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GM-CSF plays a key role in zymosan-stimulated human dendritic cells for activation of Th1 and Th17 cells

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ABSTRACT

In this study, we compared the effects of zymosan and LPS on human monocyte-derived dendritic cells. The specific effects of zymosan on the expression of several key cytokines, including granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukins (IL-1α, IL-1β and IL-12 p70) were quite distinct from the effects of LPS. Unlike activation with LPS, DCs activated by zymosan expressed little or no IL-12 p70 due to lack of expression of the p35 subunit. However, treatment with zymosan resulted in a substantial increase in Th1 and Th17 cell-polarizing capacity of DCs. Furthermore, the GM-CSF secreted by zymosan-activated DCs enhanced IL-23 production, resulting in activation of a Th17 response. GM-CSF and IL-27, rather than IL-12 p70, were both major direct contributors to the activation of a Th1 response. This signaling mechanism is distinct and yet complementary to LPS-mediated T-cell activation. We suggest that this novel zymosan-induced GM-CSF-mediated signaling network may play a key role in regulating specific immune cell type activities.

1. Introduction

The Th1/Th2 paradigm of two distinct populations of T-helper cells established by Mosmann and Coffman [1] has been the major framework used to address adaptive immunity for nearly two decades. Th1 and Th2 cells are characterized by specific cytokine signatures (e.g., interferon-γ (IFN-γ) for Th1 cells and interleukin-4 (IL-4) for Th2 cells). Recently, a paradigm shift has evolved in the study of T-helper-cell-mediated immunity. The diversity of T-helper cell populations has been expanded following the discovery of regulatory T cells (Tregs) with immunosuppressive function, and IL-17-producing effector T helper cells (Th17 cells), which can exhibit distinct functions from Th1 and Th2 cells [2,3].

Dendritic cells (DCs), the key antigen presenting cells (APCs) in the initiation of an immune response, play a central role in the harmonization of immunity and tolerance. When DCs are activated by inflammatory stimuli or infectious agents, they undergo a maturation process characterized by high expression of MHC and co-stimulatory molecules [4]. Mature DCs migrate into lymphoid tissues and subsequently acquire the capacity to activate resting, naïve, or memory T lymphocytes [5]. Cytokine production by DCs is considered to be a main contributor to the promotion of T cell polarization. IL-12 has been considered a major cytokine in the polarization of Th1 cells [6]. Recent studies indicate that IL-27 and IL-23 (both new members of the IL-12 family) are associated with the expansion and survival of Th1 cells and Th17 cells, respectively [7,8].

GM-CSF is an important hematopoietic growth factor and a multi-functional immune modulator. It is required for *in vitro* differentiation of progenitor cells into DCs [9] and has been extensively studied for use as an adjuvant in various cancer vaccine approaches [10–12]. GM-CSF cannot only increase immune responses [13] but can also alter the balance of Th1/Th2 cytokines [14]. The APC-produced GM-CSF is critical for the activation of resting T cells and the maintenance of APC function; hence, GM-CSF can effectively increase an antigen-induced immune response [14,15]. APCs treated with GM-CSF induce allogeneic T cell proliferative responses by upregulating the production of type 1 cytokines (IL-12, TNF-α and IFN-γ) while downregulating the type 2

Abbreviations: Th1, T-helper 1; Th17, T-helper 17.

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cytokines (IL-10 and IL-4) [16]. In addition, a recent study also demonstrated a novel role for GM-CSF in enhancing the expansion and survival of Th17 cells via regulation of IL-6 and IL-23 *in vivo* [17].

Zymosan is a crude cell-wall component mixture of the baker's yeast extracts from *Saccharomyces cerevisiae*, composed mainly of β-glucans (50–57%), mannans and chitins. The US Food and Drug Administration (FDA) has given these β-glucans derived from yeast extract a GRAS ("Generally Recognized as Safe") rating [18–20]. Pillemer and Ecker [21] demonstrated that zymosan could non-specifically potentiate and modulate the immune system. Zymosan can activate a broad range of cell types, including macrophages [22], granulocytes (polymorphonuclear leukocytes) [23], natural killer cells [24] and DCs [25,26]. Furthermore, it has been demonstrated that zymosan can activate the Th1 immune response when used as an adjuvant in a DNA vaccine for HIV-1 [27].

Recent studies have shown that DCs can be activated by zymosan via the induction of specific intracellular signals that stimulate TLR2 and dectin-1, initiators and modulators of various immune responses [28,29]. Some studies have shown that zymosan acts as a pro-inflammatory stimulus and promotes both pro-inflammatory (IL-2, IL-6, IL-12, TNF-α and COX-2) and anti-inflammatory (IL-10) responses in macrophages and bone marrow-derived DCs [22,28]. However, another recent study [30] showed that exposing mouse splenic DCs to zymosan resulted in the induction of a "regulatory" DC phenotype, characterized by secretion of a high level of IL-10 and little or no IL-6 or IL-12 p70, and this in turn resulted in poor activation of antigen-specific $CD4^+$ T cells, leading to immune-tolerance activity. The reason for the discrepancy between these two studies [28–30] is currently not clear, and it was speculated that it may be due to a difference in the subsets of test cells being examined in different studies. More recently, Gerosa et al. [31] reported that zymosan stimulated IL-23 rather than IL-12 p70 production in monocyte-derived DCs. Their results indicate that IL-23 and other cytokines produced by zymosan-activated DCs play a role in activating the Th17 response. Interestingly, although there is little or no IL-12 p70 production, a Th1 response can still be stimulated by zymosan-activated human DCs. Therefore, we hypothesize here that instead of IL-12 p70, some other cytokine such as GM-CSF and IL-27 may be involved in producing the Th1 response in zymosan-activated human monocyte-derived DCs.

Stimulation of the maturation and antigen-presenting activity of DCs is critical for the induction of T lymphocyte responses in immunity. Currently, various cell therapy approaches are under investigation to manipulate DCs for clinical or experimental applications [32,33]. For example, human monocyte-derived DCs from some cancer patients are clinically defective in terms of phenotypic and/or functional maturation [34,35]. There is thus a need to identify molecular or biochemical agents that can effectively induce DC differentiation and activation in a timely manner on exposure to target antigens.

These clinical considerations prompted us to investigate the effect of zymosan on human DCs. We found that both pro- and anti-inflammatory cytokines were induced in zymosan-treated human monocyte-derived DCs. Specifically, zymosan augmented the expression of GM-CSF, IL-6, IL-10, MCP-1, IL-1α, IL-1β, TNF-α, IL-23 and IL-27, but had little or no effect on IL-12 p70. The effect of zymosan on the induction of GM-CSF and the expression of unique cytokine profiles differed drastically from that of LPS. The GM-CSF secreted by zymosan-activated DCs enhanced IL-23 production. Furthermore, we found that zymosan-activated DCs can effectively prime T cells and increase their ability to produce IFN-γ and IL-17A. Together, our findings suggest that in zymosan-activated DCs, high levels of GM-CSF and IL-27 rather than IL-12 p70 have an alternative and important role in Th1 cell activation. GM-CSF may also be indirectly involved in activating the Th17 response via enhancement of IL-23 production. This novel signaling network warrants future systematic investigation for its potential clinical applications.

2. Materials and methods

2.1. Reagents

Zymosan (Sigma–Aldrich) was suspended in 0.15 M sodium chloride, placed in a boiling water bath for 1 h, then centrifuged for 30 min at 4000*g*; the supernatant was discarded and the residue was washed twice in phosphate buffered saline. The residue was then suspended at 20 mg/ml in PBS and stored at −70 °C. LPS (100 ng/ml) was obtained from Sigma–Aldrich. Recombinant GM-CSF and IL-4 were purchased from PeproTech. Purified monoclonal anti-human GM-CSF, IL-23, IL-27 and IL-12 p40 antibodies were purchased from R&D Systems.

2.2. Preparation of human DCs in primary cultures

DCs were prepared from blood samples drawn from normal healthy volunteers at the Red Cross Blood Center, Taipei, Taiwan, as previously described [36]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation with a Ficoll-Metrizoate density gradient (GE Healthcare), and Percoll gradient solution (isosmotic solution: PBS/citrate = 11:9). The PBMCs were purified with a human Monocyte Isolation Kit II by passing through a magnetic cell separation system (MACS). DCs were prepared from highly purified monocytes by culturing for 6 d in RPMI 1640 medium containing 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and supplemented with 10% fetal bovine serum (all from Invitrogen) and recombinant GM-CSF and IL-4 (each 100 ng/ml).

2.3. Beadlyte human 22-Plex multi-cytokine detection system

Measurements of the expression of a series of cytokines/chemokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p40, IL-12 p70, IL-13, IL-15, IP-10, Eotaxin, IFN-γ, TNF-α, MCP-1, MIP-1α, RANTES and GM-CSF) in culture supernatants of DCs treated with zymosan at different concentrations (1–100 μg/ml) or LPS (100 ng/ml) were conducted using the Luminex suspension array technology (Upstate) following the manufacturer's instructions. Briefly, beads pre-coated with specific anti-cytokine/chemokine antibodies served as targets or as capture reagents for targets. Reporters, also tagged with a fluorescent label, were then bound to the target. In the Luminex instrument, the beads passed rapidly through two laser beams and high-speed digital signal processors, and computer algorithms distinguished which assay was being carried on each bead while quantifying the reaction based on fluorescent reporter signals.

2.4. Mixed lymphocyte reaction (MLR)

Human $CD4^+$ T lymphocytes were isolated and purified from the heavy-density fraction of Percoll gradients followed by immunomagnetic depletion as previously described [37], using a naïve $CD4^+$ T cell isolation kit and magnetic cell sorting column (all from MACS). DCs stimulated with test agents for 24 h were washed and then co-cultured at different densities ($2–60 \times 10^2$ cells/well) with purified naïve $CD4^+$ T cells (10^5 cells/well) in 96-well microculture plates for 5 days. Cell proliferation was quantified by use of a BrdU colorimetric ELISA kit (Roche) following the manufacturer's instructions. For determination of T cell polarization, DC:T cell

co-cultures (1:4 ratio) were incubated in a 96-well plate for 1 day and T cells were subsequently expanded in 24-well plates in a medium supplemented with IL-2 (5 ng/ml). After 8 days in culture, T cells were stimulated with phorbol myristate acetate (PMA) (20 ng/ml) and ionomycin (1 μg/ml) for 6 h. To avid cytokine secretion, the Golgi vesicle disruptor brefeldin A (10 μg/ml) was added during the final 2 h of stimulation, and cells were then examined for intracellular accumulation of IFN-γ and IL-17A. T cells were fixed (2% paraformaldehyde), permeabilized (0.5% saponin), stained with FITC-conjugated mouse anti-IFN-γ or PE-Cy7-conjugated mouse anti-IL-17A mAbs, and analyzed by flow cytometry.

2.5. ELISA for cytokines

Measurement of IL-6, IL-10, IL-12 p70, TNF-α, GM-CSF, IL-23, IL-27, IL-17A and INF-γ in the supernatants of different test agent stimulated DC cultures was carried out with the DuoSet kit (R&D Systems) following the manufacturer's recommendations.

2.6. Flow cytometry analysis

DCs from 6-day cultures were collected and incubated with zymosan at various concentrations (1–100 μg/ml) or LPS (100 ng/ml). LPS and solvent (vehicle)-treated DCs were used as positive and negative controls, respectively. At 24 h post-incubation cells were harvested, washed with PBS/1% FBS, and subsequently stained for DC cell-surface markers, including CD40, CD80, CD83, CD86 and HLA-DR labeled with PE in 100 μl of PBS containing 1% FBS for 30 min at 4 °C. Cells were washed twice with PBS/1% FBS and then fixed in 1% paraformaldehyde. Cell sorting analysis was conducted using a Coulter Epics XL-MCL flow cytometer (Beckman/Coulter).

2.7. Primer design, RT-PCR and real-time PCR analyses

Total RNA was extracted from test cells using TRIzol reagent (Invitrogen). Aliquots of 1 μg RNA underwent RT-PCR with the AccessQuick RT-PCR System (Promega) according to the manufacturer's instructions at cycle numbers that yielded products within the linear range as determined for each pair of test primers. The sequences of the primers for human *IL-12 p40* were 5′-CCA AGA ACT TGC AGC TGA AG-3′ (sense) and 5′-TGG GTC TAT TCC GTT GTG TC-3′ (antisense); human *IL-12 p35* 5′-TGT TCC CAT GCC TTC ACC A-3′ (sense) and 5′-TTT GTC TGG CCT TCT GGA GC-3′ (antisense); and human *GAPDH* 5′-CAT CAC TGC CAC CCA GAA GAC TGT GGA-3′ (sense) and 5′-TAC TCC TTG GAG GCC ATG TAG GCC ATG-3′ (antisense). The PCR products were analyzed on an ethidium bromide-stained 2% agarose gel. For real-time PCR assay, reverse transcription was carried out using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's instructions. Cycling parameters were 15 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 20 s annealing at 57 °C and extension at 72 °C for 20 s. Real-time PCR was performed using a LightCycler 2.0 instrument (Roche, Mannheim, Germany).

3. Results

3.1. Morphological characteristics of zymosan-treated DCs

Effective conversion of a non-adherent monocyte phenotype to a typical immature DC (imDC) morphology characterized by irregular cell shape and multiple dendritic projections was observed in 6-day-old DC cultures (Fig. 1A). To determine the changes in cellular morphology, imDCs were treated with zymosan or LPS (positive control) for 4 h and examined microscopically. Most LPS-activated

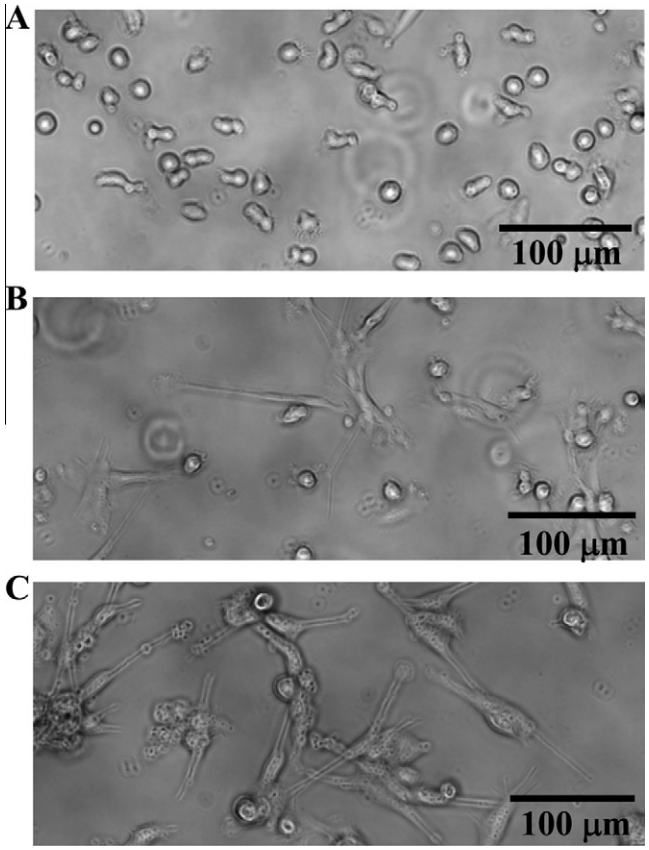

Fig. 1. Morphological differences between zymosan- and LPS-activated DCs. Morphological characteristics of human monocyte-derived DCs were examined under phase contrast microscopy. (A) Untreated immature DCs; (B) LPS-activated DCs; (C) Zymosan-activated DCs, showing abundant, intracellular punctate particles.

DCs were spindle-shaped and adhered to the culture dish (Fig. 1B). Zymosan activation resulted in the exhibition of a phenotype similar to LPS-activated DCs. In contrast however, zymosan treatment also generated more punctate particles inside test cells (Fig. 1C), as was previously observed for intracellular phagosomes in human DCs [38].

3.2. Zymosan induces phenotypic maturation of DCs

To determine whether zymosan can activate human DCs, monocyte-derived imDCs were untreated or treated with zymosan (1–100 μg/ml) or with LPS (100 ng/ml) for 24 h. Phenotypic maturation of test cells was evaluated by analyzing the expression of a specific maturation marker (CD83), costimulatory molecules (CD40, CD80 and CD86), and the peptide-presenting MHC-II molecule (HLA-DR) by flow cytometry. Substantial upregulation (3- to 5-fold) of CD40, CD80 and CD86 molecules and the maturation marker CD83 was observed following zymosan treatment. As shown in Fig. 2, zymosan slightly upregulated the expression of HLA-DR in DCs compared to that of LPS treated cells. Most of the surface marker changes were readily detectable even at low concentrations of zymosan (10–25 μg/ml) or LPS (100 ng/ml). Thus, zymosan can effectively induce the phenotypic maturation of human monocyte-derived DCs.

3.3. Analysis of cytokine expression profile in zymosan-treated DCs

Since the reported expression patterns of cytokines in hDCs treated with zymosan varied [28,30], we carefully analyzed specific cytokine expression profiles in zymosan-treated human

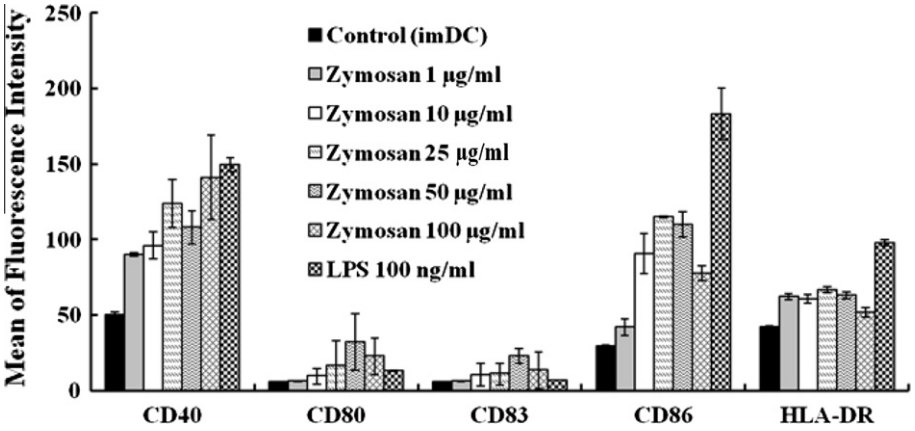

Fig. 2. Zymosan induces expression of specific DC maturation markers. Immature DCs were left untreated, or stimulated with zymosan (1–100 μg/ml) or LPS (100 ng/ml). Expression of cell surface markers (CD40, CD80, CD83, CD86 and HLA-DR) was analyzed after 24 h using flow cytometry. Untreated immature DCs served as reference for all other culture conditions. The data are representative of the results from three independent experiments.

monocyte-derived DCs. Luminex suspension array analysis revealed that zymosan treatment (1–100 μg/ml) significantly increased the expression of GM-CSF, TNF-α, IL-1α, IL-1β, MCP-1, IL-6, IL-10 and IL-12 p40 in a dose-dependant manner but had little or no effect on the expression of IL-12 p70 (Fig. 3A). Zymosan (100 μg/ml) and LPS (100 ng/ml) induced strikingly different expression of two cytokines, with high levels of GM-CSF and barely detectable levels of IL-12 p70 with zymosan treatment and the reverse with LPS treatment. The remaining 13 cytokines examined in this study, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-13, IL-15, IP-10, Eotaxin, IFN-γ, MIP-1, RANTES, did not show any significant changes after zymosan treatment (data not shown).

3.4. Confirmation of specific cytokine expression patterns in zymosan-treated DCs

We used ELISA to further verify the expression patterns of specific cytokines known to modulate DC functions. The analysis confirmed that zymosan induced the expression of several pro-inflammatory (GM-CSF, TNF-α, IL-23, IL-27 and IL-6) and anti-inflammatory (IL-10) cytokines (Fig. 3B). Under identical test conditions, we detected little or no induction of IL-12 p70 protein expression by zymosan treatment (Fig. 3B). Both assays confirmed the lack of any IL-12 P70 protein expression following zymosan treatment.

3.5. Zymosan induces the mRNA expression of p40 subunit but not p35 subunit of IL-12 p70 in DCs

To determine the underlying cause of the failure of zymosan to induce IL-12 p70, we studied its effect on the expression of biologically inactive subunits of IL-12 p70 (p40 and p35), known to be regulated differently. RT-PCR analysis of RNA samples from zymosan-treated DCs at 6 h post-stimulation revealed that the mRNA expression of p40 but not p35 was readily induced by zymosan (Fig. 4A). This result was further confirmed by subsequent analysis with a real-time PCR assay. Therefore, our results strongly suggest that zymosan cannot induce the expression of active IL-12 p70, because of its lack of induction of p35 mRNA expression in human DCs. To our knowledge, this is the first demonstration of the molecular basis for the failure of zymosan to induce IL-12 p70.

3.6. GM-CSF secreted by zymosan-activated DCs enhances IL-23 production

To clarify the relationship between GM-CSF, IL-12 P40, IL-23 and IL-27 in zymosan-activated DCs, we have analyzed their expression in zymosan-treated DCs in the presence of anti-GM-CSF, anti-IL-23, anti-IL-27 or IL-12 p40 antibodies. Anti-GM-CSF anti-body strongly inhibited the zymosan-induced expression of IL-12 P40 and IL-23 but not that of IL-27 in test DCs (Fig. 5A–D). On the other hand, anti-IL-12 p40 antibody strongly inhibited the zymosan-induced expression of IL-23 and IL-27 but not that of GM-CSF in test DCs (Fig. 5B–C). In addition, a partial inhibition of IL-12 p40 expression was detected by treatment with anti-IL-23 p19 antibody; treatment with anti-IL-27 antibody had little or no effect on IL-12 p40 and GM-CSF expression in zymosan-stimulated DCs (Fig. 5).

3.7. Zymosan increases DC allostimulatory capacity

The above results demonstrate an apparently unique and previously unreported cytokine expression profile (i.e., high levels of pro- and anti-inflammatory cytokines including GM-CSF, IL-6, TNF-α, IL-23 and IL-10, but little or no expression of IL-12 p70) in zymosan-treated DCs. To determine whether this unique cytokine expression pattern induced by zymosan could affect the allostimulatory capacity of zymosan-treated DCs, we performed a mixed lymphocyte reaction analysis. Zymosan-treated DCs were co-cultured with naïve $CD4^+$ T cells and T-cell proliferation activity measured. After 5 days of stimulation, both zymosan- and LPS-treated DCs induced similar significant increases in T-cell proliferation activity as compared with untreated DCs (Fig. 6A). Therefore, the lack of IL-12 p70 expression in zymosan-treated DCs did not interfere with their allostimulatory capacity. This result therefore suggests that other cytokines or factors expressed by zymosan-treated DCs may contribute to their T-cell stimulatory activity.

3.8. Zymosan-treated DCs preferentially induce Th1 and Th17 cell activation

To evaluate the possible T-cell polarizing capacity of DCs stimulated with zymosan, we assessed the expression of Th1, Th2 and Th17 cytokines (IFN-γ, IL-4 and IL-17A, respectively) in test cells using ELISA. T cells co-cultured with zymosan-activated DCs released significantly more IFN-γ and IL-17A than untreated DCs but showed little or no induction of IL-4 (Fig. 6B). The secretion level of IFN-γ was similar to that seen in LPS-treated DCs. However, T cells co-cultured with LPS-activated DCs failed to induce IL-17A production. Our data hence suggest that zymosan can preferentially induce Th1- and Th17-type cytokine expression in human DCs. The results on T-cell intracellular staining showed that, as compared to immature DCs, zymosan treatment of DCs increased

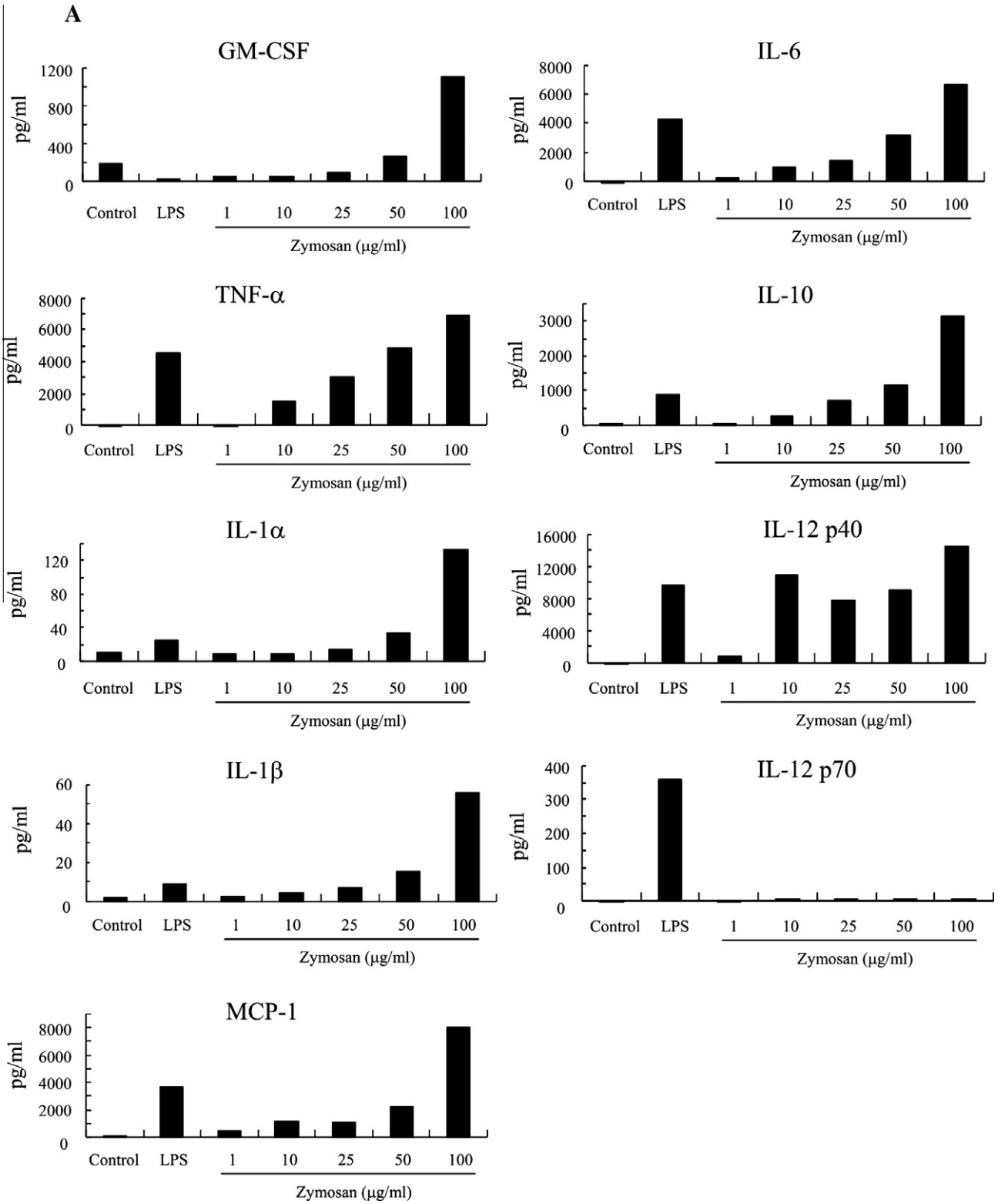

Fig. 3. Specific cytokine expression patterns in zymosan-treated DCs. (A) DCs were treated with zymosan or LPS as described in Fig. 2. At 24 h post-treatment, culture supernatants were analyzed for expression of a group of 22 cytokines/chemokines using the Beadlyte Multi-cytokine Detection System. Only cytokines or chemokines with significant change in expression level are shown. (B) Cytokine or chemokine expression profiles were analyzed using specific ELISA kits. The data in (A) and (B) are representative of the results from two and three independent experiments, respectively.

the number of IFN-γ secreting T cells (from 15.7% to 40.7%) and IL-17A secreting T cells (from 1% to 1.64%), indicating the promotion of naïve T-cell differentiation into Th1 and Th17 type cells (Fig. 6D). Since RORγt is recognized as a Th17 cell-specific transcription factor, the effect of zymosan treatment of DCs and the resultant induction of Th17 cell activation can be further confirmed by the increase of the number of RORγt positive T cells (from 0.46% to 1.61%) induced by treatment of DCs with zymosan. Together, our data hence suggest that zymosan-treated DCs can preferentially induce Th1 as well as Th17 cell activation.

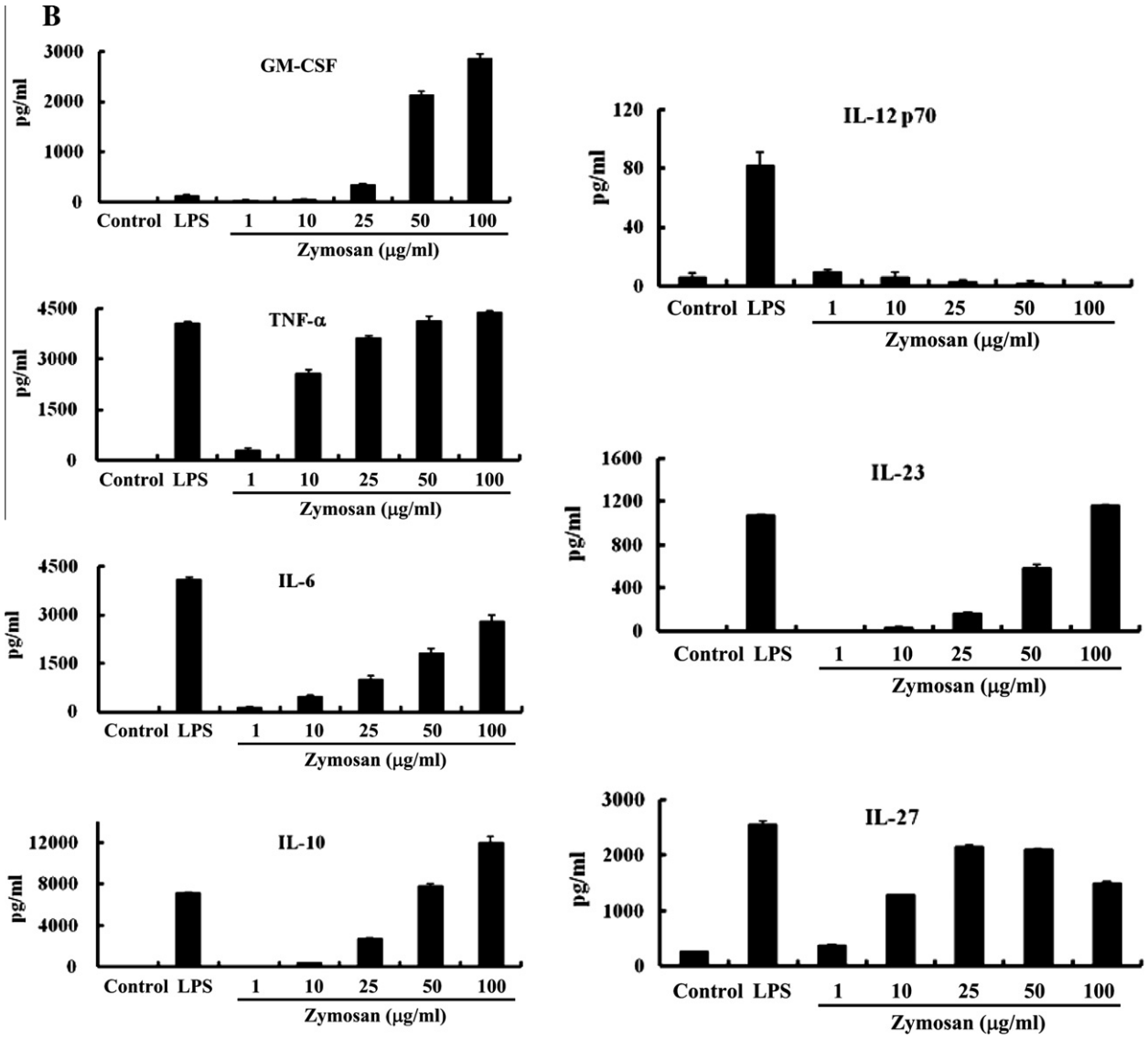

Fig. 3 (*continued*)

3.9. GM-CSF plays a key role in Th1 and Th17 immune responses induced by zymosan-treated DCs

Since zymosan-treated DCs differed from LPS-treated DCs in terms of the level of GM-CSF secretion, and previous studies have suggested that GM-CSF may play multiple roles in modification of immune cell activities such as increasing the T-cell proliferation, and Th1 and Th17 immune responses [16,17], here we tested the role of GM-CSF in the induction of Th1 and Th17 immune responses by zymosan-treated hDCs. As shown in Fig. 6C, GM-CSF-neutralizing antibodies significantly inhibited T-cell secretion of IFN-γ and IL-17A. The only effect of GM-CSF on the differentiation of Th1 and Th17 was in the production of IFN-γ. The T-cell intracellular staining assay showed that anti-GM-CSF antibody treatment decreased the number IFN-γ secreting T cells (from 40.7% to 16.6%) and anti-IL-27 anti-body treatment mainly decreased the number of IFN-γ secreting T cells (from 40.7% to 16.5%) (Fig. 6D). Therefore, in the absence of active IL-12 p70, GM-CSF and IL-27 secreted by DCs in response to zymosan may thus directly stimulate the activation of a Th1 immune response, and GM-CSF but not IL-27 may also be indirectly involved in Th17 immune response by mediating the enhancement of IL-23 production in test DCs.

4. Discussion

Immune deregulation or suppression is often a key underlying cause of various diseases, including cancers. The identification of novel immunomodulatory therapeutic agents that can improve specific immune functions is therefore important. One means to enhance specific immune function is to elevate Th1-cell function by modulating the specific activity of antigen-presenting cells. Various DC-derived cytokines with Th1 cell-polarizing capacity have been recently identified, with IL-12 being one of the most extensively investigated examples [39]. Clinical use of cytokines such as IL-12, IL-6, IL-2 and TNF-α as therapeutics is currently hampered by their chemical instability, high cost, and strong adverse effects such as septic shock, rheumatoid arthritis, and asthma [40]. Th1 cells have long been associated with the pathogenesis of autoimmune and chronic inflammatory diseases; however, in recent years, the discovery and characterization of the Th17 lineage has led to a paradigm shift in cellular immunology and immunotherapy studies. Like IL-12 in the control of the Th1 response, the induction of IL-23 leads to activation of Th17 cells, an activity that plays a pivotal role in the pathogenesis of autoimmune and chronic inflammatory diseases [41,42]. Although various strategies to use

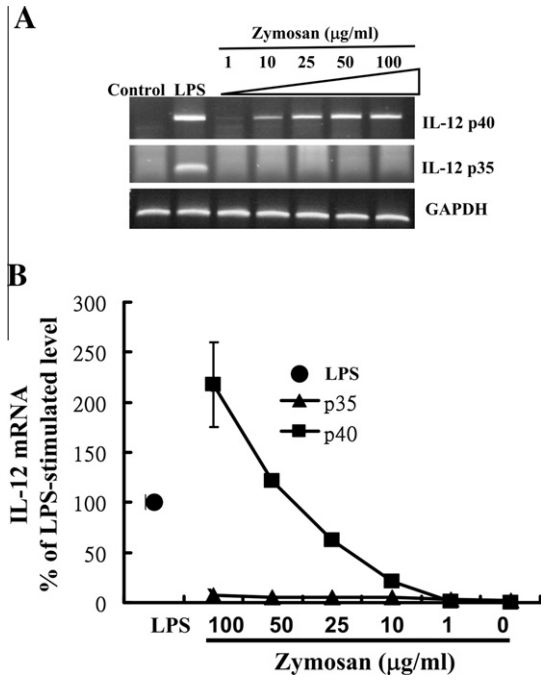

Fig. 4. Zymosan induces mRNA expression of p40 subunit but not p35 subunit of IL-12 p70 in treated DCs. Immature DCs were stimulated with zymosan, or LPS as described above in Fig. 2. At 6 h post treatment, cells were harvested and analyzed for expression of IL-12 p40 and IL-12 p35 mRNA using (A) RT-PCR and (B) real-time PCR analyses. The data are representative of the results from two independent experiments.

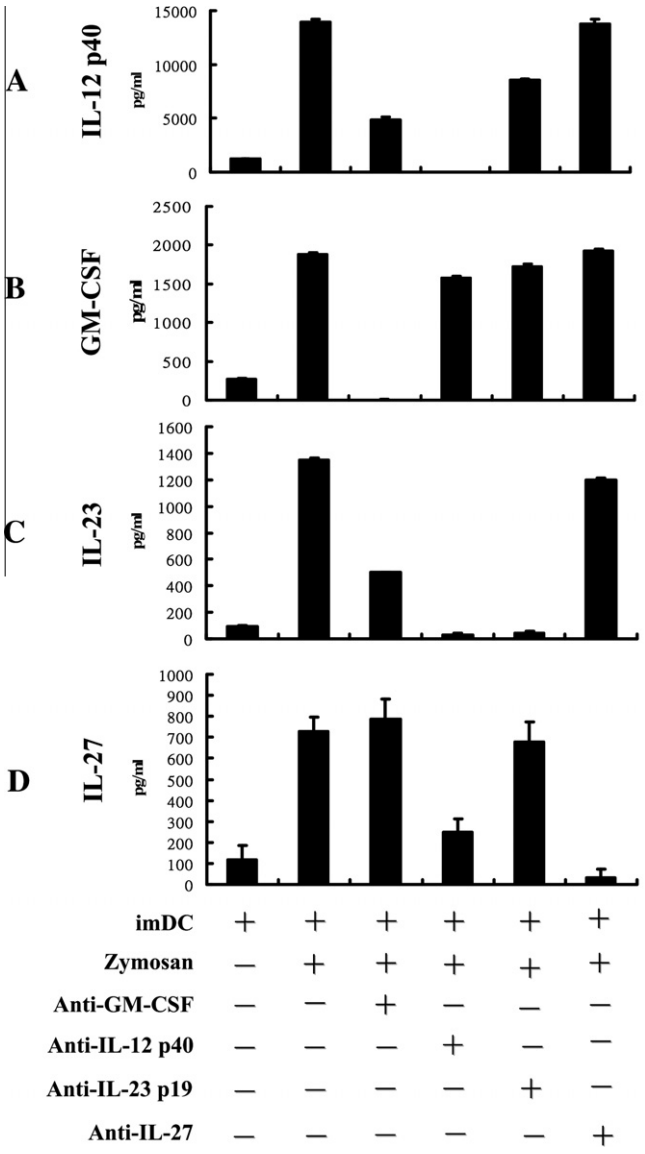

Fig. 5. GM-CSF secreted by zymosan-activated DCs can enhance IL-23 production. Immature DCs were left untreated (negative control) or stimulated with zymosan (100 μg/ml) in the presence of anti-GM-CSF or anti-IL-12 p40 antibodies. At 24 h post treatment, cell supernatants were harvested and analyzed using specific ELISA kits. The data are representative of the results from two independent experiments.

Th17 cells in clinical applications are being contemplated, the physiological roles of Th17 cells in immunity are far from thoroughly understood. Th17 cells are considered to be involved in neutrophil recruitment and to play an important role in immunity against bacteria, fungi and protozoa. In addition, it has been reported that Th17 cells have antitumor activity and can eradicate established melanomas [43]. Therefore, besides IL-12-Th1 immunity, IL-23-Th17 immunity may also play a crucial role in antitumor immunity.

In recent years, interest in developing natural products or other biologics that can confer appropriate immune-stimulatory effects has been increasing. Here, we investigated the potential of zymosan, a mixed cell-wall component of yeast *S. cerevisiae*, to stimulate the capacity of DCs to initiate the Th1 and Th17 responses. We found, as did previous reports [30], that the pro-inflammatory agent zymosan induces characteristic morphological changes and significant expression of a maturation marker (CD83), co-stimulatory molecules (CD40, CD80 and CD86), and the antigen-presenting MHC II molecule HLA-DR in monocyte-derived imDCs. We also observed that zymosan treatment conferred a significantly different cytokine expression profile in human DCs than LPS treatment by inducing the secretion of high levels of the pro- and anti- inflammatory cytokines GM-CSF, TNF-α, IL-6, IL-1α, IL-1β, IL-10, MCP-1, IL-23, IL-27 and IL-12 p40 but little or no expression of IL-12 p70, unlike with LPS treatment. Furthermore, we found that zymosan induced the mRNA expression of the p40 but not the p35 subunit of IL-12, which suggests a cause for the lack of expression of bioactive IL-12 p70.

These results of IL-12 p70 expression are consistent with previous findings, which reported the induction of only very low levels of IL-12 p70 in zymosan-treated DCs [30]. However, induction of high levels of IL-12 p70 by LPS-treated DCs was suggested to be essential, but not required, for stimulating the Th1 immune response in both human and mouse systems. Recent studies further indicate that IL-27, a new IL-12-family member, is associated with the expansion and survival of Th1 cell [7].

Previous study has also shown that the lack of expression of IL-12 p70 and high levels of IL-10 expression in zymosan-treated DCs may result in immunological tolerance [30]. IL-10 has been shown to block the up-regulation of costimulatory molecules and the induction of IL-12, thereby impairing the ability of DCs to generate a Th1 response [44]. In contrast, in our present study, zymosan-treated DCs show increased allostimulatory capacity and induce a Th1 immune response. This activity of zymosan is apparently mediated via novel cytokine signaling pathways involving IL-27 and GM-CSF, but not IL-12. In addition to the induction of a Th1 response, our results (Figs. 3B and 6C and D) also show that zymosan-treated DCs stimulate IL-23 expression and induce a Th17 response. A very similar result, recently reported by Gerosa and co-workers [31], demonstrated the induction of IL-23 and other cytokines by zymosan-activated DCs and its key role in activation of Th17 response.

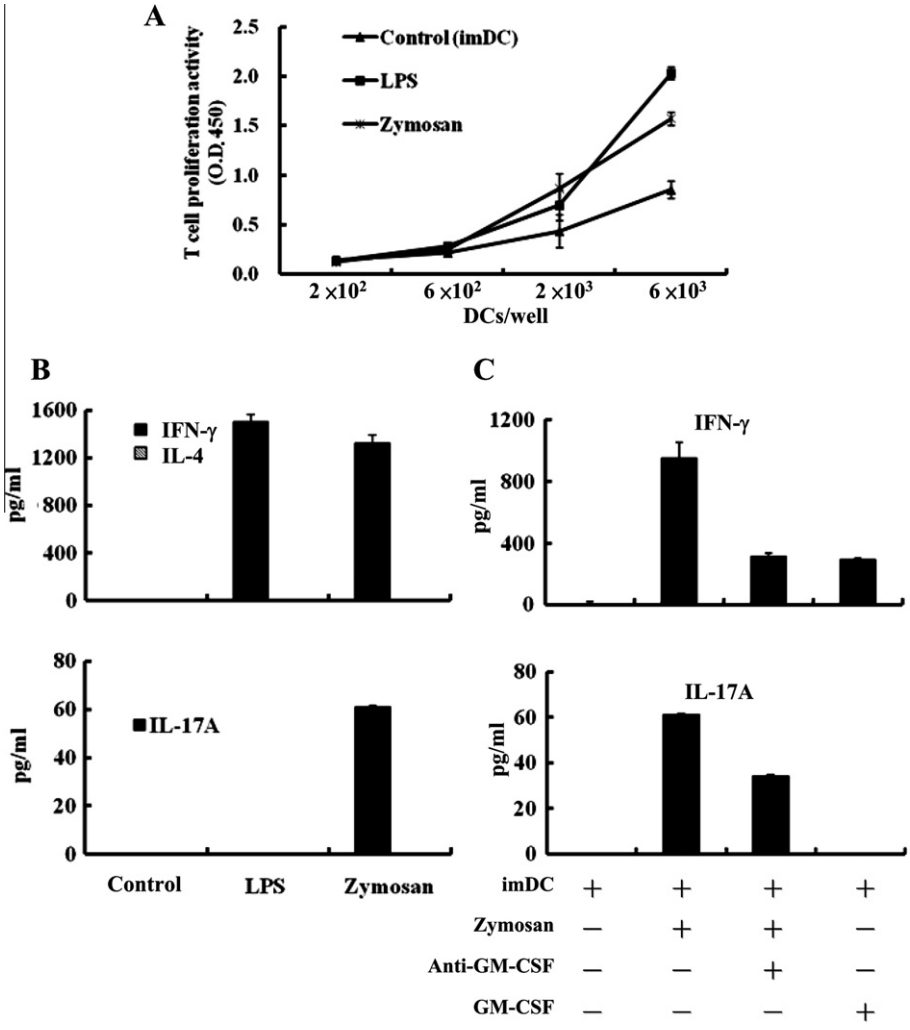

Fig. 6. GM-CSF plays a networking role in Th1 and Th17 immune responses induced by zymosan-treated DCs. Immature DCs were left untreated (negative control), or stimulated with zymosan (100 μg/ml) or LPS (100 ng/ml, as positive control). At 24 h post treatment, different cell densities of DCs were cocultured with naïve $CD4^+$ T cells (10^5/well). (A) Allogeneic $CD4^+$ T cell stimulatory activity of zymosan-activated DCs. T-cell proliferation was measured by detecting BrdU incorporation after 5 days of co-culture. (B) Induction of IFN-γ, IL-4 and IL-17A expression in T cells co-cultured with zymosan-activated DCs. Culture supernatants were collected after 5 days of co-culture and the expression of IFN-γ, IL-4 and IL-17A cytokines was measured by ELISA. (C) GM-CSF secreted by zymosan-treated DCs is involved in Th1 and Th17 cytokine secretions. Immature DCs were left untreated (negative control) or treated with zymosan (100 μg/ml) and anti-GM-CSF antibody. At 24 h post-treatment, test DCs (2×10^3 and 6×10^2) were co-cultured with naïve $CD4^+$ T cells (10^5/well). After 4 days of co-culture, IFN-γ and IL-17A cytokines were measured as described above. (D) Immature DCs were treated with zymosan (100 μg/ml) for 24 h. DCs was washed and co-cultured with T cells for 8 days in the presence of IL-2. T cells were then surface stained with CD4 antibody and intracellularly stained with RORγt antibody. A portion of test T cells were stimulated with PMA and ionomycin for 6 h before detection for cytokine expression. T cell polarization was determined by analyzing the level of intracellular accumulation of IFN-γ and IL-17A via flow cytometry. The data are representative of the results from two independent experiments.

Interestingly, our observation that zymosan-treated DCs express high levels of GM-CSF as well as IL-27 led us to speculate the possible involvement of these cytokines in the stimulation of the Th1 immune response. Our investigation of the relationship among GM-CSF, IL-23 and IL-27 in zymosan-activated DCs implies that GM-CSF, secreted by zymosan-activated DCs, may enhance IL-23 but not IL-27 activities in DCs. GM-CSF has been reported to play a key role in the development of several chronic inflammatory/autoimmune states [45]. The involvement of GM-CSF in inflammatory reactions is further demonstrated by its ability to upregulate proinflammatory cytokine expression and allogeneic T cell activation [46]. GM-CSF has recently been shown to play a critical role in enhancing generation of Th17 cells via regulation of IL-6 and IL-23 *in vivo* [17]. GM-CSF has also been shown to induce IL-12 expression in human dendritic cells and to polarize Th1 response [16]. IL-12 and IL-23 share the same subunit IL-12 p40. Our data further demonstrates that anti-GM-CSF anti-body can strongly inhibit the zymosan-activated DCs via upregulation of IL-12 p40 expression in test DCs (Fig. 5A). Therefore, we hypothesize here that GM-CSF may augment IL-23 expression in zymosan-activated DCs via upregulation of IL-12 p40 mRNA expression. This possibility needs to be evaluated by further investigation.

In our present study, zymosan-activated DCs not only induced a Th17 response but also a Th1 response. Although the same IL-23 protein level was also observed in LPS-activated DCs (Fig. 3B), LPS treatment however only induced a Th1 response (Fig. 6B), an effect that has been reported to result from the lack of IL-1β production [31]. Here, however, we showed that GM-CSF and IL-27 may both independently contribute to priming Th1 activation in zymosan-activated DCs. Moreover, in addition to zymosan-activated DCs secreting IL-23 to prime Th17 cell activation, we found that secreted GM-CSF may also indirectly contribute to the Th17 response via enhancement of IL-23 production. Putting these various findings together, we propose a possible model of the involvement of GM-CSF in zymosan-activated DCs for priming the activation of Th1 and Th17 cells (Fig. 7). We believe this novel

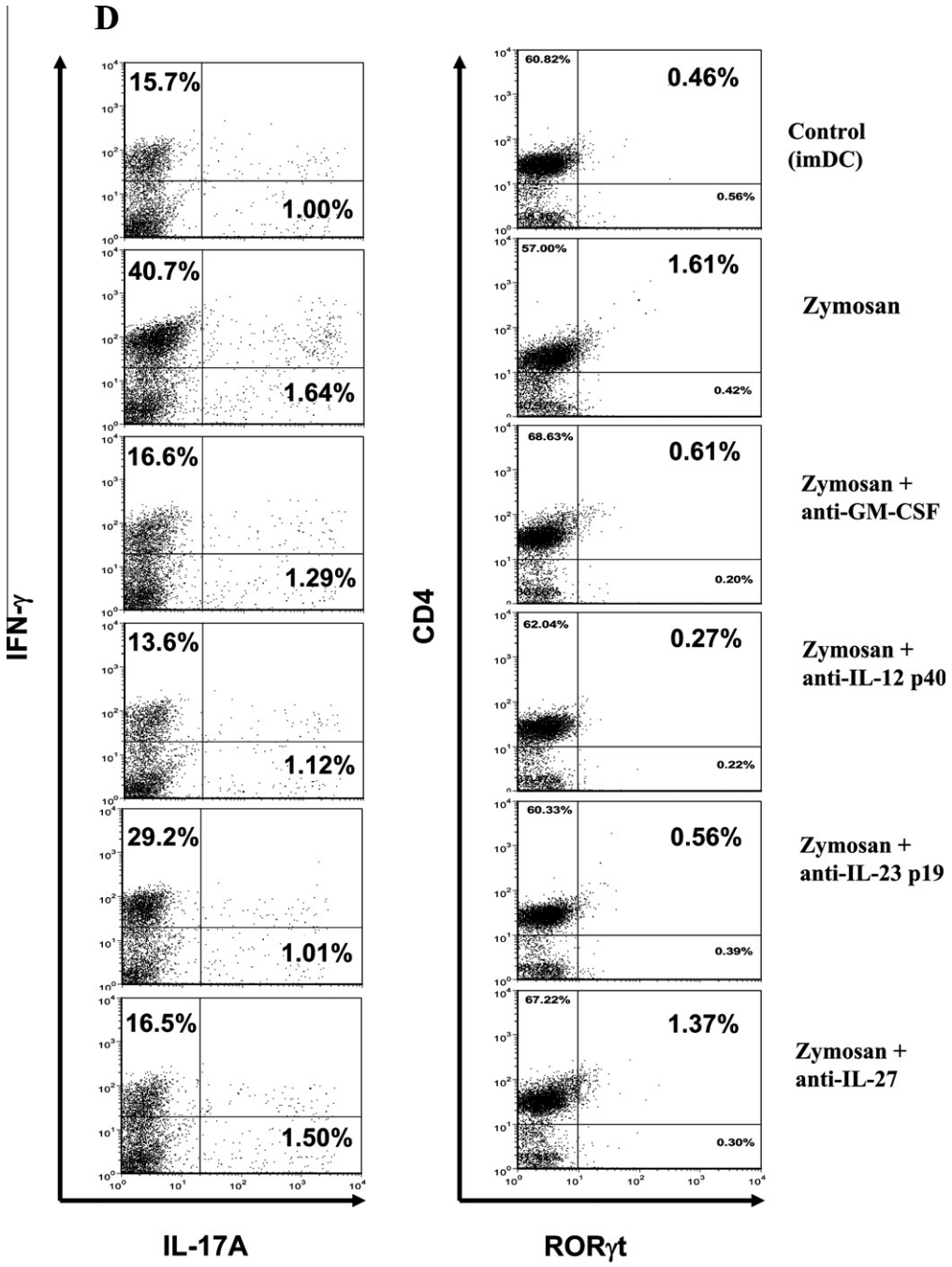

Fig. 6 (*continued*)

GM-CSF mediated network may play a key role in the activation of Th1 and Th17 immune responses.

It is important to note here that the expression of GM-CSF by zymosan-activated DC continued, even after the removal of zymosan from the culture medium, for at least another 4 days (data not shown). In the recognition of zymosan by macrophages or DCs [28], both TLR-2 and dectin-1 are known to be recruited to phagosomes, where dectin-1 binds β-glucans and TLR-2 binds to other distinct components of the yeast cell wall. The phagocytosed zymosan particles can bind to dectin-1 [47], and here were found as punctate granules inside DCs (Fig. 1C). This activity, we believe, may result in a continuous intracellular signaling activity inducing IL-23 and GM-CSF secretion, and if so, could thus explain the constitutive expression of IL-23 and GM-CSF even after the removal of zymosan suspension particles from test culture media.

GM-CSF was previously shown to induce a subset of DCs with heightened ability to phagocytose particulate materials, such as dead tumor cells, and accordingly express more costimulatory molecules [14]. This finding thus provides a novel mechanism for the action of zymosan in mediating key immune cell functions. In addition, GM-CSF is being evaluated as an adjuvant for various immunotherapy and vaccine approaches against cancer [48–50]. Our current findings on zymosan and GM-CSF activities add support for the potential application of these agents as adjuvants in cancer vaccines and other immunotherapy approaches.

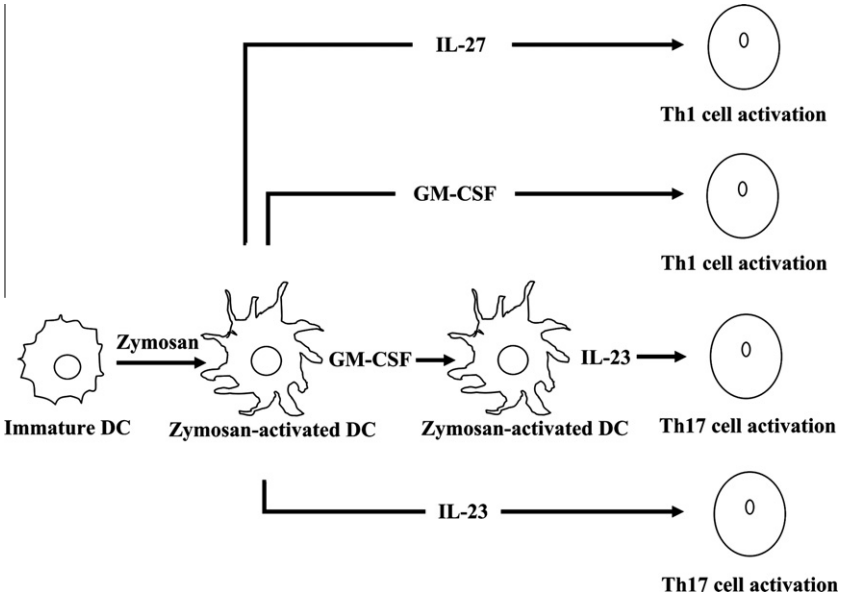

Fig. 7. Hypothetical model of a GM-CSF mediated network for zymosan-activated DCs to activate Th1 and Th17 immune responses.

5. Conclusions

In conclusion, we demonstrate that zymosan not only upregulates several maturation-associated cell-surface molecules but also confers a unique cytokine expression profile in human monocyte-derived DCs. It significantly increased the expression of GM-CSF, TNF-α, IL-1α, IL-1β, IL-6, IL-10, IL-12 p40, IL-23, IL-27 and MCP-1, but had little or no effect on the expression of IL-12 p70. Zymosan-stimulated DCs apparently induce Th1 and Th17 immune responses via these cytokines, especially GM-CSF. In terms of clinical application, zymosan is a much safer modifier than LPS: the FDA has previously given a GRAS rating for β-glucan derived from baker's yeast extract [8–20]. Zymosan is a safe and efficient modulator of human DCs and may thus have considerable potential for application as a therapeutic agent or adjuvant to enhance immune-stimulatory functions in DC or T cell based immunotherapy approaches.

Disclosures

The authors have no financial conflict of interest.

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