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Synergistic Effect of Nod1 and Nod2 Agonists with Toll-Like Receptor Agonists on Human Dendritic Cells To Generate Interleukin-12 and T Helper Type 1 Cells

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A synthetic Nod2 agonist, muramyldipeptide (MDP), and two Nod1 agonists, FK565 and FK156, mimic the bacterial peptidoglycan moiety and are powerful adjuvants that induce cell-mediated immunity, especially delayed-type hypersensitivity. In this study, we used human dendritic cell (DC) cultures to examine possible T helper type 1 (Th1) responses induced by MDP and FK565/156 in combination with various synthetic Toll-like receptor (TLR) agonists, including synthetic lipid A (TLR4 agonist), the synthetic triacyl lipopeptide Pam3CSSNA (TLR2 agonist), poly(I:C) (TLR3 agonist), and CpG DNA (TLR9 agonist). Immature DCs derived from human monocytes expressed mRNAs for Nod1, Nod2, TLR2, TLR3, TLR4, and TLR9. The stimulation of DCs with MDP and FK565 in combination with lipid A, poly(I:C), and CpG DNA, but not with Pam3CSSNA, synergistically induced interleukin-12 (IL-12) p70 and gamma interferon (IFN-γ), but not IL-18, in culture supernatants and induced IL-15 on the cell surface. In correlation with the cytokine induction, an upregulation of the mRNA expression of these cytokine genes was observed. Notably, IL-12 p35 mRNA expression increased >1,000-fold upon stimulation with lipid A plus either MDP or FK565 compared with stimulation with each stimulant alone. In contrast, for the expression of CD83 and costimulatory molecules such as CD40, CD80, and CD86, no synergistic effects were observed upon stimulation with Nod plus TLR agonists. The culture supernatants of DCs stimulated with lipid A plus either MDP or FK565 activated human T cells to produce high levels of IFN-γ, and the activity was attributable to DC-derived IL-12. These findings suggest that Nod1 and Nod2 agonists in combination with TLR3, TLR4, and TLR9 agonists synergistically induce IL-12 and IFN-γ production in DCs to induce Th1-lineage immune responses.

Freund's complete adjuvant (10), which contains killed mycobacterial cells, has been widely used as a powerful adjuvant to induce cell-mediated immunity, represented by delayed-type hypersensitivity, as well as to enhance humoral immunity against test antigens in laboratory animals. A series of studies on the mycobacterial component responsible for the unique adjuvant activity of Freund's complete adjuvant revealed that Wax D is the active entity, being composed of peptidoglycan (PGN), arabinogalactan, and mycolic acid, and thereafter the PGN moieties of various bacteria were revealed to also be active in this respect (37). In the mid-1970s, the minimal essential structure of PGN for adjuvant activity was demonstrated to be muramyldipeptide (MDP; *N*-acetylmuramyl-L-alanyl-D-isoglutamine) by use of a chemically synthesized compound (8, 24). MDP reproduced various bioactivities of PGN (39, 40), although the activities of MDP were generally weaker than those of PGN and MDP was scarcely active in some experiments. PGN activates macrophages via Toll-like receptor 2 (TLR2) (35, 42, 51), whereas MDP lacks TLR2-agonistic activity (41, 46, 49, 51).

Fleck et al. (9) reported that desmuramylpeptide (DMP) containing *meso*-diaminopimelic acid (*meso*-DAP) was also active as an adjuvant to induce cell-mediated immunity. *meso*-DAP-type PGN is found in most gram-negative bacteria and in some gram-positive bacteria, including mycobacteria, while most gram-positive bacteria such as *Staphylococcus* and *Streptococcus* strains possess L-lysine (Lys)-type PGN (34). In their report, Fleck et al. (9) suggested by mistake that Lys-type DMPs were similarly active to *meso*-DAP-type DMPs in this respect. Thereafter, French and Japanese investigators chemically synthesized adjuvant-active *meso*-DAP-type DMPs (1). In the course of the study, the Fujisawa Pharmaceutical Company generated a DMP, D-lactyl-L-alanyl-γ-D-glutamyl-*meso*-DAP-glycine, by chemically mimicking a counterpart purified from fermentation broths of *Streptomyces* strains, and this DMP was designated FK156; the company then synthesized various derivatives of FK156, among which the leading compound was FK565, or heptanoyl-D-glutamyl-*meso*-DAP-γ-D-alanine (13).

Recently, an intracellular molecule carrying nucleotide-binding oligomerization domain 2 (Nod2) was revealed to be a receptor for MDP (12, 17). Thereafter, another Nod family molecule, Nod1, was demonstrated to recognize a PGN motif containing *meso*-DAP (7, 11). We found that FK156 and FK565 were Nod1 agonists similar to γ-D-glutamyl-*meso*-DAP (45), which is the minimal active structure of a Nod1 agonist (7, 22).

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Yang et al. (50) showed a marked synergistic effect of MDP with lipopolysaccharide (LPS) of inducing interleukin-8 (IL-8) in human monocytic cells in culture. Recently, Uehara et al. (45) demonstrated synergism between synthetic Nod agonists and TLR agonists: MDP (Nod2 agonist) and DMPs such as FK156 and FK565 (Nod1 agonists) in combination with synthetic lipid A (TLR4 agonist), the synthetic lipopeptide Pam3CSSNA (TLR2 agonist), and bacterial CpG motif DNA (TLR9 agonist) synergistically induced IL-8 in human monocytic cells. It must be noted that most bacteria possess various TLR ligands including PGN which can also sense Nod1 and/or Nod2 after degradation by bacterial or host PGN-lytic enzymes. Therefore, in host innate immune responses to invading bacteria, dual signaling through extracellular TLR molecules and intracellular Nod molecules should lead to the synergistic activation of host cells.

Dendritic cells (DCs) are initiators and modulators of immune responses. Peripheral DCs are characterized by the ability to capture and process antigens, their migration to lymphoid organs, and the expression of various costimulatory molecules for antigen-specific lymphocyte activation. Cytokines secreted by DCs initiate and enhance both innate and acquired immunity (4). When DCs were activated to produce IL-12, a heterodimeric cytokine (IL-12 p70) composed of a 40-kDa subunit (p40) and a 35-kDa subunit (p35) played a critical role in the development of Th1 cells from naïve Th0 cells (43). Many studies suggest that the activation of DCs by microbial components leads to the secretion of IL-12, which subsequently induces Th1 development and gamma interferon (IFN-γ) production by T cells (44). Various TLR agonists differentially modulate IL-12 production in DCs and are involved in determining the Th1/Th2 balance (2, 20, 33). In the peripheral blood, Nod2 is highly expressed on DCs as well as on monocytes and granulocytes (15). As mentioned above, MDP and *meso*-DAP-containing DMPs are powerful adjuvants for inducing cell-mediated immunity, especially delayed-type hypersensitivity, suggesting their possible induction of Th1 responses. For this study, we examined possible synergistic effects of Nod agonists with TLR agonists on the induction of IL-12 and IFN-γ in human DCs to polarize immune responses toward Th1 development. We used mainly chemically synthesized compounds to avoid the influence of minor components in bacterial fractions.

MATERIALS AND METHODS

Reagents. A synthetic MDP and its inactive stereoisomer, *N*-acetylmuramyl-L-aranyl-L-isoglutamine (MDP-LL), and polyinosinic-poly(C) [poly(I:C)] were purchased from Sigma (St. Louis, MO). A synthetic *Escherichia coli*-type lipid A (LA-15-PP) was purchased from Daiichi Pharmaceutical Co. (Tokyo, Japan). A conventional CpG DNA, CpG DNA 1826 (TCCATGACGTTCCTGACGTT [CpG motif is underlined]) (3), was purchased from Proligo Japan Co. (Tokyo, Japan). The synthetic DMPs FK156 and FK565 (13) were supplied by Fujisawa Pharmaceutical Co. (Tokyo, Japan). A synthetic triacyl lipopeptide, *S*-[2,3-bis-(palmitoyloxy)propyl]-*N*-palmitoylpentapeptide (Pam3CSSNA; molecular weight, 1,269.8), which mimics the *N*-terminal pentapeptide of a lipoprotein derived from *E. coli* (5, 48), was prepared as described previously (29). The neutralizing anti-human IL-12 p40/p70 monoclonal antibody (MAb) C8.6 (mouse immunoglobulin G1 [IgG1]) was purchased from BD Biosciences (San Diego, Calif.). The neutralizing anti-human IL-18 MAb 125-2H (mouse IgG1) was purchased from MBL Co., Ltd. (Nagoya, Japan). Anti-HLA-DR conjugated to fluorescein isothiocyanate (FITC) (B8.12.2; mouse IgG2b), anti-CD1a conjugated to phycoerythrin (PE) (BL6; mouse IgG1), and anti-CD83–FITC (HB15a; mouse IgG2b) were purchased from Immunotech (Marseille, France). Anti-CD40–FITC (5C3; mouse IgG1), anti-CD80–FITC (MAb104; mouse IgG1), and anti-CD86–FITC (FUN-1; mouse IgG1) were purchased from BD Biosciences. Anti-CD14–PE (61D3; mouse IgG1) was obtained from eBiosciences (San Diego, Calif.). Anti-human IL-15 MAb 34505.11 (mouse IgG) was purchased from Genzyme/Techne (Minneapolis, Minn.). Other reagents were obtained from Sigma, unless indicated otherwise.

Preparation of DCs from monocyte cultures. Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood from healthy adult donors by Lympholyte-H (Cedarlane Laboratories, Ontario, Canada) gradient centrifugation at 800 × *g* for 20 min at room temperature. Human monocytes were isolated from the PBMC suspension by sorting via CD14. For sorting, the suspension was incubated for 30 min at 4°C with a superparamagnetic microbead-conjugated anti-human CD14 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) in 2 mM EDTA–0.5% bovine serum albumin–phosphate-buffered saline. Magnetic field-activated cell sorting (MACS) was performed according to the manufacturer's recommendations by passing the cells over a large cell, using MACS LS separation columns (Miltenyi Biotec) in a magnetic field. Isolated $CD14^+$ cells (monocytes; 2×10^6 cells/ml) were cultured in 24-well plates (Falcon; Becton Dickinson Labware, Lincoln Park, N.J.) in 1 ml of complete medium (RPMI 1640 medium [Nissui, Tokyo, Japan] supplemented with 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% fetal calf serum [Gibco BRL Life Technologies, Auckland, New Zealand]) supplemented with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) (100 ng/ml; PeproTech, Rocky Hill, N.J.) and recombinant IL-4 (100 ng/ml; PeproTech) at 37°C. After 3 days of culture, half of the medium in each well was exchanged. After 6 days of culture, >97% of the cells expressed characteristic DC-specific markers (CD1a and HLA-DR), as determined by flow cytometry. Cells were stimulated with various stimuli in the presence of GM-CSF (100 ng/ml) and IL-4 (100 ng/ml) in complete medium.

Preparation of purified T cells. Purified T lymphocytes were prepared using a pan-T-cell isolation kit (Miltenyi Biotec). Briefly, PBMCs were incubated with anti-CD11b, -CD16, -CD19, -CD36 and -CD56 hapten antibodies for 10 min at 4°C. After a wash, non-T cells were labeled with anti-hapten microbeads for 15 min at 4°C. Labeled cells were subsequently removed by the use of MACS LS separation columns in a magnetic field. Purified T lymphocytes were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum.

Flow cytometry. Cells were washed with phosphate-buffered saline and stained with FITC-conjugated MAbs or FITC-conjugated isotype-matched mouse IgG at 4°C for 20 min. Staining was analyzed on a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Mountain View, Calif.). Data were collected for 10,000 events, stored in the list mode, and then analyzed with Lysis II software (Becton Dickinson). The arithmetic mean was used for computation of the mean fluorescence intensity.

Measurement of IL-12 p70, IFN-γ, IL-8, IL-15, and IL-18 by ELISA. DCs were stimulated with various test specimens for 3 days in 1 ml of complete medium. The IL-12 p70, IFN-γ, IL-8, IL-15, and IL-18 levels in the culture supernatants were measured with enzyme-linked immunosorbent assay (ELISA) kits (IL-12 p70, IFN-γ, IL-8, and IL-15 kits were from PharMingen, San Diego, Calif.; IL-18 kits were from MBL Co., Ltd.) according to the manufacturers' directions. The concentrations of the cytokines were determined using the Softmax data analysis program (Molecular Devices, Menlo Park, Calif.).

RT-PCR. Total RNA from DCs or T cells was isolated using Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription (RT) of the RNA samples to cDNAs was performed with avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, Fla.) and a random primer (nonadeoxyribonucleotide mixture; Takara Bio Inc., Otsu, Japan). For cDNA preparation, 1.0 μg of RNA, 50 pmol of random primer, 5 mM $MgCl_2$, 2 μl of 10× RNA PCR buffer (Takara Bio Inc.), a 1.0 mM concentration of each deoxynucleoside triphosphate (Takara Bio Inc.), 5 U of avian myeloblastosis virus reverse transcriptase XL, and 20 U of RNase inhibitor (Takara Bio Inc.) were added to a total volume of 20 μl. The reaction mixture was incubated at 30°C for 10 min, 42°C for 30 min, and then 99°C for 5 min to inactive the reverse transcriptase, cooled at 5°C for 5 min, and stored at −20°C. The primers used for PCR were as follows: for Nod1, 5′-TGATGCTGTTTCT GCCTCTC-3′ and 5′-AATTTGACCCCTGCGTCTAG-3′; for Nod2, 5′-AGCC ATTGTCAGGAGGCTC-3′ and 5′-CGTCTCTGCTCCATCATAGG-3′; for TLR2, 5′-TCACCTACATTAGCAACAG-3′ and 5′-GATCTGAAGCATCAAT CTC-3′; for TLR3, 5′-GATCTGTCTCATAATGGCTTG-3′ and 5′-GACAGA TTCCGAATGCTTGTG-3′; for TLR4, 5′-TGGATACGTTTCCTTATAAG-3′ and 5′-GAAATGGAGGCACCCCTTC-3′; for TLR9, 5′-GTGCCCCACTTCT CCATG-3′ and 5′-GGCACAGTCATGATGTTGTTG-3′; and for β-actin, 5′-A TTGGCAATGAGCGGTTCCGC-3′ and 5′-CTCCTGCTTGCTGATCCACAT

C-3′. The primers for Nod1, Nod2, TLR2, TLR3, TLR4, TLR9, and β-actin were constructed to generate fragments of 374, 312, 368, 305, 506, 260, and 336 bp, respectively. Each PCR mixture contained 4 μl of the cDNA mixture, 1.2 μl of 10× Takara Ex *Taq* buffer (Takara Bio Inc.), 2.5 mM $MgCl_2$, and 0.1 μl of Takara Ex *Taq* (Takara Bio Inc.) in a total of volume of 20 μl. Amplification was performed as follows: for Nod1, 35 cycles of denaturation at 94°C for 60 s, annealing at 56°C for 60 s, and extension at 72°C for 60 s; for Nod2, 35 cycles at 94°C for 60 s, 58°C for 60 s, and 72°C for 90 s; for TLR2, 33 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 90 s; for TLR3, 35 cycles at 95°C for 45 s, 50°C for 45 s, and 72°C for 60 s; for TLR4, 28 cycles at 95°C for 40 s, 54°C for 40 s, and 72°C for 60 s; for TLR9, 35 cycles at 94°C for 60 s, 54°C for 60 s, and 72°C for 60 s; and for β-actin, 30 cycles at 94°C for 30 s, 55°C for 40 s, and 72°C for 60 s. Amplified samples were visualized in 2% agarose gels stained with ethidium bromide and photographed under UV light.

Real-time PCR. Fifty nanograms of cDNA was used for amplification. PCRs were performed with a Light Cycler FastStart DNA SYBR green I kit (Roche Diagnostics, Mannheim, Germany), using a Light Cycler instrument (Roche Diagnostics). Each reaction was carried out in a total volume of 20 μl in a glass capillary containing 50 ng of cDNA sample, 3 mM $MgCl_2$, 10% Light Cycler-DNA Master SYBR green I buffer, and 20 pmol of each primer. The primers used were as follows: for IL-12 p35, 5′-CTTCACCACTCCCAAAACCTG-3′ and 5′-AGCTCGTCACTCTGTCAATAG-3′; for IL-12 p40, 5′-CCACATTCC TACTTCTC-3′ and 5′-GTCTATTCCGTTGTGTC-3′; for IFN-γ, 5′-TGCAGG TCATTCAGATGTAG-3′ and 5′-AGCCATCACTTGGATGAGTT-3′; for IL-15, 5′-GCCAACTGGGTGAATGTAATA-3′ and 5′-GTGAAGAACTAACGT TAACTA-3′; and for IL-18, 5′-GCTTGAATCTAAATTATCAGTC-3′ and 5′-GAAGATTCAAATTGCATCTTAT-3′. Each cDNA sample was denatured at 95°C for 10 min, and the PCR cycling conditions were as follows: for IL-12 p35, 40 cycles at 95°C for 15 s, 56°C for 10 s, and 72°C for 21 s; for IL-12 p40, 40 cycles at 95°C for 15 s, 52°C for 10 s, and 72°C for 11 s; for IFN-γ, 40 cycles at 95°C for 15 s, 55°C for 10 s, and 72°C for 12 s; for IL-15, 40 cycles at 95°C for 15 s, 60°C for 10 s, and 72°C for 21 s; for IL-18, 40 cycles at 95°C for 15 s, 55°C for 10 s, and 72°C for 14 s; and for β-actin, 40 cycles at 95°C for 15 s, 55°C for 10 s, and 72°C for 14 s, with a single fluorescence detection point at the end of the extension segment. After PCR amplification, one cycle of increase from 65°C to 95°C at a transition rate of 0.1°C/s, with continuous detection of fluorescence, was performed to assess the specificity of the amplified PCR products. No amplification of unspecific products was observed. Data were calculated from a standard curve and expressed relative (assigning a value of 1 to medium alone) to data for β-actin mRNA as an internal standard.

Statistical analysis. All of the experiments in this study were conducted at least three times. The data shown are representative results. Experimental values are given as means ± standard deviations (SD) of triplicate assays. The statistical significance of the difference between two means was examined by one-way analysis of variance, using the Bonferroni or Dunnett method, and P values of <0.05 were considered significant.

RESULTS

Phenotypic analysis of monocyte-derived immature DCs and their mRNA expression for Nods and TLRs. To prepare DCs, human peripheral blood monocytes were cultured with IL-4 and GM-CSF for 6 days. On day 6 of culture, we detected high levels of HLA-DR and CD1a in the cells by flow cytometry (Fig. 1A), indicating that these cells were phenotypically DCs. In further flow cytometric analyses, the cells expressed practically no CD14, a high level of CD40, and low levels of CD80 and CD86, but they did not express CD83, a marker for mature DCs (Fig. 1B), indicating that they were immature DCs. We examined the mRNA expression for Nods and TLRs by RT-PCR and found that the immature DCs expressed Nod1, Nod2, TLR2, TLR3, TLR4, and TLR9 (Fig. 1C).

Synergistic effects of MDP or FK565 with synthetic lipid A or LPS on induction of IL-12 p70 and IFN-γ in DCs in culture. We examined the effects of MDP (Nod2 agonist), MDP-LL (inactive stereoisomer of MDP), FK565 (Nod1 agonist), synthetic lipid A (LA-15-PP) and LPS (TLR4 agonists), and various combinations of these stimulants to induce IL-12 p70,

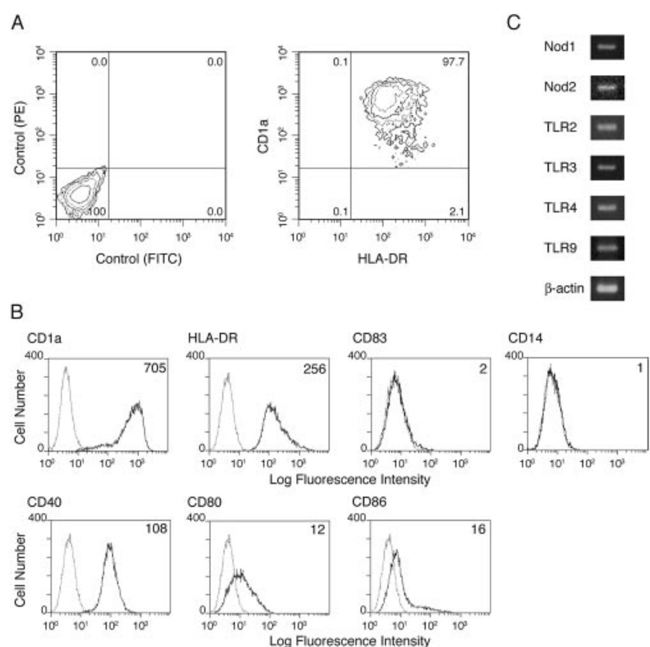

FIG. 1. Expression of Nods and TLRs in DCs derived from human monocytes. (A) Preparation of DCs from monocytes. Human monocytes were cultured in the presence of IL-4 (100 ng/ml) and GM-CSF (100 ng/ml) for 6 days. Cells were collected and stained with anti-HLA-DR (FITC) and anti-CD1a (PE) by flow cytometry to confirm the purity of DCs. (B) Phenotypes of DCs. The DCs were collected and stained with anti-CD1a, -HLA-DR, -CD83, -CD14, -CD40, -CD80, and -CD86 (solid lines) or matched isotype MAbs (dotted lines) and analyzed by flow cytometry. The results in panels A to C are representative of three independent experiments. The percentage of gated cells (A) and the mean fluorescence intensity (B) are indicated in the corner. (C) mRNA expression for Nods and TLRs in DCs. Total RNA was extracted from the cells and analyzed for Nod1, Nod2, TLR2, TLR3, TLR4, TLR9, and β-actin by RT-PCR.

IFN-γ, IL-8, IL-15, and IL-18 production in DCs in culture. In the experiments, the production of cytokines was examined after 72 h of cultivation because cytokine levels reached a maximum at 72 h (data not shown). As shown in Fig. 2A, lipid A and LPS each alone exhibited definite activity for inducing IL-12 p70 production. In contrast, MDP and FK565 were scarcely active and only slightly active, respectively. The combinatory stimulation of DCs with MDP or FK565 plus lipid A or LPS induced marked production of IL-12 p70. FK156 also exhibited similar synergistic activities with lipid A and LPS (data not shown). In this experiment, lipid A and LPS exhibited practically comparable activities. Therefore, lipid A was used in the following experiments, because commercial preparations of LPS were suggested to be contaminated with Nod1 and Nod2 ligands (16). It was previously reported that IL-12 acts directly on human monocyte-derived DCs to produce IFN-γ via the IL-12 receptor (27), suggesting the presence of an autocrine positive feedback pathway in DCs. As shown in Fig. 2B, MDP and FK565 also showed synergistic effects in combination with lipid A to induce IFN-γ production in DCs in culture. On the other hand, the combination of MDP and FK565 with lipid A showed no and only slight synergism to induce IL-8, respectively (Fig. 2C). IL-15 and IL-18 were not detected in any culture supernatants of DCs (data not shown).

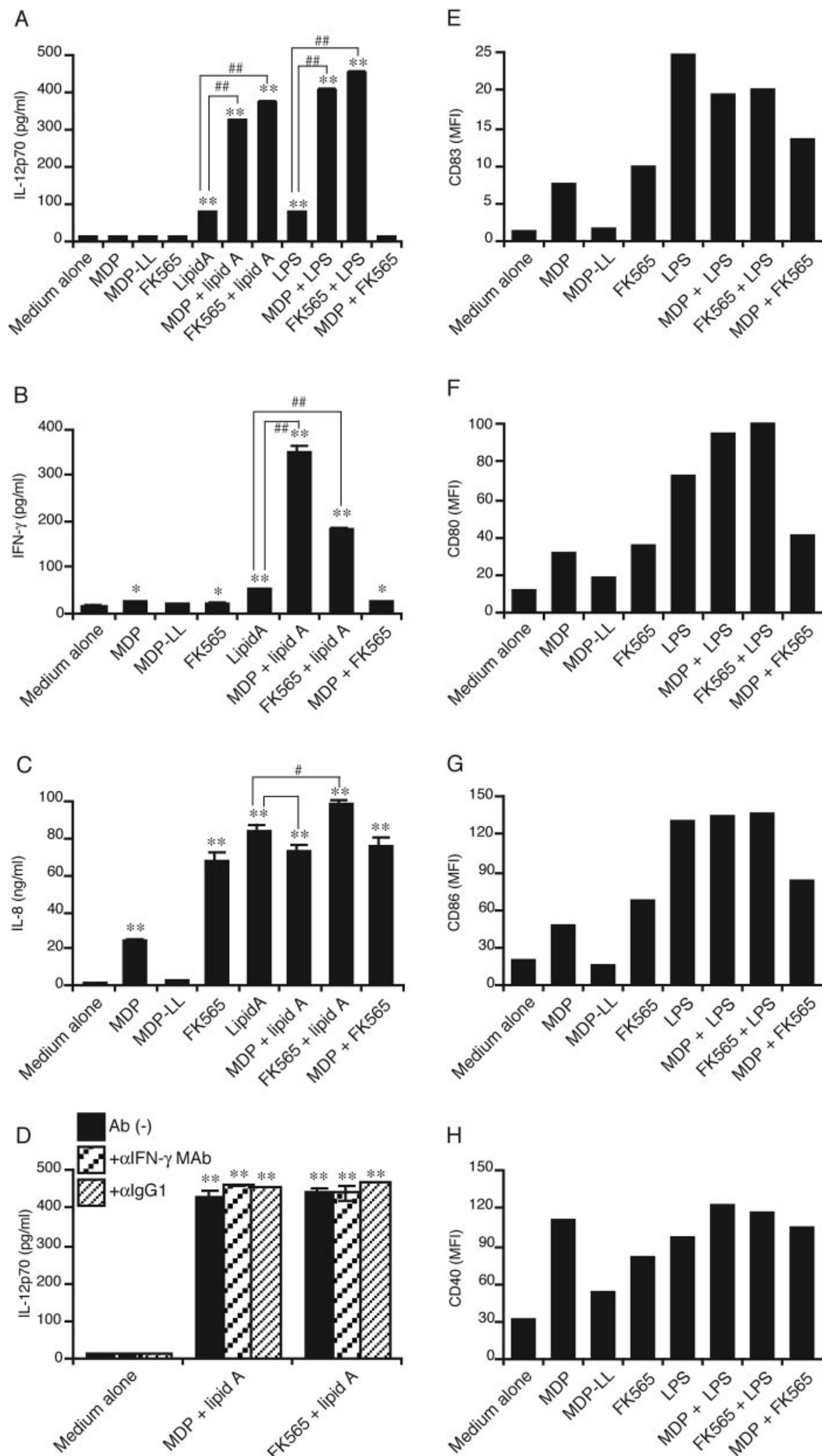

FIG. 2. Synergistic effect of MDP or FK565 with lipid A or LPS on induction of IL-12 p70 and IFN-γ, but not IL-8, and upregulation of CD83, CD80, CD86, and CD40 expression on DCs. DCs were stimulated for 72 h at 37°C with MDP (10 μg/ml), FK565 (10 μg/ml), MDP-LL (10 μg/ml), lipid A (10 ng/ml), and LPS (10 ng/ml). DCs were pretreated with an anti-IFN-γ MAb (10 μg/ml) or a matched isotype MAb (10 μg/ml) for 30 min at 37°C and then were stimulated with MDP, FK565, and LPS for 24 h (H). The amounts of IL-12 p70 (A and D), IFN-γ (B), and IL-8 (C) in the culture supernatants were analyzed by ELISA. Cells were stained with anti-CD83 (E), -CD80 (F), -CD86 (G), and -CD40 (H) or with matched isotype MAbs and analyzed by flow cytometry. The results indicate the mean fluorescence intensities (MFI). Data from ELISAs are expressed as mean values ± SD, and statistically significant differences are shown. ** and *, $P < 0.01$ and $P < 0.05$, respectively, versus medium-alone control; ## and #, $P < 0.01$ and $P < 0.05$, respectively, versus lipid A or LPS alone. The results are representative of three independent experiments.

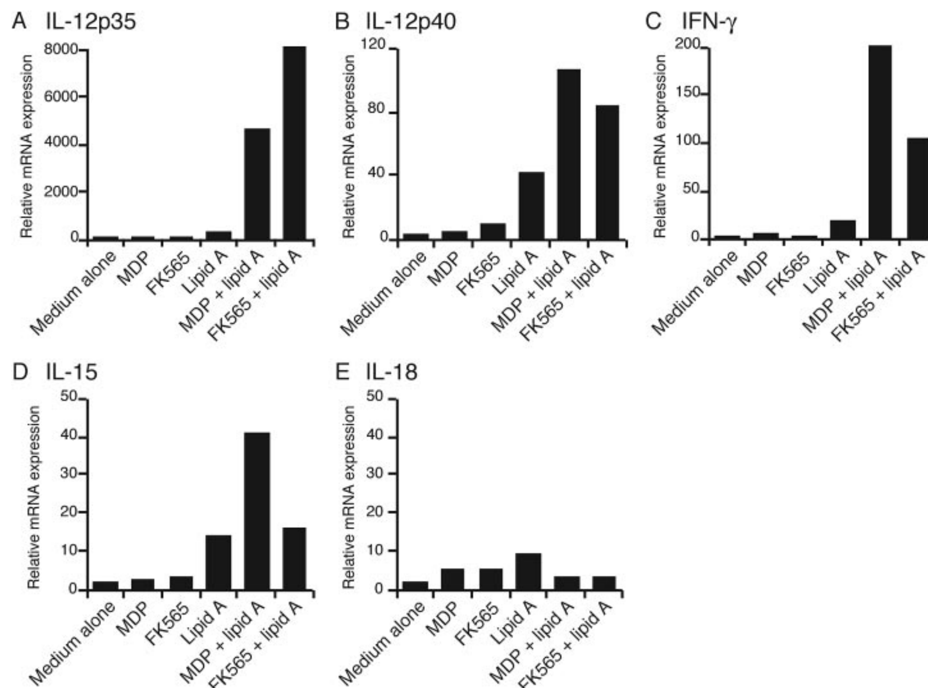

FIG. 3. Synergistic effect of MDP or FK565 with lipid A on induction of IL-12 p35, IL-12 p40, IFN-γ, and IL-15 mRNA expression in DCs. DCs were stimulated for 6 h (or 24 h for IL-15 mRNA) at 37°C with MDP (10 μg/ml), FK565 (10 μg/ml), and lipid A (10 ng/ml). Total RNA was extracted from the cells, and reverse transcription of the RNA samples to cDNAs was performed. Real-time PCR amplification was carried out using a LightCycler-FastStart DNA Master SYBR green I kit (Roche Diagnostics). The relative induction of IL-12 p35 (A), IL-12 p40 (B), IFN-γ (C), IL-15 (D), and IL-18 (E) mRNA expression was determined after normalization using β-actin. The results are representative of three independent experiments.

It must be noted that the combination of MDP and FK565 did not exhibit any synergistic effects and that MDP-LL was inactive in all respects (Fig. 2A to C). The synergistic induction of IL-12 p70 production by lipid A in combination with MDP and FK565 was unaffected in the presence of a neutralizing anti-IFN-γ MAb (Fig. 2D). Therefore, the synergistic IL-12 p70 production induced by lipid A in combination with MDP or FK565 was independent of DC-derived IFN-γ.

Concerning the expression of CD83, a mature DC marker, a definite or marked induction was observed after stimulation with MDP or FK565, respectively, and LPS (Fig. 2E). Stimulation with MDP plus LPS or FK565 plus LPS did not induce any further upregulation of CD83 than stimulation with LPS alone, probably because LPS induced enough maturation of DCs. A similar tendency was observed for the expression of the costimulatory molecules CD80 (Fig. 2F) and CD86 (Fig. 2G). Concerning CD40 upregulation in DCs, MDP and FK565 exerted comparable activities to that of LPS (Fig. 2H).

Synergistic effects of MDP and FK565 with synthetic lipid A on induction of mRNA expression of IL-12 p35, IL-12 p40, IFN-γ, and IL-15 in DCs. Next, we examined the combinatory effect of MDP and FK565 with lipid A on the induction of mRNA expression of various Th1 cytokine genes. Considering the cytokine levels, we determined the mRNA expression of IL-12 p35, IL-12 p40, IFN-γ, IL-15, and IL-18 by real-time PCR. MDP and FK565 each alone exhibited only a weak induction of the mRNA expression of these cytokines, while lipid A alone exhibited a definite induction of mRNA expression in DCs in culture (Fig. 3A to E). Stimulation with MDP plus lipid A markedly and synergistically induced the mRNA expression of IL-12 p35 (Fig. 3A), IL-12 p40 (Fig. 3B), IFN-γ (Fig. 3C), and IL-15 (Fig. 3D) but not that of IL-18 (Fig. 3E). Similar synergistic effects were also observed with FK565 plus lipid A for the mRNA expression of IL-12 p35 (Fig. 3A), IL-12 p40 (Fig. 3B), and IFN-γ (Fig. 3C), while at most an additive effect was noted for IL-15 mRNA induction (Fig. 3D). Among these Th1 cytokine gene profiles, the remarkable synergistic induction of IL-12 p35 mRNA should be noted; the combinations of MDP plus lipid A and FK565 plus lipid A induced several thousand times more IL-12 p35 mRNA than each stimulant alone (Fig. 3A). Concerning IL-18 mRNA expression, neither a synergistic nor additive effect was induced by the combination of MDP plus lipid A or FK565 plus lipid A (Fig. 3E).

Synergistic effects of synthetic Nod agonists with synthetic TLR agonists on induction of IL-12 p70 and IFN-γ production and membrane-bound IL-15 expression in DCs in culture. We further examined whether MDP and FK565 exerted synergistic effects with other TLR agonists, specifically the synthetic triacyl lipopeptide Pam3CSSNA (TLR2 agonist), poly(I:C) (TLR3 agonist), and CpG DNA (TLR9 agonist). As shown in Fig. 4A, poly(I:C) and CpG DNA as well as lipid A exhibited a definite induction of IL-12 p70 production in DCs, while the lipopeptide was inactive in this respect. Poly(I:C) and CpG DNA, similar to lipid A, exhibited marked synergistic effects with MDP and FK565 on the induction of IL-12 p70 production, whereas lipopeptides were completely inactive, even in combi-

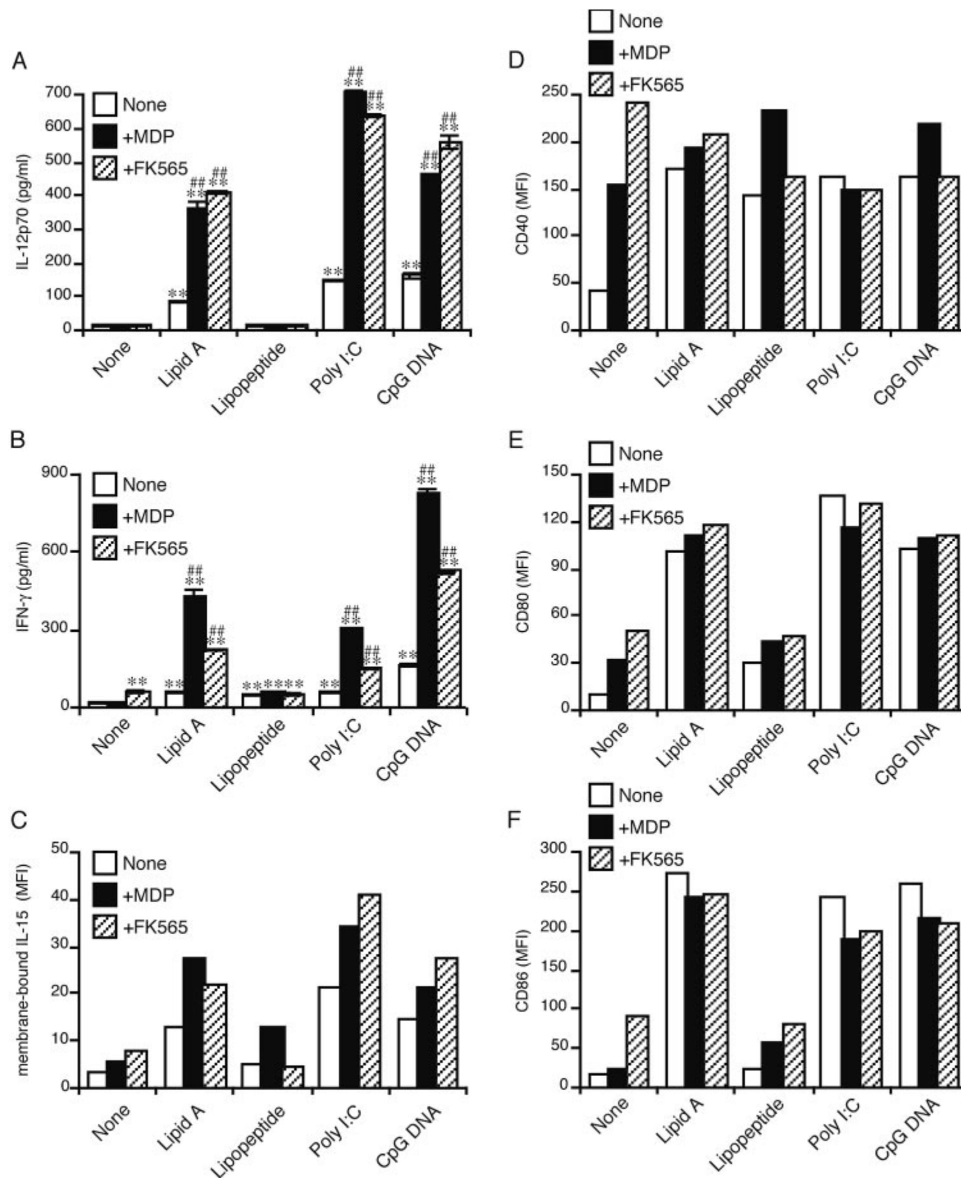

FIG. 4. Synergistic effect of Nod agonists with TLR agonists on induction of IL-12 p70 and IFN-γ production and on upregulation of membrane-bound IL-15, CD40, CD80, and CD86 expression on DCs. DCs were stimulated for 72 h at 37°C with MDP (10 μg/ml), FK565 (10 μg/ml), lipid A (10 ng/ml), Pam3CSSNA (1 μM, 1.27 μg/ml), poly(I:C) (10 μg/ml), or CpG DNA (2 μM). The amounts of IL-12 p70 (A) and IFN-γ (B) in the culture supernatants were analyzed by ELISA. Data are expressed as mean values ± SD, and significant differences are shown. ** and *, $P < 0.01$ and $P < 0.05$, respectively, versus medium alone; ## and #, $P < 0.01$ and $P < 0.05$, respectively, versus the respective TLR agonist alone. Cells were stained with anti-human IL-15 (C), -CD40 (D), -CD80 (E), and -CD86 (F) or with matched isotype MAbs and analyzed by flow cytometry. The results indicate the mean fluorescence intensities (MFI). The results are representative of three independent experiments.

nation with MDP or FK565. Similar tendencies were also observed for IFN-γ production, although the synergistic effects of MDP with lipid A, poly(I:C), and CpG DNA were clearer than those of FK565 with the respective TLR agonists (Fig. 4B). In this experiment, the lipopeptide alone exhibited slight activity but did not show any synergistic effects with MDP or FK565. The lack of synergism between the lipopeptide and MDP or FK565 on the induction of IL-12 p70 and IFN-γ was confirmed when the optimum concentration (1 μM) of lipopeptide was used (data not shown). Concerning cell-bound IL-15, lipid A, poly(I:C), and CpG DNA markedly increased its expression, and MDP and FK565 slightly increased its expression (Fig. 4C). A further increase in cell-bound IL-15 expression was induced by the stimulation of DCs with MDP or FK565 in combination with lipid A, poly(I:C), and CpG DNA. However, the lipopeptide was generally inactive, except for a slight activity in combination with MDP.

The expression of CD80 and CD86 on DCs was upregulated by lipid A, poly(I:C), and CpG DNA but not by the lipopeptide (Fig. 4E and F). These TLR agonists, including the lipopep-

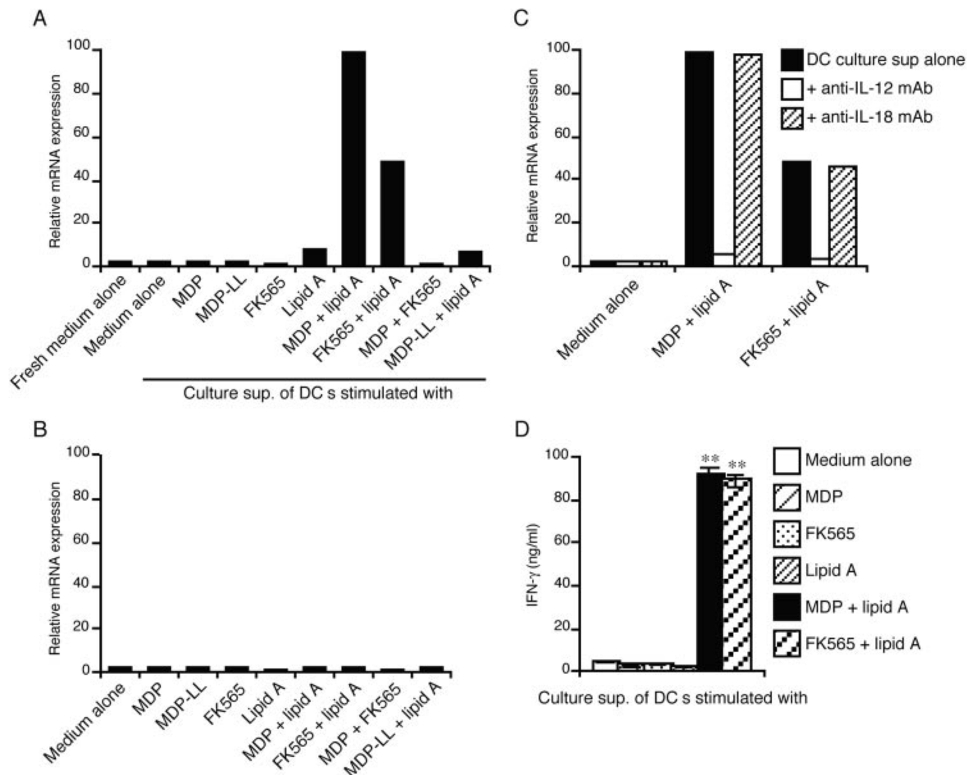

FIG. 5. Combinatory stimulation of DCs by MDP or FK565 with lipid A induces IL-12, which in turn induces IFN-γ mRNA expression in T cells. (A) DCs were stimulated for 72 h at 37°C with MDP (10 μg/ml), FK565 (10 μg/ml), MDP-LL (10 μg/ml), lipid A (10 ng/ml), or various combinations of the compounds. After cultivation, the culture supernatants of DCs were collected. Human peripheral blood T cells were stimulated with the culture supernatants. (B) As a control experiment, T cells were also stimulated with the respective stimulants for 24 h at 37°C. (C) DC culture supernatants were pretreated with an anti-IL-12 MAb (10 μg/ml) or anti-IL-18 MAb (1 μg/ml) for 30 min at 37°C. The total RNA was then extracted from the T cells (A to C), and real-time PCR analyses were carried out. The relative induction of IFN-γ mRNA expression was determined after normalization using β-actin. (D) The amount of IFN-γ in the culture supernatants was analyzed by ELISA. The results are representative of three different experiments. Data are expressed as mean values ± SD, and statistically significant differences are shown. **, $P < 0.01$ versus medium alone.

tide, as well as Nod agonists were definitely and similarly active in upregulating CD40 expression (Fig. 4D). However, no synergistic upregulation of these molecules by the TLR agonists in combination with MDP or FK565 was observed (Fig. 4D to F), probably because lipid A, poly(I:C), and CpG DNA each alone sufficiently induced the maturation of DCs and because the lipopeptide was completely inactive, except toward CD40.

Induction of IFN-γ expression by supernatants of DCs stimulated with MDP and FK565 in combination with synthetic lipid A in human T-cell cultures. The above observation that the stimulation of DCs with Nod agonists plus TLR agonists markedly induced Th1 cytokines, especially IL-12, raised the question of whether DC-derived cytokines promote the development of naïve T cells to Th1 cells, resulting in IFN-γ production. Therefore, we examined the possible induction of IFN-γ expression in human peripheral blood T cells treated with culture supernatants of DCs stimulated with MDP, FK565, synthetic lipid A, and combinations thereof. $CD56^+$ NK cells were not present in our T-cell preparation (data not shown). As expected, culture supernatants of DCs stimulated with MDP plus lipid A and FK565 plus lipid A induced a remarkable upregulation of IFN-γ mRNA in the T cells in culture, while each stimulant alone and the combination of MDP plus FK565 was scarcely active or only slightly active in this respect (Fig. 5A). MDP-LL, a stereoisomer of MDP, was inactive by itself and did not exhibit a synergistic effect with lipid A. The possibility that these stimulants in the culture supernatants of DCs directly activated T cells was negligible given the data shown in Fig. 5B. To examine which factor(s) in the supernatants of DCs is responsible for the activation of T cells to induce IFN-γ mRNA expression, a neutralization assay was performed using anti-IL-12 and anti-IL-18 MAbs. Preincubation with the anti-IL-12 MAb of culture supernatants derived from DCs stimulated with lipid A plus either MDP or FK565 completely diminished IFN-γ mRNA expression by T cells, whereas anti-IL-18 exhibited no influences on the activity of the culture supernatants (Fig. 5C), indicating that IL-12 produced by activated DCs is mainly responsible for the DC-derived Th1-inducing activity. This finding does not necessarily exclude the contribution of other factors, although the possible contribution of IL-18 was completely ruled out. Supernatants from T cells cultured with activated DC culture supernatants were subjected to IFN-γ ELISA. We clearly observed IFN-γ production from the T cells, as shown in Fig. 5D.

DISCUSSION

In this study, we showed that a Nod2-agonistic MDP and Nod1-agonistic FK565 in combination with TLR4-agonistic lipid A, TLR3-agonistic poly(I:C), or TLR9-agonistic CpG DNA, but not with TLR2-agonistic Pam3CSSNA, synergistically induced IL-12 production in human DCs in culture and that the IL-12 thus generated promoted T cells to produce IFN-γ. These findings strongly suggest that the combinatory stimulation of DCs via the Nod pathway and the TLR pathway synergistically promotes Th1-lineage immune responses. In bacterium-host interactions, host cells should be stimulated with bacterial PGN fragments, namely, a Nod1 and/or Nod2 agonist(s), in addition to various TLR agonists. Therefore, the above synergism should generally occur in host-bacterium interactions. In particular, facultative intracellular parasitic bacteria, represented by mycobacteria, intracellular Nod1, and/or Nod2 should be stimulated strongly by *meso*-DAP-type PGN. It must be noted that a suitable vehicle such as a water-in-oil emulsion was required for Nod1 and Nod2 adjuvants to induce cell-mediated immunity, especially Th1-mediated delayed-type hypersensitivity. These vehicles might be useful for the adjuvants to interact with intracellular Nod receptors. Our findings present a possible mechanism by which PGN-related Nod adjuvants induce Th1-lineage immune responses.

IL-12 derived from DCs plays a central role in the development of Th1 cells producing IFN-γ (25). The culture supernatants of DCs stimulated with MDP plus lipid A and FK565 plus lipid A synergistically induced IFN-γ protein production by T cells (Fig. 5D), indicating that DC-derived IL-12 p70 induced Th1 differentiation and/or activation. Since T cells were prepared by depleting non-T cells, the T cells likely consist of naïve T cells and activated/memory T cells. Thus, it cannot be totally excluded that IFN-γ is produced not only by naïve T cells but also by activated/memory Th1 cells. The combination of IL-12 with either IL-18, IFN-γ, or IL-15 synergistically induced IFN-γ in T cells (6, 28, 30, 38). The induction of IFN-γ mRNA expression in T-cell cultures in response to culture supernatants of DCs stimulated with either MDP or FK565 plus lipid A was completely inhibited by an anti-IL-12 MAb, whereas an anti-IL-18 MAb did not have any influence (Fig. 5C), indicating that DC-derived IFN-γ-inducing activity is attributable to IL-12 and not IL-18. It must be noted, however, that this finding does not rule out the possible involvement of other cofactors such as IFN-γ and IL-15, as suggested above. Concerning IL-15, cell surface molecules on DCs were detected, but cell-free IL-15 was not detected in the DC-derived culture supernatants in this study; a slight upregulation of cell surface IL-15 was induced by MDP, FK565, and lipopeptide (each alone), and a definite upregulation of cell surface IL-15 was induced by lipid A, poly(I:C), and CpG DNA (each alone) and by MDP and FK565 in combination with lipid A, poly(I:C), and CpG DNA (Fig. 3D and 4C). Therefore, the involvement of IL-15 in the IFN-γ-inducing ability of DC-derived supernatants was negligible, but the possibility of interactions between DCs and T cells via IL-15 and the IL-15 receptor still remained.

Various TLR agonists by themselves induced low levels of IL-12 in DCs in culture, and the induction of higher levels of IL-12 in response to TLR agonists was attained in the presence of costimulants such as IFN-γ and CD40 ligand (21, 23, 26, 36). It must be considered that both IFN-γ and CD40 ligand are consequences of Th1 responses. Therefore, this study is the first report of the production of high levels of IL-12 in DC cultures followed by Th1 responses induced by only bacterial components without other stimuli. Previous reports showed that in vitro-differentiated monocyte-derived DCs in the presence of IL-4 and GM-CSF do not express TLR9 (19). However, monocyte-derived DCs expressed TLR9 in this study (Fig. 1C) and produced IL-12 p70 and IFN-γ in response to CpG DNA (Fig. 4A and B). Since in vitro-differentiated monocyte-derived DCs contain $<2\%$ $CD1a^-$ $HLA\text{-}DR^+$ cells, which are characterized as plasmacytoid DCs (18) (Fig. 1A), it cannot be totally excluded that CpG DNA-mediated activities may be caused by the presence of a small number of plasmacytoid DCs (Fig. 4A and B).

In this study, the TLR2-agonistic lipopeptide Pam3CSSNA did not show a synergistic induction of IL-12 p70 and IFN-γ with MDP or FK565 in DCs in culture (Fig. 4). On the other hand, marked synergism was observed between Pam3CSSNA and MDP or FK565 for IL-8 induction in human monocytic THP-1 cells in culture (45). Although monocyte-derived DCs expressed TLR2 (Fig. 1C) and produced a low but significant level of IFN-γ upon stimulation with Pam3CSSNA (Fig. 4B), the cells did not produce IL-12 p70 (Fig. 4A). Consistent with our data, triacyl-type lipopeptides induced little or no IL-12 p70 expression in human monocyte-derived DCs (2) and mouse bone marrow-derived DCs (33) and also only weakly induced CD80 and CD86 expression in human DCs (2). In these previous reports, the authors suggested that TLR4, TLR5, and TLR9 ligands induced Th1 polarization, while TLR2 ligands induced Th2 polarization. In this context, Re and Strominger reported that TLR2 stimulation by Pam3Cys failed to induce IL-12 p70 due to a rapid release of IL-10 that is responsible for the inhibition of IL-12 p35 and IFN-γ induction in human DCs (31, 32). Watanabe et al. (47) reported that the Nod2 signal downregulated Th1 responses induced by TLR2 signaling, but not by other TLR signals. IL-12 p70 production from wild-type mouse splenocytes by Pam3Cys was decreased in the presence of MDP by a reduction of c-Rel activation (47). Furthermore, IL-12 p35 and p40 gene transcription was critically dependent on c-Rel activity in mouse DCs (14). These findings, in conjunction with our findings, strongly suggest that the Nod1 as well as Nod2 signal augments Th1 polarization induced by various TLR signals, except for TLR2 signaling, which inhibits the induction of Th1 cytokines due to IL-10 production.

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This report is dedicated to a pioneer in PGN and muramylpeptide research, S. Kotani (Osaka University), who is now deceased.

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