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Combined Stimulation of Toll-Like Receptor 5 and NOD1 Strongly Potentiates Activity of NF-κB, Resulting in Enhanced Innate Immune Reactions and Resistance to *Salmonella enterica* Serovar Typhimurium Infection

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Pathogen recognition receptors (PRRs) are essential components of host innate immune systems that detect specific conserved pathogen-associated molecular patterns (PAMPs) presented by microorganisms. Members of two families of PRRs, transmembrane Toll-like receptors (TLRs 1, 2, 4, 5, and 6) and cytosolic NOD receptors (NOD1 and NOD2), are stimulated upon recognition of various bacterial PAMPs. Such stimulation leads to induction of a number of immune defense reactions, mainly triggered via activation of the transcription factor NF-κB. While coordination of responses initiated via different PRRs sensing multiple PAMPS present during an infection makes clear biological sense for the host, such interactions have not been fully characterized. Here, we demonstrate that combined stimulation of NOD1 and TLR5 (as well as other NOD and TLR family members) strongly potentiates activity of NF-κB and induces enhanced levels of innate immune reactions (e.g., cytokine production) both *in vitro* and *in vivo*. Moreover, we show that an increased level of NF-κB activity plays a critical role in formation of downstream responses. In live mice, synergy between these receptors resulting in potentiation of NF-κB activity was organ specific, being most prominent in the gastrointestinal tract. Coordinated activity of NOD1 and TLR5 significantly increased protection of mice against enteroinvasive *Salmonella* infection. Obtained results suggest that cooperation of NOD and TLR receptors is important for effective responses to microbial infection *in vivo*.

Innate immunity is the first line of host organism defense against invading pathogens. Innate immune responses are triggered via detection of conserved pathogen-associated molecular patterns (PAMPs) presented by pathogens by specific pathogen recognition receptors (PRRs) expressed on different types of host cells. Various PRRs have been identified in mammals, including transmembrane Toll-like receptors (TLRs), C-type lectin receptors (CLRs), cytosolic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and others (1). The critical role played by PRRs in formation of efficient protective immunity has been demonstrated in a number of animal models in which particular PRR genes are knocked out or their expression is knocked down. In these types of TLR- and NOD-defective animals, responses to the specific PAMP recognized by the targeted PRR are impaired (as evidenced by lack of induction of antimicrobial peptides, proinflammatory cytokines, etc.). In addition, TLR- and NOD-defective animals showed increased susceptibility to various bacterial infections (2–5).

Under conditions of real infection, it is likely that several types of PRRs are simultaneously activated by multiple PAMPs presented by the pathogen (6). The physiological response to combined activation of several types of PRRs could be significantly different from that induced by activation of an individual PRR. Indeed, recent studies showed synergistic effects when members of two families of PRRs were activated simultaneously: transmembrane TLR receptors (TLR2, TLR4, or TLR5 recognizing bacteria-derived molecules such as lipoteichoic acid, lipopolysaccharide, and flagellin, respectively) and cytosolic NLRs (NOD1 and NOD2 recognizing fragments of bacterial peptidoglycan: D-glutamyl-meso-diaminopimelic acid [iE-DAP] and muramyl dipeptide [MDP], respectively) (7, 8). *In vitro* studies showed that combined stimulation of NOD-like (NOD1 or NOD2) and Toll-like (TLR2, TLR3, or TLR4) receptors significantly increased (up to 5- to 10-fold) secretion of a number of cytokines, including interleukin-1α (IL-1α), IL-1β, IL-8, IL-10, IL-12p70, tumor necrosis factor alpha (TNF-α), etc., by bone marrow-derived macrophages (BMDM) and peripheral blood mononuclear cells (PBMC) (9, 10). It was also shown that TLR4-tolerized macrophages remained responsive to NOD1 and NOD2 stimulation, as evidenced by production of IL-6 and TNF-α (9). In addition, mice deficient in both NOD1 and NOD2 showed decreased resistance to *Listeria monocytogenes* after induction of TLR4-mediated tolerance in contrast to wild-type animals (9). Taken together, these experiments demonstrate interaction between NOD and TLR signaling pathways in production of immune responses; however, the molecular mechanisms

Received 30 April 2013 Returned for modification 24 May 2013
Accepted 23 July 2013
Published ahead of print 29 July 2013
Editor: A. J. Bäumler
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.00525-13.

doi:10.1128/IAI.00525-13

underlying such interaction and whether this interaction translates into enhanced efficacy of antibacterial immune responses have not been established.

NF-κB is a key regulator of immune responses, controlling expression of numerous proteins that contribute to various immune reactions such as cytokines (IL-6, IL-8, TNF-α, CCR5, etc.), cytokine receptors (CCR2, CCR7, IL2RA, etc.) antimicrobial factors (IER-3, DEFB4, lactoferrin, CRP, etc.), adhesion molecules (selectin E and VCAM1), and antiapoptotic proteins (Bcl-Xl, Bcl2L1, and Bcl2A1) (11). NF-κB is activated downstream of both TLR and NOD receptors and is, therefore, a likely candidate for the main mediator of synergistic effects of combined NOD and TLR stimulation (11).

In this study, we showed a critical role of NF-κB in production of enhanced levels of immune effectors (e.g., cytokines and antimicrobial peptides) after combined stimulation of members of the NLR and TLR receptor families, primarily NOD1 and TLR5, *in vitro* in human THP-1 cells. Moreover, using transgenic mice carrying an NF-κB-dependent luciferase (Luc) reporter gene in their germ line, we studied (using live imaging and *ex vivo* tissue analyses) the kinetics and organ specificity of NF-κB activation after administration of NOD1 and TLR5 agonists as single agents or in combination. These experiments demonstrated potentiation of both NF-κB activation and production of downstream effectors such as cytokines and antimicrobial peptides when NOD1 and TLR5 receptors were stimulated simultaneously and showed that enhancement of cytokine production required NF-κB activity. *In vivo*, the synergistic effect of combined NOD1 and TLR5 (NOD1+TLR5) stimulation was seen in only a subset of analyzed organs and was strongest in the small intestine. This synergy translated into significant enhancement of mouse resistance to infection with enteroinvasive *Salmonella*.

MATERIALS AND METHODS

Mice. Inbred BALB/c female mice weighing 18 to 20 g purchased from the Pushchino nursery (Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Pushchino, Russia) were used for cytokine and survival assays. *Salmonella* infection of female BALB/c mice was done at the Gamaleya Institute (Moscow, Russia) under biosafety level 2 (BSL-2)-equivalent conditions using NIH-approved ethical standards. For *in vivo* detection of NF-κB activity, 6- to 8-week-old BALB/c-Tg(*IκBα-luc*)Xen mice (Jackson Laboratory, Bar Harbor, ME) carrying an NF-κB-inducible luciferase reporter gene (Xenogen, Alameda, CA) were used at Roswell Park Cancer Institute (Buffalo, NY, USA) following protocols approved by the Roswell Park Cancer Institute IACUC. The mice were fed a complete pelleted laboratory chow and had access to food and tap water *ad libitum*.

Reagents. The following PRR ligands were used: synthetic TLR2 and TLR5 agonists CBLB612 and CBLB502, respectively (Cleveland BioLabs, Inc. Buffalo, NY, USA), and NOD1 and NOD2 agonists C12-iE-DAP and L18-MDP, respectively (InvivoGen, San Diego, CA, USA). Lipopolysaccharide (LPS) purified by gel filtration chromatography was purchased from Sigma-Aldrich (USA).

A Bradford protein assay kit was purchased from Bio-Rad (USA), complete protease inhibitor cocktail tablets were from Roche Diagnostics (Deutschland GmbH, Mannheim, Germany), and tissue protein extraction reagent (T-PER) was purchased from Pierce (Rockford, IL, USA). Inhibitors celastrol, triptolide, gefitinib, and dexamethasone (DEX) were obtained from (InvivoGen, San Diego, CA, USA).

Cultured cells. THP1-XBlue-CD14 cells (InvivoGen, USA) derived from THP-1 human acute monocytic leukemia cells were maintained at approximately 1×10^6 cells/ml in RPMI 1640 (Gibco, USA) medium, supplemented with 10% fetal calf serum (Thermo scientific, USA), 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 0.1 M $NaHCO_3$ (all from PanEco, Russia), 200 μg/ml Zeocin (a formulation containing phleomycin D1), and 250 μg/ml G418 (both from InvivoGen, USA) at 37°C with 5% CO_2.

Mouse infection studies. *Salmonella enterica* serovar Typhimurium IE147 strain was the kind gift of L. N. Nesterenko and Y. M. Romanova (N. F. Gamaleya Research Institute for Epidemiology and Microbiology, Moscow, Russia). Bacteria were cultured overnight in LB broth at 37°C with shaking at 300 rpm. An overnight culture of *S.* Typhimurium was washed with phosphate-buffered saline (PBS) and adjusted to 2.5×10^7 CFU/ml. The number of CFU was determined on the next day by counting colonies that grew on *Salmonella-Shigella* (SS) agar (Himedia, India). Female BALB/c mice were injected subcutaneously (s.c.) with PBS or with CBLB502 (1 μg/mouse), C12-iE-DAP (200 μg/mouse), or their combination 9 h before oral administration of 5×10^6 CFU (0.2 ml) of *S.* Typhimurium. The time period between PRR agonist injection and *S.* Typhimurium infection was selected based on previous experiments for induction of maximum protective effect (data not shown). For the survival experiment, mice (10 mice per group) were monitored for 35 days. For determination of bacterial load, spleens were isolated from mice (10 mice per group) at 3, 6, and 9 days after bacterial infection and homogenized in 0.5 ml of PBS using a FastPrep 24 device (MP Biomedicals, USA). Aliquots (100 μl) of diluted or undiluted homogenates were plated in duplicate on SS agar (Himedia, India). After overnight incubation at 37°C, colonies were counted manually.

***In vivo* and *ex vivo* NF-κB luminescence assays.** Female BALB/c-Tg(*IκBα-luc*)Xen reporter mice were injected s.c. with PBS vehicle or with CBLB502 (1 μg/mouse) or C12-iE-DAP (200 μg/mouse) alone or in combination. At 2, 4, 6, 8, or 10 h after PRR agonist injection, mice were injected intraperitoneally (i.p.) with D-luciferin (3 mg/mouse; Promega, USA) and anesthetized using 2.5% isoflurane. Luminescence images were captured 2 min after D-luciferin injection using an IVIS Imaging System, 100 series (Caliper Life Sciences, USA), with an integration time of 10 s. In parallel, luciferase activity was also measured in tissue (liver, spleen, kidney, lung, and small and large intestine) homogenates prepared at the same time points following PRR agonist treatment as described above. Small and large intestine sections (approximately 3 cm long) were dissected 2 cm below the stomach (referred to as duodenum) and 5 cm below the cecum (referred to as colon), correspondingly. Sections were surgically isolated from omentum and feces and washed in ice-cold PBS. Organ homogenates were prepared at +4°C in 1× Reporter Lysis Buffer (Promega, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich, USA) using a FastPrep 24 device with Lysing Matrix A (MP Biomedicals, USA). All homogenates were normalized (10 mg) by protein concentration using Bradford reagent (Sigma-Aldrich, USA). To detect luciferase activity, aliquots of homogenates (50 μl) were mixed with 50 μl of Bright-Glo Luciferase Assay Buffer containing luciferin substrate in a 96-well plate (all from Promega, USA). Plates were briefly vortexed and then immediately read using a Wallac 1420 plate reader (PerkinElmer, USA).

***In vitro* NF-κB assay.** Briefly, secreted embryonic alkaline phosphatase (SEAP) activity was determined as follows. Aliquots of culture medium were clarified by centrifugation at 14,000 × *g* for 2 min, heated at 65°C for 5 min to inhibit endogenous phosphatase activities, adjusted to 1× SEAP assay buffer (0.5 M carbonate, pH 9.8, 0.5 mM $MgCl_2$), and then incubated at 37°C for 10 min in a 96-well culture dish. Fifty microliters of 60 μM *p*-nitrophenylphosphate (Sigma-Aldrich, USA) dissolved in SEAP assay buffer (prewarmed to 37°C) was added to the mixture (to a final volume of 200 μl). The A_{405} of the reaction mixture was read in a Wallac 1420 plate reader (PerkinElmer, USA). SEAP activity is given in milliunits (mU) per ml. One milliunit is defined as the amount of phosphatase that hydrolyzes 1.0 pmol of *p*-nitrophenylphosphate per min, and this corresponds to an increase of 0.04 mU per min.

Western blot analysis. For evaluation of p65 nuclear translocation, THP1-XBlue-CD14 cells were seeded in duplicate in 6-cm dishes at a density of 1×10^6 cells/ml. The next day, cells were treated with NOD1 and TLR5 agonists, alone or in combination, or left untreated. Thirty minutes after addition of agonists, cells were harvested, and nuclear and cytoplasmic cell extracts were prepared using an NE-PER kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. For detection of PRR expression levels, total protein extracts from THP-1 cells were prepared using radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail (all, Sigma-Aldrich, USA). For *in vivo* detection of antimicrobial peptides, small intestine homogenates were prepared 16 h after s.c. administration of PRR agonists as described below (see the paragraph "*Ex vivo* cytokine/chemokine assay"). All samples were normalized based on total protein concentrations measured using Bradford reagent (Sigma-Aldrich, USA). Samples were mixed with 2× Laemmli sample buffer (Sigma-Aldrich, USA) and run under denaturation conditions on 15% polyacrylamide gels. Proteins were transferred to nitrocellulose Hybond C membranes (GE Healthcare, USA) using a semidry Trans-Blot Transfer Cell (Bio-Rad, USA). Primary antibodies were anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-p65 (Santa Cruz Biotechnology, CA, USA), anti-TLR5, anti-NOD1, and anti-beta-defensin-3 (Santa Cruz Biotechnology, USA). Horseradish peroxidase (HRP)-conjugated secondary anti-rabbit and anti-mouse antibodies were from GE Healthcare (GE Healthcare, Germany). Antibody-bound protein bands were visualized using an enhanced chemiluminescence (ECL) detection kit and cassette on Hyperfilm membrane (GE Healthcare, Germany).

***Ex vivo* cytokine/chemokine assay.** For *ex vivo* cytokine/chemokine assays, BALB/c mice were injected s.c. with PBS or with CBLB502 (1 μg/mouse), C12-iE-DAP (200 μg/mouse), or their combination. Two hours later, samples of small intestine, large intestine, and peripheral blood from tail vein were collected. Tissue samples were placed immediately into ice-cold T-PER extraction buffer containing complete protease inhibitor (Sigma-Aldrich, USA) and homogenized using a FastPrep 24 device with Lysing Matrix A (MP Biomedicals, USA). The homogenates were centrifuged at 12,000 rpm for 12 min at +4°C. All supernatant samples were normalized (to 10 mg/ml) by total protein concentration as measured using Bradford reagent (Sigma-Aldrich, USA). Peripheral blood samples were incubated for 20 min at +37°C for clot formation. Serum samples were obtained using subsequent centrifugation at 1,000 rpm for 10 min. The levels of 20 cytokines and chemokines (IL-1α, -2, -4, -5, -6, -10, -13, -17, -21, -22, -27, gamma interferon [IFN-γ], TNF-α, CXCL1/keratinocyte-derived chemokine [KC], monocyte chemotactic protein 3 [MCP-3], MCP-1, macrophage inflammatory protein 1α [MIP-1α], MIP-1β, RANTES, and granulocyte-macrophage colony-stimulating factor [GM-CSF]) were measured in the prepared mouse serum and small and large intestine homogenate samples using mouse Th1/Th2 and chemokine bead-based FlowCytomix kits (Bender MedSystems GmbH, Austria) according to the manufacturer's instructions.

***In vitro* cytokine/chemokine assay.** THP1-XBlue-CD14 cells carrying an NF-κB-dependent SEAP reporter gene were seeded in 96-well plates at 10^5 cells per well in complete RPMI 1640 medium. Eighteen hours after cells were treated with PRR ligands, plates were centrifuged at 1,000 rpm for 10 min, and culture supernatants were collected. Levels of 19 cytokines and chemokines (IL-1β, -2, -4, -5, -6, -8, -9, -10, -12p70, -13, -17A, -22, IFN-γ, TNF-α, MCP-1, MIP-1α, MIP-1β, G-CSF, and monokine induced by IFN-γ [MIG]) were measured in the prepared supernatants in triplicate using human Th1/Th2/Th9/Th17/Th22 and chemokine bead-based FlowCytomix kits (Bender MedSystems GmbH, Austria) according to the manufacturer's instructions.

Statistical analysis. The data shown are representative results. Experimental values are given as the means ± standard deviations (SD) of triplicate assays. Groups were compared using Student's *t* test. In the mouse infection study Kaplan-Meier survival curves were compared using a log rank test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Combined NOD1 and TLR5 stimulation has a synergistic effect on NF-κB activation in THP-1 cells. Previous work showed that costimulation of NODs (NOD1 and NOD2) and TLRs (TLR2, TLR4, and TLR9) in various cell types resulted in enhanced production of several cytokines, such as interleukin-1β (IL-1β), IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) compared to stimulation of a single type of PRR (9, 10, 12–14). However, the molecular mechanism(s) underlying these enhanced responses were not defined. Since NF-κB controls expression of many cytokines and is activated by both TLR5 and NOD1 (11), we hypothesized that synergistic activation of NF-κB was responsible for the enhanced effect of costimulation with TLR5 and NOD1 (TLR5+NOD1) on cytokine production. We tested this hypothesis *in vitro* using THP1-XBlue-CD14 cells expressing endogenous NOD1 and TLR5 receptors (Fig. 1A) along with an introduced secreted embryonic alkaline phosphatase (SEAP) reporter gene controlled by an NF-κB-dependent promoter. These reporter cells were left untreated or treated with NOD1 and TLR5 agonists alone or in combination. For stimulation of NOD1, we used C12-iE-DAP, an acylated derivative of iE-DAP (D-glutamyl-meso-diaminopimelic acid, the moiety in bacterial molecules recognized by NOD1) (7). For activation of TLR5, we used CBLB502, a pharmacologically optimized derivative of the natural TLR5 agonist, bacterial flagellin (15). Based on SEAP expression in cells treated with C12-iE-DAP or CBLB502 as a single agent over a wide range of concentrations, the NOD1 agonist was a much weaker NF-κB activator than the TLR5 agonist (Fig. 1B)]. Interestingly, however, when used in combination with the TLR5 agonist, concentrations of NOD1 ligand that did not produce substantial NF-κB-dependent responses by themselves markedly increased (up to 3.88-fold) the already strong NF-κB-dependent response generated by TLR5 stimulation. Similarly, addition of CBLB502 at suboptimal concentrations significantly potentiated the NF-κB response after NOD1 stimulation (up to 6.76-fold). For many tested doses (from 10 pg/ml up to 100 μg/ml), the level of NF-κB-dependent reporter expression detected after combined stimulation of TLR5 and NOD1 was greater than the sum of the levels seen in cells with stimulation of only one receptor type. It is important to note that detected levels of NF-κB activity after combined stimulation of TLR5 and NOD1 could be reached using up to 100-fold higher doses of isolated agonist or in some cases could not be reached at all. These results indicate that costimulation of TLR5 and NOD1 receptors has a synergistic, rather than additive, effect on activation of NF-κB.

The results obtained in the SEAP reporter system were confirmed using a second, non-reporter-based method to assess NF-κB activation: Western blot analysis of nuclear translocation of the p65 subunit of NF-κB (Fig. 1C). In this experiment, suboptimal concentrations of TLR5 (10 ng/ml) and NOD1 (100 ng/ml) agonists applied to THP-1 cell cultures as single agents had no effect or a minimal effect on p65 nuclear accumulation, whereas their combination induced substantial p65 translocation.

It is noteworthy that combined stimulation of other TLRs (TLR2, TLR4, and TLR5) with either NOD1 or NOD2 produced similar synergistic effects on NF-κB activation (see Fig. S1 in the supplemental material). Therefore, positive cross talk between

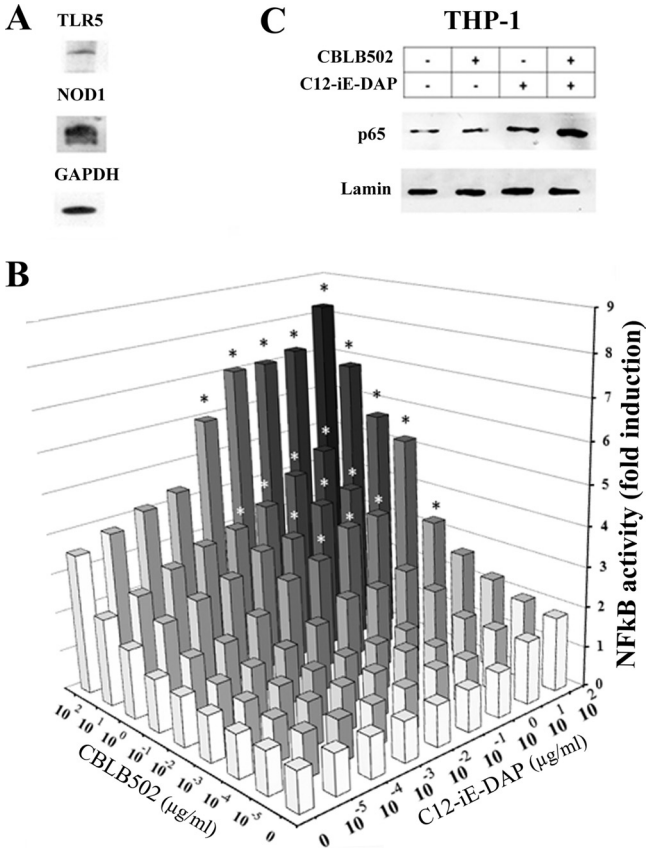

FIG 1 Combined stimulation of NOD1 and TLR5 receptors leads to enhanced NF-κB activity in THP1-XBlue-CD14 cells. (A) Western blot analysis of NOD1, TLR5, and GAPDH expression in THP1-XBlue-CD14 cells. (B) NF-κB activity (NF-κB-dependent SEAP reporter gene expression) in THP1-XBlue-CD14 cells treated for 18 h with the indicated doses (μg/ml) of C12-iE-DAP and CBLB502 (alone or in combination). Results are expressed as the fold increase in NF-κB activity relative to intact (untreated) cells (values are mean from three independent experiments, each performed in duplicate). Means were compared using the Student *t* test. Asterisks indicate significant differences ($P < 0.05$) in NF-κB activity between treatment with combined agonists and treatment with CBLB502 or C12-iE-DAP alone. (C) Detection of p65 nuclear translocation as an indicator of NF-κB activation in THP1-XBlue-CD14 cells left untreated or treated with C12-iE-DAP (100 ng/ml) or CBLB502 (10 ng/ml) alone or in combination. Thirty minutes after the addition of PRR agonists, nuclear protein extracts were prepared and assessed by Western blotting using anti-p65 and anti-lamin (loading control) antibodies.

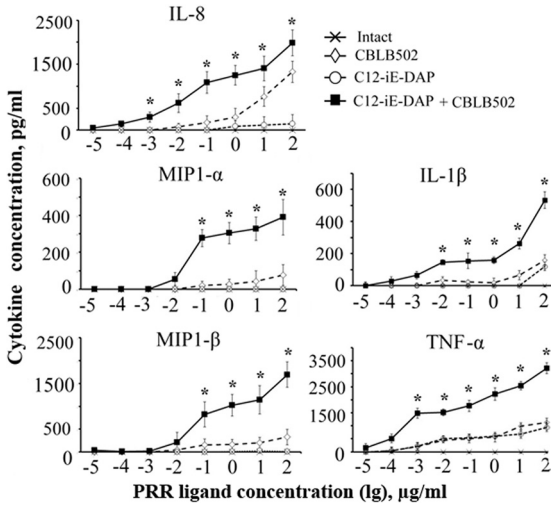

FIG 2 Combined stimulation of NOD1 and TLR5 receptors leads to enhanced cytokine production in THP1-XBlue-CD14 cells. Concentrations of IL-1β, IL-8, MIP-1α, MIP-1β, and TNF-α in culture supernatants of THP1-XBlue-CD14 cells treated with C12-iE-DAP, CBLB502, and their combination, as indicated, for 18 h. Intact cells (no treatment) were used as controls. For combined activation of NOD1 and TLR5, C12-iE-DAP was used at a fixed concentration of 1 μg/ml in combination with the concentrations of CBLB502 indicated on the *x* axis of the figure. Asterisks indicate significant differences ($P < 0.05$) in cytokine production levels between treatment with combined agonists and treatment with CBLB502 or C12-iE-DAP alone.

members of the TLR and NOD receptor families appears to be a general phenomenon that is not restricted to TLR5 and NOD1.

NF-κB plays a critical role in production of enhanced levels of cytokines after combined stimulation of TLR5 and NOD1 receptors. It was previously shown that combined stimulation of TLR2, TLR4, or TLR9 with NOD1 or NOD2 significantly enhanced production of IL-8 in cultured THP-1 cells (13). Here, we examined whether combined NOD1 and TLR5 stimulation enhanced cytokine production in THP-1 cells compared to stimulation of only NOD1 or TLR5. Levels of 19 cytokines (see Materials and Methods) were measured in culture supernatants collected 18 h after addition of C12-iE-DAP and/or CBLB502 to cell cultures using bead-based FlowCytomix kits. For isolated stimulation of NOD1 and TLR5 receptors, we used C12-iE-DAP and CBLB502, respectively, in final concentrations ranging from 10 pg/ml to 100 μg/ml. For combined stimulation, we used a fixed suboptimal C12-iE-DAP concentration (1 μg/ml) previously determined to provide maximal enhancement of the NF-κB response when used in combination with different concentrations of CBLB502. As seen for NF-κB activation, combined stimulation of NOD1 and TLR5 receptors resulted in significant enhancement of production of 5 of the 19 analyzed cytokines compared to stimulation of either single receptor type. Cytokines that showed a synergistic (greater than additive) response to combined TLR5+NOD1 stimulation included IL-1β (up to a 4.4-fold increase), IL-8 (up to a 7.8-fold increase), MIP-1α (up to a 13.2-fold increase), MIP-1β (up to a 6.2-fold increase), and TNF-α (up to a 4.1-fold increase) in comparison to isolated stimulation (Fig. 2).

Having demonstrated that costimulation of TLR5 and NOD1 leads to both potentiation of NF-κB activity and production of cytokines, we next tested whether NF-κB was required for the effect on cytokines. This was accomplished by using several inhibitors for blocking the RIP2-TAK1-IKK-NF-κB pathway: (i) gefitinib for inhibition of the upstream common signal kinase of TLR and NOD receptors (RIP2) (16), (ii) celastrol for inhibition of the RIP2-activated TAK1 signal kinase (17), (iii) triptolide for inhibition of NF-κB transcriptional activation (18), and (iv) synthetic glucocorticoid dexamethasone (DEX) to inhibit the activity of both the NF-κB and mitogen-activated protein kinase (MAPK)–AP-1 pathways (19). Celastrol (5 μM), triptolide (10 nM), gefitinib (10 μM), and DEX (100 μg/ml) were added in final concentrations to cultures of THP-1 cells 1 h prior to addition of PRR agonists (C12-iE-DAP at 1 μg/ml and/or CBLB502 at 1 μg/ml). Eighteen hours later, levels of NF-κB activity (NF-κB-dependent SEAP reporter expression) and cytokine production were assessed (Fig. 3A and B). IL-1β and TNF-α were measured since their production was found to be strongly enhanced by combined

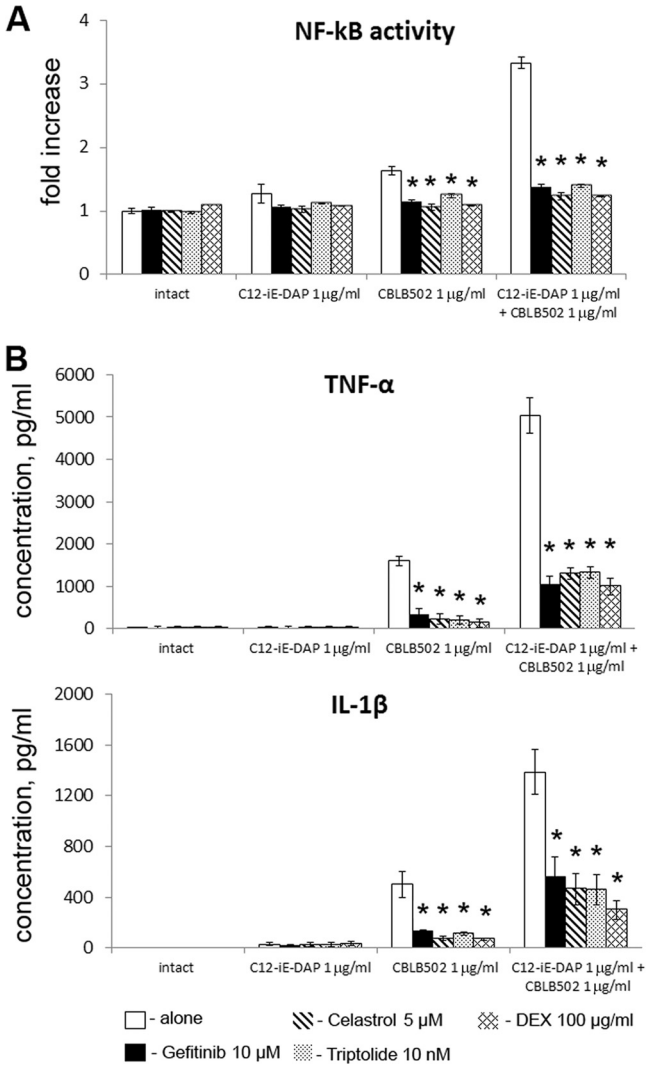

FIG 3 Role of NF-κB in enhanced cytokine production after combined stimulation of NOD1 and TLR5 receptors in THP1-XBlue-CD14 cells. (A) NF-κB activity (SEAP reporter expression) in THP1-XBlue-CD14 cells treated for 18 h with C12-iE-DAP (1 μg/ml), CBLB502 (1 μg/ml), or their combination without (alone) or with the inhibitor gefitinib (10 μM), celastrol (5 μM), triptolide (10 nM), or DEX (100 μg/ml), as indicated. Data are presented as the mean fold change relative to untreated cells (no PRR agonists and inhibitors) in three independent experiments, each performed with duplicates. Error bars indicated standard deviations (SD). Asterisks indicate significant differences ($P < 0.05$) in NF-κB responses between inhibitor-treated and untreated cells. (B) Concentrations of IL-1β and TNF-α in the culture supernatants of THP1-XBlue-CD14 cells treated with C12-iE-DAP (1 μg/ml), CBLB502 (1 μg/ml), or their combination without (alone) or with the inhibitor gefitinib (10 μM), celastrol (5 μM), triptolide (10 nM), or DEX (100 μg/ml), as indicated. Data are presented as the means ± SD (error bars) from three independent experiments, each performed with duplicates. Asterisks indicate significant differences ($P < 0.05$) in cytokine production levels between inhibitor-treated and untreated cells.

stimulation of NOD1 and TLR5. The results of this experiment showed that nontoxic concentrations of NF-κB inhibitors abrogated not only potentiation of the NF-κB response but also production of IL-1β and TNF-α. These data show the important role of enhancement of NF-κB activity in potentiation of cytokine production levels after combined TLR5 plus NOD1 stimulation. An inhibitor of both AP-1 and NF-κB pathways, DEX, was slightly more efficient in reducing production of IL-1β and TNF-α, reflecting a possible role of the MAPK–AP-1 pathway in the potentiation effect of cytokine production (9).

Combined NOD1 and TLR5 stimulation potentiates NF-κB activation *in vivo*. To determine whether the synergistic activation of NF-κB by combined NOD1 and TLR5 stimulation that was observed *in vitro* also occurs *in vivo*, we used BALB/c-Tg(*IκBα-luc*)Xen reporter mice carrying an NF-κB-dependent luciferase reporter gene. NF-κB activity in these mice can be detected by live imaging of bioluminescence in intact sedated animals after administration of NF-κB-activating agents (PRR ligands, UV radiation, etc.) and luciferin (20).

NF-κB-Luc transgenic mice were injected s.c. with 100 μl of C12-iE-DAP (200 μg/ml) or CBLB502 (1 μg/ml) alone or in combination. Doses of individual agonists resulting in suboptimal activation of NF-κB activity were determined in preliminary experiments (data not shown). Control mice were injected s.c. with 100 μl of PBS. NF-κB activity was detected by live imaging of mice at 2, 4, 6, 8, or 10 h after administration of PRR agonists by i.p. injection of D-luciferin 2 min before imaging. The obtained data showed that the NOD1 agonist C12-iE-DAP induced significantly lower levels of NF-κB activity than the TLR5 agonist CBLB502 (Fig. 4A). When used as single agents, both PRR agonists caused maximal reporter induction at 2 to 4 h postinjection. The combination of C12-iE-DAP and CBLB502 also induced maximal NF-κB reporter activity at 2 to 4 h after administration. However, compared to single-agonist treatments, combined-agonist treatment produced more sustained NF-κB activation, which was strong even at 10 h after agonist injection, when the signals induced by either agent alone were practically undetectable. These imaging data also revealed strong enhancement of NF-κB activity in the abdominal region of mice that received the combination of agonists.

To obtain more precise quantitative results, we measured luciferase activity in tissue lysates of livers, spleens, kidneys, lungs, and small and large intestines isolated from NF-κB-Luc transgenic mice at different times after administration of PRR agonists (Fig. 4B). This showed that the degree of enhancement of reporter activation induced by combined stimulation of NOD1 and TLR5 compared to stimulation of either receptor type alone varied greatly between different organs. There was no significant enhancement detected in the liver and spleen with administration of combined agonists compared to administration of CBLB502 alone. In contrast, the luciferase signal in small intestines, large intestines, lung, and kidney was significantly higher in mice treated with combined NOD1 and TLR5 agonists than in mice treated with either agonist alone. Maximal levels of enhancement of NF-κB activation by the combination of agonists was detected in the small intestines. In this tissue, NF-κB showed synergistic activation at 4, 6, 8, and 10 h following combined PRR agonist treatment. The greatest effect was seen at the 10-h time point, with NF-κB showing 2.76-fold, 8.24-fold, and 60-fold activation (relative to PBS treatment) in samples from CBLB502-treated, C12-iE-DAP-treated, and CBLB502- and C12-iE-DAP-treated mice, respectively. Thus, combined TLR5+NOD1 stimulation led to induction of NF-κB that was 7.5-fold stronger than the sum of the induction produced by treatment with CBLB502 and C12-iE-DAP separately. It is notable that the maximal synergistic effect in the small intestine was observed at 10 h after agonist administra-

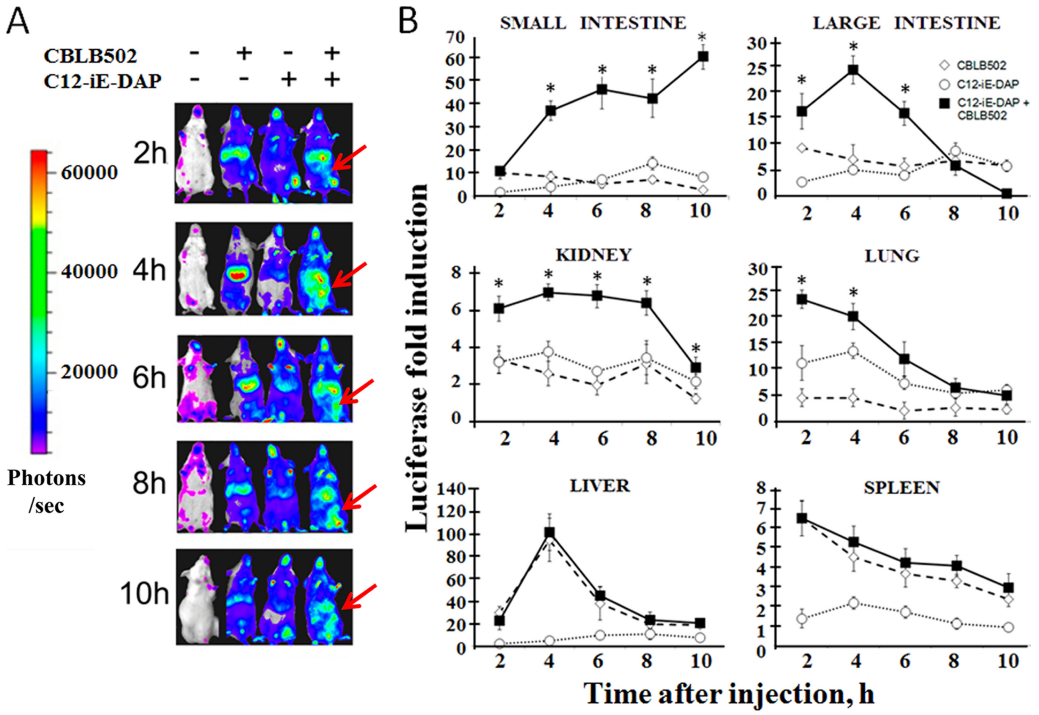

FIG 4 Kinetics of NF-κB-luciferase activity in NF-κB-Luc transgenic mice exposed to TLR5 and NOD1 ligands. (A) *In vivo* imaging of NF-κB-luciferase activity in live NF-κB-Luc transgenic mice at 2, 4, 6, 8, or 10 h after s.c. injection of PBS or of CBLB502 (1 μg/mouse), C12-iE-DAP (200 μg/mouse), or their combination. Mice were anesthetized and injected i.p. with D-luciferin prior to imaging. Pseudocolored images reflect the intensity of bioluminescence (i.e., NF-κB activity) according to the scale shown on the left side of the figure, with red indicating the most intense light emission and purple indicating the weakest signal. Three additional experiments gave results similar to those shown here. Red arrows indicate enhancement of the NF-κB-dependent luciferase reporter signal in the abdominal region of mice that received CBLB502 and C12-iE-DAP in combination. (B) NF-κB activity in tissue homogenates prepared from NF-κB-Luc transgenic mice at the indicated time points after injection of 200 μg/mouse C12-iE-DAP alone, 1 μg/mouse CBLB502 alone, or their combination. Control mice were injected with PBS. Results are expressed as the fold increase in luciferase activity relative to PBS-treated control animals. Each point represents the mean of three mice per group ± SD (error bars). Data shown are pooled from two independent experiments. Asterisks indicate significant differences ($P < 0.05$) in NF-κB activity levels between combined agonists administration and CBLB502 or C12-iE-DAP alone.

tion; at this late time point, NF-κB activity had significantly declined in all other tissues. Synergistic activation of NF-κB was also observed in the large intestine, kidney, and lung; however, its scale and duration were lower than in the small intestine. To determine whether the high level of NF-κB activity reached after combined stimulation of NOD1 and TLR5 could be achieved by using higher doses of TLR5 agonist as a single agent, we treated reporter mice with a range of ascending CBLB502 doses (from 0.04 μg to 25 μg/mouse in 5-fold increments) for 4 h (Fig. 5). In most of the studied organs, including kidney, spleen, large intestine, and liver, the levels of reporter activation reached by combined stimulation of NOD1 and TLR5 could be achieved by using higher doses of CBLB502 as a single agent. Significant enhancement of NF-κB activity in lungs after combined stimulation of NOD1 and TLR5 was likely due to strong NF-κB activation in response to C12-iE-DAP administration (the effect of C12-iE-DAP alone at 4 h is shown in Fig. 4B). In the small intestine, however, the enhanced level of NF-κB activity induced by combined stimulation of NOD1 and TLR5 could not be reached by much higher doses (25-fold increment) of CBLB502 administered as a single agent and could not be explained by an additive response to C12-iE-DAP (Fig. 4B).

Combined NOD1 and TLR5 stimulation potentiates local and systemic levels of cytokine and antimicrobial peptide production. Having demonstrated that combined NOD1 and TLR5 stimulation leads to synergistic activation of NF-κB in mouse small intestines, we examined its biological outcome in terms of cytokine production *in vivo*. For this purpose, BALB/c mice were injected s.c. with 100 μl of C12-iE-DAP (200 μg/ml) or CBLB502 (1 μg/ml) alone or in combination. Control mice were injected s.c. with 100 μl of PBS. Two hours after administration of PRR ligands, mice were euthanized, and small intestine, liver, and blood samples were isolated for measurement of cytokine levels in tissue homogenates and blood serum using bead-based immunoassays as described above. In mouse serum, production of 3 of the 20 measured cytokines (IL-6, IL-22, and TNF-α) was enhanced following combined TLR5+NOD1 stimulation compared to stimulation of either receptor alone (Fig. 6A). In mouse small intestine samples, 6 of the 20 measured cytokines (IL-5, -6, -13, -21, -22, and TNF-α) showed enhanced production following combined TLR5+NOD1 stimulation (Fig. 6B). In both serum and small intestine samples, combined TLR5+NOD1 stimulation had a synergistic effect (greater than the additive effect of single-receptor stimulation) on production of at least some of the induced cytokines, including IL-6 in serum and IL-5, IL-13, and TNF-α in small intestines.

In contrast to what was observed in serum and small intestine samples, none of the analyzed cytokines showed enhanced production in liver samples from mice treated with combined TLR5+NOD1 agonists compared to each agonist alone (data not shown). These data demonstrate that combined stimulation of

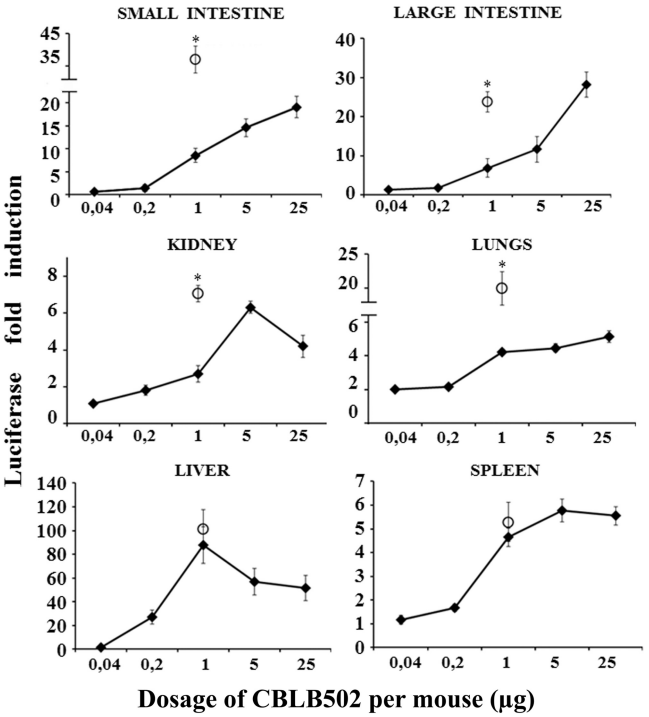

FIG 5 NF-κB-dependent luciferase reporter activity in NF-κB-Luc transgenic mice injected with different doses of CBLB502 versus combined injection of CBLB502 and C12-iE-DAP. Luciferase levels were measured in tissue homogenates prepared 4 h after injection of NF-κB-Luc reporter mice with 0.04 μg, 0.2 μg, 1 μg, 5 μg, or 25 μg of CBLB502 per mouse as indicated on the *x* axis. Results are expressed as the mean fold change in luciferase activity relative to the mean in PBS-treated control mice ($n = 5$ mice per group). Open circles represent the fold induction of luciferase 4 h after combined injection of CBLB502 (1 μg/mouse) and C12-iE-DAP (200 μg/mouse). Error bars indicate SD. Asterisks indicate significant differences ($P < 0.05$) in NF-κB activity levels between administration of combined agonists and of CBLB502 alone.

NOD1 and TLR5 receptors has both local, organ-specific (particularly in the small intestine) effects as well as systemic (blood serum) effects involving synergistic cytokine production. The observed correlation between enhancement of cytokine production and enhancement of NF-κB activation (see above) is consistent with potentiation of the cytokine response being NF-κB dependent.

As an additional indicator of the effect of combined TLR5+NOD1 stimulation on NF-κB-dependent immune responses, we evaluated production of beta-defensin-3, which was previously shown to be induced during bacterial infection (21). Using Western blot analysis of small intestine homogenates, we found that stimulation of TLR5 with CBLB502 induced production of beta-defensin-3. There was no production of beta-defensin-3 following stimulation of NOD1 with C12-iE-DAP; however, combined stimulation of NOD1 and TLR5 led to increased production of these peptides relative to that observed with TLR5 stimulation alone (Fig. 6C). Taken together, these results clearly indicate that combined stimulation of TLR5 and NOD1 receptors potentiates NF-κB activation and downstream immune responses not only *in vitro* but also *in vivo*.

Combined TLR5 and NOD1 stimulation enhances resistance of mice to *Salmonella* infection. The results described above suggested that combined TLR5 and NOD1 stimulation might lead to more effective antimicrobial defense responses than stimulation of either receptor alone, particularly in the small intestine. To test this possibility *in vivo*, we used an enteropathogenic bacterial strain, *Salmonella enterica* serovar Typhimurium. BALB/c mice were injected s.c. with PBS or with C12-iE-DAP (200 μg/ml), CBLB502 (1 μg/ml), or their combination 9 h before oral infection with 5×10^6 CFU of *S.* Typhimurium in a volume of 0.5 ml. Bacterial loads were monitored in spleens isolated 3, 6, and 9 days after infection (Fig. 7A). The bacterial loads in spleens of mice treated with C12-iE-DAP or CBLB502 alone were not significantly different from those in spleens from PBS-treated mice. In contrast, spleens from mice treated with a combination of C12-iE-DAP and CBLB502 to stimulate both NOD1 and TLR5 had 10-fold fewer bacterial CFU. This reduced bacterial load resulted in significantly prolonged mouse survival: on day 35 after infection, 80% of mice treated with both C12-iE-DAP and CBLB502 were alive, compared to only 20% of mice treated with PBS or either PRR agonist alone (Fig. 7B). Therefore, in this animal model of enteropathogenic bacterial infection, combined stimulation of NOD1 and TLR5 receptors is critically important for efficient bacterial clearance and improved mouse survival. These results illustrate the key role that synergistic activation of immune responses stemming from stimulation of multiple PRRs likely plays in a broad spectrum of scenarios of *in vivo* pathogen exposure.

DISCUSSION

Microorganisms contain various molecular structures that stimulate specific PRRs expressed by host cells. Multiple PRRs with different cellular localizations are activated by any given microorganism, and this leads to production of an appropriate immune response (6). This redundancy in stimulation of PRRs and the multiplicity of activated host signaling pathways provide a number of biological advantages for the host, the importance of which is indicated by the fact that pathogens frequently acquire tools to alter PRR-mediated stimulation of immune responses. Thus, during microbial infection, PRR-mediated immune responses can be blocked by either direct microbial inhibition of PRR activation or PRR-mediated signaling pathways (22, 23) or by induction of PRR tolerance via prolonged exposure to PRR ligands (24). Under conditions of suppressed PRR signaling, activation of different PRRs could be crucial for induction of a minimal immune response sufficient for effective pathogen clearance (9). Another potential advantage of combined detection of multiple pathogen structures by different types of PRRs could be enhancement of the intensity of the generated immune responses compared to single PRR stimulation.

There are several reports in the literature demonstrating synergy between members of at least two different families of PRRs: NLRs (NOD1 and NOD2) and TLRs (TLR2, TLR3, TLR4, TLR7, and TLR9) (9, 10, 12–14). Most of the reported data show that combined stimulation of NOD and TLR receptors by corresponding agonists results in enhancement of cytokine production levels in various cell lines. These studies raised several important questions related to the interplay between NLRs and TLRs, including the following: (i) what molecular mechanism(s) underlies enhancement of cytokine production after combined stimulation of NOD and TLR receptors, (ii) whether the synergy between TLR and NOD receptors is relevant to all members of the TLR and NOD receptor families or restricted to certain receptor combinations, (iii) whether other (noncytokine) immune effector mole-

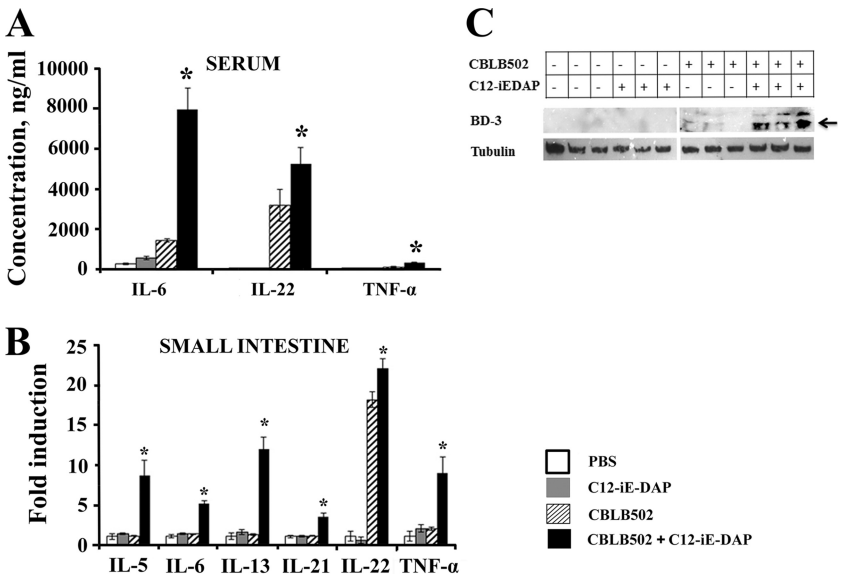

FIG 6 Combined stimulation of NOD1 and TLR5 receptors results in potentiation of cytokine production in mouse serum and small intestine homogenates. (A and B) Mice ($n = 5$ per group) were injected s.c. with PBS or with CBLB502 (1 μg/mouse), C12-iE-DAP (200 μg/mouse), or their combination. Blood and small intestine samples were collected 2 h after PRR ligand administration. (A) Concentrations of IL-6, IL-22, and TNF-α were measured in blood serum. The mean absolute cytokine concentration ± SD is shown. Asterisks indicate significant differences ($P < 0.05$) in NF-κB activity levels between administration of combined agonists and of CBLB502 alone. (B) Concentrations of IL-5, IL-6, IL-13, IL-21, IL-22, and TNF-α were measured in small intestine homogenates. The mean fold change in cytokine concentration relative to the mean concentration in PBS-treated animals is shown. Error bars indicate SD. Asterisks indicate significant differences ($P < 0.05$) in NF-κB activity levels between administration of combined agonists and of CBLB502 alone. (C) Mice ($n = 3$ per group) were injected s.c. with PBS or with CBLB502 (1 μg/mouse), C12-iE-DAP (200 μg/mouse), or their combination. Homogenates were prepared from small intestine samples collected 16 h after PRR ligand administration and used for Western blot analysis of beta-defensin-3 (BD3) and tubulin (loading control). The arrow indicates beta-defensin-3. The experiment was repeated three times.

cules are synergistically activated following combined stimulation of TLR and NOD receptors, and (iv) whether the synergy resulting from combined stimulation of NOD and TLR receptors results in enhanced antimicrobial protection *in vivo*. The objective of our work was to address these issues.

First, since both NOD and TLR receptors activate the transcription factor NF-κB, which serves as a major regulator of immunity (11), we investigated whether combined stimulation of members of the TLR and NOD receptor families enhanced NF-κB activation relative to stimulation of each receptor alone. In these experiments, we used THP-1 cells, which naturally express multiple TLR and NOD receptors, allowing for combined stimulation of TLR2, -4, and -5 and NOD1 and NOD2 receptors. Therefore, this study is the first report indicating that combined stimulation of NOD1+TLR5, as well as combined stimulation of NOD1 and TLR2 or TLR4 or of NOD2 and TLR2, TLR4, or TLR5, leads to significant greater-than-additive potentiation of NF-κB activation (see Fig. S1 in the supplemental material). This indicated that synergy between TLR and NOD receptors is likely a common characteristic of all members of those receptor families. These experiments also implicated NF-κB as the likely mediator of subsequent enhanced immune responses. We confirmed this by evaluating cytokine production in THP-1 cells treated with single or combined NOD1 and TLR5 agonists in the presence or absence of the different NF-κB inhibitors (celastrol and triptolide) and an inhibitor of the common upstream kinase RIP2 (gefitinib). Using inhibitors, we showed that production of IL-1β and TNF-α could be blocked by inhibiting the NF-κB pathway. Therefore, this is the first report showing that the effect of enhanced cytokine production triggered by combined activation of NOD and TLR receptors *in vitro* is mediated by enhanced NF-κB activation. However, the most significant (up to 2.7-fold) inhibition of cytokine production was observed using DEX, which shows that MAPK could also be implicated in the potentiation effect after combined stimulation of NOD1 and TLR5.

Importantly, we also demonstrated that combined stimulation of NOD1 and TLR5 led to potentiation of NF-κB activation *in vivo*. Transgenic NF-κB-Luc reporter mice allowed us to show that such potentiation occurs in an organ-specific manner *in vivo*. NF-κB activity in liver and spleen was not enhanced after combined administration of the NOD1 agonist C12-iE-DAP and the TLR5 agonist CBLB502 compared to injection of CBLB502 alone. This result might be explained in part by the fact that liver is a primary target organ of CBLB502, showing rapid and intense NF-κB activation following *in vivo* administration of the drug (25). Potentiation of NF-κB activity following combined NOD1+TLR5 stimulation was observed in kidney, small intestine, and large intestine samples but was most prominent in small intestine. In this tissue, the level of NF-κB activation induced by combined treatment with CBLB502 (1 μg/mouse) and C12-iE-DAP (200 μg/mouse) was even higher than that induced by much greater doses of CBLB502 administered as a single agent (25 μg/mouse). Correlating with this potentiation of NF-κB activation, we also observed enhanced production of a subset of analyzed cytokines (IL-5, IL-6, IL-13, IL-21, IL-22, and TNF-α) and analyzed antimicrobial peptide (beta-defensin-3) in small intestine samples from mice treated with combined NOD1 and TLR5 agonists compared to samples from mice treated with each agonist alone. In addition to these local effects in the small intestine, potentiation of systemic (serum) cytokine levels was observed for

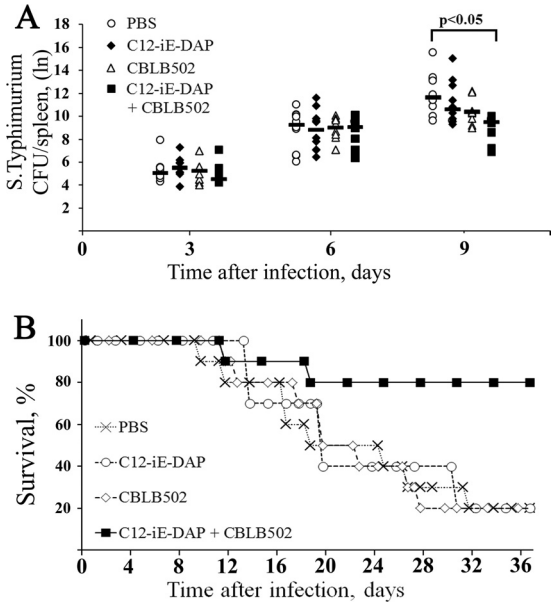

FIG 7 Combined stimulation of TLR5 and NOD1 results in improved immunity against *Salmonella* serovar Typhimurium *in vivo*. (A) *S.* Typhimurium titers in the spleens (CFU/organ) of mice injected s.c. with PBS or with CBLB502 (1 μg/mouse), C12-iE-DAP (200 μg/mouse), or their combination 9 h prior to oral infection with a lethal dose (5×10^6 CFU) of *Salmonella* serovar Typhimurium. Bacterial loads were monitored in spleens isolated at 3, 6, and 9 days after infection by spleen homogenates on LB medium. Symbols represent individual mice, and the horizontal bars indicate per-group means ($n = 10$ mice/group). *P* values were calculated with Student's *t* test. Bacterial loads were statistically significant ($P < 0.05$) between administration of combined agonists and administration of PBS. (B) Kaplan-Meier survival curves for groups of mice treated with PBS, CBLB502, C12-iE-DAP, or CBLB502 plus C12-iE-DAP ($n = 10$/group) as described for panel A. *P* values were calculated with a log rank test. Survival of the mice administered a combination of CBLB502 and C12-iE-DAP versus administration of PBS, CBLB502, or C12-iE-DAP was determined to be significantly different ($P < 0.05$).

IL-6, IL-22, and TNF-α. Notably administration of other combinations of PRR ligands (TLR2/NOD1, TLR2/NOD2, TLR4/NOD1, TLR4/NOD2, and TLR5/NOD2) also greatly enhanced cytokine expression in small intestine compared to expression with each agonist alone (see Fig. S2 in the supplemental material).

The findings described above suggest that TLR5 and NOD1 cooperate to provide an effective immune defense in the small intestine. Previous studies demonstrated an important role for NOD receptors in host defense at mucosal surfaces (5), but synergy between NOD and TLR receptors was not investigated. Here, using a mouse model of infection with the enteroinvasive bacteria *S.* Typhimurium, we showed that combined stimulation of NOD1 and TLR5 produced a much stronger immune response in the gastrointestinal tract than stimulation of either receptor alone. This was indicated by significantly reduced bacterial load and prolonged mouse survival. The observed protective effect was not connected with changes in cytokine response in small intestine. We found no significant difference in cytokine expression levels (such as TNF-α and IL-1α) in *Salmonella*-infected animals and animals pretreated with combinations of TLR and NOD ligands before infection (see Fig. S3 in the supplemental material). According to our published data (25), we suppose that the protective effect observed after combined treatment of TLR5 and NOD1 ligands most probably connected with mobilization of immune cells to the organ (changes in tissue homeostasis) and changes in their functionality but not with changes in cytokine levels.

In summary, we showed that synergy between NOD and TLR receptors, which leads to enhanced activation of NF-κB and production of NF-κB-dependent immune effectors, translates into a meaningful biological effect in animals challenged by microbial infection. In general, cross talk between pattern recognition signaling pathways may allow a host to distinguish between real infections and isolated PAMPs and mount the most effective immune response (Fig. 8 shows a model of the synergistic activity).

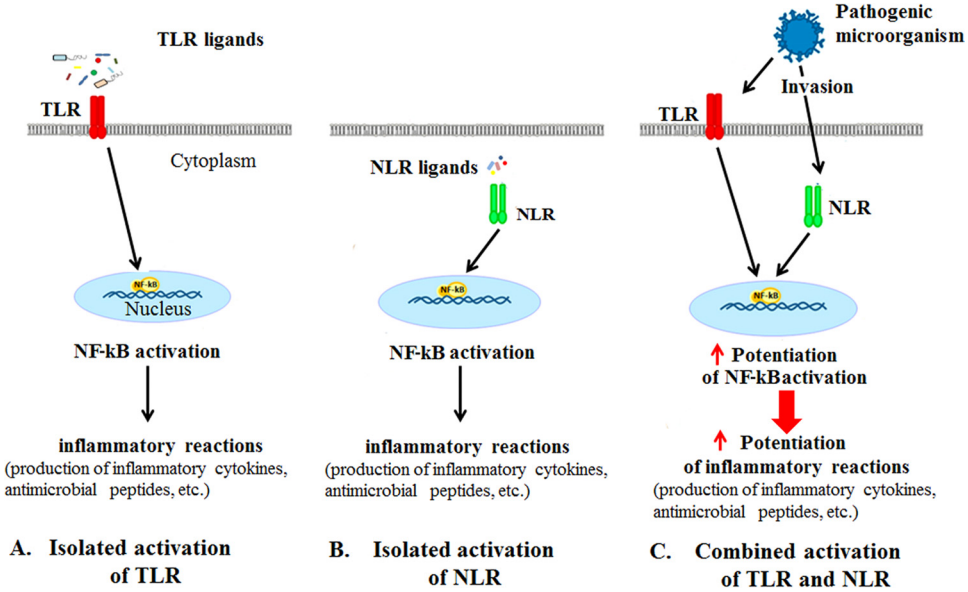

FIG 8 Schematic illustration of the synergistic activity of TLR and NOD receptors. Isolated activation of TLR or NOD receptors by specific PAMPs leads to induction of NF-κB-dependent immune responses. Pathogens present multiple PAMPs, which leads to combined stimulation of multiple PRRs, such as TLR and NOD receptors. Activation of multiple PRR-dependent signaling pathways results in enhanced NF-κB activation and enhanced NF-κB-regulated immune responses.

Further studies are required to elucidate the precise molecular mechanism(s) underlying the synergistic effects of combined TLR and NOD signaling. Such studies might add to our understanding of host-bacteria interactions including bacterial infections and possible beneficial roles of normal bacterial flora. Ultimately, this work may lead to exploration of specific combinations of NOD and TLR agonists as new immunostimulatory drugs capable of efficient microbial clearance and/or enhancement of the immunogenicity of vaccines.

ACKNOWLEDGMENTS

We thank Patricia Stanhope-Baker for help with manuscript editing and insightful suggestions and Inna Dolzhikova for technical assistance.

This work was funded by grants from Cleveland BioLabs, Inc., and NIH (R01AI080446 and RC2AI087616) to A.V.G. and by the Ministry of Education and Science of Russia (grant 8797) to D.V.S.

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