γ concentrations were reduced by roughly 4-fold in sera of the mice infected with the Δp60 bacterial strain (Fig. 3A). This was despite the recovery of similar numbers of bacterial CFU from the livers of the infected animals at this early time point during the infection (Fig. 3B). We therefore conclude that, rather than impede IFN-γ production, bacterial expression of p60 enhances serum levels of this potent immunoregulatory cytokine. Furthermore, the likely source of this IFN-γ was NK cells or memory CD8+ T cells, which are both known to produce IFN-γ at early (18–48 h) times after infection (26–28). Conversely, activation of Ag-specific T cell responses occurs at least 3–4 days after infection of naive animals with L. monocytogenes (3).

The effects of p60 expression on several other host cytokine responses were also evaluated (Table I). Although serum TNF-α, IL-10, and IL-1β were below the limits of detection by ELISA at 22–26 h of infection with either bacterial strain, significant quantities of serum IL-12/23p40, IL-12p70, and IL-6 were observed. Thus, although serum IL-12/23p40 concentrations were independent of bacterial p60 expression, both IL-12p70 and IL-6 concentrations were significantly increased in mice infected with p60-expressing bacteria. Although not conclusive, these results are consistent with the notion that increased IL-12p70 production may enhance innate IFN-γ production by NK cells and/or memory CD8+ T cells. Conversely, IL-6 production is known to attenuate cellular responses to IFN-γ during Mycobacterium. tuberculosis infection (29, 30). Thus, the overall enhanced production of socs1, 3, il10, and IL-6 would be expected to impact the response of infected cells to subsequent IFN-γ stimulation. In this light, we speculate that p60-dependent hyperinduction of IFN-γ primarily affects cell types other than L. monocytogenes-infected macrophages. Given the pleotropic effects of IFN-γ on cell survival and trafficking, such imbalances may well contribute to the ability of p60 to enhance L. monocytogenes expansion in IFN-γ-responsive mice.

FIGURE 2. Expansion of L. monocytogenes in IFN-γR−/− mice is unique to p60-deficient bacteria, and infected cells up-regulate expression of factors that suppress responses to IFN-γ. A, Complementation of mutant L. monocytogenes growth is a unique feature of the Δp60 mutant. CI values were calculated from individual B6 and B6,IFN-γR−/− mice 64 h after infection with 1:1 ratios of the Erm-sensitive wt strain and each of the indicated mutant ErmR bacterial strains. The mutant strains tested were engineered with in-frame deletions of the L. monocytogenes virulence factors p60, PlcB, NamA, or ActA. Symbols represent results from individual mice, and bars indicate mean CI values calculated from each group of three to five mice. Bacterial expression of p60 independent up-regulation of host genes that impede induction of these genes was also seen during infection with wt L. monocytogenes during infection with mutant strains. C57BL/6 mice were infected with 10^4 CFU of wt or Δp60 bacterial strains for 22 h, and serum concentrations of this cytokine in C57BL/6 BMMS were harvested from 3 x 10^6 total macrophages at each time point after infection and treated with DNase. Following reverse transcription, the abundance of the indicated cDNAs was estimated by semiquantitative RT-PCR using primers designed to span exons in gapdh, socs1, socs3, and il-10.

FIGURE 3. Serum IFN-γ concentrations are enhanced by infection with p60-expressing L. monocytogenes. C57BL/6 mice were infected with 10^4 CFU of wt or Δp60 bacterial strains for 22 h, and serum concentrations of IFN-γ were determined using ELISA (A). The differences in serum concentrations between infections with wt and Δp60 bacteria were significant using the t test (p < 0.007). B, Livers were homogenized to determine bacterial CFU. Shown are means ± SD. UN, Uninfected.
NK cell activation is enhanced in mice infected with Δp60 L. monocytogenes

To determine the cellular source of p60-dependent IFN-γ production, we used intracellular IFN-γ staining to detect and enumerate IFN-γ-producing NK and T cell populations directly ex vivo (i.e., with no in vitro stimulation). Over the course of six experiments, we consistently observed that a smaller proportion of NK1.1+ CD3− NK cells were activated by the Δp60 L. monocytogenes infection varied from 25.7 to 76.2% in this data set. In contrast, only a very small proportion of T cells produced IFN-γ when infected with Δp60 L. monocytogenes (Fig. 4). The differences shown in this figure were statistically significant (p < 0.003), and likely underrepresent the importance of bacterial p60 expression in the stimulation of NK cell activation. Thus, an average of 29.7% fewer NK cells were activated by the Δp60 strain after 20–26 h of infection. The proportion of NK cells responding to wt L. monocytogenes infection varied from 25.7 to 76.2% in this data set. In contrast, only a very small proportion of T cells produced IFN-γ at this early time in the infection (Fig. 4, bottom), and differences in the proportion of responding T cells between the wt and Δp60 bacterial infections were not statistically significant (p < 0.14). Because the time point studied is too early for the activation of Ag-specific T cell responses in these naive mice, the IFN-γ+ T cells we observed are presumably memory T cells, which have previously been shown to produce IFN-γ in response to IL-12 production.

![Figure 4](image-url) **FIGURE 4.** Bacterial expression of p60 enhances NK cell IFN-γ production during early infection. Splenocytes were harvested from C57BL/6 mice 24 h after infection with wt or Δp60 L. monocytogenes strains and analyzed for intracellular IFN-γ. Live splenocytes were gated for NK1.1+ CD3− (top) and NK1.1+ CD3− (bottom) cells. In these mice, infection with Δp60 bacteria induced ~27% fewer NK cells to produce IFN-γ than did wt bacteria (p < 0.008), whereas innate IFN-γ production by CD3+ cells was not significantly different (p < 0.14). Shown is a representative sample from a total of six experiments. Averaged across these experiments, ~29% fewer NK cells were activated upon infection with Δp60 bacteria.

![Figure 5](image-url) **FIGURE 5.** Complementation of Δp60 L. monocytogenes with the p60 gene restores NK cell IFN-γ production to wt levels. A. Splenocytes were harvested at 24 h after infection of three C57BL/6 mice per group with wt L. monocytogenes, Δp60, and a complemented Δp60 strain that expresses the p60 gene from the pPL2 integrational vector (Δp60 + p60). Splenocytes were stained, gated on NK1.1+ CD3−, and analyzed for intracellular IFN-γ. As shown, NK cell activation was reduced during infection with the Δp60 L. monocytogenes strain, but restored to wt levels in mice infected with the Δp60 + p60 L. monocytogenes strain. B, Mean percentages ± SD of gated NK cells from each group of mice that stain positive for intracellular IFN-γ. These data include the animals depicted in A. Differences between wt and Δp60 infections, as well as Δp60 and Δp60 + p60 infections were significant (p < 0.001), whereas those between wt and Δp60 + p60 strains were not significant (p < 0.10). C, Serum IFN-γ levels were enhanced by expression of p60 in L. monocytogenes. Serum was collected at 24 h after infection of mice with wt, Δp60, and Δp60 + p60 L. monocytogenes and analyzed by ELISA for IFN-γ. Shown are means ± SD for each group of the three mice from above. Differences between wt and Δp60 and between Δp60 and Δp60 + p60 were not significant (p > 0.02), whereas those between wt and Δp60 + p60 were not (p < 0.26). Significance was determined using Student’s t test.

![Table 1](image-url) **Table I.** Serum cytokine concentrations ~24h after infection of C57BL/6 mice with the respective bacterial strains

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Bacterial Strain</th>
<th>Mean Serum Concentration (pg/ml)</th>
<th>SD</th>
<th>p (wt vs Δp60)</th>
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</thead>
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<tr>
<td>IL-12/23p40</td>
<td>wt</td>
<td>7092.8</td>
<td>78.7</td>
<td>~0.1</td>
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<tr>
<td></td>
<td>Δp60</td>
<td>5445.8</td>
<td>183.3</td>
<td>~0.05</td>
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<td>IL-12p70</td>
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<td>1507.9</td>
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<td></td>
<td>Δp60</td>
<td>886.7</td>
<td>126.6</td>
<td>~0.05</td>
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<tr>
<td>IL-6</td>
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<tr>
<td></td>
<td>Δp60</td>
<td>303.3</td>
<td>43.8</td>
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* Bold values indicate statistically significant differences based on t tests.

With regard to NK cell activation, we found that both the proportion of activated NK cells and the concentrations of serum IFN-γ were restored to nearly wt levels when we infected mice with a Δp60 L. monocytogenes strain in which p60 expression had been restored by genetic complementation (Fig. 5). This finding clearly implicated bacterial expression of the p60 protein as contributing to the enhanced activation of NK cells and enhanced serum IFN-γ concentrations. However, our failure to observe p60-dependent IFN-γ production by (memory) T cells suggests that the increased IL-12p70 production associated with bacterial expression of p60 does not entirely account for the increased NK cell response.

Purified p60 protein does not directly promote NK cell activation

To determine whether the p60 might directly elicit NK cell activation during the early stages of L. monocytogenes infection, we asked whether purified p60 protein could stimulate NK cell IFN-γ production in vitro. For this experiment, we engineered and purified a recombinant, mature, p60 protein containing an N-terminal...
hexahistadine tag. The purified His-p60 protein was active for PGN-digesting activity in a zymography assay (Fig. 6A), indicating that the N-terminal His-tag does not impede the catalytic activity of the protein. As a source of cells enriched for NK cells and depleted of macrophages, we used NWNA splenocytes. Approximately two-thirds of splenic B cells and phagocytic cells were depleted in these experiments, as judged by FACS analysis, whereas T cells and NK cells were enriched by a factor of ~2–3 (data not shown). When the NWNA cells were incubated alone or coincubated with His-p60, we failed to detect NK cell production of IFN-γ, as judged by intracellular cytokine staining (Fig. 5B). Conversely, >50% of the NK1.1+CD3− population responded to treatment with PMA and ionomycin by producing IFN-γ (Fig. 6B).

The failure to see direct effects of p60 on NK cell activation strongly supports the idea that the NK cell-stimulating effects of bacterial p60 expression are indirect. Hence, we speculate that p60 acts by generating bioactive muropeptides that stimulate host muropeptide receptors to up-regulate NK cell activating ligands and/or cytokines on infected immune or stromal cells. Proof that p60 acts indirectly on infected cells to enhance NK cell activation awaits the development of sensitive assays to measure NK cell activation by infected cells in vitro.

Discussion

We illustrate a requirement for the abundantly secreted PGN-digesting autolysin, p60, in subversion of innate IFN-γ responses during early stages of L. monocytogenes infection. This novel observation extends our previous findings that specific bacterial autolysins contribute to L. monocytogenes pathogenicity (17) and lends support to the model that they do this through modulation of host innate immune responses. Our findings indicate that p60 expression enhances IL-6, IL-12p70, and IFN-γ production within the first ~24 h of in vivo bacterial infection. Although we did not expect to observe activation of naive, Ag-specific T cells at this early time point in the infection, the Forman and Bancroft groups have previously shown that memory T cells can be activated by IL-12 to produce innate IFN-γ within 24–48 h of L. monocytogenes infection (27, 28). Such activation is independent of TCR signals and is thought to exert a protective effect. NK cell activation, however, can occur independent of IL-12. Interestingly, the increased serum IFN-γ seen in the context of bacterial p60 expression was associated with an increased NK cell activation, but not an increase in IFN-γ production by (memory) T cells. These findings suggest that p60 triggers NK cell activation through IL-12-independent mechanisms and/or that memory T cell activation is less sensitive to fluctuations in serum IL-12p70 concentrations.

Because purified His-p60 did not induce NK cell activation, we thus propose that bacterial expression of p60 by cytosolic L. monocytogenes induces an increased expression of IL-12 and/or cell surface Ags recognized by NK cell-activating receptors (31, 32). However, we were unable to detect IL-12p70 and the production of IL-12p40 was not significantly affected by p60 expression in the supernatants of BMM infected with L. monocytogenes in vitro. Interestingly, others have previously reported that Ab-mediated depletion of NK cells impairs L. monocytogenes replication in infected host tissues (33, 34). Although the basis for the apparently antiprotective effects of NK cell activation is not known, our results suggest that such activation is actively enhanced by the bacterium through the production of specific virulence-promoting factors.

Regarding the mechanism for the effects of p60 on the host cell, we previously showed that the C-terminal catalytic domain was required to complement expansion of the Δp60 strain (17), suggesting that the ability of p60 to digest PGN is important for this process. It is increasingly recognized that PGN and muropeptide fragments of intact PGN stimulate a variety of host receptor systems, including TLR2, NOD1, NOD2, and several mammalian PGRPs (35, 36). We and others have thus proposed that p60 and perhaps other bacterial autolysins that contribute to the pathogenicity of other Gram-positive bacteria can manipulate or alter host immune responses through their effects on the degradation of intact PGN or bioactive muropeptides (17, 36). Here, we have excluded a role for TLR2 in p60-mediated enhancement of in vivo bacterial expansion, although TLR2 appears to play a protective role in immunity to systemic L. monocytogenes infections (37). Preliminary experiments with CARD4/NOD1-deficient mice have yielded identical results (not shown), which would appear to exclude this muropeptide receptor as well. Furthermore, in vivo data published by Kobayashi et al. (38) argue against a role for NOD2 during in vivo expansion of wt L. monocytogenes following a systemic infection. Thus, although the predicted cleavage of the isoglutamate-mesodiaminopimelate peptide bond in L. monocytogenes PGN by

**FIGURE 6.** Purified His-p60 protein digests L. monocytogenes PGN but fails to activate NK cells in vitro. A, Zymogram showing ability of His-p60 protein (>95% pure) to digest L. monocytogenes cells. An 8% polyacrylamide gel was cast with 2% heat-killed L. monocytogenes cells (HKLm). Proteins were separated by SDS-PAGE and renatured to reveal p60 endopeptidase activity at ~60 kDa. Lane 1, molecular mass ladder; lane 2, BSA; lane 3, purified His-p60. B, Treatment of NWNA cells with His-p60 fails to trigger IFN-γ production. His-p60 was added to cultures of NWNA cells at 10 mg/ml. Controls included unstimulated NWNA cells and cells stimulated with PMA plus ionomycin to activate protein kinase C and calcium flux to induce IFN-γ production. At 20 h after the start of stimulation, NWNA cells were treated with brefeldin A to block cytokine secretion, followed by surface staining for CD3, F480, and NK1.1 and intracellular staining for IFN-γ at 24 h after stimulation. Representative FACS plots gated on CD3+ F480+ NK1.1+ NWNA cells are shown from two to three independent samples per treatment and two separate experiments.
p60 would be predicted to enhance production of ligands for NOD2 and destroy ligands for NOD1, the loss of either receptor is not sufficient to restore p60-independent bacterial expansion. These results thus suggest that p60 or p60-dependent mureopeptides target other host innate receptors or that the more relevant effect of the catalytic activity of p60 is the release of bacterial cytosolic components, rather than mureopeptides. For example, recent work from Stetson and Medzhitov (39) suggests that L. monocytogenes and other bacteria release their DNA into the cytosol of infected host cells, which stimulates expression of ifnβ and il6 independent of TLR and NOD1/2 signaling. The nature of the novel cytosolic receptor that detects L. monocytogenes DNA and whether p60 or other autolysins might contribute to DNA release remain to be determined.

Although our work indicates that the induction of NK cell activation and IFN-γ production are significant in vivo consequences of bacterial p60 expression, the mechanisms by which such processes might enhance bacterial expansion are not immediately clear. Indeed, one might expect increased IFN-γ production to tilt host responses to a Th1 phenotype and thus increase the intensity of the antibacterial response during the development of T cell responses to the infection. However, it is increasingly clear that immunoregulatory cytokines exert pleiotropic effects, not all of which may be beneficial to limiting the replication of bacteria or other pathogens. For instance, it was recently shown that enhanced IL-6 signaling caused by a hyperresponsive mutant gp130 receptor (Y759F) promotes pathological induction of autoimmune arthritis (40), and this same mutation was previously associated with increased susceptibility to mortality and bacterial growth during a L. monocytogenes infection (41), suggesting potential deleterious effects of an overzealous IL-6 response. IL-6 production has also been linked to suppression of T cell responses during M. tuberculosis infection (29). Similarly, Badovinac et al. (42, 43) showed that IFN-γ reduces the survival of Listeria-specific CD8+ T cells and hence reduces the protection mediated by such cells. Our experiments have not addressed the effects of p60 on T cell responses to L. monocytogenes infection. However, because we have focused on early events in the infection process, it is likely that the anti-inflammatory effects of IFN-γ seen in our studies reflect altered regulation of immune cell recruitment, function, or survival. Indeed, our finding that up-regulation of socs1, 3, and il10 occurs independent of p60 in infected macrophages suggests that the bacterium may redirect innate IFN-γ responses toward uninjured innate immune cells. Hence, p60 may create a localized information overload that paralyzes uninjured immune cells and thus permits enhanced bacterial growth, survival and/or dissemination from foci of infection. Later in the infection, immune cells may learn to overcome this paralysis, for example, through the down-regulation of the IFN-γ receptor that is known to occur on Th1-committed CD4+ T cells (44).

It is not known whether other bacterial pathogens actively enhance NK cell activation. However, like L. monocytogenes, M. tuberculosis is an intracellular bacterial pathogen that benefits from hyperproduction of IFNβ and suppression of IFN-γ signaling in infected cells via mechanisms that include IL-6 production, TLR hyperstimulation, and a myeloid differentiation factor 88-dependent mechanism that involves PGN (30, 45–48). Interestingly, the genomes of M. tuberculosis and related bacteria contain several genes with regions of homology to the catalytic domain or other regions of L. monocytogenes p60, and two p60 homologues were recently implicated in the intracellular survival of Mycobacterium marinum (49). Likewise, NK cells play at most a minor role in protective immunity to M. tuberculosis and Mycobacterium avium (50, 51) and appear to be detrimental during infection with other bacterial species and with the parasite Brugia malayi (52–54). In M. avium infection, it is also noteworthy that a recent study showed up-regulation of SOCS1 and SOCS3 and a consequent suppression of IFN-γ-dependent activation in infected human macrophages (55). Thus, the scenario described here for L. monocytogenes may also occur in the context of other intracellular bacterial infections. It will be important to further define how p60-like proteins and mureopeptides contribute to NK cell activation and other aspects of immune modulation during L. monocytogenes infection, given that such studies may also have an impact on our understanding of the mechanisms used by other important intracellular pathogens to establish acute and chronic infections.

In summary, our studies suggest that during the early stages of infection bacteria actively induce IFN-γ production by NK cells, and such hyperinduction enhances bacterial replication in IFN-γ-responsive hosts. Our results further suggest that bacterial pathogens may segregate the protective from the deleterious effects of IFN-γ production during innate stages of intracellular bacterial infections. Further study of bacterial mechanisms for subverting innate IFN-γ responses may reveal strategies to selectively enhance the beneficial effects of endogenous or therapeutic IFN-γ in the treatment of infections, immunodeficiencies, and neoplasia.

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Disclosures

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