20. For the LIA1 locus, deletion occurred at the following frequencies after injection of dsRNA: 0 in 34 at 3.5 hours, 1 in 50 at 4.5 hours, 9 in 257 at 6 hours, 11 in 20 at 7.5 hours, and 5 in 18 at 10 hours after mixing cells for mating. Deletion was not detected after injection of single-stranded RNA at similar concentrations at the 6-hour stage (0 in 115 for the sense strand and 0 in 108 for the antisense strand).
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Nod1 Detects a Unique Muropeptide from Gram-Negative Bacterial Peptidoglycan

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Although the role of Toll-like receptors in extracellular bacterial sensing has been investigated intensively, intracellular detection of bacteria through Nod molecules remains largely uncharacterized. Here, we show that human Nod1 specifically detects a unique diaminopimelate-containing *N*-acetylglucosamine–*N*-acetylmuramic acid (GlcNAc-MurNAc) tripeptide motif found in Gram-negative bacterial peptidoglycan, resulting in activation of the transcription factor NF-κB pathway. Moreover, we show that in epithelial cells (which represent the first line of defense against invasive pathogens), Nod1 is indispensable for intracellular Gram-negative bacterial sensing.

Innate immunity to bacterial pathogens relies on the specific sensing of pathogen-associated molecular patterns (PAMPs) by pattern recognition molecules. In mammals, Toll-like receptors (TLRs) represent the most extensively studied class of pattern recognition molecules, which have been shown to sense various PAMPs such as lipopolysaccharide (LPS), peptidoglycan, lipoproteins, double-stranded RNA, and CpG DNA (*1, 2*). Although TLRs are mainly expressed at the plasma membrane, it has been proposed that the Nod molecules—a family of intracellular proteins including Nod1/CARD4 and Nod2/CARD15—could represent a new group of pattern recognition molecules that sense bacterial products within the cytoplasmic compartment, thus allowing detection of intracellular invasive bacteria (*3–9*). We recently showed that Nod1 senses the presence of the Gram-negative pathogen *Shigella flexneri* within the cytoplasmic compartment of epithelial cells (*6*), and we hypothesized that the PAMP detected was LPS, because commercial preparations of LPS were shown to activate Nod1 (*5*). However, because LPS often contains bacterial cell wall contaminants, we investigated in more detail the identity of the molecular motif that is actually detected by Nod1.

Addition of a commercial preparation of *Escherichia coli* LPS (10 μg) to Nod1-overexpressing human embryonic kidney (HEK) 293 cells potentiated the level of Nod1-dependent NF-κB activation by a factor of ~5 (Fig. 1A). By contrast, highly purified *E. coli* LPS (10 μg) or lipid A (10 μg) did not stimulate the Nod1 pathway (Fig. 1A), although they activated macrophages (*10*). We aimed to identify the nature of this LPS contaminant responsible for Nod1 activation. Lipoproteins have been identified as the major contaminants of LPS preparations responsible for TLR2 signaling after stimulation with commercial *E. coli* LPS (*11*). We were unable to stimulate the Nod1 pathway by addition of either synthetic lipopeptide or Lpp, the most abundant lipoprotein in *E. coli* (Fig. 1B). Moreover, boiling or proteinase K treatment of the commercial LPS was not sufficient to abolish Nod1 signaling (*10*).

Peptidoglycans from *E. coli*, *S. flexneri*, *Neisseria meningitidis*, *Bacillus subtilis*, and *Staphylococcus aureus* were purified according to experimental procedures specifically designed for Gram-positive or Gram-negative bacteria (*12, 13*). The harsh purification steps used to purify these peptidoglycans eliminate possible contaminants (fig. S1). Strikingly, we observed that peptidoglycan preparations from Gram-negative bacteria could stimulate the Nod1 pathway, whereas the two Gram-positive peptidoglycan preparations tested here could not (Fig. 1C). Moreover, by using a mutant form of Nod1 that lacks the C-terminal leucine-rich repeats, we observed that Nod1 leucine-rich repeats play a critical role in the sensing of Gram-negative peptidoglycan (fig. S2). Therefore, these results strongly suggest that Nod1 is an intracellular pattern recognition molecule that specifically senses Gram-negative peptidoglycan through the leucine-rich repeat domain.

To identify the peptidoglycan motif detected by Nod1, we analyzed muropeptides from *N. meningitidis* by reversed-phase high-performance liquid chromatography (HPLC) after peptidoglycan digestion with a muramidase. Indeed, the major peptidoglycan fragments naturally released by Gram-negative bacteria are muropeptides (*14, 15*). This analysis allowed for the separation of muropeptides according to the number of amino acids of the peptidic chain linked to the amino sugars, the degree of polymerization of the peptidic chain, or natural modifications such as O-acetylation or dehydration of the amino sugars (Fig. 2A). Individual muropeptides were collected and tested for their ability to activate the Nod1 pathway. Surprisingly, only two fractions (3 and 17) contained muropeptides able to activate Nod1 (Fig. 2B). Mass spectroscopy analysis revealed that fraction 3 is a muropeptide with a mass/charge ratio *m*/*z* of 893 and the active molecule in fraction 17 is a muropeptide with *m*/*z* of 873 (*16*). The 893 *m*/*z* molecule is consistent with a reduced muropeptide *N*-acetylglucosamine (GlcNAc or "G") β-1,4–linked to *N*-acetylmuramic acid (MurNAc or

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"M"), substituted with a tripeptide group (Fig. 2C); the 873 *m/z* molecule corresponds to the same muropeptide naturally dehydrated on the MurNAc moiety (anhydro-MurNAc). The tripeptide group substituted on the MurNAc, L-Ala-D-Glu-mesoDAP (where DAP is diaminopimelate), is therefore the same in fractions 3 and 17. HPLC analyses and biological assays were carried out on muropeptides isolated from *S. flexneri* with similar findings (*10*).

To gain more insight into the molecular pattern sensed by Nod1, we compared the activation of the Nod1 pathway by GM-dipeptide, GM-tripeptide, and GM-tetrapeptide. Equivalent amounts (10 ng) of GM-dipeptide, GM-tripeptide (from fraction 3), and GM-tetrapeptide [fraction 6 (*16*)] were tested for their ability to activate the Nod1 pathway. We observed that Nod1 specifically detects GM-tripeptide but not GM-dipeptide or GM-tetrapeptide (Fig. 2D). Because Nod1 is closely related to Nod2, we also tested these peptidoglycan products for Nod2 detection. Our previous findings and

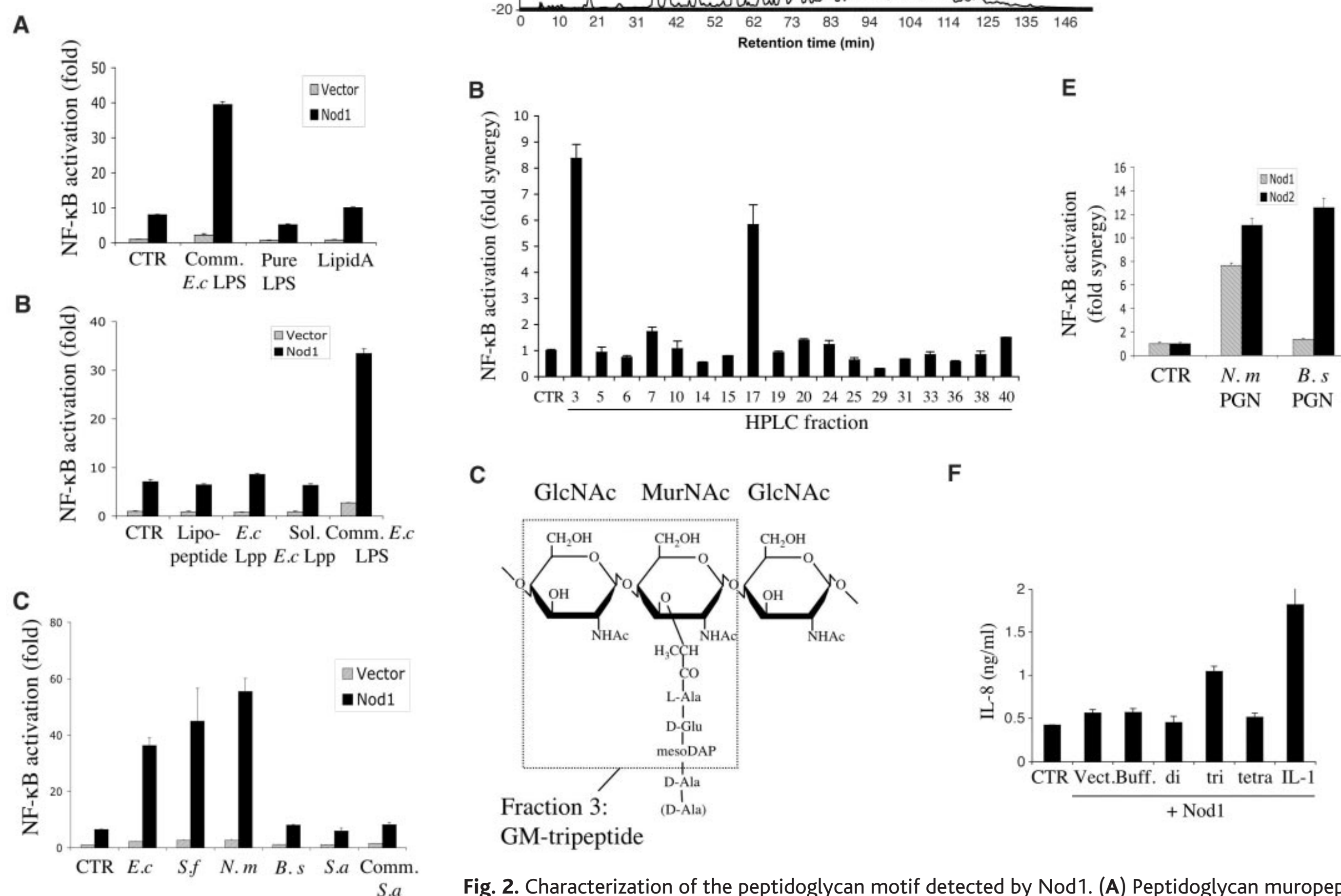

Fig. 1. The Nod1 pathway is stimulated by commercial LPS and Gram-negative bacterial peptidoglycans. (**A**) Stimulation of the Nod1 pathway by commercial *E. coli* LPS but not protein-free pure LPS or lipid A. (**B**) Lipopeptide or *E. coli* lipoproteins (lipidated and delipidated, i.e., "Lpp" and "soluble *E.c* Lpp") fail to activate the Nod1 pathway. (**C**) Nod1 mediates NF-κB responsiveness to Gram-negative peptidoglycans (*E. coli*, *S. flexneri*, *N. meningitidis*) but not Gram-positive peptidoglycans [*B. subtilis*, *S. aureus*, or commercial *S. aureus* peptidoglycan (Comm. *S.a*)]. LPS, lipid A, and lipoprotein preparations were used at 10 μg/ml; peptidoglycan preparations were used at 1 μg/ml. CTR, unstimulated.

Fig. 2. Characterization of the peptidoglycan motif detected by Nod1. (**A**) Peptidoglycan muropeptides from *N. meningitidis* were separated by reversed-phase HPLC and then analyzed by mass spectroscopy (*16*). N.D. (a), not determined for this particular HPLC fractionation, but found to be O-acetylated dimeric GM-tetrapeptide in later HPLC fractionation and mass spectroscopy analyses. This fraction was also negative for Nod1 stimulation in additional experiments (*10*). N.D. (b), fraction unknown. (**B**) 1% of the volume of each individual HPLC fraction was tested for stimulation of the Nod1 pathway, showing that fraction 3, corresponding to GM-tripeptide, activates Nod1. Fraction 17, corresponding to a mixture of dimeric GM-tetrapeptide (does not activate Nod1) and anhydrous GM-tripeptide, activates Nod1. (**C**) Schematic representation of *N. meningitidis* peptidoglycan detailing the Nod1- active motif from fraction 3 (GM-tripeptide) as determined by mass spectroscopy. (**D**) Equivalent amounts (10 ng) of GM-dipeptide (GM-di; synthetic source), GM-tripeptide (GM-tri), or GM-tetrapeptide (GM-tetra) were tested for stimulation of Nod1-, Nod2-, or TLR2-dependent activation of NF-κB. (**E**) Synergistic activation of Nod1 or Nod2 by *N. meningitidis* and *B. subtilis* peptidoglycans. (**F**) Production of the proinflammatory chemokine IL-8 in HeLa epithelial cells stimulated with GM-tripeptide in the presence of Nod1 but not GM-dipeptide or tetrapeptide. IL-1 (10 ng/ml) stimulation of IL-8 production is shown as a positive control. Buff, buffer diluent of the muropeptides. All muropeptides added were at 30 ng/ml. $P < 0.005$ for GM-tripeptide compared to vector alone by Student's *t* test.

those of Inohara *et al*. had shown that Nod2 recognizes M-dipeptide (*17, 18*). Here, however, we found that Nod2 detects GM-dipeptide in addition to M-dipeptide but does not detect GM-tripeptide or GM-tetrapeptide (Fig. 2D), which suggests that these two Nod molecules both sense peptidoglycan but require distinct molecular motifs to achieve detection. We also observed that TLR2 could not detect any of the muropeptides tested (Fig. 2D).

The peptidoglycan motif sensed by Nod2, GM-dipeptide, is found in all bacteria, hence Nod2 may be a general sensor of peptidoglycan degradation products (*17, 18*). In contrast, the additional requirement of mesoDAP for Nod1 sensing explains why Nod1 detects only those peptidoglycans purified from Gram-negative bacteria (see Fig. 1C) as compared to the Gram-positive peptidoglycans tested. Indeed, whereas *S. aureus* peptidoglycan contains lysine instead of mesoDAP, *B. subtilis* contains ~95% amidated diaminopimelate (fig. S3). Furthermore, *S. aureus* peptidoglycan does not have any detectable amounts of GM-tripeptide (*12*). In addition, these findings indicate that the Nod1 sensing system requires an exposed mesoDAP, because GM-tetrapeptide (which also contains mesoDAP) is not sensed by Nod1.

We next observed that Nod2, which does not require mesoDAP to achieve peptidoglycan detection, could sense both *B. subtilis* and *N. meningitidis* peptidoglycans (Fig. 2E). In contrast, even if the *B. subtilis* peptidoglycan shares some structural similarities with mesoDAP-containing peptidoglycans (fig. S3), Nod1 does not detect *B. subtilis* peptidoglycan (Fig. 2E, also shown in Fig. 1C). In addition to NF-κB activation, we found that sensing of GM-tripeptide by Nod1 also leads to the production of the proinflammatory chemokine interleukin-8 (IL-8) (Fig. 2F), which is one of the major cytokines produced by epithelial cells infected with Gram-negative bacteria (*19, 20*). Taken together, our results show that for Nod1-dependent activation of NF-κB and IL-8, the naturally occurring peptidoglycan structural requirements include GM linked to a tripeptide and a terminal mesoDAP amino acid in which both carboxy groups play a major role.

Previous studies stressed the pivotal role of epithelial cells as the first line of defense against bacterial pathogens at mucosal surfaces. We first prepared extracts from various Gram-negative or Gram-positive bacteria and determined the relative peptidoglycan content of these extracts (fig. S4A). We then added these bacterial extracts extracellularly and showed that they were unable to activate NF-κB in HEK293 epithelial cells (Fig. 3A), confirming that these cells do not display an endogenous TLR2/4 sensing system. The only exception was *Salmonella typhimurium* extract; in this case NF-κB activation is likely to involve TLR5 (*21*), because extracts from a flagellin-deficient *S. typhimurium* strain were unable to stimulate the NF-κB pathway (Fig. 3A). A digitonin-based permeabilization technique was then used to elicit entry of bacterial products into the cytoplasm, allowing us to directly compare the ability of bacterial products from either invasive or noninvasive bacteria to activate the NF-κB pathway. We observed that extracts from several Gram-negative bacteria were able to stimulate the NF-κB pathway, whereas those from the four Gram-positive bacteria were not (Fig. 3A). The specific activation of NF-κB by only Gram-negative extracts was confirmed in two other epithelial cell lines (HeLa and Caco-2) by microinjection of bacterial products from either *S. flexneri* or *S. aureus*, followed by detection of the NF-κB p65 subunit nuclear translocation by immunofluorescence (Fig. 3B) (fig. S4B). Therefore, these data show that epithelial cells sense Gram-negative but not Gram-positive bacterial products when presented to the cytoplasmic compartment. These findings are consistent with the fact that the released peptidoglycan motifs from the Gram-negative bacteria tested here all contain GM-tripeptide with a terminal mesoDAP, and that released Gram-positive bacterial peptidoglycan products lack this structure. In the case of *Listeria monocytogenes*, the peptidoglycan contains mesoDAP; however, the peptidoglycan degradation products have not yet been characterized. Of interest, the major peptidoglycan hydrolase in *L. monocytogenes* is a *N*-acetylmuramoyl-L-alanyl-amidase that cleaves the bond between the peptidoglycan sugar backbone and the peptidic chains. Therefore, *L. monocytogenes* is more likely to release free peptidic chains and amino sugars than substantial amounts of muropeptides (*22*).

This signaling pathway is independent of MyD88, a key adaptor protein of the TLR/IL-1 pathway (*23*), because a dominant-negative form of MyD88 was unable to block the activation of the NF-κB pathway induced in digitonin-permeabilized cells by extracts from Gram-negative bacteria, including *S. typhimurium* ΔF, *S. flexneri*, and *E. coli* (fig. S5A) (*10*). By contrast, using a dominant-negative form of Nod1 (DN-Nod1), we blocked NF-κB activation induced in digitonin-permeabilized cells by bacterial products from *S. flexneri*, *S. typhimurium*, and *E. coli* (fig. S5B). Several reports have shown that Nod1 activates the NF-κB pathway through the recruitment of Rip2 (*6, 24–26*). Accordingly, we observed that a dominant-negative form of Rip2 also blocked the NF-κB pathway induced in digitonin-permeabilized cells by extracts from Gram-negative bacteria (fig. S5C) (*10*).

Taken together, these results strongly suggest that Nod1 is the major intracellular sensor of bacterial products in epithelial cells. However, Nod2 as well as Nod1 are expressed by these cells, both at the level of protein (fig. S6) and mRNA (*10*), so we cannot rule out the possibility that Nod2 or other intracellular pattern recognition molecules might play a role in this detection

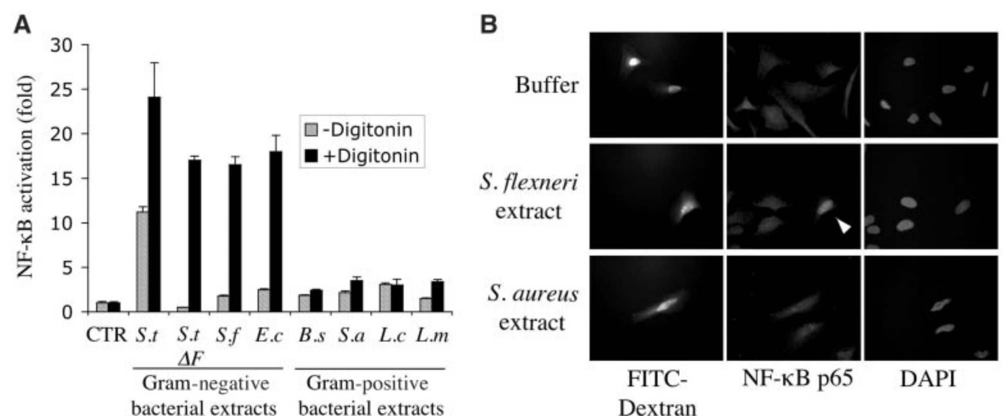

Fig. 3. Intracellular detection of Gram-negative but not Gram-positive bacterial products in epithelial cells. (**A**) Extracts from Gram-negative (*S.t*, *Salmonella typhimurium*; *S.t* ΔF, *S. typhimurium*–delta flagellin; *E.c*, *Escherichia coli*; *S.f*, *Shigella flexneri*) and Gram-positive (*B.s*, *Bacillus subtilis*; *S.a*, *Staphylococcus aureus*; *L.c*, *Lactobacillus casei*; *L.m*, *Listeria monocytogenes*) bacteria were added to HEK293 cells permeabilized or not by digitonin (10 μg/ml); after 4 hours, NF-κB activity was measured with an NF-κB–luciferase reporter assay. (**B**) HeLa cells were microinjected with either Dextran–fluorescein isothiocyanate only (buffer) or with bacterial extracts and stained for NF-κB p65; 100% of cells microinjected with Gram-negative bacterial supernatants show translocated NF-κB, whereas no active cells were observed with the Gram-positive bacterial supernatants. DAPI (4′,6′-diamidino-2-phenylindole) stain shows position of nuclei. Arrows point to translocated NF-κB p65 in the nuclei of activated cells.

system. Therefore, we examined intracellular bacterial sensing in isolated intestinal epithelial cells from mice deficient in Nod1. In epithelial cells from wild-type mice, intracellular presentation of Gram-negative but not Gram-positive bacterial supernatants led to the activation of NF-κB, consistent with our findings in three epithelial cell lines studied here (Fig. 4) (fig. S7). In contrast, cells from the Nod1-deficient mice were not activated by bacterial supernatants (Fig. 4) (fig. S7), although tumor necrosis factor α (TNFα) could still efficiently stimulate these cells (Fig. 4). These observations suggest that Nod2 is nonfunctional in epithelial cells in steady-state conditions. These findings, therefore, show that Nod1 is the crucial intracellular sensor of bacterial products in epithelial cells and that induction of the Nod1-dependent proinflammatory pathway depends on the ability of a bacterial pathogen—either invasive or extracellular—to translocate Gram-negative peptidoglycan to the intracellular environment.

In *Drosophila*, the Toll pathway detects both Gram-positive bacteria and fungi, whereas the Imd pathway is specific to Gram-negative bacterial sensing (*27*). Recently, two peptidoglycan recognition proteins (PGRPs) have been shown to play a key role in the discriminatory detection of bacteria in *Drosophila* (*28–31*). PGRP-SA is involved in Gram-positive bacterial recognition in the Toll pathway, whereas PGRP-LC acts upstream of Imd in Gram-negative bacterial sensing. This discriminatory sensing by *Drosophila* is based on differences in peptidoglycan motifs (*32*).

Our studies have shown that in mammalian cells, Nod1-dependent detection of bacteria relies on the sensing of a Gram-negative peptidoglycan motif. Indeed, our findings show that GM-tripeptide and GM-dipeptide constitute a new class of bacterial PAMPs, which are recognized differentially by Nod1 and Nod2, respectively. These peptidoglycan motifs are naturally occurring degradation products released from bacteria during growth. Therefore, the peptidic composition of the peptidoglycan degradation products, either released by the bacteria or processed by the host cell in the lysosomal compartment, is critical in defining the host response to bacterial infection. In this respect, the characterization of the peptidoglycan motifs sensed by Nod1 and Nod2 suggests that these two molecules have complementary and nonoverlapping functions that contribute to innate immunity. Moreover, our results show that Nod1 is likely the sole sentinel molecule in the epithelial barrier allowing intracellular detection of bacteria through peptidoglycan sensing, thereby highlighting its key role in innate immune defense.

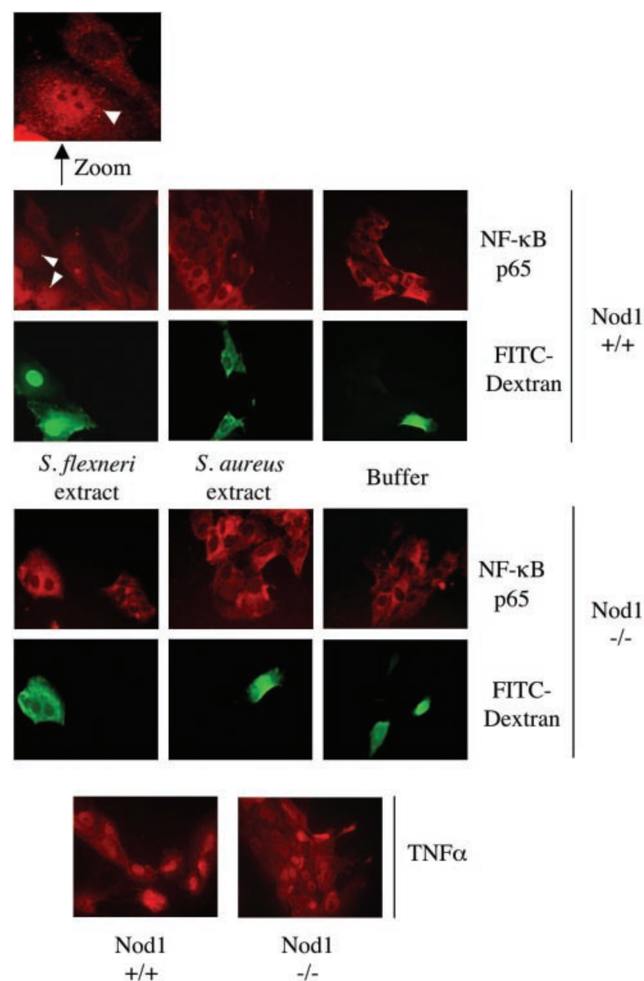

Fig. 4. Intestinal epithelial cells from mice deficient in Nod1 do not respond to bacterial supernatants. Microinjection of Gram-negative but not Gram-positive bacterial supernatants activates NF-κB in isolated intestinal epithelial cells from wild-type mice, as observed by nuclear translocation of the p65 subunit of NF-κB (as shown in Fig. 3B). In contrast, cells from Nod1-deficient mice are not activated by bacterial supernatants, although TNFα (10 ng/ml) can efficiently stimulate NF-κB nuclear translocation in these cells. Whereas no active cells were observed with Gram-positive bacterial supernatant microinjection, 98% of wild-type cells microinjected with Gram-negative bacterial supernatants showed translocated NF-κB in the nucleus. In the case of the Nod1-deficient cells, no active cells were observed in either case. Extracellular addition of either Gram-negative or Gram-positive bacterial supernatants failed to stimulate NF-κB nuclear translocation in wild-type or Nod1-deficient cells (*10*). Experiments were repeated three times independently with similar results.

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