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Aspergillus fumigatus Cell Wall α-(1,3)-Glucan Stimulates Regulatory T-Cell Polarization by Inducing PD-L1 Expression on Human Dendritic Cells

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Background. Human dendritic cell (DC) response to α-(1,3)-glucan polysaccharide of *Aspergillus fumigatus* and ensuing $CD4^+$ T-cell polarization are poorly characterized.

Methods. α-(1,3)-Glucan was isolated from *A. fumigatus* conidia and mycelia cell wall. For the analysis of polarization, DCs and autologous naive $CD4^+$ T cells were cocultured. Phenotype of immune cells was analyzed by flow cytometry, and cytokines by enzyme-linked immunosorbent assay (ELISA). Blocking antibodies were used to dissect the role of Toll-like receptor 2 (TLR2) and programmed death-ligand 1 (PD-L1) in regulating α-(1,3)-glucan-mediated DC activation and T-cell responses. DCs from TLR2-deficient mice were additionally used to consolidate the findings.

Results. α-(1,3)-Glucan induced the maturation of DCs and was dependent in part on TLR2. "α-(1,3)-Glucan-educated" DCs stimulated the activation of naive T cells and polarized a subset of these cells into $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs). Mechanistically, Treg stimulation by α-(1,3)-glucan was dependent on the PD-L1 pathway that negatively regulated interferon-gamma (IFN-γ) secretion. Short α-(1,3)-oligosaccharides lacked the capacity to induce maturation of DCs but significantly blocked α-(1,3)-glucan-induced Treg polarization.

Conclusions. PD-L1 dictates the balance between Treg and IFN-γ responses induced by α-(1,3)-glucan. Our data provide a rationale for the exploitation of immunotherapeutic approaches that target PD-1–PD-L1 to enhance protective immune responses to *A. fumigatus* infections.

Keywords. *Aspergillus fumigatus*; α-(1,3)-glucan; dendritic cells; programmed death-ligand 1; regulatory T cells.

Aspergillus fumigatus is the most ubiquitous respiratory human fungal pathogen. In the immunocompetent individual, *A. fumigatus* causes a number of diseases, such as lung/sinus aspergilloma and allergic bronchopulmonary aspergillosis, whereas in the immunocompromised condition it causes fatal invasive aspergillosis.

In addition to macrophages and neutrophils that have a prominent role in the control of *Aspergillus* infections, dendritic cells (DCs) also exhibit anti-*Aspergillus* functions. DCs internalize both *A. fumigatus* conidia and hyphae, and undergo maturation to instruct $CD4^+$ T-cell response to fungi [1–5]. Different families of pattern recognition receptors (PRRs) on DCs participate in the recognition of *A. fumigatus* antigens [3, 6, 7].

The *A. fumigatus* cell wall is a complex and dynamic structure, and a major source of pathogen-associated molecular patterns (PAMPs). Polysaccharides constitute approximately 90% of the cell wall and are mainly composed of branched β-(1,3)-glucan, α-(1,3)-glucan, chitin, galactomannan, and morphotype-specific galactosaminogalactan [8]. α-(1,3)-Glucan is the key amorphous component of *A. fumigatus* cell wall and represents approximately 40% of cell wall polysaccharides in the mycelia and 19% in the conidia [9]. Murine models of aspergillosis have shown that α-(1,3)-glucan induces innate as well as adaptive immune responses [10]. However, the human DC response towards α-(1,3)-glucan and ensuing T-cell polarization have not been analyzed yet.

Here, we show that α-(1,3)-glucan induces activation of human DCs with a capacity to polarize $CD4^+CD25^+FoxP3^+$ regulatory T-cell (Treg) responses. Blocking experiments suggest that α-(1,3)-glucan-induced programmed death-ligand 1 (PD-L1) on DCs mediate Treg polarization. α-(1,3)-Glucan could promote Th1 (interferon-gamma, IFN-γ) responses only when PD-L1 was blocked, suggesting that PD-L1 acts as a negative regulator of α-(1,3)-glucan-mediated protective immune responses. The polymeric nature of α-(1,3)-glucan is critical for the induction of DC activation as short α-(1,3)-oligosaccharides lack the DC stimulatory ability. However, short

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α-(1,3)-oligosaccharides efficiently inhibit α-(1,3)-glucan-mediated Treg polarization.

MATERIALS AND METHODS

Reagents

PE-conjugated MAbs to CD80, CD83, CD273, CD275, CD127, IL-4; FITC-conjugated MAbs to CD86, CD274, CD1a, CD25, IFN-γ; APC-conjugated MAbs to CD83, HLA-DR; Alexa 700-conjugated MAbs to CD4 were from BD Biosciences. PE-conjugated MAb to CD40 and CD252 were from Beckman Coulter and Biolegend, respectively. PE-conjugated MAbs to IL-17A, APC-conjugated MAbs to FoxP3, Fixable Viability Dye eFluor 506, anti-TLR4, anti-PD-L1, and isotype-control MAbs were from eBioscience. Blocking MAbs to human TLR-2, Dectin-1, and DC-SIGN were from R&D Systems. Mannan and anti-β-actin antibody were from Sigma–Aldrich. Recombinant mouse GM-CSF was from PeproTech. Anti-Ser536-phospho-NF-κB p65, anti-Thr202/Tyr204-ERK1/2, anti-NF-κB-p65, and anti-ERK1/2 were from Cell Signaling Technology. HRP-conjugated anti-rabbit IgG was obtained from Jackson ImmunoResearch.

A. fumigatus

The clinical isolate strain CBS144.89 was maintained on 2% malt-agar slants at ambient temperature [11]. Conidia were harvested from 10–12-day-old malt-agar slants using 0.05% Tween–water. Swollen conidia were prepared by incubating dormant conidia in Sabouraud liquid medium at 37°C in a shaken incubator for 5 h, followed by collecting and washing with water. For mycelia, 1000 conidia in Sabouraud liquid medium were loaded into IBIDI plates, incubated at 37°C for 20 h, and washed with water. All the conidial morpotypes were *para*-formaldehyde fixed [11].

Isolation of α-(1,3)-Glucan From *A. fumigatus* Morphotypes and Preparation of α-(1,3)-Glucan Oligosaccharides

Alkali-soluble fraction was obtained from the conidial/mycelial cell wall [12] and incubated in 100 mM sodium *meta*-periodate for 4 days at 4°C in the dark. Excess of periodate was destroyed by adding ethylene glycol. After dialysis against water, the products were reduced overnight in 100 mM NH_4OH containing 20 mg $NaBH_4$. Excess reagent was removed upon repeated codistillations with methanol. Further, Smith degradation was performed with 10% acetic acid at 100°C for 1 h and the resultant product was freeze-dried [10]. We treated the material with a recombinant β-(1,3)-glucanase [13] and found that β-(1,3)-oligomers accounting for 3–5%. We repeated β-(1,3)-glucanase treatment until no more β-(1,3)-oligomers were released from the α-(1,3)-glucan preparation. From this α-(1,3)-glucan, oligo-α-(1,3)-mixtures of various sizes (degree of polymerization [DP] DP6-10 and DP8-14) were prepared and their purity was checked by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and gas–liquid chromatography (GC) analyses [14], and they were >98% pure.

α-(1,3)-Glucan was depolymerized by recombinant α-(1,3)-glucanase [15]; briefly, α-(1,3)-glucan in 10 mM acetate buffer, pH 5.5 was mixed with recombinant α-(1,3)-glucanase (from *Penicillium purpurogenum* expressed in *Pichia pastoris*) and incubated at 37°C.

Isolation of β-(1,3)-Glucan From *A. fumigatus*

β-(1,3)-Glucan was isolated from the alkali-insoluble cell wall fraction, as described earlier with modifications [10, 16]. Briefly, alkali-insoluble fraction was subjected to periodate oxidation for 4 days and subjected to chitin deacetylation with 30% NaOH to obtain β-(1,3)-glucan. Purity of the β-(1,3)-glucan was checked by gas–liquid chromatography.

Generation and Treatment of DCs with Polysaccharides and Oligosaccharides

DCs were generated from peripheral blood monocytes [17] by using buffy bags of healthy donors (Centre Necker-Cabanel, EFS, Paris; INSERM-EFS ethical committee permission N°12/EFS/079). Immature DCs (0.5×10^6/mL) were cultured in the presence of GM-CSF and IL-4 alone or with α-(1,3)-glucan (0.5 or 1 µg), β-(1,3)-glucan (1 µg), α-(1,3)-oligos (DP6-10 or DP8-14, 1 µg each), dormant or swollen conidia (0.5×10^6) or mycelia (from 2500 conidia) for 48 h.

After 48 h of incubation, cell-free culture supernatants were analyzed for DC cytokines (IL-12, IL-6, IL-10, IL-1β, TNF-α; ELISA Ready-SET-Go, eBioscience) and DCs were processed for flow cytometry. The samples were acquired using LSR II (BD Biosciences) and the data were analyzed by BD FACS DIVA and Flowjo.

For blocking PRRs, DCs were preincubated with MAbs to TLR-2, Dectin-1, DC-SIGN, TLR-4 (10 µg/0.5×10^6 cells), isotype-control antibodies or with mannan (1 mg/mL) followed by stimulation with α-(1,3)-glucan.

For blocking PD-L1, α-(1,3)-glucan-treated DCs (0.5×10^6) were washed, incubated with anti-PD-L1 or isotype-control MAbs (10 µg) for 2 h, and subjected to coculture with naive $CD4^+$ T cells.

DC-naive $CD4^+$ T-Cell Coculture

DCs were cocultured with $CD4^+CD45RA^+CD25^-$ autologous naive T cells (0.1×10^6) at 1:10 ratio for 5 days in serum-free X-VIVO medium. To investigate the inhibitory effect of short oligosaccharides towards α-(1,3)-glucan-mediated $CD4^+$ T-cell polarization, DCs pretreated with DP6-10 α-(1,3)-oligo mixture were stimulated with 1 µg α-(1,3)-glucan for 48 h. Cells were washed and cocultured with autologous naive T cells at 1:10 ratio for 5 days.

The cell-free culture supernatants were analyzed for T-cell cytokines (IFN-γ, IL-4, and IL-17A; ELISA Ready-SET-Go). $CD4^+$ T cells were activated with phorbol myristate acetate (PMA) (50 ng/mL/0.5 million cells) and ionomycin (500 ng/mL/0.5 million cells), along with GolgiStop for 4 h. For the analysis of $CD4^+$ T-cell polarization (Th1, Th2, Th17, and Treg), surface staining was

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performed with fluorescence-conjugated MAbs to CD4, CD127, and CD25. Cells were then fixed, permeabilized using an intracellular staining kit (eBioscience), and incubated at room temperature with fluorescence-conjugated MAbs to FoxP3, IFN-γ, IL-4, and IL-17A. Samples were processed for flow cytometry.

Mice

Wild-type (C57BL/6J), *tlr4*$^{-/-}$ (C3H/HeJ), and *tlr2*$^{-/-}$ (B6.129-*Tlr2*^{tm1Kir}/J) mice (the Jackson Laboratory) were maintained in the Central Animal Facility, Indian Institute of Science, Bangalore, India.

Generation of Murine Bone-Marrow-Derived DCs

Bone marrow from tibia and femur were collected by flushing with ice-cold Dulbecco's Modified Eagle's Medium (DMEM)–10% fetal bovine serum, followed by washing with DMEM. The cells (1×10^6 cells/mL) were suspended in DMEM–10% fetal bovine serum and 20 ng/mL rmGM-CSF. The cells were supplemented on day 3 and 5 with GM-CSF-containing fresh medium. On day 7, nonadherent cells were collected, analyzed, and used for experiments.

Immunoblotting

α-(1,3)-Glucan-stimulated bone-marrow-derived DCs (BM-DCs) were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 µg/mL of each aprotinin, leupeptin, pepstatin, 1 mM Na3VO4, and 1 mM NaF). Equal amount of protein from each cell lysate was resolved on 12% dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) by the semidry transfer method. Nonspecific binding was blocked with Tris-buffered saline, 0.1% Tween 20 (TBST)–5% nonfat dry milk powder. The blots were incubated with primary antibody followed by anti-rabbit-HRP antibody in 5% bovine serum albumin (BSA). After washing in TBST, immunoblots were developed with enhanced chemiluminescence detection system (Perkin Elmer). β-Actin was used as loading control.

Statistical Analysis

Statistical analyses were performed by two-way parametric Student's t-test, two-way nonparametric Mann–Whitney test, or one-way ANOVA with Tukey's multiple comparison post-test as indicated. $P < .05$ was considered significant.

RESULTS

A. fumigatus Cell Wall α-(1,3)-Glucan Induces Maturation and Activation of Human DCs

α-(1,3)-Glucan from *A. fumigatus* mycelia induced maturation of DCs characterized by significantly higher expression of CD83, costimulatory molecules CD80, CD86, CD40, and antigen-presenting molecule HLA-DR (Figure 1A). Further, α-(1,3)-glucan from both conidial and mycelial cell walls had similar ability to induce DC maturation (data not shown). A comparative study revealed that α-(1,3)-glucan, swollen conidia, and mycelia have equivalent capacity to stimulate DC maturation (Supplementary Figure 1*A*). In accordance with our earlier observation [11], dormant conidia did not induce DC maturation.

Activated DCs secrete a large array of immunoregulatory cytokines that are crucial for T-cell polarization. α-(1,3)-Glucan induced small quantities of TNF-α, IL-6, IL-10, and IL-12p70 (Figure 1B). However, cytokine analysis clearly demonstrated that as compared to α-(1,3)-glucan, swollen conidia and mycelia are highly stimulatory and induced huge amounts of various DC cytokines (Supplementary Figure 1*B*).

We confirmed that induction of DC maturation by α-(1,3)-glucan preparation was due to its polymeric nature as enzymatic depolymerization of α-(1,3)-glucan resulted in abrogation of DC maturation (Figure 1C). α-(1,3)-Glucan-induced maturation of DCs was dose dependent (Figure 1D).

TLR2 Plays an Important Role in α-(1,3)-Glucan-induced Maturation of DCs

Previous reports have demonstrated that TLRs (particularly TLR2) and C-type lectin receptors play a crucial role in sensing *A. fumigatus* PAMPS and mounting antifungal immunity [3, 6, 7, 18–25]. Therefore, we explored the PRR implicated in the signaling of DC maturation by α-(1,3)-glucan. Blocking of lectin receptors by mannan, or anti-Dectin-1, anti-DC-SIGN antibodies as well as TLR4 blockade did not affect α-(1,3)-glucan-mediated maturation of DCs (data not shown). However, blockade of TLR-2 on DCs prior to stimulation with α-(1,3)-glucan significantly reduced the expression of costimulatory and antigen-presenting molecules (Figure 2A).

To further confirm the role of TLR2 in α-(1,3)-glucan-mediated activation of DCs, we resorted to BM-DCs from *tlr2*$^{-/-}$ mice. DCs from *tlr4*$^{-/-}$ mice were used as the control. We demonstrate that ERK 1/2 and NF-κBp65 activation by α-(1,3)-glucan are dependent on TLR2 as DCs from *tlr2*$^{-/-}$ mice, but not *tlr4*$^{-/-}$ mice, showed severely compromised activation of these pathways (Figure 2B). Together, these results indicate that TLR2 plays a crucial role in mediating activation of DCs by α-(1,3)-glucan.

α-(1,3)-Glucan-matured DCs Polarize Treg Responses

We explored the ability of α-(1,3)-glucan-matured DCs to induce $CD4^+$ T-cell polarization from naive T cells. Analysis of $CD4^+$ T cells based on the surface expression of CD25 and CD127, intracellular staining for cytokines (IFN-γ, IL-4, IL-17A), and transcription factor (FoxP3) revealed that α-(1,3)-glucan-matured DCs stimulated activation of naive T cells and a proportion of these cells differentiated into $CD4^+CD25^+CD127^-$ $FoxP3^+$ Tregs (Figure 3A, B). However, Th1, Th2, and Th17 responses were not significantly altered (Figure 3C). These data indicate that although the majority of $CD4^+$ T cells were CD25 positive and activated, these T cells had

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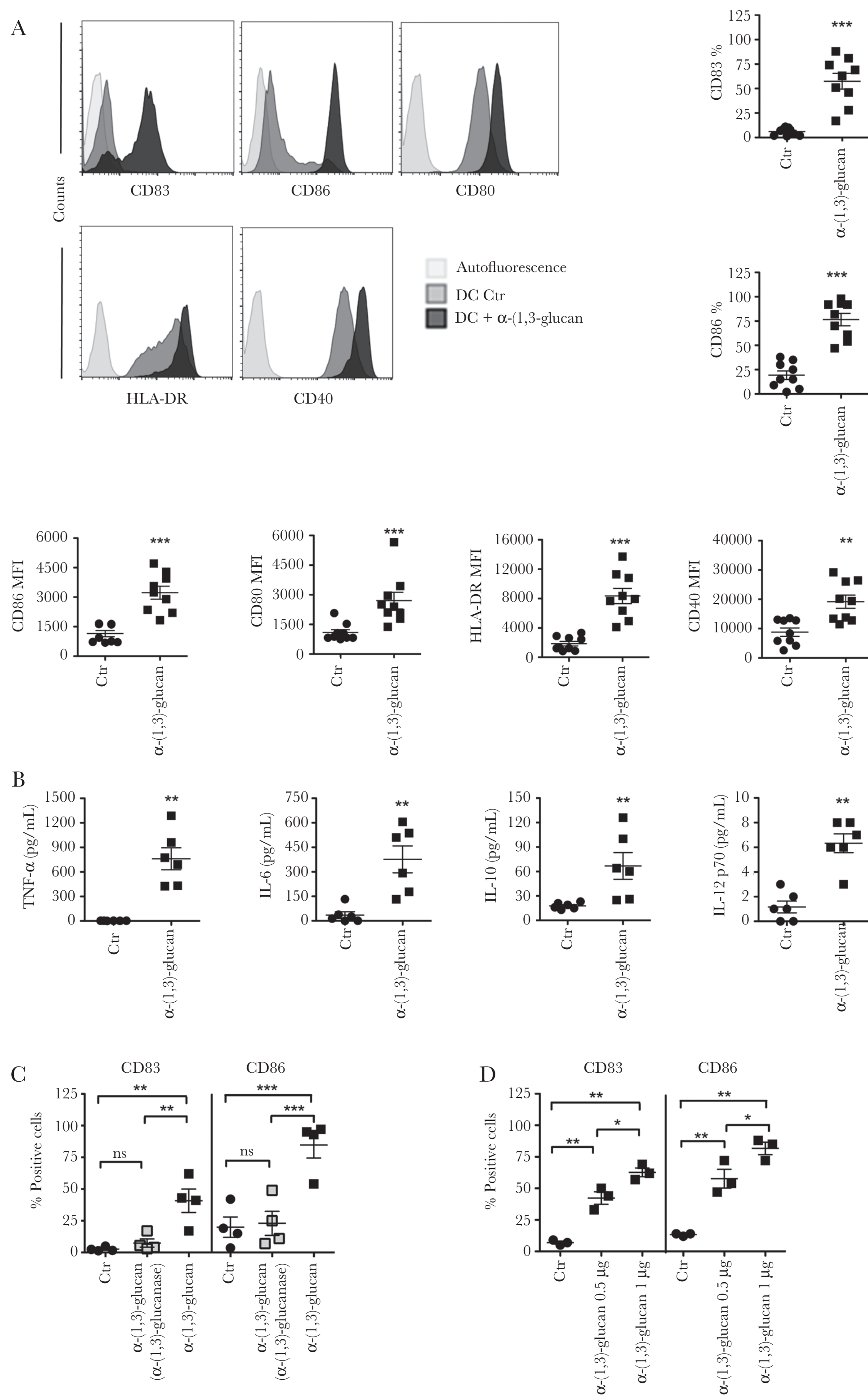

Figure 1. α-(1,3)-Glucan from *A. fumigatus* mycelial cell wall stimulates maturation of human DCs and cytokine secretion. *A, B,* Monocyte-derived DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with α-(1,3)-glucan (1 μg) for 48 h. Phenotypic analysis of DCs was performed by flow cytometry. *A,* Representative histograms showing the expression of various markers on DCs, percent DCs positive for CD83 and CD86 (mean ± SEM, n = 9 donors), and median fluorescence intensities (MFI) of expression of CD86, CD80, HLA-DR, and CD40 (mean ± SEM, n = 9 donors) are presented. *B,* The amounts of TNF-α, IL-6, IL-10, and IL-12p70 in the cell-free culture supernatants (mean ± SEM, n = 6 donors). ** $P < .01$; *** $P < .001$, as determined by two-way nonparametric Mann–Whitney test. *C,* Enzymatic depolymerization of α-(1,3)-glucan results in abrogation of DC maturation. Percent DCs positive for CD83 and CD86 are presented (mean ± SEM, n = 4 donors). *D,* α-(1,3)-Glucan induces maturation of DCs in a dose-dependent manner. DCs were cultured in GM-CSF and IL-4 alone (Ctr) or α-(1,3)-glucan (0.5 and 1 μg) for 48 h. Percent DCs positive for CD83 and CD86 are presented (mean ± SEM, n = 3 donors). * $P < .05$; ** $P < .01$; *** $P < .001$; ns, not significant as analyzed by one-way ANOVA test.

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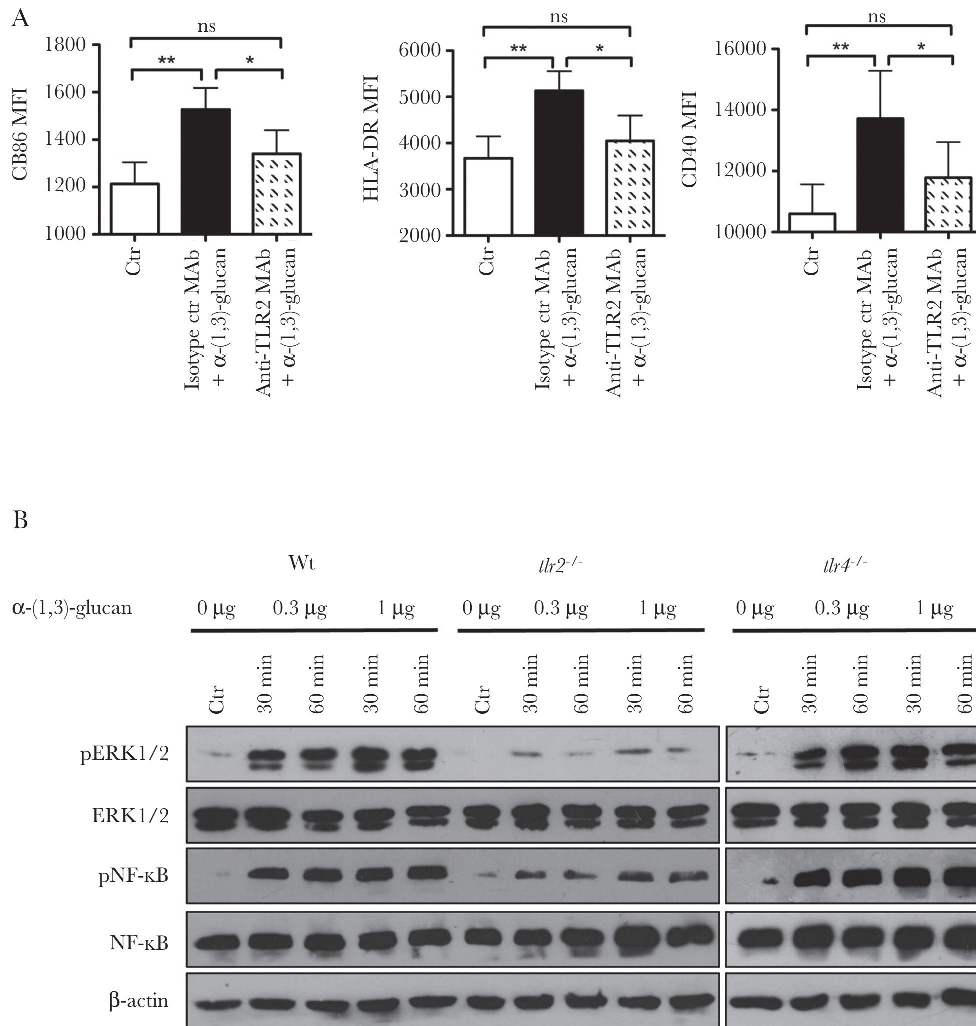

Figure 2. TLR2 plays a critical role in the induction of DC activation by **α**-(1,3)-glucan. *A,* DCs were cultured in GM-CSF and IL-4 alone (Ctr) or in the presence of anti-TLR2 blocking monoclonal antibodies or isotype control antibodies. After 1 h blocking, DCs were stimulated with **α**-(1,3)-glucan. The MFI of expression of CD86, HLA-DR, and CD40 are presented (mean ± SEM, n = 5). * $P < .05$; ** $P < .01$; ns, not significant as analyzed by one-way ANOVA test. *B,* BM-DCs from Wt (wild-type, C57BL/6J), *tlr2*$^{-/-}$ (B6.129-*Tlr2tm1Kir*/J) and *tlr4*$^{-/-}$ (C3H/HeJ) mice were stimulated with **α**-(1,3)-glucan (0, 0.3, and 1 µg) for 30 and 60 min. Phosphorylation of ERK1/2 (pERK1/2) and NF-**κ**Bp65 (pNF-**κ**B) was analyzed by western blot. **β**-Actin served as loading controls.

undergone incomplete differentiation. Quantification of various T-cell cytokines in the DC-CD4$^+$ T-cell coculture supernatants further validated these results. We found that α-(1,3)-glucan-matured DCs induced minimal quantities of Th1 (IFN-γ), Th2 (IL-4), and Th17 (IL-17A) cytokines (Figure 3D).

Further, we show that unlike α-(1,3)-glucan, both swollen conidia and mycelia promote robust Th1 responses (Supplementary Figure 2). However, all these stimuli exhibited similar Treg polarizing capacity. None of these stimuli induced Th17 and Th2 responses.

α-(1,3)-Glucan-induced PD-L1 Expression on the DCs Dictates the Balance Between Treg and IFN-γ Responses

We then explored the mechanism of α-(1,3)-glucan-induced Treg responses. Analysis of various costimulatory molecules on DCs that are implicated in Treg expansion revealed that α-(1,3)-glucan induced significantly higher expression of PD-L1 (CD274) on DCs (Figure 4A) whereas other costimulatory molecules (PD-L2, OX-40L, and ICOSL) implicated in Treg polarization were not induced (data not shown). To validate the role of PD-L1 in α-(1,3)-glucan-induced Treg responses, we employed blocking antibodies and found that PD-L1 blockade led to significant inhibition of α-(1,3)-glucan-induced Tregs (Figure 4B, C). Of interest, secretion of protective Th1 cytokine IFN-γ was significantly enhanced upon PD-L1 blockade (Figure 4D), while IL-17 and IL-4 remained at minimal levels (data not shown). The data thus indicate that PD-L1 on DCs dictates the balance between Treg and IFN-γ responses induced by α-(1,3)-glucan.

Short α-(1,3)-Oligosaccharides Lack the Capacity to Induce DC Activation

The consequence of innate cell stimulation by PAMPs is determined by the avidity with which these PAMPs are recognized by PRRs. Therefore, we analyzed whether short

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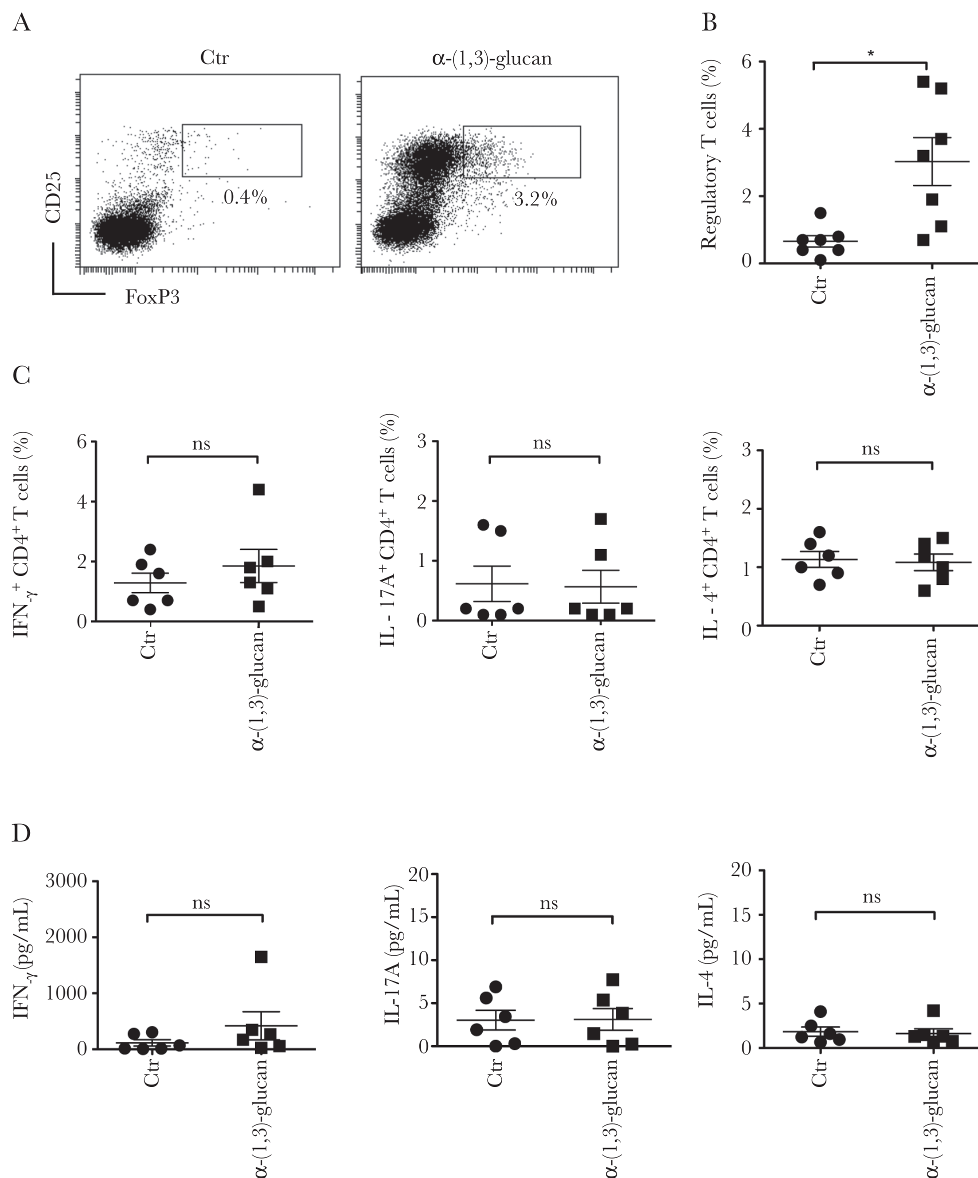

Figure 3. **α**-(1,3)-Glucan-matured DCs polarize predominantly Treg response. Monocyte-derived DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with **α**-(1,3)-glucan from *A. fumigatus* mycelia for 48 h. DCs were washed and cocultured with autologous naive $CD4^+$ T cells at 1:10 ratio for 5 days. The $CD4^+$ T cells were analyzed for the polarization of various T-cell subsets. *A,* Representative dot-plot of Tregs showing CD25 and FoxP3 expression. *B,* Treg polarization induced by "**α**-(1,3)-glucan-educated" DCs (mean ± SEM, n = 7 donors). *C,* Polarization of Th1, Th17, and Th2 responses by **α**-(1,3)-glucan as analyzed by intracellular staining for IFN-**γ**, IL-17A, and IL-4, respectively, by flow cytometry (mean ± SEM, n = 6 donors). *D,* Amount of secretion of $CD4^+$ T-cell cytokines IFN-**γ**, IL-17A, and IL-4 in the cell-free culture supernatants of DC–T cell cocultures (mean ± SEM, n = 6 donors). * $P < .05$; ns, not significant as determined two-way nonparametric Mann–Whitney test.

α-(1,3)-oligosaccharides retain the ability to induce DC activation. We show that short α-(1,3)-oligosaccharide mixtures (DP 6-10 or 8-14) fail to induce activation of DCs as analyzed by the phenotype (Figure 5A) and cytokines secreted (Figure 5B). These results thus demonstrate that the polymeric nature of the α-(1,3)-glucan is critical for inducing DC activation.

Short α-(1,3)-Oligosaccharides Inhibit α-(1,3)-Glucan-mediated Treg Polarization

The lack of induction of DC maturation by α-(1,3)-glucan short oligosaccharides raises an interesting possibility that they might interfere with stimulatory functions of α-(1,3)-glucan. To confirm this, DCs were pretreated with α-(1,3)-oligosaccharides of DP6-10 followed by stimulation with α-(1,3)-glucan and analysis of T-cell polarization. As shown in Figure 6A, short α-(1,3)-oligosaccharides interfered with Treg-polarizing capacity of α-(1,3)-glucan while frequency of other T-cell subsets (Figure 6B) and secretion of IFN-γ, IL-17, and IL-4 (Figure 6C) were not altered.

α-(1,3)-Glucan and β-(1,3)-Glucan Distinctly Polarize Th Responses

As β-(1,3)-glucan is one of the major immunogenic motifs of the *A. fumigatus* cell wall, we compared the abilities of

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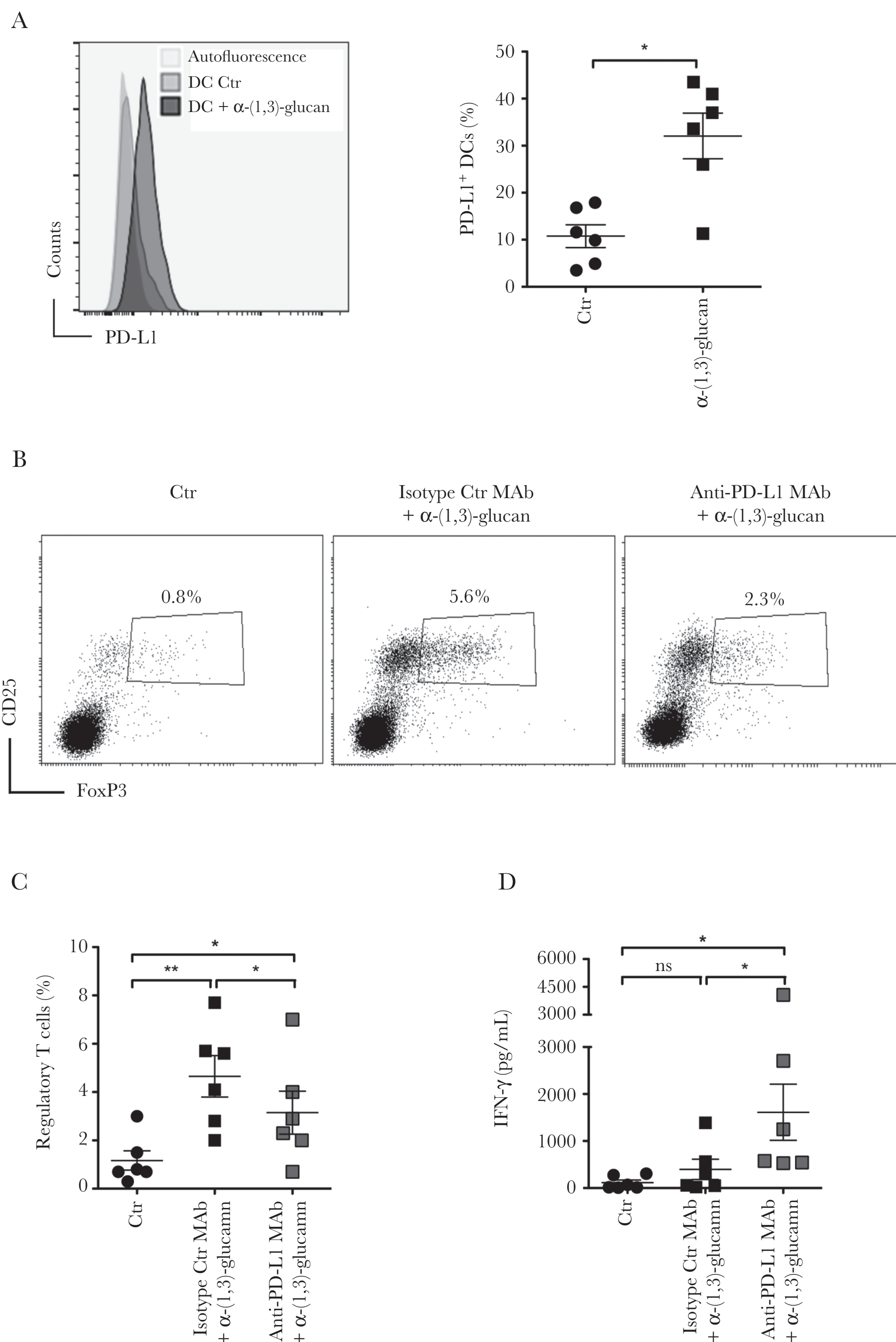

Figure 4. α-(1,3)-Glucan-induced PD-L1 expression on DCs dictates the balance between Treg and IFN-γ responses. Monocyte-derived DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with α-(1,3)-glucan from *A. fumigatus* mycelia for 48 h. *A,* Expression of PD-L1 (CD274) on DCs as analyzed by flow cytometry. Representative histograms and mean ± SEM values of 6 donors are presented. * $P < .05$; as determined by two-way nonparametric Mann–Whitney test. *B–D,* DCs were washed and incubated with blocking monoclonal antibodies to PD-L1 or isotype control antibodies and subjected to coculture with autologous naive CD4+ T cells at 1:10 ratio. *B, C,* Repercussion of PD-L1 blockade towards Treg response in DC:T cell cocultures. Representative dot-plot and mean ± SEM values from 6 donors are presented. *D,* Modulation of IFN-γ secretion in DC-T cell cocultures upon PD-L1 blockade (mean ± SEM, n = 6 donors). * $P < .05$; ** $P < .01$; ns, not significant as determined by one-way ANOVA test.

α-(1,3)-glucan and β-(1,3)-glucan in inducing DC-mediated T-cell polarization. In contrast to α-(1,3)-glucan, β-(1,3)-glucan induced both Treg and Th1 polarization (Figure 7A–C) thus confirming the protective role of β-(1,3)-glucan in *Aspergillus* infection. However, unlike in vivo data from mice [10, 26], β-(1,3)-glucan did not polarize human Th17 response from

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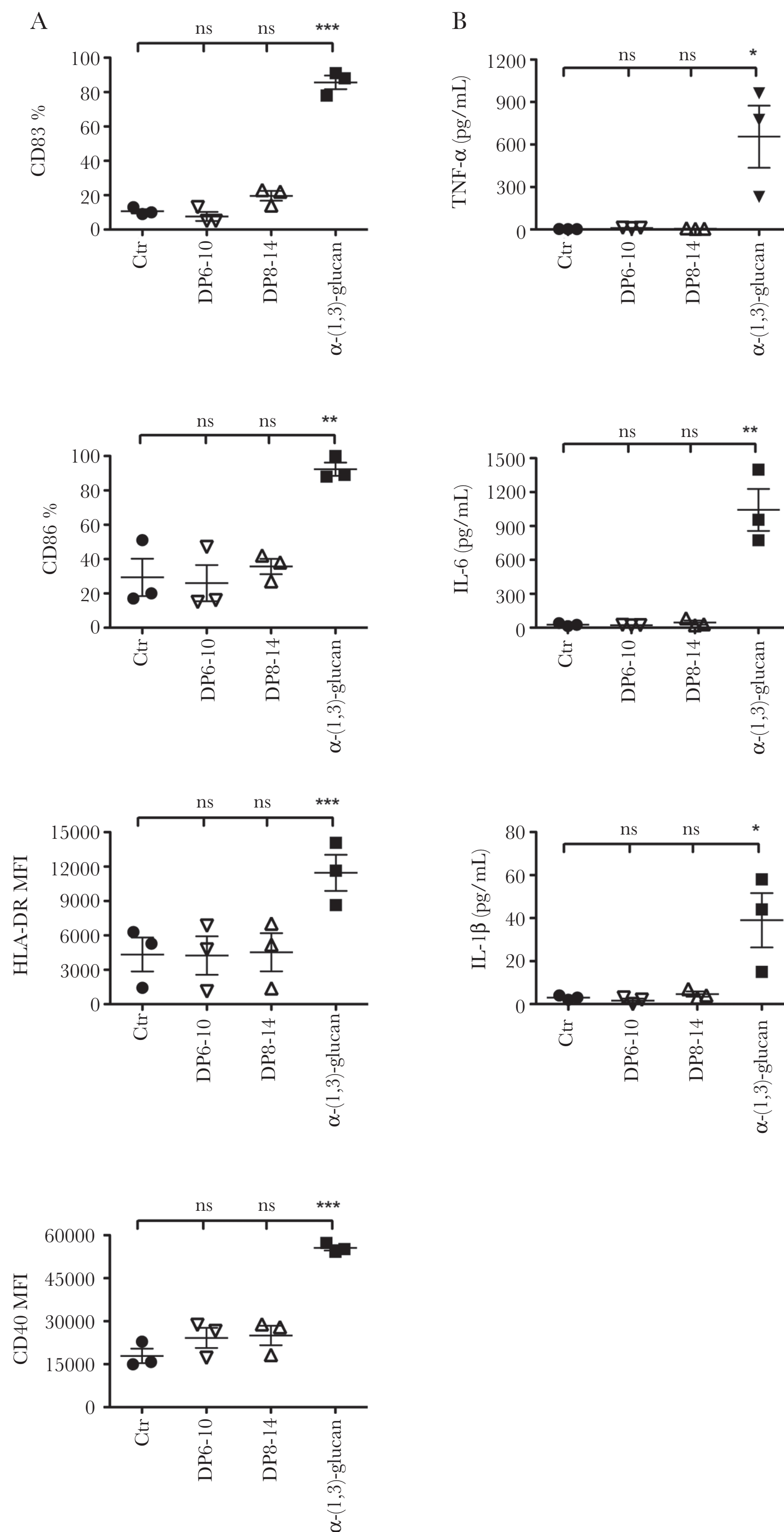

Figure 5. Short α-(1,3)-oligosaccharides lack the capacity to induce maturation of DCs. DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with α-(1,3)-oligosaccharides (DP6-10 or DP8-14) or α-(1,3)-glucan for 48 h. *A,* The expression of CD83, CD86 (% positive cells), HLA-DR, and CD40 (MFI) on DCs (mean ± SEM, n = 3 donors). *B,* The amount of TNF-α, IL-6, and IL-1β in the cell-free culture supernatants (mean ± SEM, n = 3 donors). * $P < .05$; ** $P < .01$; *** $P < .001$; ns, not significant as determined by one-way ANOVA test.

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naive T cells. In fact, our data are in line with a previous report that *A. fumigatus* is a poor inducer of Th17 response in humans, both in vitro and in vivo [27].

The equivalent Treg polarization induced by α-(1,3)-glucan and β-(1,3)-glucan suggests that these polysaccharides induce PD-L1 on DCs to a similar extent. In fact, both α-(1,3)-glucan and β-(1,3)-glucan significantly induced PD-L1 on DCs (Figure 7D, left). However, as compared to α-(1,3)-glucan that induced small quantities of Th1-polarizing cytokine IL-12p70, β-(1,3)-glucan induced significantly higher amounts of IL-12p70 in DCs (Figure 7D, right). Thus, our data indicate that discrepancies in the ability to induce DC-derived IL-12p70 contributed to differential polarization of Th responses by α-(1,3)-glucan and β-(1,3)-glucan.

DISCUSSION

Although *A. fumigatus* conidial surface rodlet-melanin layers cover underlying cell wall polysaccharides [11, 28, 29], remodeling of cell wall during conidial germination exposes these polysaccharides, which in turn activate host defense mechanisms. Several reports have demonstrated that β-(1,3)-glucan is a major immunogenic motif of germinated *A. fumigatus* conidia and hyphae that is recognized by Dectin-1 on innate cells [3, 22, 23]. A subsequent report illustrated that Dectin-1–mediated

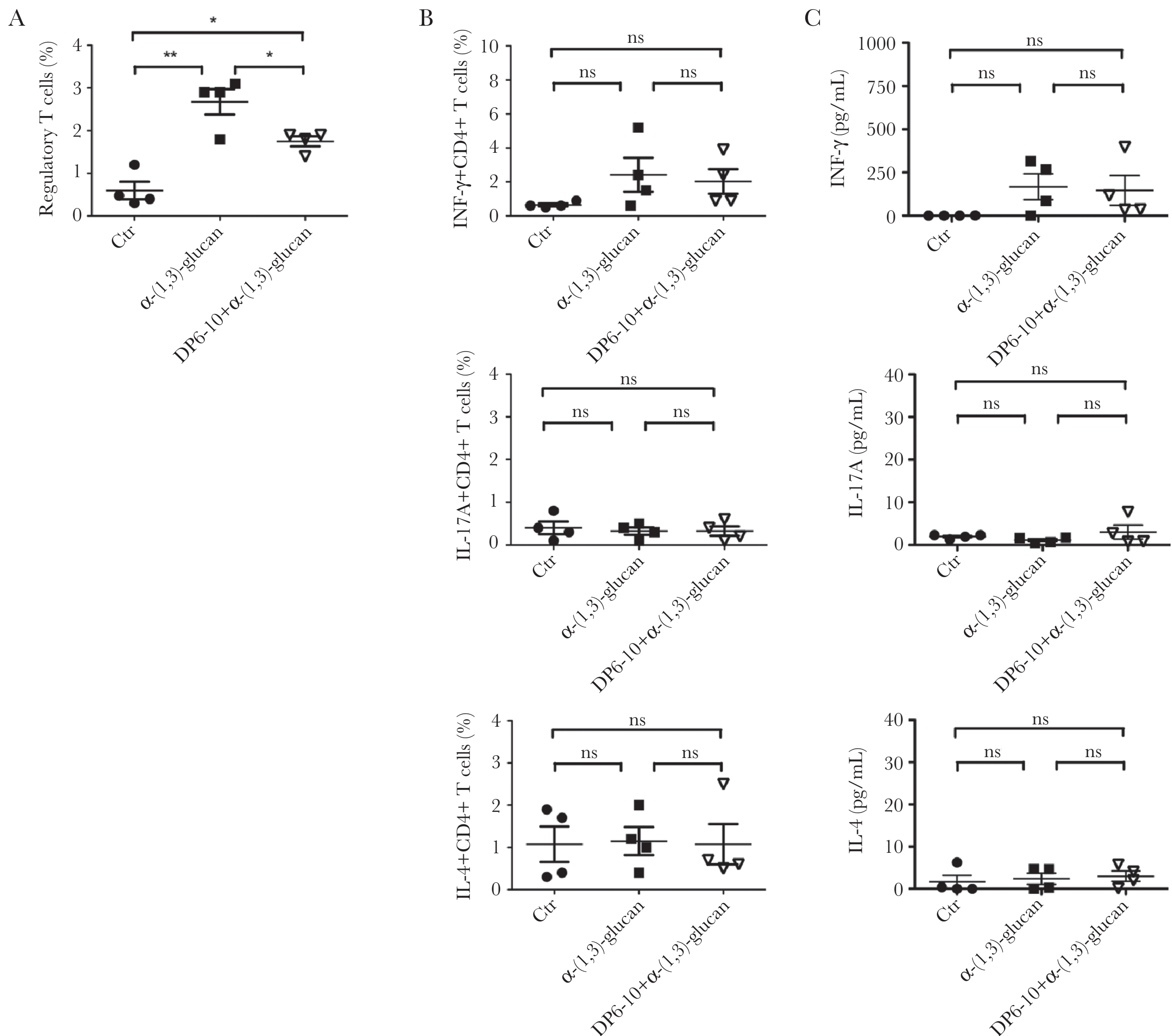

Figure 6. Short α-(1,3)-oligosaccharides inhibit α-(1,3)-glucan-mediated Treg polarization. DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with α-(1,3)-oligosaccharides (DP6-10) for 30 min followed by stimulation with α-(1,3)-glucan for 48 h. DCs were washed and cocultured with autologous naive CD4+ T cells at 1:10 ratio for 5 days. The CD4+ T cells were analyzed for the polarization of various T-cell subsets. *A,* Frequency of Tregs, and (*B*) frequency of Th1, Th17, and Th2 subsets (mean ± SEM, n = 4 donors). *C,* Amount of secretion of CD4+ T-cell cytokines IFN-γ, IL-17A, and IL-4 in the cell-free culture supernatants of DC–T cell cocultures (mean ± SEM, n = 4 donors). * $P < .05$; ** $P < .01$; ns, not significant as determined by one-way ANOVA test.

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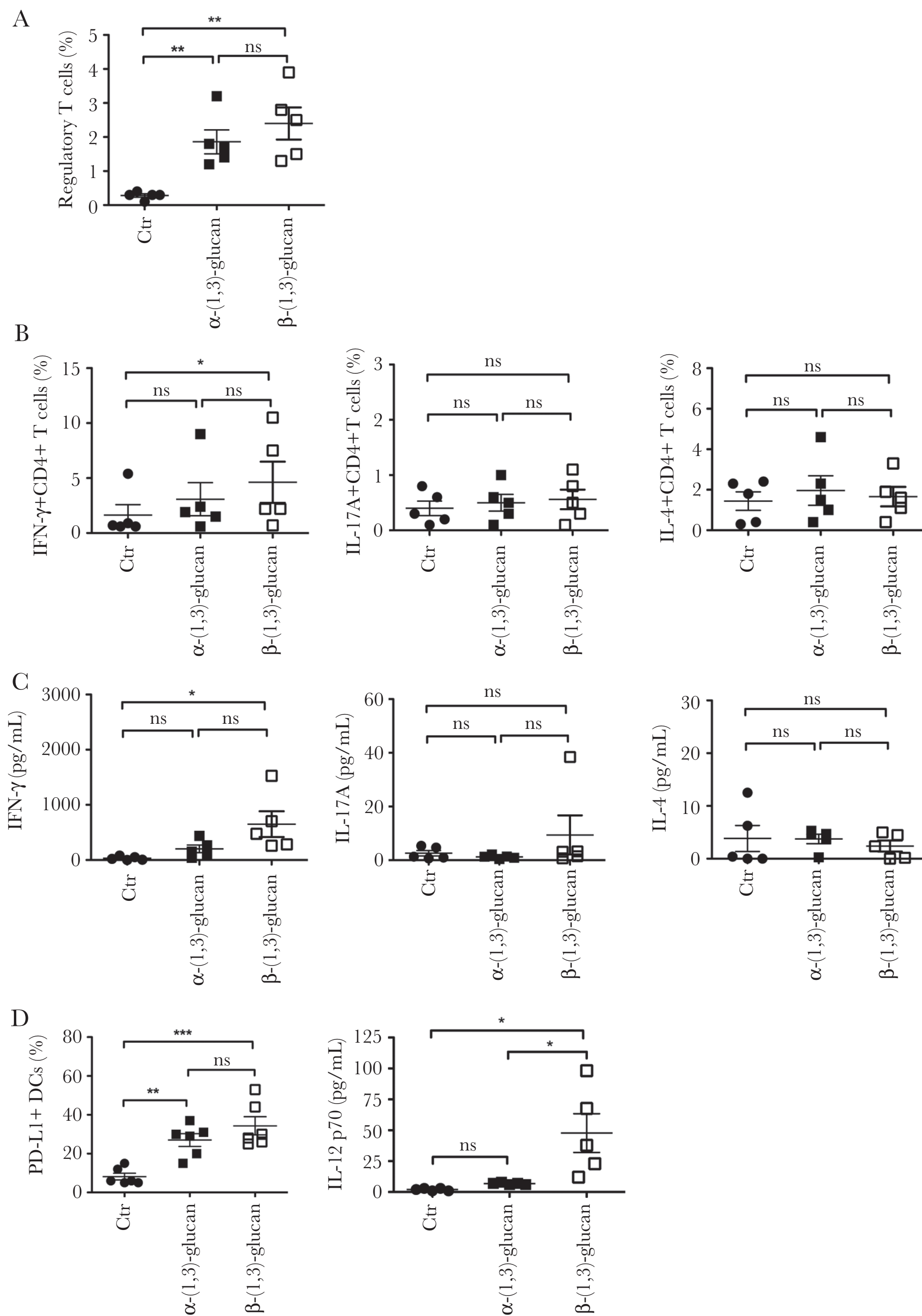

Figure 7. α-(1,3)-Glucan and β-(1,3)-glucan distinctly polarize Th responses. Monocyte-derived DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with α-(1,3)-glucan or β-(1,3)-glucan from *A. fumigatus* mycelia for 48 h. DCs were washed and cocultured with autologous naive CD4+ T cells at 1:10 ratio for 5 days. The CD4+ T cells were analyzed for the polarization of various T-cell subsets. *A,* Treg polarization (mean ± SEM, n = 5 donors) induced by α-(1,3)-glucan and β-(1,3)-glucan. *B,* Polarization of Th1, Th17, and Th2 responses by α-(1,3)-glucan and β-(1,3)-glucan as analyzed by intracellular staining for IFN-γ, IL-17A, and IL-4, respectively, by flow cytometry (mean ± SEM, n = 5 donors). *C,* Amount of secretion of CD4+ T-cell cytokines IFN-γ, IL-17A, and IL-4 in the cell-free culture supernatants of DC–T cell cocultures (mean ± SEM, n = 5 donors). *D,* Induction of PD-L1 (CD274) and IL-12p70 in DCs by α-(1,3)-glucan and β-(1,3)-glucan (mean ± SEM, n = 5–6 donors). * $P < .05$; ** $P < .01$; *** $P < .001$; ns, not significant as determined by one-way ANOVA test.

signaling in murine innate cells enhances Th17 responses to *A. fumigatus* by diminishing IL-12 and IFN-γ [26]. Our previous work demonstrated that *A. fumigatus* cell wall β-(1,3)-glucan induces Th1/Th17 responses, whereas galactomannan activates Th2/Th17 responses in mice [10]. On the other hand, galactosaminogalactan is an immunosuppressive polysaccharide [30,

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31] and inhibits Th1/Th17 cytokines in human peripheral blood mononuclear cells (PBMC) through the induction of IL-1RA [31]. *Candida albicans*-derived chitin induces anti-inflammatory IL-10 in human PBMC and this IL-10 might be responsible for the lack of induction of Th1 and Th17 responses to chitin [32]. In contrast, chitin from *A. fumigatus* showed both pro- and anti-inflammatory properties; in the presence of other PAMPs, chitin showed synergistic proinflammatory properties, whereas in the presence of serum immunoglobulins, it induced IL-1RA [33].

Our present study shows that α-(1,3)-glucan, yet another major cell wall component of *A. fumigatus*, promotes activation of naive T cells (based on the expression of CD25) and is in line with the enhanced expression of HLA-DR and costimulatory molecules CD80, CD86, and CD40 on DCs. Moreover, a fraction of these activated $CD4^+$ T cells differentiated into Tregs, while other effector T-cell subsets were not significantly altered. Together, these data are indicative of incomplete differentiation of T cells due to inability of α-(1,3)-glucan to induce large amounts of T-cell polarizing cytokines and associated high expression of PD-L1 on DCs. Although antigen-specific T-cell clones from healthy donors produced IFN-γ and IL-17 upon β-(1,3)-glucan stimulation [10], we found that "β-(1,3)-glucan-educated" DCs promoted Treg and Th1 but not Th17 polarization, suggesting that Th17 response to β-(1,3)-glucan in vivo is contributed by other APCs. In support of this proposition, monocyte-derived $CD11c^+$ DCs in mice were reported to inhibit *A. fumigatus*-specific Th17 differentiation [26]. Together, these data imply that cell wall polysaccharides, as well as innate cell subsets, diversify the immune response to *A. fumigatus* by their distinct abilities to polarize specific $CD4^+$ T-cell responses.

DCs play a key role in the polarization of $CD4^+$ T-cell responses. Interaction between PD-L1, OX-40L, ICOSL on DCs and PD-1, OX-40, ICOS on $CD4^+$ T cells have been shown to induce Tregs [34–37]. We found that α-(1,3)-glucan did not induce OX-40L and ICOSL on the DCs, ruling out the involvement of these costimulatory molecules in Treg polarization. However, α-(1,3)-glucan induced high expression of PD-L1 on DCs, the interaction of which with PD-1 promotes Treg responses and efficiently suppresses Th1 response [38]. Further, interaction of PD-L1 with PD-1 on T cells leads to the activation of SHP1/2 that suppresses STAT1, resulting in the abrogation of IFN-γ production. Thus, low-level induction of IL-12p70 in association with high PD-L1 expression on the DCs explains low Th1 polarization by "α-(1,3)-glucan-educated" DCs. In fact, Treg and Th1 polarization induced by β-(1,3)-glucan was associated with high expression of PD-L1 on DCs and production of large amounts of DC-derived IL-12p70. However, IL-12 was decreased in murine DCs when exposed to *Pseudallescheria boydii*-derived α-glucan [39]. This could be attributed to the fact that *P. boydii*-derived α-glucan contains linear α-(1,4)-linkages with side chains substituted at carbon-6 position of glucose, whereas *A. fumigatus*-derived glucan is α-(1,3)-glucan with an average of 1% intra-chain α-(1,4)-linked glucose residues. This re-enforces the fact that the linkage pattern of a polysaccharide plays a crucial role in the kind of immune stimulatory mechanism.

Our previous work with a vaccination model demonstrated that α-(1,3)-glucan renders protection against *A. fumigatus* and was associated with Th1 (IFN-γ) and Treg responses [10]. However, we found that α-(1,3)-glucan-pulsed human DCs polarized predominantly Tregs. The IFN-γ production was low; clear Th1 response was observed only upon PD-L1 blockade on DCs. The discrepancy in the results might be attributed to various factors, including differences in experimental systems, expression pattern of PD-L1 and other PRRs, or due to the protocol used to isolate α-(1,3)-glucan during previous work. Upon encounter with α-(1,3)-glucan in vivo, the final immune response will depend not only on the relative degree of stimulation of particular innate immune cells but also on their diversity, cooperation of other innate cells, nonimmune cells and PRRs, and the cellular localization.

Among TLRs, innate recognition of *A. fumigatus* is mediated mainly by TLR4 and TLR2 [18–21, 40]. Thus, TLR2 occupies a unique place in *A. fumigatus* pathogenesis. However, a specific receptor that recognizes *A. fumigatus* α-(1,3)-glucan has not been identified so far. Our data indicate that TLR2 is implicated in the cross-talk between α-(1,3)-glucan and the human DCs. There was significant reduction in the expression of DC costimulatory molecules upon TLR2 blockade prior to stimulation with α-(1,3)-glucan and was further substantiated by signaling events in $tlr2^{-/-}$ DCs. In line with our observation, *P. boydii* cell wall-derived α-glucan induced cytokines from mouse macrophages and BM-DCs in a TLR2-dependent mechanism [39].

By using functional assays, we show that α-(1,3)-oligosaccharides are not stimulatory. Importantly, the short α-(1,3)-oligosaccharides efficiently blocked α-(1,3)-glucan-mediated Treg response. These data fit well with the "fibril hypothesis" wherein soluble short oligosaccharides recognize a single PRR without inducing an immune response, whereas fibrillar polysaccharides bind to several PRRs leading to increased avidity, receptor clustering, triggering of signaling events, and activation of immune response [8].

Many pathogens evade protective immune responses by exploiting the PD-L1-PD-1 pathway [34]. Our data show that *A. fumigatus* could exploit this pathway through its PAMPs like α-(1,3)-glucan to selectively mediate Treg responses. In humans, *A. fumigatus*-specific Tregs represent approximately 0.1% among $CD4^+$ T cells. A high Treg/memory conventional T-cell ratio in normal individuals indicates that Tregs control effector T-cell response to *A. fumigatus* [41]. In fact, although Tregs prevent infection-associated inflammation and tissue damage, they also dampen protective immune response to pathogens and enhance their persistence [42]. Therefore, restraining the Treg responses represents a promising approach to boost protective response against *A. fumigatus* infection. Our data indicate that PD-L1-PD-1 axis could be targeted to enhance protective immunity to *A. fumigatus* infections.

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In addition to causing T-cell exhaustion and anergy, the PD-L1-PD-1 pathway inhibits IFN-γ and blockade of this pathway leads to enhanced IFN-γ response [43] and inhibition of Treg expansion [36, 44]. Th1 response plays an important role in curtailing *A. fumigatus* invasion [45, 46] and murine models have shown that innate cell-primed CD4+ T cells migrate to airways [47]. Patients with invasive aspergillosis secrete low levels of IFN-γ and administration of IFN-γ, adjunct to antifungal therapy, significantly enhances clinical improvements [48]. Adoptive transfer experiments in experimental invasive aspergillosis also provide therapeutic utility of IFN-γ-producing Th1 cells [49]. Restoration of protective T-cell response by blocking the PD-L1-PD-1 axis has emerged as an important therapeutic intervention in oncology [50]. Accordingly, blockade of PD-L1 on "α-(1,3)-glucan-educated" DCs led to enhanced IFN-γ secretion without modulation of Th2 and Th17 responses. Our results thus provide a rationale for exploiting PD-L1-PD-1-targeted immunotherapeutics to enhance protective immune response to *A. fumigatus* infections in patients and in α-(1,3)-glucan vaccination settings.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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