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Recognition of Peptidoglycan from the Microbiota by Nod1 Enhances Systemic Innate Immunity

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Introductory Paragraph

Humans are colonized by a large bacterial flora (the microbiota) essential for the development of the gut immune system[1-3]. A broader role for the microbiota as a major modulator of systemic immunity has been proposed[4,5], however, evidence and mechanism have remained elusive. We show that the microbiota is a source of peptidoglycan that systemically primes the innate immune system, enhancing killing by bone marrow-derived neutrophils of two important pathogens: *Streptococcus pneumoniae* and *Staphylococcus aureus*. This requires signaling via the pattern recognition receptor Nod1 (which recognizes *meso*DAP-containing peptidoglycan found predominantly in Gram-negative bacteria), but not Nod2 (which detects peptidoglycan found in Gram-positive and Gram-negative bacteria) or Tlr4 (which recognizes lipopolysaccharide)[6,7]. We demonstrate translocation of peptidoglycan from the gut to neutrophils in the bone marrow and show levels in sera correlate with neutrophil function. *In vivo* administration of Nod1 ligands is sufficient to restore neutrophil function after microbiota depletion. *Nod1*$^{-/-}$ mice show increased susceptibility to early pneumococcal sepsis, demonstrating a role for Nod1 in priming innate defenses facilitating a rapid response to infection. These data establish a mechanism for systemic immunomodulation by the microbiota and highlight potential adverse consequences of microbiota disruption, by broad-spectrum antibiotics, on innate immune defense to infection.

Humans are colonized by approximately 10^{13}–10^{14} bacteria, residing primarily on the mucosal surfaces of the host[8]. Understanding host-bacteria relationships has generally

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Author Contributions

T.B.C designed the research, performed the experiments, analyzed the data and wrote the manuscript, K.M.D performed experiments, analyzed data and contributed to the manuscript, E.S.L and A.Y.Z performed experiments and analyzed data, Y.Y. contributed vital reagents, J.N.W. designed the research, analyzed the data and wrote the manuscript.

focused on pathogenesis, however, infections are relatively rare and the predominant host-bacteria interactions are at worst benign, and often beneficial. Local effects of the microbiota, on gut homeostasis[9] and gut immune development[10,11], have been delineated, however, few studies have addressed the systemic effect of the microbiota[12]. A broader influence of the microbiota in the regulation of systemic immunity, due to the translocation of microbial products from the luminal side of the mucosa into the circulation, has been proposed[4,13]. However, evidence to support such an effect remains preliminary and an understanding of the mechanisms involved is lacking. Neutrophils are a critical component of the innate immune system and are often the initial defense against extracellular pathogens[14]. We have previously shown that local recognition of peptidoglycan, by the pattern recognition receptor Nod1 enhances killing of complement-opsonized *S. pneumoniae* by neutrophils[15] (**Fig. 1a**). To ascertain whether this was a general mechanism of neutrophil activation we investigated opsonophagocytic killing of another major pathogen, *S. aureus*, which in contrast to *S. pneumoniae*, requires the oxidative burst for effective killing[16]. Neutrophils from the peritoneal cavity of mice administered (i.p.) heat-killed *Haemophilus influenzae*, a Gram-negative bacterium with *meso*DAP-containing peptidoglycan, showed enhanced killing of *S. aureus* in an *ex vivo* opsonophagocytic assay, compared to neutrophils from unstimulated control mice (**Fig. 1b**). However, neutrophils from *Nod1*$^{-/-}$ mice were refractory to stimulation with heat-killed *H. influenzae*, confirming that local recognition of peptidoglycan by Nod1 enhances opsonophagocytic killing of both *S. pneumoniae* and *S. aureus*. This demonstrates that Nod1 has a broad influence on neutrophil function activating both oxidative and non-oxidative mechanisms of killing. Interestingly, we observed that neutrophils from *Nod1*$^{-/-}$ mice were not only unresponsive to stimulation by exogenous peptidoglycan, but also showed defects in their basal level of killing in the absence of additional stimulation (comparison of *S. pneumoniae* and *S. aureus* killing by neutrophils from unstimulated control wild-type and *Nod1*$^{-/-}$ mice) (**Fig. 1a,b**). We hypothesized that recognition of microbial products under basal conditions (in the absence of infection) systemically primes the innate immune system, enhancing neutrophil function, and that the host microbiota provides this stimulus. To investigate this hypothesis, mice were administered broad-spectrum antibiotics and the systemic effect of microbiota depletion on bone marrow-derived neutrophil function assessed. We confirmed by flow cytometry that, analogously to neutrophil-enriched PECs, the cell population enriched from the bone marrow interacting with bacteria were Ly6G^{+} neutrophils, and this interaction was dependent on opsonization of bacteria by complement (data not shown). Neutrophils isolated from the bone marrow of antibiotic treated mice and tested in an *ex vivo* opsonophagocytic assay, showed a significant reduction in killing of *S. pneumoniae* and *S. aureus*, compared to those from non-antibiotic treated control mice (**Fig. 1c,d**). Bone marrow-derived neutrophils from mice maintained germ-free also showed reduced killing of *S. pneumoniae* and *S. aureus*, compared to those from previously germ-free conventionalized mice, confirming the systemic role of the microbiota in enhancing neutrophil function (**Fig. 1e,f**). To define the mechanism by which the microbiota exerts this effect, we treated mice deficient in pattern recognition receptors for different microbial products (*Nod1*$^{-/-}$, *Nod2*$^{-/-}$ and *Tlr4*$^{-/-}$) with broad-spectrum antibiotics. Bone marrow-derived neutrophils from antibiotic treated *Tlr4*$^{-/-}$ and *Nod2*$^{-/-}$ mice showed a significant reduction in the killing of both *S. pneumoniae* and *S. aureus* compared to non-antibiotic

treated mice, showing that neither lipopolysaccharide nor MDP from the microbiota are important for systemic innate immune activation in this model (**Fig. 1g,h**). In contrast, bone marrow-derived neutrophils from *Nod1*$^{-/-}$ mice were not only defective in killing *S. pneumoniae* and *S. aureus* but were also unresponsive to signals from the microbiota, showing that recognition of peptidoglycan from the microbiota by Nod1 is critical for maintaining a basal level of immune activation. Next we addressed how the microbiota could communicate systemically to sites distal to the gut, and whether peptidoglycan from the gut microbiota was translocated from the luminal side of the mucosa into the host circulation under basal conditions (in the absence of infection). The translocation of microbial products has been documented previously, however, their effects have generally been considered in the context of compromised barrier function due to viral or bacterial infection[17,18]. In addition, specific mechanisms for the uptake of peptidoglycan fragments from the colonized mucosa have been proposed[19,20]. Formerly germ-free mice were colonized with *E. coli* with [^{3}H]-DAP-labeled peptidoglycan. *E. coli* colonized the gut stably over three days (**Fig. 2a**) and peptidoglycan was detected systemically in sera and levels correlated with those in feces (**Fig. 2a,b**). This indicates that during colonization peptidoglycan is constantly turned over and either excreted or translocated across the gut mucosa into the circulation. Furthermore, during colonization peptidoglycan accumulated in the bone marrow (**Fig. 2b**), and could be detected in the neutrophil fraction (72 hours post-oral inoculation, 0.043 ± 0.014% of total CPM of inoculum per 10^{9} neutrophils). To demonstrate the activity of translocated peptidoglycan, sera from either antibiotic treated, non-antibiotic treated, germ-free or previously germ-free conventionalized mice, was added to a bioassay using HEK293T cells carrying an NF-κB-luciferase reporter, co-transfected with either a Nod1 or Nod2 construct. In this bioassay, addition of mouse sera elicited both Nod1 and Nod2-dependent NF-κB activation, confirming the presence of translocated peptidoglycan (**Fig. 2c,d**). Sera from germ-free and antibiotic treated mice elicited significantly less Nod1 and Nod2-dependent NF-κB activation than sera from previously germ-free conventionalized and non-antibiotic treated mice, respectively (**Fig. 2c,d**). This demonstrates that the decrease in Nod1-dependent neutrophil killing correlated with systemic levels of Nod1-activating peptidoglycan fragments. To determine whether Nod1 ligands alone were sufficient to restore innate immunity after microbiota depletion, mice were treated with broad-spectrum antibiotics and then administered (i.p.) Mur*N*AcTri$_{DAP}$ (the Nod1 ligand), MDP, or vehicle controls. Bone marrow-derived neutrophils from mice treated with Mur*N*AcTri$_{DAP}$, but not MDP, showed increased killing of *S. pneumoniae*, compared to vehicle control, confirming that fragments of peptidoglycan found systemically, recognized by Nod1, are sufficient to activate neutrophils after microbiota depletion (**Fig. 3a**). Additionally we confirmed the expression of *Nod1* in bone marrow-derived neutrophils by RT-PCR (data not shown). We have previously shown that neutrophils are required to control sepsis from pneumococcal infection of the upper respiratory tract[21]. To investigate the importance of Nod1 in priming innate immunity, WT and *Nod1*$^{-/-}$ mice were challenged intranasally with *S. pneumoniae*. *Nod1*$^{-/-}$ mice were more susceptible to early sepsis (day 3, $P = 0.0176$; and day 4, $P = 0.0437$) than WT, however, there was no difference in the overall survival between *Nod1*$^{-/-}$ and WT animals (>day 5) (**Fig. 3b)**. This suggests that *Nod1*$^{-/-}$ mice have delayed responses to infection due to a lack of innate immune priming rather than any intrinsic defects. We then determined if

neutrophils could be directly activated by peptidoglycan. HL-60 cells differentiated to a neutrophil-like phenotype were treated with Mur*N*AcTri$_{DAP}$, MDP, or vehicle controls. Treatment with Mur*N*AcTri$_{DAP}$, but not MDP, increased killing of *S. pneumoniae*, compared to vehicle control (**Fig. 3c**), demonstrating that peptidoglycan recognized by Nod1, but not Nod2, can directly enhance neutrophil function. This enhancement by Mur*N*AcTri$_{DAP}$ required the ability of Nod1 to activate the transcription factor NF-κB (**Fig. 3c**). Neutrophils isolated from human whole blood also showed a significant increase in killing of *S. pneumoniae* after treatment with Mur*N*AcTri$_{DAP}$ (**Fig. 3d**).

Previous work[22-24], and data presented here, has shown that microbial products derived from the microbiota are found systemically, however, there is little appreciation of how they effect the host. Depletion of the microbiota significantly reduced systemic peptidoglycan levels which correlated with a reduction in killing of *S. pneumoniae* and *S. aureus* by bone marrow-derived neutrophils. We demonstrate that peptidoglycan recognized by Nod1 was sufficient to restore neutrophil function in the absence of the microbiota, and in the absence of Nod1 signaling there is a failure to prime the innate immune system when examined both *in vitro* and *in vivo*. This reveals a novel role for peptidoglycan in priming systemic innate immunity, and for Nod1 as a homeostatic regulator. Our data challenges the traditional view of innate immunity as quiescent in the absence of infection, and activated only upon pathogen recognition[25]. We propose that the microbiota constantly modulates the innate immune system, priming it, thus facilitating a rapid response to pathogens, such as *S. pneumoniae* and *S. aureus*, that might otherwise quickly overwhelm innate defenses. This systemic effect of microbial products means that disruption of the microbiota, with a concomitant lowering of systemic immune activation, may have profound consequences on the host rendering it more susceptible to infections. This may be a factor in the prevalence of secondary infections which occur after broad-spectrum antibiotic therapy[26]. There is increasing discussion regarding the beneficial, or “probiotic”, role of the microbiota, however, the mechanism by which the microbiota exerts these effects are vague and inferred. Our data provides a direct example and mechanism for a probiotic activity of the microbiota, demonstrating that translocated microbial products found systemically benefit the host by enhancing systemic innate immune function.

Materials and Methods

Bacterial strains

S. pneumoniae P1121 (a type 23F isolate), *S. pneumoniae* P1547 (a type 6A isolate) and *H. influenzae* 636 were grown as previously described[15,27]. *S. aureus* 8325-4 was grown in tryptic soy broth at 37 °C with shaking.

Mouse strains

Mice were used at 6–10 weeks and housed in accordance with Institutional Animal Care and Use Committee protocols. Wild-type C57Bl/6 and congenic *Nod2*$^{-/-}$ (C57Bl/6) and *Tlr4*$^{-/-}$ (C57Bl/10ScN) mice were from the Jackson Laboratories. *Nod1*$^{-/-}$ mice were provided by Millennium Pharmaceuticals. C57Bl/6 mice were bred and maintained germ-free in one

Trexler flexible film isolator in the University of Pennsylvania gnotobiotic facility. Germ-free mice were conventionalized by housing in non-germ-free conditions for >3 weeks.

Cell culture

HL-60 cells were cultured in RPMI 1640 medium (GIBCO) + 10% FCS (GIBCO), 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (GIBCO) in a humidified atmosphere of 5% CO_2, at 37 °C. HL-60 cells were differentiated to a neutrophil-like phenotype by addition of 1.3% DMSO to medium for 7 days.

Microbiota depletion

Mice were provided with broad-spectrum antibiotics (ampicillin 1 g ml^{-1}, Mediatech; neomycin sulphate 1 g ml^{-1}, Calbiochem; metronidazole 1 g ml^{-1}, Fluka; and vancomycin 0.5 g ml^{-1}, Sigma) in drinking water for 1 week.

Isolation of neutrophils from the peritoneal cavity

We obtained neutrophils by lavage of the peritoneal cavity with PBS + 20 mM EDTA, of mice administered (i.p.) 1 ml PBS + 10% casein for 24 hours, and again 2 hours prior to cell harvest, as described previously[15]. PECs from unstimulated control mice were elicited with casein alone, PECs from stimulated mice were elicited with casein containing heat-inactivated (65 °C for 30 minutes) *H. influenzae* 636 (1×10^7 cells per animal), stimulation protocol modified from[28]. We enriched neutrophils from PECs by Ficoll density gradient centrifugation using Mono-poly resolving medium according to the manufacture's instructions (MP Biomedicals). Flow cytometry of enriched cells showed that >90% were $Ly6G^+$.

Isolation of neutrophils from bone marrow

We isolated neutrophils from the hind limbs of mice. Bones were washed with 70% ethanol, then Hank's buffer ($-Ca^{2+}$ and $-Mg^{2+}$) (Mediatech) + 0.1% gelatin. Bones were flushed with Hank's buffer ($-Ca^{2+}$ and $-Mg^{2+}$) + 0.1% gelatin and enriched neutrophils by Ficoll density gradient centrifugation using Mono-poly resolving medium.

Opsonophagocytic killing assay

We assessed opsonophagocytic killing by neutrophils using an *ex vivo* assay as described previously[15]. A 200 μl assay in Hank's buffer ($+Ca^{2+}$ and $+Mg^{2+}$) + 0.1% gelatin, was composed of 1×10^2 PBS-washed bacteria, 1×10^5 neutrophils in Hank's buffer ($+Ca^{2+}$ and $+Mg^{2+}$) (GIBCO) + 0.1% gelatin and 20 μl of fresh serum from either mouse or baby rabbit as a source of complement. Assays were incubated for 45 minutes at 37 °C with rotation. NF-κB signaling was inhibited by 6-amino-4-(4-phenoxyphenylethylamino) quinazoline (CALBIOCHEM), as indicated. Bacterial viability was determined in serial dilutions.

Bioassay for detection of peptidoglycan

HEK293T cells were cultured in DMEM + 10% FCS, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin in a humidified atmosphere of 5% CO_2, at 37 °C. Cells were seeded at a density of 6×10^4 cells per well and transiently transfected using FuGENE 6 Transfection

Reagent (Roche) according to the manufacturer's instructions. Transfection reactions contained 25 ng pNF-κB-luc (Stratagene), and either 50 ng pcDNA3-Nod1-FLAG[29], 50 ng pCMV-Tag-Nod2[30], or 50 ng empty vector control plasmid (pcDNA3 or pCMV-Tag2C) (Stratagene). Sera was added directly to cells with transfection reagents, at a final dilution of 1:125. 24 hours post-transfection luciferase expression was measured using a Luciferase Assay System (Promega) according to the manufacturer's instructions. Control assays using MDP and Mur*N*AcTri$_{DAP}$ added to sera from germ-free mice confirmed Nod1- and Nod2-dependent NF-κB activation specificity, respectively.

Preparation of peptidoglycan fragments

MDP and Mur*N*AcTri$_{DAP}$ were synthesized as described previously[15,31].

Flow cytometry

For flow cytometry, we used antibodies to mouse Ly6.G (BD Biosciences), CD45 (BioLegend) and F4/80 (eBioscience). *S. pneumoniae* P1121 were labeled with FITC (1 mg ml^{-1}) for 30 minutes at 37 °C for bacterial uptake assays. Cells were washed and resuspended in Hank's buffer (−Ca^{2+} and −Mg^{2+}) + 0.1% gelatin and diluted 1:10.

Analysis of Nod1 expression by RT-PCR

Bone marrow-derived neutrophils, enriched by Ficol density gradient centrifugation, were further purified by FACS. The Ly6G^{+}, CD45^{+} and F4/80^{-} cell population was sorted to isolate neutrophils. RNA was isolated using an RNeasy mini kit (QIAGEN) according to the manufacture's instructions, and cDNA reverse transcribed using a high capacity reverse transcription kit (Applied Biosystems). We confirmed expression of *Nod1* in bone marrow-derived neutrophils and HL-60 cells by RT-PCR (data not shown). PCR primers are described in supplementary methods.

Statistical analysis

Difference between groups was compared by the unpaired Student's *t*-test (GraphPad Prism 4). *P*-values <0.05 were considered significant. The Kaplan-Meier logrank test was used to compare survival between groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BM	bone marrow

BSA	bovine serum albumin
CFU	colony forming units
CPM	counts per minute
DAP	diaminopimelic acid
DMEM	Dulbecco's Modified Eagle Medium
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HEK293T	human embryonic kidney 293T cells
HL-60	human promyelocytic leukemia cells
i.p.	intraperitoneal
LB	Luria broth
MDP	*N*-acetylmuramyl-L-alanyl-γ-D-glutamate
Mur*N*AcTri$_{DAP}$	*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-*meso*-2,6-diaminopimelic acid
NF-κB	nuclear factor-kappa B
Nod1	nucleotide-binding oligomerization domain containing 1
Nod2	nucleotide-binding oligomerization domain containing 2
PBS	phosphate buffered saline
PEC	peritoneal exudates cell
r.p.m.	revolutions per minute
SEM	standard error of the mean
Tlr4	toll-like receptor 4
WT	wild-type

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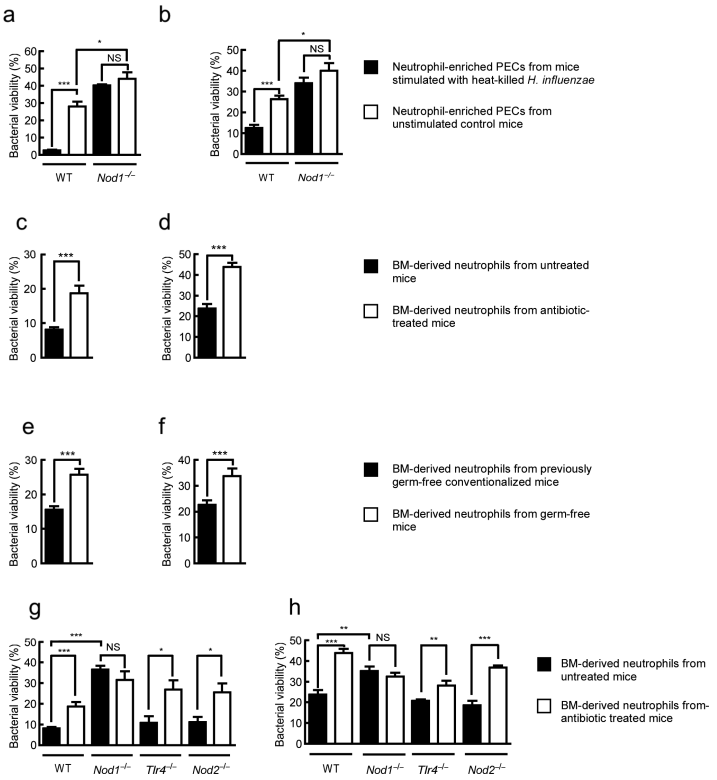

Figure 1. The microbiota enhances neutrophil function via Nod1 signaling

(**a,b**) Killing of *S. pneumoniae* (**a**) and *S. aureus* (**b**) by neutrophil-enriched PECs obtained from WT and *Nod1*$^{-/-}$ mice stimulated with heat-killed *H. influenzae* (i.p.) and unstimulated control mice. (**c,d**) Killing of *S. pneumoniae* (**c**) and *S. aureus* (**d**) byneutrophils harvested from bone marrow of WT mice treated with broad-spectrum antibiotics and untreated control mice. (**e,f**) Killing of *S. pneumoniae* (**e**) and *S. aureus* (**f**) by neutrophils harvested from bone marrow of germ-free WT mice and previously germ-free conventionalized mice. (**g,h**) Killing of *S. pneumoniae* (**g**) and *S. aureus* (**h**) by neutrophils harvested from bone marrow of WT, *Nod1*$^{-/-}$, *Tlr4*$^{-/-}$, and *Nod2*$^{-/-}$ mice treated with broad-spectrum antibiotics and untreated control mice. For all assays bacterial viability is expressed relative to control assays without neutrophils. Values represent at least three independent experiments performed in triplicate ± SEM. **P*< 0.05, ***P*<0.01 and ****P*<0.001.

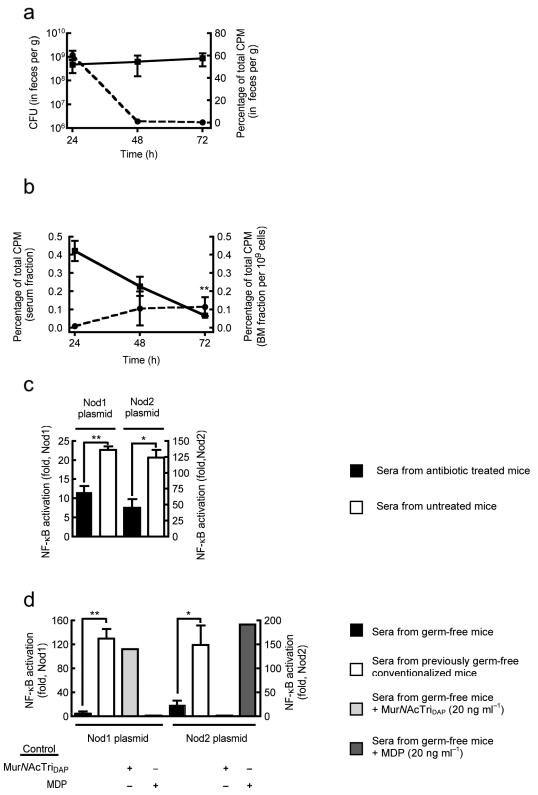

Figure 2. Peptidoglycan is present systemically in sera and bone marrow cells, and levels are decreased in the absence of the microbiota

(**a,b**) Detection of translocated peptidoglycan in blood, feces and bone marrow cell samples from mice inoculated with [^{3}H]-DAP labeled *E. coli* via oral gavage, by liquid scintillation counting. CFU in feces (—) and CPM in feces (– –) (**a**); CPM in serum fraction (—) and CPM in bone marrow fraction (– –) (**b**). Data is relative to CPM of total inoculum, time is post-oral inoculation. Statistical comparison of peptidoglycan accumulation in bone marrow cells at 72 hour is relative to 24 hour time point. (**c,d**) A HEK293T cell bioassay to detect peptidoglycan recognized by Nod1 and Nod2 in the sera of antibiotic treated and non-antibiotic treated WT mice (**c**); and conventionalized and germ-free WT mice (**d**). HEK293T cells were co-transfected with a NF-κB-luciferase reporter and either a Nod1 or Nod2 construct; luciferase expression was measured 24 h post-transfection and considered a measure of NF-κB activation. The specificity of NF-κB activation was confirmed using sera from germ-free mice with MDP and Mur*N*AcTri$_{DAP}$ added *in vitro* to control Nod1 and Nod2 bioassays. Values represent fold increase in luciferase expression above empty vector controls, and are based on three independent experiments performed in triplicate ± SEM. **P*< 0.05, ***P*<0.01.

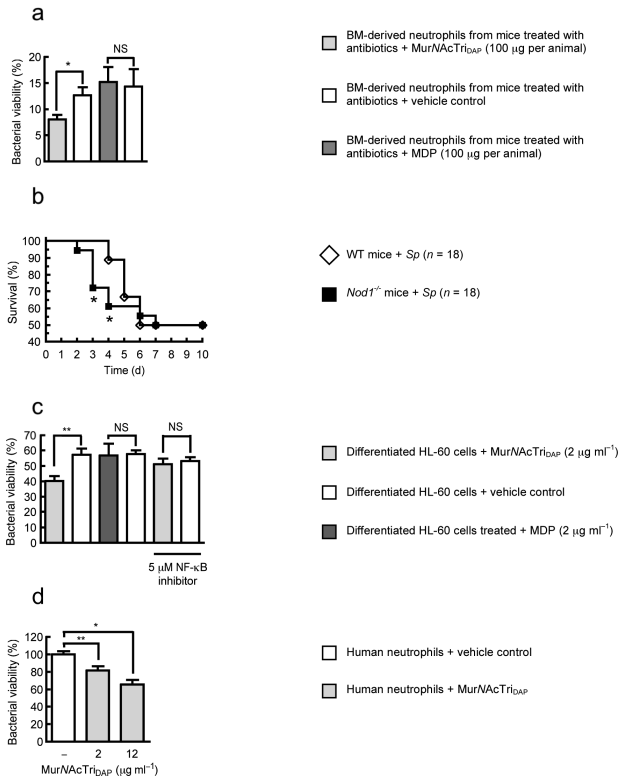

Figure 3. Peptidoglycan recognized by Nod1 restores neutrophil function after microbiota depletion and can activate neutrophils directly via NF-κB signaling, and Nod1 is required for early responses during pneumococcal sepsis

(**a**) Killing of *S. pneumoniae* P1121 by neutrophils from WT mice treated with broad-spectrum antibiotics and then administered (i.p., day 7) either Mur*N*AcTri$_{DAP}$, MDP, or vehicle controls. 24 hours after stimulation neutrophils were harvested from the bone marrow and their ability to kill *S. pneumoniae* compared. Bacterial viability is expressed relative to control assays without neutrophils. (**b**) Survival of *Nod1*$^{-/-}$ mice and WT mice challenged intranasally with 5×10^7 CFU of *S. pneumoniae* P1547. (**c**) Killing of *S. pneumoniae* by differentiated HL-60 cells treated with either Mur*N*AcTri$_{DAP}$, MDP, or vehicle controls for 4 hours. For NF-κB inhibition, cells were pretreated with 6-amino-4-(4-phenoxyphenylethylamino) quinazoline for 2 hours at 37 °C prior to stimulation with peptidoglycan. Bacterial viability is expressed relative to control assays without neutrophils. (**d**) Killing of *S. pneumoniae* by human neutrophils treated with Mur*N*AcTri$_{DAP}$ for 2 hours, bacterial viability is shown relative to vehicle control treatment. Values represent at least three independent experiments performed in triplicate ± SEM. **P*< 0.05, ***P*<0.01.