Nod1 acts as an intracellular receptor to stimulate chemokine production and neutrophil recruitment in vivo

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Nod1 is a member of family of intracellular proteins that mediate host recognition of bacterial peptidoglycan. To characterize immune responses mediated by Nod1, synthetic ligand compounds possessing enhanced ability to stimulate Nod1 were developed to study the function of Nod1. Stimulation of epithelial cells with Nod1 stimulatory molecules induced chemokines and other proinflammatory molecules that are important for innate immune responses and recruitment of acute inflammatory cells. Administration of Nod1 ligands into mice induced chemokines and recruitment of acute inflammatory cells, an activity that was abolished in Nod1-null mice. Microarray analysis revealed that Nod1 stimulation induces a restricted number of genes in intestinal epithelial cells compared with that induced by tumor necrosis factor (TNF) α. Nod1 stimulation did not induce TNFα, interleukin 12, and interferon γ, suggesting that the primary role of Nod1 is to induce the recruitment of immune cells. These results indicate that Nod1 functions as a pathogen recognition molecule to induce expression of molecules involved in the early stages of the innate immune response.

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Abbreviations used: AcAiQ, D-Ala-Gln-octadecyl ester; AcMTP, MurNAc-L-Ala-γ-D-Glu-L-Lys(stearoyl)-OH; DAP, diaminopimelic acid; GlcNAc, *N*-acetyl-glucosamyl; HEK, human embryonic kidney; iE-DAP, γ-D-glutamyl-*meso*-DAP; iQ-DAP, γ-D-glutaminyl-*meso*-DAP; MurNAc, *N*-acetyl-muramyl; PGN, peptidoglycan; TLR, Toll-like receptor.

Recognition of bacterial components by host-specific molecules is the first step in the defense against invading bacteria (1). In the initial encounter with pathogenic bacteria, epithelial surfaces provide a physical barrier but also produce several molecules that are microbicidal or that inhibit bacterial growth (1). A second line of defense is mediated through host cellular receptors that recognize molecules uniquely expressed by bacteria, and upon activation they induce innate and adaptive immune responses to effectively eliminate the invading microbe. Toll-like receptors (TLRs) are a major class of host molecules that are activated by microbial products, including LPS, lipoproteins, and bacterial DNA (1, 2). In addition to TLRs that recognize bacterial ligands at the plasma membrane and luminal side of intracellular vesicles, a family of proteins called Nods, or Caterpiller, provide sensing of bacteria in the cytosol (2–4). The Nod protein family is comprises more than 20 members, including Nod1, Nod2, Cryopyrin, and Ipaf. The majority of Nod proteins are composed of an amino-terminal effector domain involved in downstream signaling, a centrally located nucleotide-binding oligomerization domain, and carboxyl-terminal leucine-rich repeats.

Nod1 is the founding member of the Nod protein family, which is expressed in multiple tissues and cells, including intestinal epithelia (2, 5). The core structure of the ligand recognized by Nod1 is a peptidoglycan (PGN)-specific dipeptide, γ-D-glutamyl-*meso*-diaminopimelic

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acid (iE-DAP), which exists as insoluble PGN, soluble PGN fragments, and intermediates produced during PGN biosynthesis (6, 7). There is evidence that Nod1 mediates host recognition of bacteria or bacterial fractions containing soluble PGN-related molecules with the essential iE-DAP dipeptide (8–12). Upon recognition of its bacterial ligand, Nod1 induces activation of NF-κB and mitogen-activated protein kinase, two signaling pathways that are involved in cellular responses against bacteria (5–13). Activation of intracellular signaling through Nod1 is mediated by RICK, a caspase recruitment domain–containing kinase that interacts with Nod1 (5, 13–15). However, the immune responses elicited by Nod1 stimulation are poorly characterized.

Bacteria containing Nod1 stimulatory molecules also possess TLR ligands, including LPS, that induce innate immune responses in host cells (1). There is evidence that Nod and TLR proteins act cooperatively to modulate immune responses against bacteria. For example, stimulation of Nod1 alone induces the secretion of very low levels of IL-6 and TNFα in macrophages, but the response is greatly enhanced by co-stimulation with LPS (6). Recent reports also suggest that Nod1 plays a role in intestinal immunity by regulating innate immune responses of epithelial cells infected with invasive enterobacteria and *Helicobacter pylori* (10, 11). The intestinal lumen contains a large number of resident bacteria that live in a symbiotic relationship with the host (16). Commensal bacteria express a wide array of stimulatory TLR ligands, but the organism has developed mechanisms to avoid innate recognition and harmful inflammatory responses at intestinal sites (17). These include a lack or low levels of TLRs such as TLR2 and TLR4 in intestinal epithelial cells and expression of molecules that inhibit TLR signaling (17). Given that Nod1 is functionally expressed in intestinal epithelial cells (5, 9, 10, 18), we hypothesized that stimulation of Nod1 by bacterial products might be sufficient to induce immune responses that are important for host defense against invading bacteria. In this paper, we demonstrate that stimulation of cultured cells and mice with bacterial products containing the essential iE-DAP dipeptide induces innate immune responses in a Nod1-dependent manner.

RESULTS

Addition of peripheral structures to the core dipeptide recognized by Nod1 does not result in increased stimulatory activity

Stimulation of host cells by iE-DAP alone induces very low levels of IL-6 and TNFα secretion (6). Furthermore, high doses of iE-DAP, compared with TLR ligands, are required to induce chemokines in intestinal epithelial SW620 and oral epithelial HSC-2 cells (19, 20). The high dose of Nod1 ligand required for Nod1 stimulation has hampered the analysis of Nod1-mediated immune responses in vitro and in vivo. To circumvent this problem, we sought to develop synthetic compounds that posses an enhanced ability to stimulate Nod1. Previous reports suggest that the peripheral structures of iE-DAP–containing molecules (Fig. 1 A) affect their ability to stimulate Nod1 (6, 7). Therefore, we initially characterized the structural requirement for Nod1 stimulatory activity using a published bioassay with human embryonic kidney (HEK) 293T cells transiently expressing Nod1 (6). The monomer disaccharide tetrapeptide *N*-acetyl-glucosamyl (GlcNAc)-*N*-acetyl-muramyl (MurNAc)-L-Ala-γ-D-Glu-*meso*DAP-D-Ala was Nod1 stimulatory, whereas the dimeric disaccharide tetrapeptide resulting from one linkage between the ε-amino residue in *meso*DAP and the α-carboxyl residue in D-Ala was not (Fig. 1 B). We also found that the stimulatory activity of the tripeptide L-Ala-γ-D-glutaminyl-*meso*-DAP (iQ-DAP) was less than the dipeptide iQ-DAP (Fig. 1 C). These results indicate that NH_2-terminal peptide expansion of the core dipeptide recognized by Nod1 does not result in higher stimulatory ability.

Development of synthetic compounds with high Nod1 stimulatory activity

Based on the results given in Fig. 1, we used the dipeptide iE-DAP as a seed structure to develop compounds with an enhanced ability to stimulate Nod1. Consistent with cytosolic localization of Nod1 (5), microinjection or calcium phosphate–mediated incorporation of Nod1 ligands into cells enhances the ability of the ligands to stimulate Nod1 (6, 8, 12). Furthermore, *H. pylori* is reported to inject Nod1 ligand through a type IV secretion system into host cells (11). These observations suggest that hydrophobic acylation of Nod1 ligands may improve their membrane permeability and ability to stimulate Nod1. To test this hypothesis, we developed synthetic compounds containing various acyl residues at the NH_2 terminus of iE-DAP (X_1 in Fig. 1 A). *N*-myristoyl (C-14) iE-DAP, designated here as KF1B, as well as other acyl iE-DAP compounds (not depicted) were found to specifically stimulate Nod1 (Fig. 2 A), but not Nod2 or TLR4/MD-2, signaling using the HEK293T bioassay (Fig. 2 B). These results indicate that acylation of the iE-DAP dipeptide does not affect ligand recognition or receptor specificity. Further analysis showed that KF1B exhibited a several hundredfold higher ability to induce Nod1-dependent NF-κB activation than the original dipeptide iE-DAP, as determined by the amount of compound required for achieving 50% of maximum activity (Fig. 2 A). Testing of additional compounds revealed that pentadecanoyl (referred as KFC15; C-15) and palmitoyl (referred as KFC16; C-16) iE-DAP also possessed a similar ability to stimulate Nod1 as KF1B (unpublished data). Although efficient stimulation of Nod1 by natural ligands requires the coexistence of calcium phosphate particles under our culture conditions (6, 8), we found that KF1B no longer requires calcium phosphate particles to stimulate Nod1 signaling, which was consistent with our hypothesis that the enhanced ability of KF1B to stimulate Nod1 is caused by increased accessibility to the cytosol (Fig. 2 C).

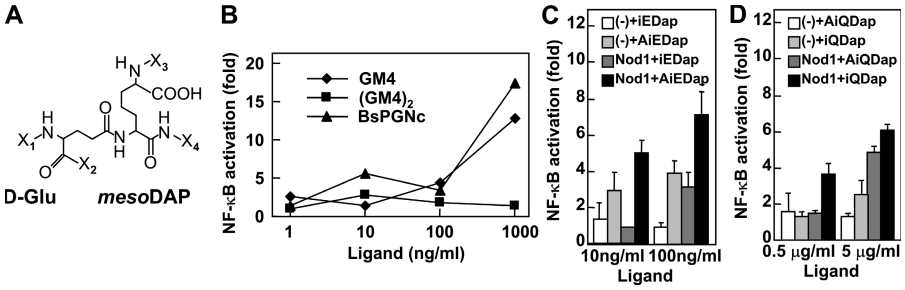

Figure 1. Structures of core Nod1 ligand and the effects of amino acid modifications on the Nod1 stimulatory activity. (A) Core structure of Nod1 ligands. The structure of core Nod1 ligand iE-DAP is shown with its peripheral residues (X_1 to X_4). The activity of the Nod1 ligand without modification is given as one unit. The tripeptide in C and D contain L-Ala at the X_1 position; KF1B, KFC15, and KFC16 have myristoyl [$CH_3(CH_2)_{12}$-C=O], pentadecanoyl [$CH_3(CH_2)_{13}$-C=O], and palmitoyl [$CH_3(CH_2)_{14}$-C=O], respectively, at the X_1 position. iQ-DAP has amino residue at the X_2 position; a cross-bridge to another PGN chain occurs at X_3. The amino residues at the X_4 position varies dependent on the bacterial species and composition of PGN intermediates. (B) Nod1 stimulatory activity of purified PGN fragments from *B. subtilis*. 10^5 HEK293T cells were transfected with the Nod1 expression plasmid together with NF-κB–dependent and –independent reporter plasmids as described previously (reference 4). 8 h after transfection, cells were treated with the indicated amounts of whole soluble Cellosyl-treated PGN fragments, GlcNAc-MurNAc-L-Ala-γ-D-Glu-*meso*DAP-D-Ala (GM4), or dimeric GlcNAc-MurNAc-L-Ala-γ-D-Glu-L-*meso*DAP-D-Ala cross-bridged between *meso*DAP and D-Ala [$(GM4)_2$]. 24 h after transfection, NF-κB–dependent transcription activity was determined as described previously (reference 4). (C) Nod1 stimulatory activity of synthetic di/tripeptides. HEK293T cells were transfected with the Nod1 expression plasmid (Nod1) or control vector (−). 8 h after transfection, cells were treated with the indicated amounts of synthetic iE-DAP or AiE-DAP (L-Ala-iE-DAP-D-Ala). 24 h after transfection, NF-κB–dependent transcription activity was determined. (D) Nod1 stimulatory activity of synthetic di/tripeptides. HEK293T cells were transfected with the Nod1 expression plasmid (Nod1) or control vector (−). 8 h after transfection, cells were treated with the indicated amounts of iQ-DAP or L-Ala-γ-D-Glu-*meso*DAP-D-Ala (AiQ-DAP). 24 h after transfection, NF-κB–dependent transcription activity was determined. The results shown are given as means ± SD of triplicate cultures and are representative of three experiments.

Nod1-dependent enhancement of LPS-induced cytokine secretion by KF1B in macrophages and DCs

To determine if KF1B enhances LPS-induced cytokine production through endogenous Nod1, human monocytic MonoMac6 cells that express Nod1 were stimulated with the iE-DAP–derived compounds and controls. Stimulation of MonoMac6 cells with KF1B alone at concentrations as high as 2,500 ng/ml induced neither the secretion of IL-6 nor IL-1β (Fig. 3, A and B). However, KF1B possessed a higher ability to enhance LPS-induced production of these

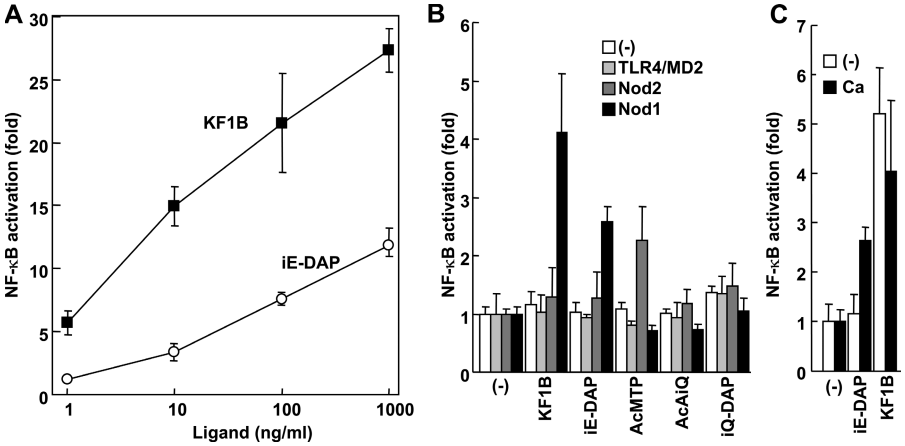

Figure 2. Development of synthetic acyl Nod1 ligands. (A) Enhanced ability of synthetic acyl compound KF1B to stimulate Nod1. HEK293T cells were transfected with the Nod1 expression plasmid. 8 h after transfection, cells were treated with the indicated amounts of the ligand. 24 h after transfection, NF-κB–dependent transcription activity was determined. (B) Nod1-dependent NF-κB activation induced by the acyl Nod1 ligands. HEK293T cells were transfected with the expression plasmid of Nod1, Nod2, or TLR4 and MD2 (TLR4/MD2) or control vector (−) as described in Fig. 1. 8 h after transfection, cells were treated with 5 ng/ml KF1B, iE-DAP, AcMTP, AcAiQ, or iQ-DAP. 24 h after transfection, NF-κB–dependent transcription activity was determined. (C) Requirement of calcium phosphate particles for iE-DAP, but not KF1B, to stimulate Nod1. HEK293T cells were first transfected with Nod1 expression plasmid and reporter constructs. 8 h after transfection, calcium phosphate particles were removed by changing the medium. 9 h after transfection, the cells were stimulated with 1 ng/ml Nod1 ligands, iE-DAP, and KF1B in the absence and presence of calcium phosphate particles. 24 h after transfection, NF-κB–dependent transcription activity was determined. The results shown in the figures are given as means ± SD of triplicate cultures and are representative of five experiments.

cytokines than the original iE-DAP (Fig. 3, A and B). KF1B was effective in stimulating LPS-induced responses at concentrations as low as 25 ng/ml and was comparable to MurNAc-L-Ala-γ-D-Glu-L-Lys(stearoyl)-OH (AcMTP), a modified ligand of Nod2 (21–23). As observed with MonoMac6 cells, stimulation of BM-derived mouse macrophages and DCs with KF1B alone did not induce the secretion of CCL2/MCP-1 (Fig. 3 C), IL-1β, and IL-12 (not depicted). However, KF1B enhanced LPS-induced CCL2 secretion from DCs (Fig. 3 C) and IL-6 secretion from macrophages (Fig. 3 D). Notably, the response of macrophages with KF1B was abolished in Nod1-deficient macrophages (Fig. 3 D), demonstrating that KF1B requires Nod1 for enhancement of CCL2 secretion.

Nod1 stimulation through KF1B induces chemokine expression and NF-κB activation in intestinal epithelial cells

Given that Nod1 is expressed in the intestinal epithelium, we tested whether KF1B alone can induce cellular responses in epithelial cells. We found that KF1B alone, but not *meso*-DAP, Induced production of IL-8 in two human intestinal epithelial cell lines, LoVo and SW620 (Fig. 4, A–C). In contrast, LoVo cells did not respond after stimulation with LPS, CpG, and polyIC (Fig. 4, A and B), which was consistent with the lack of TLR signaling in most intestinal epithelial cells (17). In addition, KF1B induced NF-κB in LoVo cells as determined with an NF-κB–dependent reporter assay (Fig. 4 D). ELISA, real-time PCR, and immunoblotting analyses showed that KF1B also induced CXCL1/Groα and CD83, two molecules that are important for the recruitment and interaction of immune cells (Fig. 4, E–G).

Nod1 and TNF-α stimulation induced common and specific gene induction in epithelial cells

To further characterize events induced by Nod1 stimulation in LoVo intestinal cells, we determined global gene expression changes using a cDNA microarray containing ∼20,000 genes (Table I). At 60 min after stimulation, KF1B induced 11 genes at >2-fold and 41 genes at >1.6-fold as compared with unstimulated cells (Table I). Further analysis at 3 and 24 h after stimulation revealed induction of 6 and 17 genes at >1.6-fold levels, respectively. The intestinal epithelium is the first line of defense against invasive bacteria and mediates innate immune responses, at least in part through TNFα. Therefore, we next examined the gene expression profile induced by KF1B and TNFα in LoVo cells. TNFα induced 37 genes at >1.6-fold levels at 1 h after stimulation (Table I). Notably, 11 of these genes induced by TNFα stimulation were also induced by KF1B (Table I). The great majority of genes induced by both KF-1B and TNFα are known to be up-regulated through NF-κB and proinflammatory molecules (Table I), which is consistent with the observation that KF1B and TNFα induce gene expression through NF-κB signaling. The genes induced by KF1B and TNFα included molecules involved in innate immune responses (CXCL1, MBL2, and CD83) and negative feedback regulators of inflammatory and apoptotic pathways (NFKBIA/IκBα, ATF3, TNFAIP3/A20, CFLAR/CLARP, and BIRC3/c-IAP2). Transient induction of CXCL1 mRNA in the early stage of Nod1 stimulation is consistent with the ability of KF1B to induce CXCL1 secretion (Fig. 4 E). Nod1 stimulation did not induce proinflammatory cytokines, including TNFα and IL-1s, suggesting that Nod1 mediates a restricted pattern of immune responses. The microarray results also revealed a

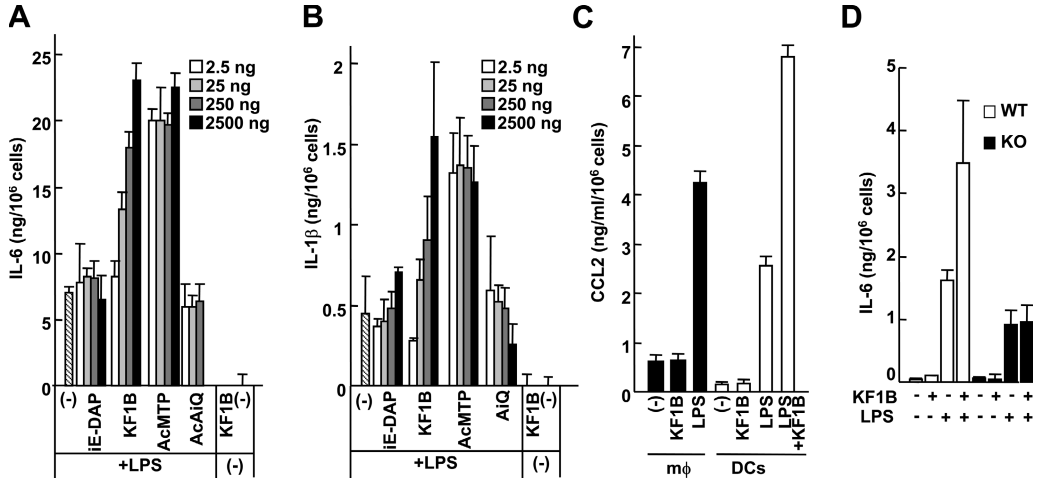

Figure 3. Enhancement of LPS-induced IL-6, IL-1β, and CCL2 secretion by Nod1 stimulation in monocytic MonoMac6, macrophages, and DCs. MonoMac6 cells were treated with the indicated amounts per milliliter of compounds in the absence (−) and presence (+LPS) of 0.7 ng/ml *E. coli* O55:B5 LPS for 24 h. The levels of IL-6 (A) and IL-1β (B) in the medium were determined by ELISA. (C) BM-derived macrophages (mφ) were treated with 2 μg/ml KF-1B and/or *E. coli* O55:B5 LPS, and BM-derived DCs were treated with 2 μg/ml KF-1B and/or 10 ng/ml *E. coli* O55:B5 LPS for 24 h (D) BM-derived mφ from WT and Nod1-deficient (KO) mice were treated with 2 μg/ml KF-1B and/or 10 ng/ml *E. coli* O55:B5 LPS for 24 h. The levels of CCL2 (C) and IL-6 (D) in the medium were determined by ELISA. The results shown are given as means ± SD of triplicate cultures and are representative of three experiments.

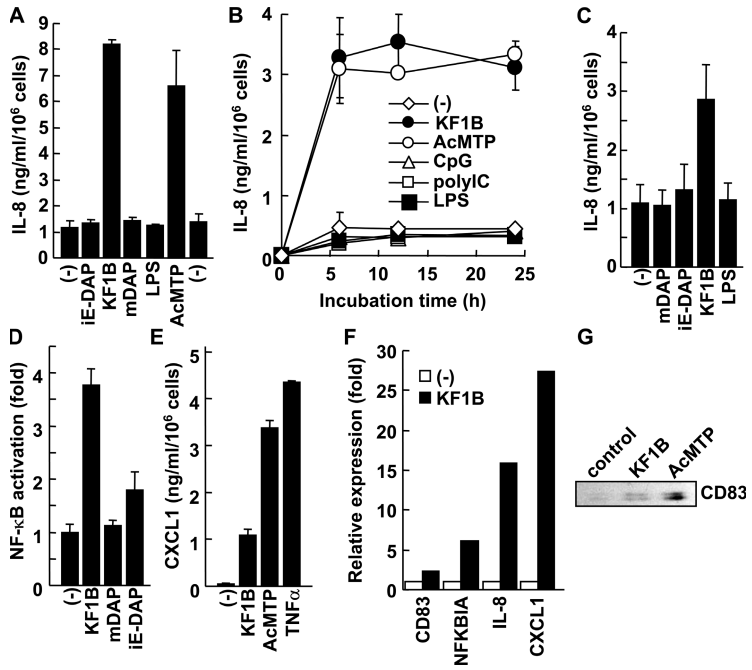

Figure 4. Cytokine secretion from human intestinal epithelial LoVo cells. LoVo cells were treated with 2 μg/ml of each ligand, except 200 ng/ml for AcMTP and 10 ng/ml TNFα. The levels of IL-8 (A) and CXCL1 (E) were determined by ELISA. (B) LoVo cells were treated with 20 μg/ml KF1B, 5 μg/ml AcMTP, 1 μM CpG, 5 μg/ml polyIC, and 5 μg/ml *E. coli* 055:B5 LPS. The levels of IL-8 in medium at the indicated times were determined by ELISA. (C) SW620 cells were treated with 5 μg/ml of the indicated ligands. The secretion levels of IL-8 were determined by ELISA. (D) LoVo cells were treated transfected with NF-κB–dependent and –independent reporter by Lipofectamine 2000. 8 h after transfection, cells were treated with 2,000 ng/ml KF1B, iE-DAP, or control *meso*DAP or left untreated (−). (F) The mRNA levels of CD83, NFKBIA, IL-8, and CXCL1 from cells treated with 2 μg/ml KF1B (closed bars) for 60 min or left untreated (open bars) were determined by real-time PCR analysis. (G) CD83 in lysates from LoVo cells treated with 2,000 ng/ml KF1B for 24 h was detected by immunoblotting with anti-CD83 antibody. The results shown are given as means ± SD of triplicate cultures and were representative of five experiments.

group of genes that are induced specifically by Nod1 or TNFα stimulation. 31 genes were induced by Nod1 but not TNFα stimulation. These genes included signaling molecules of TLR and IL1R pathways (MAP3K7/TAK1 and MAIL/Iκ-Bζ), components of ubiquitin/proteasome pathway (UBE2M, UBE2D3, RYBP, and WSB1) and 6 novel genes, whereas TNFα stimulation induced 37 unique genes including signaling molecules of Ephrin pathways (EFNA1 and EPHA2), transcription factors (EGR1, FOSL2/Fra2, and ELF3), and innate immune factors (PLAU/plasminogen activator and TNFAIP2a; Table I). These results suggest that Nod1 and TNFα mediate unique and overlapping innateimmune responses in epithelial cells.

Nod1 stimulation induces chemokine production and neutrophil recruitment in vivo

To test whether KF1B mediates Nod1-dependent immune responses in vivo, we stimulated WT and Nod1-deficient mice with KF1B i.p. and measured production of chemokines and cytokines in the animals. Administration of KF1B induced rapid production of CCL2 (Fig. 5 A) and CXCL2/Groβ in the serum (Fig. 5 B). KF1B was as effective as LPS or MDP in inducing secretion of CCL2 (Fig. 5 A). Notably, serum secretion of CCL2 in response to KF1B was abolished in Nod1-deficient mice (Fig. 5 A), indicating that production of CCL2 is totally dependent on Nod1 in vivo. The lack of response of Nod1-deficient mice was specific in that they produced CCL2 after administration of LPS or MDP (Fig. 5 A). Because Nod1 is highly expressed in airways and the intestine (5), we also tested whether intranasal and oral administration of KF1B could induce immune responses. Consistent with the expression of Nod1 in these organs, production of CCL2 was induced in WT mice but not in Nod1-deficient mice after intranasal and oral administration of mice with KF1B (Fig. 5 C). In contrast to the production of chemokines, administration of KF1B did not induce secretion of IFN-γ, IL-3, CSF2 (M-CSF), IL-12 (Fig. 5 D), and IL-4, -10, -18, and -1β (not depicted) in the serum or tissues, including spleen, liver, and kidney, when compared with the levels found in control mice (Fig. 5 D). Further analysis did not reveal any production of these cytokines at 24 h after Nod1 stimulation (unpublished data), suggesting that the lack of response is not caused by delayed production of these cytokines. These findings are consistent with our results in vitro that Nod1 stimulation induces a restricted number of immune response genes. Because Nod1 stimulation induced chemokine secretion in vitro and in vivo, we next tested whether Nod1 stimulation can induce recruitment of specific

Table I. Genes induced by Nod1 and TNFα stimulation

KF1B (1 h)		TNFα (1 h)		KF1B (3 h)		KF1B (24 h)	
Gene	Fold	Gene	Fold	Gene	Fold	Gene	Fold
NFKBIA	3.45	***NFKBIA***	10.53	***BIRC3***	3.28	*P4HA1*	2.02
CXCL1	2.67	*CLTCL1*	5.37	***NFKBIA***	3.00	Novel	1.92
CFLAR	2.42	***TNFAIP3***	5.12	***CD83***	1.96	*BNIP3L*	1.91
SKI	2.25	***CXCL1***	4.68	***TNFAIP3***	1.85	Novel	1.90
HNRPC	2.25	Unknown	4.35	*MBL2*	1.78	*HSD11B2*	1.85
NUP43	2.13	*MBL2*	4.24	*VPS13A*	1.62	*ANGPTL4*	1.84
AHCYL1	2.04	***BIRC3***	4.07			*DDIT4*	1.82
HIPK3	2.04	*SKI*	3.98			*ANGPTL4*	1.81
ATF3	2.03	*MCF2*	3.51			*WSB1*	1.76
RYBP	2.02	Novel	2.92			*GBE1*	1.76
TCERG1	2.00	***ELF3***	2.92			*WSB1*	1.75
H3F3B	1.97	***EFNA1***	2.92			*ELK3*	1.74
Novel	1.93	***CD83***	2.75			*EGLN1*	1.73
RNF32	1.86	***TNFAIP3***	2.69			*PLOD2*	1.69
WSB1	1.85	***JUN***	2.44			***ADM***	1.68
WSB1	1.82	Novel	2.40			*CYLN2*	1.67
PRDX3	1.81	***PLAU***	2.33			*PIM1*	1.62
CXCL1	1.79	***TNFAIP2***	2.31			*SERPINB1*	1.62
MAIL	1.78	***EFNA1***	2.29				
Novel	1.76	Novel	2.28				
DC36	1.76	***CFLAR***	2.27				
JUN	1.75	*PDGFB*	2.26				
Novel	1.75	*PLK3*	1.93				
RAP2A	1.73	*EPHA2*	1.89				
MAP3K7	1.73	*TIEG*	1.85				
UBE2M	1.73	*EGR1*	1.85				
PHLDA1	1.71	*SF1*	1.82				
NCK1	1.70	***IFNGR2***	1.81				
STK17A	1.70	***BIRC1***	1.79				
SEC23A	1.70	Novel	1.76				
DDX18	1.70	*DUSP5*	1.73				
CCDC6	1.69	*ARRDC3*	1.71				
RAGE	1.69	*VMP1*	1.71				
Novel	1.69	***CXCL1***	1.71				
PCF11	1.69	Novel	1.69				
Novel	1.69	***MAP3K8***	1.67				
ETEA	1.68	Novel	1.65				
NFS1	1.67	***ATF3***	1.64				
PPA2	1.67	***FOSL2***	1.64				
UBE2D3	1.67	***EGR1***	1.61				
Novel	1.66	Novel	1.60				
Novel	1.65						

LoVo cells were treated with 2 μg/ml KF1B and 10 ng/ml TNFα for indicated times. Genes induced by KF1B at >1.6-fold ratio between control and treated cells are shown. The genes that were commonly induced by Nod1 and TNFα stimulation and those that are known to be induced by NF-κB, proinflammatory TNFα, and/or LPS (46) are underlined and bolded, respectively.

types of immune cells into the peritoneal cavity after i.p. administration of the synthetic Nod1 ligand. 16 h after administration, Nod1 stimulation was found to induce recruitment of $Gr1^+$ neutrophils but not lymphocytes, macrophages, or other cell types as determined by morphological (Fig. 6, A and B) and flow cytometric analyses (Fig. 6 C). The effect was Nod1 dependent in that the recruitment of neutrophils induced by KF1B was abolished in Nod1-deficient mice (Fig. 6).

FK1B did not increase the number of macrophages or lymphocytes in the peritoneal cavity (Fig. 6 B). The recruitment of neutrophils mediated through Nod1 stimulation is consistent with the induction of CXCLs by KF1B. To determine whether resident cells found in the peritoneal cavity respond to KF1B, we prepared intraperitoneal cells and stimulated them with the Nod1 ligand. We found no detectable secretion of CXCL1 after stimulation of KF1B even though the cells responded to LPS (Fig. 6 E). Notably, analysis of peritoneal cavity tissues that are rich in mesothelial cells revealed induction of CXCL1 expression after KF1B stimulation in vivo (Fig. 6 F). These results suggest that mesothelial cells and/or other cells present in the tissue from the peritoneal cavity are the main sources of CXCL1.

DISCUSSION

We previously demonstrated that Nod1 is essential for host response against iE-DAP–containing small molecules (5). These iE-DAP–containing molecules include intermediates and degradation products of PGN (5, 6, 12). In this paper, we show that the monomeric disaccharide tetrapeptide was Nod1 stimulatory, whereas the dimeric disaccharide tetrapeptide resulting from one linkage between the ε-amino residue in *meso*DAP and the α-carboxyl residue in D-Ala was not. This linkage bond is important for the cross-linking among PGN chains (24). The percentage of linked/free PGN chains varies among bacterial species and growth conditions in certain bacteria (24). For example, ∼80% of the ε-amino acid residue of DAP remains free in *Escherichia coli* (24). Thus, our finding that cross-linking between DAP and D-Ala eliminates Nod1-mediated host recognition suggests that the ability of bacterial PGN fragments to stimulate Nod1 varies depending on species and growth conditions of bacteria. Both hosts and bacteria have amidases that cleave the bond between the polysaccharide and oligopeptide chains of PGN, and bacteria also have enzymes that generate various oligopeptide species during the synthesis and remodeling of PGN (23). A tripeptide L-Ala-iE-DAP was reported to possess a higher ability to stimulate Nod1 than dipeptide iE-DAP using purified fractions (7). We confirmed the latter result by comparing the Nod1 stimulatory activity of L-Ala-iE-DAP and iE-DAP using synthetic equivalents (Fig. 2 C). However, we found that the stimulatory activity of the tripeptide L-Ala-iQ-DAP was less than that elicited by the dipeptide iQ-DAP (Fig. 2 D). Therefore, the association between NH_2-terminal extension of the core structure and increased ability to stimulate Nod1 is not a general feature of Nod1 ligands.

Bacterial- and synthetic iE-DAP–containing molecules are known to induce innate and acquired immune responses (25). However, because of the relatively poor immunostimulatory ability of iE-DAP–containing molecules, compared with that of LPS and MDP-related molecules (see Fig. 4 D) (6, 19, 20), it has been difficult to investigate the role of Nod1 stimulation in immune responses. In this paper we developed acyl compounds that possess an enhanced ability to stimulate Nod1. These molecules, including myristoyl (C-14), pentadecanoyl (C-15), and palmitoyl (C-16) iE-DAP compounds (referred to as KF1B, KFC15, and KFC16, respectively), were found to exhibit a several hundredfold stronger ability to stimulate Nod1 than the original iE-DAP. Because these fatty acyl residues naturally exist in host membrane lipids, their potent stimulatory ability could be explained by increased translocation across the plasma membrane and delivery into the cytosol through cellular machinery that may include lipid flippases or related factors (26). Using these compounds, we investigated the immune response induced upon Nod1 stimulation. We found that intestinal epithelial cell lines were highly responsive, whereas macrophages, DCs, and splenocytes did not respond to Nod1 ligands alone under our experimental conditions. These results are consistent with the tissue expression profile of Nod1 that showed high levels of Nod1 expression in epithelial tissues such as the intestine (5). Several genes induced by Nod1 stimulation, including CXCL1, CD83, and MBL2, are known to be involved in the recruitment of immune cells and complement factors after bacterial infection. Similarly, our analysis in mice revealed that secretion of CCL2 was induced in serum and multiple tissues upon Nod1 stimulation in vivo. These results suggest that Nod1 functions as a pathogen recognition receptor to induce expression of proteins that play a critical role in the early stages of the innate immune response.

Nod1 is an intracellular protein, and, thus, recognition of bacteria through Nod1 would signal potentially harmful invasion of epithelial surfaces such as that lining the gut. In the case of the intestine, the mucosa is exposed to a large concentration of commensal bacteria that express a wide array of TLR ligands. However, inappropriate innate recognition and inflammatory responses are avoided, at least in part, by the lack of TLR signaling in the surface intestinal epithelium (17). A model suggested by these results is that after invasion of the epithelium by pathogenic bacteria, TLR-positive innate immune cells are recruited, though Nod1-induced chemokines and stimulation of these cells results in the production of cytokines such as TNFα that amplify the host response. Thus, although both TLR and Nod proteins recognize bacterial products, they may play distinct roles in bacterial clearance. Intestinal epithelial cells are also likely to play an important role in this amplification loop of the host defense through the induction of genes that are unique to TNFα signaling. Comprehensive microarray analysis revealed that Nod1 and TNFα induced a different profile of gene expression that include both common and specific genes in epithelial cells. These observations suggest that primary and secondary responses against bacteria in intestinal epithelial cells are different. The genes induced by Nod1, but not TNFα, include TAK1 and Iκ-Bζ, which are critical factors in TLR and IL-1R pathways (27–29). Co-stimulation with Nod1 and Nod2 enhances TLR signaling (6, 30). The enhancement of TLR signaling by Nod1 might be due, at least in part, to the induction of TAK1 and Iκ-Bζ. Nod1 stimulation also induced several novel genes that have unknown functions. Further studies are required to reveal the

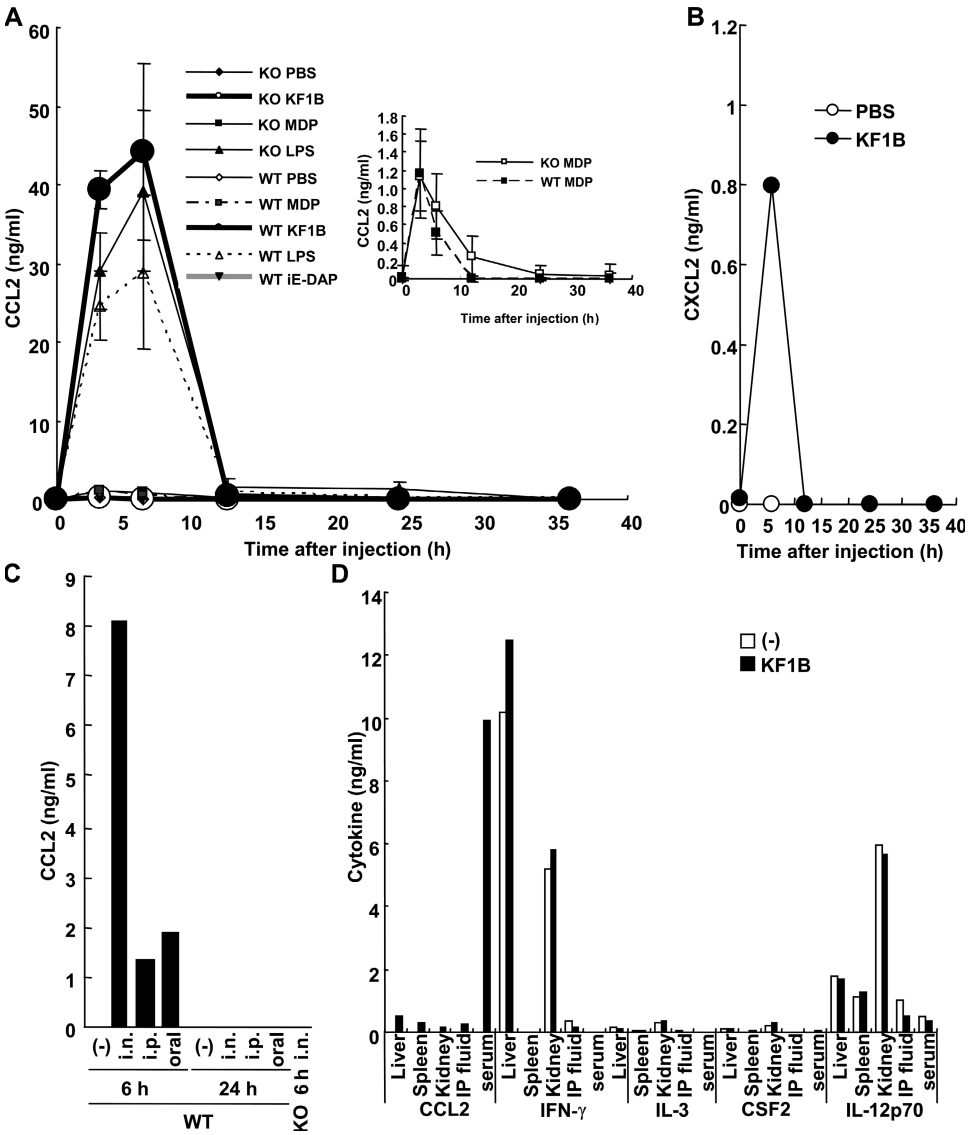

Figure 5. Nod1-dependent CCL2 induction in vivo. (A) WT and Nod1-deficient mice were administrated i.p. with 200 μg KF1B, iE-DAP, MDP, *E. coli* O55:B5 LPS, or PBS alone. The serum level of CCL2 was determined by ELISA. Because the relative levels of CCL2 induced by MDP was lower than those by KF1B, the CCL2 levels induced by MDP are also given (inset). The CCL2 levels are given with means ± SD from three mice. (B) The serum CXCL2 level from mice stimulated with 50 μg KF1B or PBS alone at indicated times after i.p. injection was determined by ELISA. Representative data from three independent experiments are shown. (C) The serum levels of CCL2 from WT and Nod1-deficient (KO) mice treated with 50 μg by a different administration method (i.n. [intranasal], i.p., and oral route) at 6 and 24 h were determined by ELISA. Representative data from four independent experiments are shown. (D) cytokine levels in tissues from mice stimulated with 200 μg KF1B or PBS alone (−) 6 h after i.p. injection was determined by ELISA. Representative data from three independent experiments are shown. The results are representative of at least three experiments.

physiological role of these Nod1-responsible genes. The molecular basis that accounts for the induction of unique genes by Nod1 and TNFα signaling is unclear. A possible explanation is that differences in gene expression reflect differential activation of intracellular signaling molecules. For example, RICK, a downstream factor of Nod1, has been shown to activate NF-kB through TRAF6, whereas TRAF2 is critical for TNFα signaling (31–33).

Nod1 gene variants have been recently associated with the development of asthma (34). Moreover, genetic variations in Nod1 and Nod2 are associated with increased susceptibility to Crohn's disease (35–37). The Crohn's disease–associated Nod2 mutations are defective in their response to muramyl dipeptide (38, 39). Because both Nod1 and Nod2 share the same downstream components for signaling, the defective response observed in cells harboring Nod2 mutations could be rescued by Nod1 stimulation. Thus, ligands such as KF1B could be useful for the development of novel approaches to treat Nod1- and Nod2-associated inflammatory diseases.

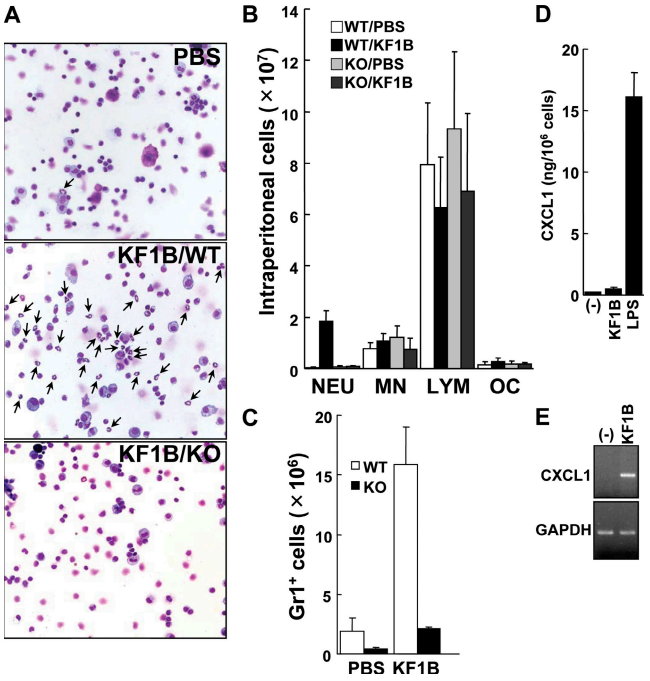

Figure 6. Specific recruitment of neutrophils by Nod1 stimulation. WT and Nod1-deficient mice were i.p. administrated with 50 μg KF1B or control PBS. 24 h after injection, i.p. fluid was obtained and analyzed by morphological assay after Diff staining. (A) The randomly chosen representative views of i.p. cells from WT mice injected with PBS and KF1B and from Nod1-deficient (KO) mice injected with KF1B are shown. (B) The numbers of neutrophils (NEU), macrophages (MN), lymphocytes (LYM), and other cell types (OC) were counted and given as means ± SD from independent experiments using three mice. (C) The numbers of $GR1^+$ cells from WT and Nod1-deficient (KO) mice with KF1B were determined by flow cytometry. (D) In vitro secretion of CXCL1 from i.p. cells. The i.p. cells from WT mice were stimulated with 50 μg/ml KF1B, control LPS, or left alone for 24 h. CXCL1 levels were determined by ELISA. (E) Induction of CXCL1 mRNA in peritoneal tissue. Tissue from the peritoneal membrane was isolated from WT mice 1 h after stimulation by i.p. injection of PBS (−) or 50 μg KF1B. The CXCL1 and control GADPH mRNA were detected by RT-PCR. The results are representative of three experiments.

MATERIALS AND METHODS

Bioassay for Nod1 ligand activity. Ligand-dependent NF-κB activation was determined using HEK293T cells transiently expressing Nod1, Nod2, or TLR4 and MD2 in the presence of pBxIV-luc and pEF1BOS-β-gal as described previously (6). In brief, HEK293T cells were transfected with expression plasmids by the calcium phosphate method, and cells were treated with medium containing various ligands 8 h after transfection. 24 h after transfection, ligand-dependent NF-κB activation was determined with a reporter assay. LoVo cells were transfected with pBxIV-luc and pEF1-BOS-β-gal (4) by Lipofectamine 2000 (Invitrogen), and cells were treated with medium containing 2 μg/ml KF1B, 0.2 μg/ml AcMTP, or 10 ng/ml TNFα 3 h after transfection. 24 h after transfection, ligand-dependent NF-κB activation was determined with a reporter assay.

Other materials. Highly purified fractions of whole soluble Cellosyl-treated PGN fragments (GlcNAc-MurNAc-L-Ala-γ-D-Glu-*meso*DAP-D-Ala, shown as fraction 3 in reference 31, or dimeric GlcNAc-MurNAc-L-Ala-γ-D-Glu-L-*meso*DAP-D-Ala cross-bridged between *meso*DAP and D-Ala, shown as fraction 21 in reference 41) from *Bacillus subtilis* were prepared as described previously (41). In brief, dibenzyl-2,6-diaminopimelate (dibenzyl-DAP) was first connected to 2-chlorotrityl resin, and the remaining amino group of DAP was reacted with Fmoc-D-Glu-OBn. After deprotection of Fmoc group, the acyl group (C-14, C-15, or C-16) was introduced to the liberated amino group to give the corresponding acylated iE-DAP on the resin. After cleavage from the resin with 10% trifluoroacetic acid, cleavage of the remaining protecting groups gave KF1B, KFC15, or KFC16, respectively. Synthetic compounds iE-DAP, iQ-DAP, CpG, and polyIC were described previously (5). Synthetic Ac-(6-O-stearoyl)-MurNAc-L-Ala-γ-D-Glu (AcMDP), AcMTP, and D-Ala-Gln-octadecyl ester (AcAiQ) were obtained from Bachem. *E. coli* O55:B5 LPS was purchased from Sigma-Aldrich. The LPS preparation that lacked contamination of substantial Nod1 and Nod2 stimulatory activity in the HEK293T bioassay was used in this study.

Cytokine assay. Human LoVo, SW620, and MonoMac6 cells were cultured and maintained in RPMI 1640 medium (Invitrogen). Macrophages and DCs were derived from BM using CSF1 and CSF2, respectively, as previously described (29). Cells were treated with the indicated amounts per milliliter of various ligands for the times shown in the figure legends. The cytokine and chemokine levels were determined by sandwich ELISA kits using specific antibodies (R&D Systems).

In vivo induction of cytokines and immune cell recruitment. 8-wk-old C57BL/6 (B6) WT and $Nod1^{-/-}$ mice in B6 background were maintained at the University of Michigan Animal Facility. B6 background $Nod1^{-/-}$ mice used in this study were generated by backcrossing at least six times with the parental B6 strain. Mice were administrated with the indicated amounts of various ligands by routes described in the figure legends. At the times indicated in the figures, sera and organs were collected from killed mice, and homogenates of organs were prepared by sonication with PBS (Invitrogen) containing 1% Triton X-100 and a mixture of protease inhibitors (Sigma-Aldrich), followed centrifugation at 100,000 *g* for 10 min. The cytokine and chemokine levels in sera and tissue homogenates from mice were determined by ELISA. For recruitment of immune cells at 24 h after i.p. administration of KF1B, peritoneal cells were collected from killed mice by PBS lavage. After centrifugation (Cytospin 2; Shandon Ltd.), cells were fixed and stained with a staining kit (Diff; Dade Behring Inc.) according to the manufacturer's recommendations. Cell types were determined by standard morphological features of stained cells. The number of $GR1^+$ cells in 10^5 cells was determined by flow cytometry (FACScan II; BD Biosciences) as previously described (41). The mouse studies were approved by the University of Michigan Committee on the Use and Care of Animals. For estimation of CXCL1 mRNA, total RNA was isolated from peritoneal tissue of WT mice using Trizol (Invitrogen). In brief, several 1-cm^2 fragments of the peritoneal membrane were isolated and immediately homogenized with Trizol solution with and without KF1B stimulation. To assess mRNA expression, a semi-quantitative RT-PCR method was used, as previously described (42). CXCL1 and control GAPDH gene fragments (355 and 191 bp, respectively) were amplified by 35 cycles of the PCR with 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min using gene-specific primers (CXCL1, 5′-CACCTCAAGAACATCCAGAGCT-3′ and 5′-CAAGCAGAACTGAACTACCATCG-3′; GAPDH, AACGACCCCTTCATTGAC-3′ and 5′-CCACGACATACTCAGCAC-3′) and subjected to 2% agarose electrophoresis.

Gene microarray analysis. LoVo cells were treated with 2 μg/ml KF1B, AcMTP, 10 ng/ml TNFα, or left alone for the times indicated in the figures. Total RNA was prepared, and Cy3- and Cy5-labeled cDNA was synthesized from total RNA as described previously (43). Differential expression of 20,000 human genes (Invitrogen) was determined by hybridization of the probes with their cDNAs, followed by an analysis using a microarray scanner (GenePix 4000A; Molecular Devices) as described previously (43).

Real-time PCR and immunoblotting analysis of ligand-induced gene profiling change. Quantitative real-time PCR was performed using gene-specific primers (CD83, 5′-TTTAATGGCCGGCTGGAAATG-3′

and 5′-TTTAGCCCATGCAACAGCCTTGTG-3′; NFKBIA, 5′-CGCGACGGGCTGAAGAAGG-3′ and 5′-ATGGCCAAGTGCAGGAACGAG-3′; IL8, 5′-CAAGGGCCAAGAGAATATCCGAACT-3′ and 5′-TATCACATTCTAGCAAACCCATTCAA-3′; and CXCL1, 5′-CTCAATCCTGCATCCCCCATAGTTA-3′ and 5′-GTGGCCTCTGCAGCTGTGTCTCT-3′) and SYBR green dye with a real-time PCR system (model 7300; Applied Biosystems) as previously described (44, 45). The mean calculated quantity of the target gene for each sample was then divided by the mean calculated quantity of the housekeeping genes *GAPDH* and *HMBS* corresponding to each sample to give a relative expression of the target gene for the sample (46). All reactions were performed in duplicate. Immunoblotting analysis of CD83 in 50 μg total protein lysate from cells treated with 2 μg/ml KF1B, AcMTP or left alone was performed using anti-CD83 monoclonal antibody (BD Biosciences). Equal loading of proteins were confirmed by reversible staining with Ponceau S (Sigma-Aldrich) before immunodetection.

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