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Distinct Patterns of Dendritic Cell Cytokine Release Stimulated by Fungal β-Glucans and Toll-Like Receptor Agonists▽

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β-Glucans derived from fungal cell walls have potential uses as immunomodulating agents and vaccine adjuvants. Yeast glucan particles (YGPs) are highly purified *Saccharomyces cerevisiae* cell walls composed of β1,6-branched β1,3-D-glucan and free of mannans. YGPs stimulated secretion of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) in wild-type murine bone marrow-derived myeloid dendritic cells (BMDCs) but did not stimulate interleukin-12p70 (IL-12p70) production. A purified soluble β1,6-branched β1,3-D-glucan, scleroglucan, also stimulated TNF-α in BMDCs. These two β-glucans failed to stimulate TNF-α in Dectin-1 (β-glucan receptor) knockout BMDCs. Costimulation of wild-type BMDCs with β-glucans and specific Toll-like receptor (TLR) ligands resulted in greatly enhanced TNF-α production but decreased IL-12p70 production compared with TLR agonists alone. The upregulation of TNF-α and downregulation of IL-12p70 required Dectin-1, but not IL-10. Gamma interferon (IFN-γ) priming did not overcome IL-12p70 reduction by β-glucans. Similar patterns of cytokine regulation were observed in human monocyte-derived dendritic cells (DCs) costimulated with YGPs and the TLR4 ligand lipopolysaccharide. Finally, costimulation of BMDCs with YGPs and either the TLR9 ligand, CpG, or the TLR2/1 ligand, Pam_3CSK_4, resulted in upregulated secretion of IL-1α and IL-10 and downregulated secretion of IL-1β, IL-6, and IFN-γ-inducible protein 10 but had no significant effects on IL-12p40, keratinocyte-derived chemokine, monocyte chemotactic protein 1, or macrophage inflammatory protein α, compared with the TLR ligand alone. Thus, β-glucans have distinct effects on cytokine responses following DC stimulation with different TLR agonists. These patterns of response might contribute to the skewing of immune responses during mycotic infections and have implications for the design of immunomodulators and vaccines containing β-glucans.

The immune response following an encounter with a microbe is heavily influenced by interactions between host pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) (28). β-Glucans, especially β-(1-3)-glucans, are the major component of the cell walls of most fungi (5). Indeed, β-glucans comprise up to 60% of the dry weight of the fungal cell wall and are considered to be a major PAMP involved in host-fungus interactions (48). Dectin-1, the major receptor for β-glucans, is a C-type lectin that is highly expressed on dendritic cells (DCs). Its expression can also be detected in macrophages, monocytes, and neutrophils (6, 45). The cytoplasmic tail of Dectin-1 contains an immunoreceptor tyrosine-based activation (ITAM)-like motif (1). Upon binding of β-glucan to Dectin-1's extracellular lectin binding domain, the tyrosine residue within the cytoplasmic ITAM motif is phosphorylated (21, 38). This results in recruitment of spleen tyrosine kinase (Syk) and caspase recruitment domain protein 9 (Card9) (19). Although the exact signal transduction pathways have not been elucidated, these events can lead to activation of nuclear factor of activated T cells (NFAT) (16), mitogen-activated protein kinases (11), and nuclear factor kappa B (NF-κB) (2, 9, 18, 38, 42, 53), leading to cytokine production.

In addition to Dectin-1, other receptors have been reported to bind β-glucans, including complement receptor 3 (CR3, a heterodimer of CD11b and CD18), lactosylceramide, scavenger receptors, and CD5 (12, 49). Host PRRs also recognize other fungal components: mannose receptor (MR) and dendritic cell intercellular adhesion molecule 3 (ICAM3)-grabbing nonintegrin (DC-SIGN) recognize mannans (30), Toll-like receptor 2 (TLR2) recognizes phospholipomannan (25), TLR4 recognizes O-linked mannans (35), and TLR9 detects fungal DNA (33, 37). During the course of a fungal infection, multiple PAMPs are likely to be stimulated by host PRRs. The final response will depend not only on the relative degree of stimulation of the individual receptors but also on whether receptor costimulation is additive, synergistic, or antagonistic. Such considerations may also be critical for vaccine design.

Here, we examined the ability of purified fungal β-glucans, alone and in combination with TLR agonists, to stimulate cytokine production in mouse and human DCs. To mimic the range of β-glucans encountered during a fungal infection, both particulate and soluble β-glucans were studied. Yeast glucan particles (YGPs) derived from *Saccharomyces cerevisiae* are composed of β1,6-branched β1,3-D-glucan and served as the particulate β-glucan. Scleroglucan (SCG), a linear β-1,3-glucan with one β-1,6-D-glucose side chain every three residues, served as the soluble stimulus. We found that the combination of β-glucans and TLR agonists had inhibitory, neutral, or stim-

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ulatory effects depending upon the individual cytokine studied. The information gathered here has important implications for our understanding of how immune responses to fungal pathogens develop, as well as for the design of vaccines and immunomodulators containing β-glucans.

MATERIALS AND METHODS

Chemicals and cell culture. Chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. The TLR agonists used in the experiments (and their final concentrations) were Pam_3CSK_4 (a triacylated synthetic lipoprotein) (10 μg/ml) (Invivogen, San Diego, CA), poly(I:C) (10 μg/ml) (Invivogen), lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (0.1 μg/ml), imiquimod (2 μg/ml) (Invivogen), and CpG 1826 (2 μg/ml) (Coley, Ottawa, Ontario, Canada). To make it an ultrapure TLR4 ligand, LPS was further purified from the original Sigma stock by two treatments with deoxycholate followed by phenol extraction and ethanol precipitation (22). SCG, derived from the filamentous fungus *Sclerotium rolfsii*, was obtained from Cargill (Minneapolis, MN). It was solubilized in dimethyl sulfoxide and added to the medium immediately before use. RPMI 1640 medium was purchased from Invitrogen Life Technologies (Carlsbad, CA). R10 medium is defined as RPMI 1640 medium supplemented with 10% fetal bovine serum (Tissue Culture Biologicals, Tulare, CA), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Invitrogen), 0.5 μg/ml amphotericin B, and 55 nM 2-mercaptoethanol (Invitrogen). Human myeloid DC (hDC) medium is the same as R10 medium except that it contains less fetal bovine serum (5%) than R10 medium. Cells were incubated at 37°C in humidified air supplemented with 5% CO_2.

YGPs. Yeast glucan particles were prepared from baker's yeast cells (*S. cerevisiae*) by a series of alkaline and acidic extraction steps. Briefly, the yeast cells were collected by centrifugation, washed free of growth medium in water, suspended in 1 M NaOH, and heated at 90°C for 1 h. Centrifugation and hot alkali extraction were then repeated. The alkali-extracted particles were then suspended in water at pH 4.5 and heated at 75°C for 1 h, followed by successive washes of the particles with water (three times), isopropanol (four times), and acetone (two times). YGPs were dried and then resuspended in 0.9% saline, briefly sonicated, counted on a hemocytometer, and stored in aliquots at −20°C until use. YGPs are 2 to 4 microns in size, hollow, porous cell wall ghosts composed of β-1,3 and β-1,6 glucan and a small fraction of chitin (23). One microgram of YGPs contains approximately 5×10^5 particles.

DTAF labeling of YGPs. YGPs (5 mg/ml) were incubated with dichlorotriazinylaminofluorescein (DTAF) (0.25 mg/ml) in 0.1 M borate buffer (pH 10.8) overnight at 37°C in the dark. Unreacted DTAF was then quenched by incubation with 1 M Tris (pH 8.3) for 30 min. YGPs were extensively washed in sterile water, incubated overnight in 70% ethanol, washed three times with sterile phosphate-buffered saline (PBS), and counted.

Mice. Wild-type C57BL/6 and interleukin-$10^{-/-}$ (IL-$10^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Dectin-$1^{-/-}$ mice were a gift from Gordon D. Brown (University of Cape Town, South Africa) (46). MyD88$^{-/-}$ mice were a gift from Shizuo Akira (Osaka University, Osaka, Japan). All knockout mice were backcrossed to a C57BL/6 background for 8 to 12 generations before use. Mice were specific pathogen free, and all animal procedures were conducted under a protocol approved by The University of Massachusetts Medical School Institutional Use and Care of Animals Committee.

Mouse BMDCs. Bone marrow-derived DCs (BMDCs) were generated as previously described (26, 31) with slight modifications. Briefly, bone marrow cells obtained from the tibiae and femurs of 8- to 12-week-old mice were cultured in R10 medium supplemented with 10% granulocyte-macrophage colony-stimulating factor (GM-CSF) conditioned medium from the mouse GM-CSF-secreting J558L cell line. Cells were fed with fresh GM-CSF-supplemented R10 medium on days 3 and 6. On day 8, nonadherent cells were collected and purified with the Magnetic Cell Separation system (MACS technology) using CD11c$^+$ magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol.

Uptake and binding of YGPs by BMDCs. CD11c$^+$ magnetic bead-purified BMDCs in 400 μl medium (10^5/well) were incubated for 2 h at 37°C in 24-well, cell culture-treated plates with or without 1 mg/ml laminarin. For the binding assay, plates were cooled down at 4°C for 1 h before DTAF-labeled YGPs (3×10^5/well) were added to each well. After 2 h of incubation at 4°C for the binding assay or 1 h of incubation at 37°C for the uptake assay, the wells were washed three times with Dulbecco's PBS (DPBS) (ice-cold DPBS for binding assay and room temperature DPBS for uptake assay) (Lonza, Allendale, NJ) to remove free YGPs and then examined under bright-field and epifluorescence microscopy. Confocal microscopy was performed on a Leica SP2 acousto-optical beam splitter confocal microscope (Leica Microsystems, Bannockburn, IL) using 35-mm glass bottom dishes (MatTek Corp., Ashland, MA), as in previous studies (52).

Monocyte-derived hDCs. Human myeloid DCs were obtained as previously described (39, 52). Briefly, peripheral blood samples were obtained from healthy volunteers following informed consent using a protocol approved by the University of Massachusetts Medical School Institutional Review Board. Peripheral blood mononuclear cells (PBMCs) were purified from blood by Lymphoprep gradient (Accurate Chemical & Scientific Corp., Westbury, NY) using Leukosep tubes (Greiner Bio-One, Germany). The PBMC layer was isolated and washed three times with hDC medium. PBMCs were then added to a six-well tissue culture plate and cultured for 2 h at 37°C to allow monocyte adherence, and then nonadherent cells were gently removed by washing. hDCs were cultured for 7 days with 50 ng/ml recombinant human IL-4 (Peprotech, Rocky Hill, NJ) and 150 ng/ml recombinant human GM-CSF (Sargramostim; Bayer, Wayne, NJ). The cells were then positively selected for CD1c (BDCA-1) expression using magnetically labeled CD1c antibodies (Miltenyi Biotec).

IFN-γ priming of mouse BMDCs or hDCs. Mouse BMDCs or hDCs were primed with 1,000 U/ml of recombinant mouse gamma interferon (IFN-γ) (Peprotech) or human IFN-γ (Peprotech) for 18 h before purification and further treated for 4 h after purification. Medium containing IFN-γ was replaced with fresh medium before stimulation with β-glucans and/or TLR agonists.

Cytokine assays. BMDCs or hDCs (10^5/well in 96-well, flat-bottom, cell culture-treated plates) were incubated with the indicated stimuli in 200 μl R10 medium for 24 h. To minimize the potential for LPS contamination, 20 μg/ml polymyxin B was added to all wells, except for those that specifically received LPS. The concentrations of tumor necrosis factor alpha (TNF-α) and interleukin-12p70 (IL-12p70) in DC supernatants were determined using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols (eBioscience, San Diego, CA). The lower limit of accurate detection for the ELISAs was 31 pg/ml. The concentrations of IL-1α, IL-1β, IL-6, IL-10, IL-12p40, IL-13, IFN-γ-inducible protein 10 (IP-10), keratinocyte-derived chemokine (KC), monocyte chemotactic protein 1 (MCP-1), and macrophage inflammatory protein 1α (MIP-1α) were analyzed on a Bio-Plex Luminex-100 station at the Baylor NIAID Luminex Core (Dallas, TX). The lower limit of accurate detection was 20 pg/ml.

Statistics. GraphPad Prism software was used for statistical analyses. When comparing three or more groups, a one-way analysis of variance with a Tukey multiple correction test was performed. For the experiments comparing two groups (see Fig. 2B, 4, 6, 7, and 8), a two-tailed unpaired Student *t* test was performed. Bonferroni's correction was applied when making multiple comparisons. Statistical significance was defined as a P value of <0.05.

RESULTS

Dependence of Dectin-1 on uptake and binding of fluorescent YGPs. Initial experiments examined whether the β-glucan receptor Dectin-1 contributed to the phagocytosis of YGPs by BMDCs. BMDCs from wild-type C57BL/6 mice efficiently took up DTAF-labeled YGPs (Fig. 1A). However, the uptake of YGPs by BMDCs was greatly impaired in BMDCs derived from Dectin-$1^{-/-}$ mice (Fig. 1B) or if wild-type BMDCs were incubated with 1 mg/ml laminarin (Fig. 1C). Laminarin is a low-molecular-weight β-glucan which is an antagonist of β-glucan receptors, including Dectin-1. Virtually no uptake of YGPs was mediated by Dectin-$1^{-/-}$ DCs incubated with laminarin (Fig. 1D). YGPs appeared to be fully phagocytosed (internalized) by phase-contrast microscopy. This impression was confirmed by confocal microscopy (data not shown). At 4°C, YGPs bound avidly to BMDCs derived from wild-type mice (Fig. 1E) but not those from Dectin-$1^{-/-}$ mice (Fig. 1F). These results identify Dectin-1 as the major receptor responsible for the uptake of YGPs by BMDCs.

Cytokines in BMDCs stimulated by β-glucans. DC production of the cytokines TNF-α and IL-12p70 are thought to critically influence the nature of the inflammatory and immunological responses during the course of an infection. There-

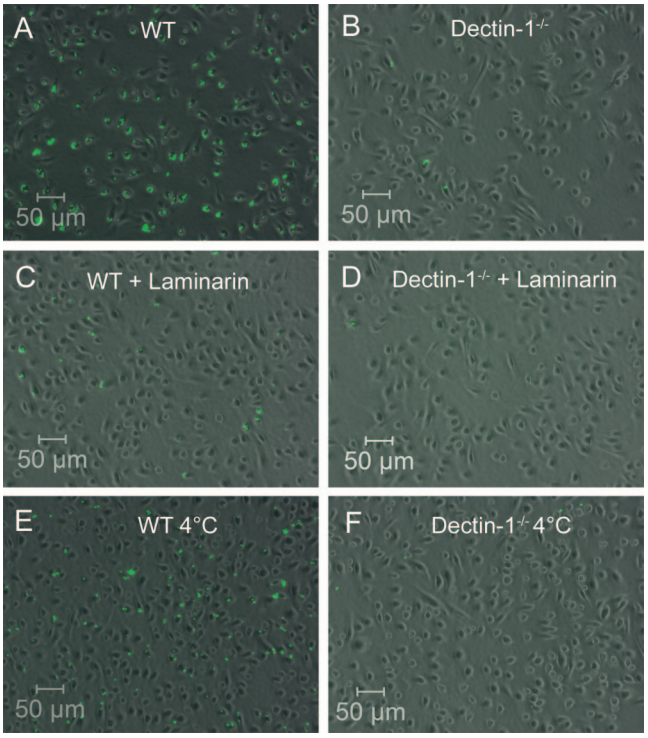

FIG. 1. Recognition of YGPs by BMDCs. DTAF-labeled YGPs (3×10^5) were incubated for 1 h at 37°C or 2 h at 4°C with 10^5 wild-type (WT) or Dectin-$1^{-/-}$ BMDCs in the presence or absence of laminarin (1 mg/ml). The cells were then washed to remove extracellular YGPs and examined by phase-contrast and epifluorescence microscopy. Representative merged images from three independent experiments are shown.

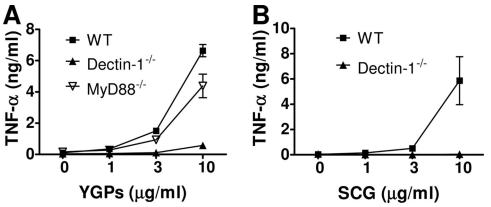

FIG. 2. Dectin-1 dependence of TNF-α production in BMDCs stimulated by β-glucans. (A) $CD11c^+$ magnetic bead-purified BMDCs from wild-type (WT), Dectin-$1^{-/-}$, or MyD$88^{-/-}$ mice were incubated with YGPs (1, 3, or 10 μg/ml) for 24 h, supernatants were collected, and mouse TNF-α levels were measured by ELISAs. Values shown are means ± standard errors (SE) (error bars) for three independent experiments performed in triplicate. The values for WT and Dectin-$1^{-/-}$ BMDCs at 3 and 10 μg/ml YGPs were significantly different with a P value of <0.001. The values for WT and Dectin-$1^{-/-}$ BMDCs at 1 μg/ml YGPs were significantly different with a P value of <0.01. The values for WT and MyD$88^{-/-}$ BMDCs at 3 and 10 μg/ml YGPs were significantly different with a P value of <0.05 and <0.01, respectively. (B) As in panel A, except BMDCs from WT and Dectin-$1^{-/-}$ mice were incubated with SCG. Results are means ± SE (error bars) for three independent experiments performed in triplicate. The values for WT and Dectin-$1^{-/-}$ BMDCs at 1, 3, and 10 μg/ml SCG were significantly different with a P value of ≤0.01.

fore, we next examined secretion of these two cytokines following stimulation of BMDCs with YGPs. YGPs potently stimulated TNF-α secretion from wild-type BMDCs in a dose-dependent manner (Fig. 2A). However, the levels of IL-12p70 were undetectable. In Dectin-$1^{-/-}$ BMDCs, YGPs failed to stimulate appreciable TNF-α production (Fig. 2A). In BMDCs from MyD88 (which is a downstream adapter molecule for many TLRs, including TLR2, TLR4, TLR7/8, and TLR9) knockout mice, YGPs stimulated slightly reduced amounts of TNF-α compared with wild-type BMDCs.

To examine whether the effects seen with YGPs are observed with other β-glucan preparations, the soluble β-glucan, SCG, was studied. SCG stimulated similar patterns of TNF-α in wild-type and Dectin-$1^{-/-}$ BMDCs as observed with YGPs (Fig. 2B). Again, as with YGPs, SCG did not stimulate detectable amounts of IL-12p70. These data show that β-glucans stimulate BMDCs to secrete the proinflammatory cytokine TNF-α in a Dectin-1-dependent, MyD88-independent manner. However, both β-glucan preparations tested failed to stimulate BMDCs to secrete IL-12p70.

Costimulation of BMDCs with YGPs and TLR agonists. During the course of a natural fungal infection, β-glucans combine with other PAMPs, including TLR agonists, to stimulate immune cells (34). Moreover, addition of TLR agonists could enhance the immunomodulatory and adjuvant effects of β-glucans. Therefore, TNF-α and IL-12p70 secretion was determined in BMDCs derived from wild-type, Dectin-$1^{-/-}$, and MyD$88^{-/-}$ mice costimulated with YGPs and selected TLR agonists. Pam_3CSK_4 (TLR2/1 agonist), poly(I:C) (TLR3 agonist), LPS (TLR4 agonist), imiquimod (TLR7 agonist), and CpG (TLR9 agonist) each stimulated TNF-α from wild-type BMDCs (Fig. 3A). Combining YGPs with the TLR agonists resulted in further increases in TNF-α secretion. Combining the TLR ligand with YGPs resulted in synergy, defined as TNF-α secretion that was greater than the sum of two stimuli given individually.

In contrast, IL-12p70 concentrations in the supernatants decreased when YGPs were combined with TLR agonists (Fig. 3B). The effects combining YGPs and TLR agonists had on upregulation of TNF-α and downregulation of IL-12p70 were profoundly blunted in Dectin-$1^{-/-}$ BMDCs (Fig. 3C and D). Substitution of MyD$88^{-/-}$ BMDCs for wild-type cells resulted in complete loss of cooperative stimulation for those TLR agonists totally dependent on MyD88 (Pam_3CSK_4, imiquimod, and CpG) (Fig. 3E). Incomplete loss was observed with LPS, which is partially dependent upon MyD88. No significant effect was seen with poly(I:C), which is MyD88 independent. IL-12p70 release from MyD$88^{-/-}$ BMDCs was near or below the detection level of the ELISA kit (Fig. 3F).

Costimulation of BMDCs with SCG and TLR agonists. In order to demonstrate that the effect of upregulation of TNF-α and downregulation of IL-12p70 is not dependent on the particulate nature of YGPs, the costimulation experiments were repeated using the soluble β-glucan, SCG. Both wild-type and Dectin-$1^{-/-}$ BMDCs were studied. Similar results were obtained with SCG (Fig. 4) as observed with YGPs (Fig. 3), suggesting regulatory effects mediated by β-glucans are not dependent on particulate interactions.

Costimulation of IFN-γ-primed BMDCs with YGPs and TLR agonists. IFN-γ potently primes DCs to increase production of IL-12p70 following stimulation with PAMPs. Therefore, we next examined whether priming BMDCs with IFN-γ

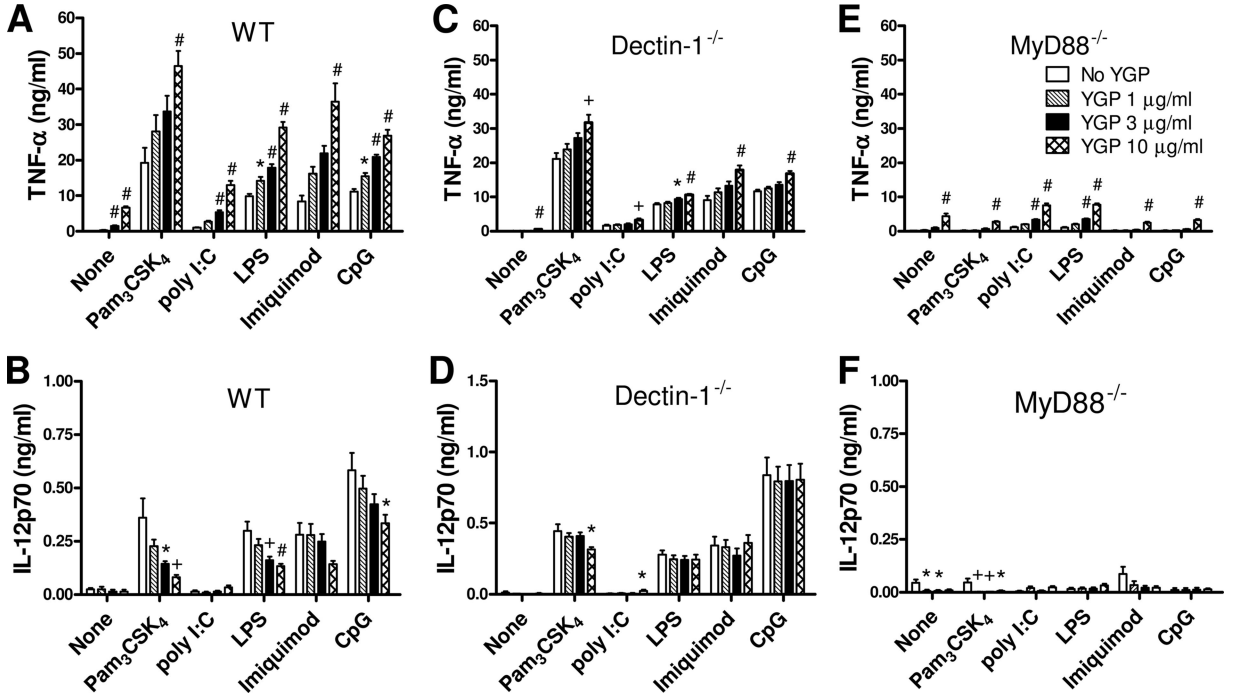

FIG. 3. TNF-α and IL-12p70 secretion by wild-type, Dectin-1$^{-/-}$, and MyD88$^{-/-}$ BMDCs stimulated with YGPs and/or TLR agonists. YGPs (1, 3, or 10 μg/ml) and/or TLR agonists were incubated with CD11c^{+} magnetic bead-purified wild-type (WT), Dectin-1$^{-/-}$, or MyD88$^{-/-}$ BMDCs. Supernatants were collected 24 h later, and mouse TNF-α (A, C, and E) and IL-12p70 (B, D, and F) levels were measured by ELISAs. The TLR agonists are shown below the *x* axes of panels A to F. Values shown are means ± standard errors (error bars) for three independent experiments performed in triplicate. The values for BMDCs not stimulated with YGPs were significantly different from the values for BMDCs stimulated with the indicated concentration of YGP ($P < 0.05$ [*], $P < 0.01$ [+], and $P < 0.001$ [#]).

would reverse the inhibitory effect of β-glucans on IL-12p70 stimulation. As expected, IFN-γ priming of wild-type and Dectin-1$^{-/-}$ BMDCs boosted secretion of IL-12p70 in response to stimulation with Pam_3CSK_4, LPS, imiquimod, and CpG (compare Fig. 5 with Fig. 3), but costimulation with YGPs still profoundly downregulated IL-12p70 secretion in IFN-γ-primed wild-type BMDCs stimulated with these TLR agonists (Fig. 5A). For the IFN-γ-primed Dectin-1$^{-/-}$ BMDCs, minimal to no β-glucan-mediated downregulation of IL-12p70 was found (Fig. 5B), similar to that in nonprimed Dectin-1$^{-/-}$

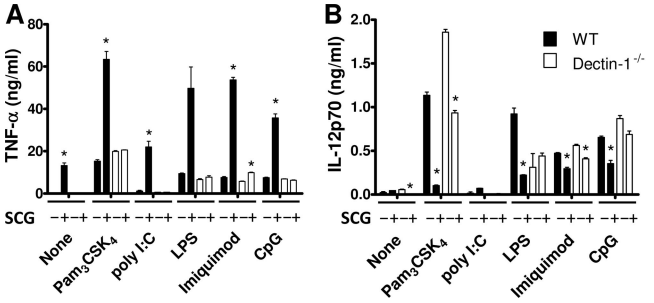

FIG. 4. TNF-α and IL-12p70 secretion by BMDCs from wild-type and Dectin-1$^{-/-}$ mice following stimulation with SCG and/or TLR agonists. CD11c^{+} magnetic bead-purified BMDCs from wild-type (WT) and Dectin-1$^{-/-}$ mice were incubated with SCG (10 μg/ml) and/or the indicated TLR agonists. Supernatants were collected 24 h later, and the levels of TNF-α (A) and IL-12p70 (B) were determined by ELISAs. Values shown are means ± standard errors (error bars) for a representative experiment of two experiments performed in triplicate. The TLR agonists are shown below the *x* axes. Results for SCG alone are also presented in Fig. 2B. Values for each condition with SCG (+) and without SCG (−) that were significantly different ($P < 0.002$) are indicated by an asterisk above the bar.

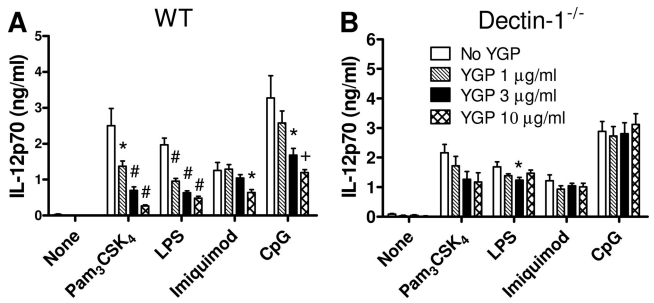

FIG. 5. IL-12p70 secretion by IFN-γ-primed BMDCs from wild-type and Dectin-1$^{-/-}$ mice following stimulation with YGPs and/or TLR agonists. YGPs (1, 3, or 10 μg/ml) and/or TLR agonists were incubated with IFN-γ-primed, CD11c^{+} magnetic bead-purified BMDCs from wild-type (WT) or Dectin-1$^{-/-}$ mice. Supernatants were collected 24 h later, and mouse IL-12p70 concentrations were measured by ELISAs. The TLR agonists are shown below the *x* axes. Values shown are means ± standard errors (error bars) for three independent experiments performed in triplicate. The values for BMDCs not stimulated with YGPs were significantly different from the values for BMDCs stimulated with the indicated concentration of YGP ($P < 0.05$ [*], $P < 0.01$ [+], and $P < 0.001$ [#]).

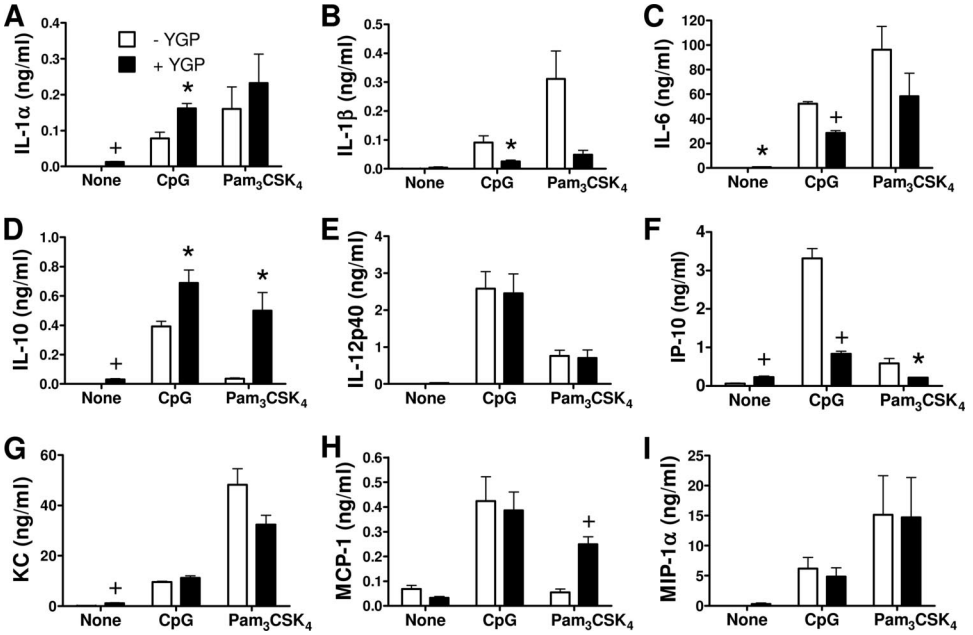

FIG. 6. Cytokine secretion by wild-type BMDCs following stimulation with YGPs and/or TLR agonists. YGPs (10 μg/ml) and/or Pam_3CSK_4 or CpG were incubated with $CD11c^+$ magnetic bead-purified wild-type BMDCs. Supernatants were collected 24 h later, and mouse IL-1α (A), IL-1β (B), IL-6 (C), IL-10 (D), IL-12p40 (E), IP-10 (F), KC (G), MCP-1 (H), MIP-1α (I), and IL-13 (data not shown; levels below the detection limit [20 pg/ml]) levels were measured. The TLR agonists are shown below the *x* axes. For the group not stimulated (None) and the groups stimulated with YGPs, CpG, and YGPs plus CpG, the values shown are means ± standard errors (SE) (error bars) for three independent experiments performed in triplicate. For the groups stimulated with Pam_3CSK_4 and Pam_3CSK_4 plus YGPs, the values shown are means ± SE for two independent experiments performed in triplicate. The values for BMDCs not stimulated with YGPs (− YGP) were significantly different from the values for BMDCs stimulated with YGP (+ YGP) ($P \leq 0.01$ [*] and $P < 0.001$ [+]).

BMDCs (Fig. 3D). These results suggest that inhibition of IL-12p70 production by β-glucans is not restored by IFN-γ priming.

Differential stimulation of cytokines and chemokines by YGPs and/or TLR agonists. The nature of the stimulated inflammatory response is predicted to depend upon many inflammatory mediators besides TNF-α and IL-12p70. Therefore, using Luminex technology, we examined the cytokine and chemokine profiles of BMDCs stimulated with YGPs in the presence and absence of TLR agonists (CpG and Pam_3CSK_4). YGPs alone (10 μg/ml) induced little to no detectable secretion of IL-1α, IL-1β, IL-6, KC, IL-10, IL-12p40, IL-13, MCP-1, IP-10, and MIP-1α (Fig. 6). CpG alone induced detected levels of all cytokines and chemokines tested, except for IL-13 (Fig. 6). Compared with CpG alone, the coadministration of YGPs and CpG resulted in upregulation of IL-1α (Fig. 6A) and IL-10 (Fig. 6D) and downregulation of IL-1β (Fig. 6B), IL-6 (Fig. 6C), and IP-10 (Fig. 6F) and had no significant effects on IL-12p40 (Fig. 6E), KC (Fig. 6G), MCP-1 (Fig. 6H), and MIP-α (Fig. 6I). Similar results were obtained following stimulation with YGPs and Pam_3CSK_4, except for MCP-1 where upregulation was observed.

Downregulation of IL-12p70 by YGPs and TLR agonists is not dependent on IL-10. The finding that costimulation of BMDCs with YGPs and TLR agonists increased the levels of IL-10 in supernatants compared with the levels when the BMDCs were stimulated with the TLR agonists alone raised the possibility that the downregulation of IL-12p70 responses was mediated by IL-10. To examine this possibility, BMDCs from $IL\text{-}10^{-/-}$ mice were stimulated with YGPs and TLR agonists, and the concentrations of TNF-α and IL-12p70 were determined. Similar patterns were seen as with the wild-type BMDCs; the addition of YGPs to TLR agonists stimulated higher TNF-α responses (Fig. 7A) but reduced concentrations of IL-12p70 for Pam3CSK4, imiquimod, and CpG (Fig. 7B).

Costimulation of IFN-γ-primed hDCs with YGPs and TLR agonists. Species-specific differences may be observed by com-

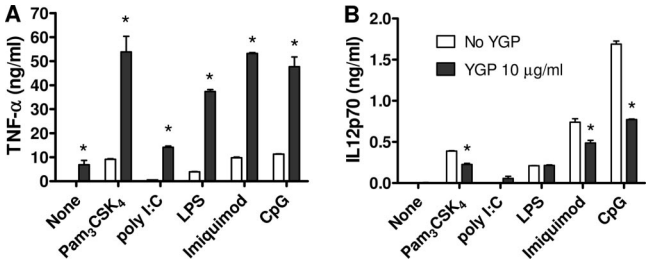

FIG. 7. TNF-α and IL-12p70 secretion by BMDCs from $IL\text{-}10^{-/-}$ mice following stimulation with YGPs and/or TLR agonists. $CD11c^+$ magnetic bead-purified BMDCs from $IL\text{-}10^{-/-}$ mice were incubated with YGPs (10 μg/ml) and/or the indicated TLR agonists. Supernatants were collected 24 h later, and the levels of TNF-α (A) and IL-12p70 (B) were determined by ELISAs. Values shown are means ± standard errors (error bars) for a representative experiment of three independent experiments performed in triplicate. The values for BMDCs not stimulated with YGPs were significantly different ($P < 0.02$) from the values for BMDCs stimulated with YGP as indicated by an asterisk above a bar.

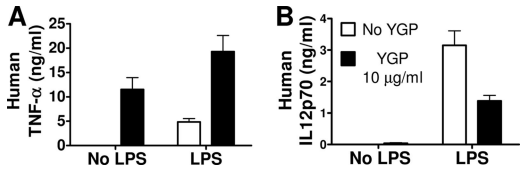

FIG. 8. TNF-α and IL-12p70 secretion by IFN-γ-primed hDCs following stimulation with YGPs and/or LPS. YGPs (10 μg/ml) and/or LPS were incubated with IFN-γ-primed, $CD11c^+$ magnetic bead-purified hDCs. Supernatants were collected 24 h later, and human TNF-α (A) and IL-12p70 (B) levels were measured by ELISAs. Values shown are means ± standard errors (error bars) for four independent experiments performed in triplicate. The values for BMDCs not stimulated with YGPs were significantly different ($P < 0.002$) from the values for BMDCs stimulated with YGP for all conditions except IL-12p70 release in the group given no LPS.

paring stimulated cytokine responses to immunomodulators. Therefore, to ascertain that the results obtained with murine BMDCs were applicable to humans, IFN-γ-primed hDCs were stimulated with YGPs and/or LPS. YGPs stimulated hDCs to secrete TNF-α but not IL-12p70, whereas LPS stimulated both cytokines (Fig. 8). The combination of YGPs and LPS leads to upregulation of TNF-α and downregulation of IL-12p70, which is the same pattern that was observed for mouse BMDCs.

DISCUSSION

DCs are the major antigen-presenting cells of the mammalian immune system. As fungal cell walls are rich in β-glucans, it is likely that the nature of the immune response will be influenced by the interaction between DCs and β-glucans. The data presented herein establish that while DCs recognize β-glucans, the cytokine response is selective in the sense that only some cytokines are stimulated. Most notably, while β-glucans stimulated strong TNF-α production, levels of IL-12p70 were undetectable. Both particulate and soluble β-glucans stimulated similar cytokine responses. This is important because cduring the course of a fungal infection, both in situ and shed β-glucans may be available to interact with Dectin-1 (41, 43).

Studies comparing responses in wild-type and Dectin-$1^{-/-}$ mice established that Dectin-1 is the major β-glucan receptor on BMDCs. First, phagocytosis of YGPs by Dectin-$1^{-/-}$ BMDCs was markedly reduced. Second, Dectin-$1^{-/-}$ BMDCs secreted nearly undetectable amounts of TNF-α following stimulation with YGPs or SCG. Nevertheless, because Dectin-$1^{-/-}$ BMDCs exhibited some laminarin-inhibitable phagocytosis and TNF-α release, the possibility that other β-glucan receptors may be involved cannot be excluded. As noted above, in addition to Dectin-1, β-glucans reportedly are recognized by CR3, lactosylceramide, scavenger receptors, and CD5 (12, 49). Moreover, stimulation of human CD5 with β-glucans was recently shown to induce mitogen-activated protein kinase activation and cytokine release (49).

The fungal cell wall is complex, and there is much interspecies and intraspecies variation, including differences in the amounts of β-glucans that are surface exposed and thus available to interact with Dectin-1 (43, 50). Studies using whole fungi as stimulants, while biologically relevant, are nevertheless difficult to interpret because the contributions of the individual cellular components are not easily dissected. The widely used cell wall derivative of *S. cerevisiae*, zymosan, contains multiple PAMPs, including β-glucans, mannans, and ligands for TLR2 and TLR4 (40, 44). This complexity greatly hinders the interpretation of experimental results using zymosan as a model β-glucan. In contrast, YGPs, while also derived from *S. cerevisiae*, are considerably purer than zymosan. By biochemical analysis, YGPs are composed of >85% glucan polymers, ~2% chitin, and <1% lipids and protein, with the remainder primarily ash and moisture (23). Stimulation of BMDCs with YGPs was dependent on Dectin-1 but independent of MyD88, TLR2 (data not shown), and the mannose receptor (data not shown).

It has been proposed that signals from TLR agonists are necessary for efficient initiation of a $CD4^+$ T-cell response by DCs (4). Our data suggest that the situation may be more complicated for fungi because β-glucans modulate the cytokine response to TLR agonists. Remarkably, depending upon the cytokine studied, additive, antagonistic, and indifferent effects were seen when the combinations of β-glucans and PAMPs were compared with the responses stimulated by individual PAMPs.

IL-12p70 is a heterodimer composed of p40 and p35 subunits. Both soluble and particulate sources of β-glucans (SCG and YGPs) downregulated BMDC-mediated secretion of IL-12p70 stimulated by the TLR agonists, Pam_3CSK_4, LPS, imiquimod, and CpG. However, the β-glucans did not affect secretion of the IL-12p40 subunit. The possibility of the formation of inhibitory p40 homodimers cannot be excluded (20). However, we speculate that the downregulatory effect of β-glucans on IL-12p70 secretion stimulated by TLR agonists is most likely due to reduced IL-12p35 production. While regulation of the IL-12p35 subunit is not well defined, it is secreted only as part of a heterodimer with IL-12p40 (47, 51). Recently, it was demonstrated that β-glucans downregulated IL-12p35 mRNA levels in DCs treated with Pam_3CSK_4 but not LPS (10, 17).

It is tempting to speculate though that the mechanisms responsible for downregulation of IL-12p70 following ligation of Dectin-1 may be similar to that seen following Fcγ receptor ligation (15). Of particular note is that Dectin-1 and Fcγ receptors have ITAM-like motifs in their cytoplasmic tails. In addition, Ca^{2+} fluxes are observed following ligation of both of these receptors (15, 53). Regardless of the mechanism, β-glucan-induced downregulation of IL-12p70 was observed in both human and murine DCs and was not overcome by IFN-γ priming. Recently, it was demonstrated that in the presence of antigen-stimulated CD8 T cells, the β-glucan curdlan stimulated BMDCs to produce IL-12p70 (29). Thus, there appear to be feedback mechanisms whereby T cells can, under certain conditions, prime DCs for a IL-12p70 response to β-glucans.

IL-12p70 is a key cytokine involved in initiating T helper 1 (Th1)-type $CD4^+$ T-cell responses (32). Therefore, the inhibitory effects of β-glucans on BMDC IL-12p70 production could have consequences vis-à-vis Th skewing during the course of a fungal infection. Indeed, fungal infections are often associated with Th17-type responses (24, 27). Another member of the IL-12 superfamily, IL-23, is important for propagating Th17 responses (27). IL-23 is a heterodimer that shares the p40 subunit with IL-12p70 but has a unique p19 subunit. Following

stimulation of BMDCs with YGPs and/or TLR agonists, we could not detect significant levels of IL-23 secretion by ELISA (detection limit of 31 pg/ml; data not shown). However, Dennehy et al. did detect low levels of IL-23 following stimulation of BMDCs with β-glucans (10). Moreover, they found that while β-glucans did not stimulate p19 mRNA, the combination of β-glucans plus Pam_3CSK_4 stimulated higher levels of p19 mRNA than Pam_3CSK_4 alone did. Nevertheless, because the levels of secreted IL-23 are low to undetectable, even in the presence of TLR agonists, these results raise the possibility that other stimuli besides β-glucans contribute to the Th17 skewing seen during fungal infections.

As noted above, fungi have multiple PAMPs that are recognized by host PRRs (34). Other examples of cross talk among fungal PAMPs have been demonstrated. *Cryptococcus neoformans* mannoproteins act synergistically with CpG to enhance TNF-α and IL-12p70 release in BMDCs (8). Moreover, zymosan cooperates with the TLR2 agonist Pam_3CSK_4 to increase TNF-α secretion and upregulate IL-12p40 mRNA (14). Furthermore, curdlan synergizes with TLR2 and TLR4 agonists to boost TNF-α and IL-10 production in human monocytes and macrophages (13). We also observed synergistic increases in IL-10 production when BMDCs were stimulated with β-glucans and TLR2 and TLR9 agonists. As IL-10 is generally anti-inflammatory, this may serve as a mechanism for the host to minimize damage from an overly exuberant immune response following fungal invasion.

In addition to their potential to affect the outcome of fungal infections, the propensity of fungal PAMPs to stimulate cross talk has implications for the design of vaccines and immunomodulators containing β-glucans. β-Glucans have been proposed as antigen delivery systems (3) and (despite a lack of definitive data) are touted as supplements to boost immune responses (36). Our data emphasize that the DC cytokine response following β-glucan stimulation is quite intricate, particularly when the β-glucans are combined with other PAMPs. In vivo responses are likely to be even more complex, as opsonization with complement and anti-β-glucan antibodies contribute additional ligands (7, 23). Further understanding of the combined responses to β-glucans and PAMPS is critical if β-glucans are to be safely and effectively used as components of vaccine adjuvants and immunomodulators.

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