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Synergism between Curdlan and GM-CSF Confers a Strong Inflammatory Signature to Dendritic Cells

Lin Min,* Siti Aminah Bte Mohammad Isa,† Wee Nih Fam,* Siu Kwan Sze,* Ottavio Beretta,‡ Alessandra Mortellaro,‡ and Christiane Ruedl*  

A simultaneous engagement of different pathogen recognition receptors provides a tailor-made adaptive immunity for an efficient defense against distinct pathogens. For example, cross-talk of TLR and C-type lectin signaling effectively shapes distinct gene expression patterns by integrating the signals at the level of NF-κB. In this study, we extend this principle to a strong synergism between the dectin-1 agonist curdlan and an inflammatory growth factor, GM-CSF. Both together act in synergy in inducing a strong inflammatory signature that converts immature dendritic cells (DCs) to potent effector DCs. A variety of cytokines (IL-1β, IL-6, TNF-α, IL-2, and IL-12p70), costimulatory molecules (CD80, CD86, CD40, and CD70), chemokines (CXCL1, CXCL2, CXCL3, CCL12, CCL17), as well as receptors and molecules involved in fugal recognition and immunity such as Mincl, dectin-1, dectin-2, and pentraxin 3 are strongly upregulated in DC treated simultaneously with curdlan and GM-CSF. The synergistic effect of both stimuli resulted in strong IκBα phosphorylation, its rapid degradation, and enhanced nuclear translocation of all NF-κB subunits. We further identified MAPK ERK as one possible integration site of both signals, because its phosphorylation was clearly augmented when curdlan was coapplied with GM-CSF. Our data demonstrate that the immunomodulatory activity of curdlan requires an additional signal provided by GM-CSF to successfully initiate a robust β-glucan-specific cytokine and chemokine response. The integration of both signals clearly prime and tailor a more effective innate and adaptive response against invading microbes and fungi. The Journal of Immunology, 2012, 188: 1789–1798.

Dendritic cells (DCs) are the key regulators of immune responses linking innate and adaptive components of the immune system. As sentinels of the mammalian immune system, they are among the first to detect, capture, and process invading infectious agents for the scrutiny of T cells (1). Hence, the optimal collaboration of DCs and T cells is absolutely crucial for successful immunity.

The hallmark of a DC life is a complex maturation process, which involves the upregulation of MHC and costimulatory molecules and secretion of several proinflammatory cytokines. This phenotypic shift dramatically increases their immunostimulatory potential, rendering them the most potent APCs of the immune system (2). Therefore, mature DCs can be considered as supervaccines that are indeed being developed for this purpose to fight against infections and tumors.

A variety of stimuli control and trigger efficiently DC maturation. Pathogen-induced DC activation is mediated via TLR-dependent as well as TLR-independent pathways (3–5). Maturation is also promoted by proinflammatory cytokines, endogenous danger signals, chemokines/chemokine receptor triggering, as well as by feedback signals from cells of the innate and acquired immune system (2). Distinct combinations of various stimuli will differentially affect DC migratory behavior, their capacity to initiate T cell activation, and, ultimately, T cell polarization (6). Recently, we identified GM-CSF as a potent DC activator (7), providing a new face for an old factor with a well-known supporting role in DC development and recruitment (8). Signaling through the GM-CSFR in ex vivo-purified DCs upregulates the expression of costimulatory molecules more efficiently than any tested TLR agonists alone. Combined with a variety of microbial stimuli, GM-CSF supports the formation of potent effector DCs capable in secreting a variety of proinflammatory cytokines that guide the differentiation of T cells during the immune response (7).

In this study, we analyzed in particular the effect of GM-CSF in combination with curdlan on the maturation process of DCs. Curdlan, a linear nonionic homopolymer of β-glucose with (1→3) glucosidic linkages obtained from Alcaligenes faecalis, has gained attention from the pharmaceutical industry due to its immunomodulatory function as well as its suitability as a protein drug delivery vehicle (9, 10). Its receptor, dectin-1 (or Clec7a), a member of the C-type lectin family, is expressed on macrophages and myeloid-related DCs and, when engaged, controls fungal infections (11) by activating several intracellular signaling pathways, like NF-κB, MAPK, and NFAT (5). Independently of dectin-1’s physiological role in antifungal response, DCs stimulated through dectin-1 influence many arms of the adaptive immunity, including Th CD4+ T cells, cytotoxic CD8+ T cells, and B cells (12, 13). Dectin-1 signaling in human monocyte-derived DCs triggers NF-κB activation through two independent signaling pathways, one through Syk and the second through the kinase Raf-1. Both signaling pathways converge at the level of NF-κB activation and regulation to control antifungal adaptive immunity (14).

*School of Biological Sciences, Nanyang Technological University, Singapore 637551; and †Singapore Immunology Network, A*Star, Singapore 138648

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The microarray data presented in this article have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE32986.

Address correspondence and reprint requests to Prof. Christiane Ruedl, School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551. E-mail address: Ruedl@ntu.edu.sg

Abbreviations used in this article: BM, bone marrow; DC, dendritic cell; FLT3L, FLT3 ligand; PRR, pattern recognition receptor.

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Our transcriptional profiling revealed robust synergistic effect between curdlan and GM-CSF. In fact, the integration of both signals strongly affected the DC maturation program, resulting in an increased upregulation of several costimulatory molecules, in a potent proinflammatory cytokine and chemokine release as well as in a superior capacity in polarizing Th cells. How curdlan and GM-CSF signaling pathways integrate to synergistically enhance proinflammatory cytokine secretion is still elusive. In this study, we show that both signaling pathways, when initiated simultaneously, result in enhanced IkBo degradation and increased NF-κB nuclear translocation. We furthermore identified the MAPK kinase ERK as one of the possible targets activated upon GM-CSF and curdlan costimulation. These results show that GM-CSF intensifies and sustains the curdlan-mediated DC activation and that the integration of both signals allows a more effective response to potential invading pathogens than each ligand alone.

Materials and Methods

Mice

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME), bred, and maintained in the animal facility of the Nanyang Technological University under specific pathogen-free conditions. All animal experiments were carried out within institutional guidelines.

Reagents

Curdlan was purchased from Wako and dissolved in 0.15 M NaOH at a concentration of 10 mg/ml and further diluted in the culture medium to the final required concentration (300 to 1 μg/ml). ATP was purchased from InvivoGen (San Diego, CA). The following Abs were used for Western blotting: anti–IL-1β Ab (R&D Systems), anti-NLRP3 (Alexis), anti-caspase-1 (Santa Cruz Biotechnology), and anti–caspase-11 (BioLegend, San Diego, CA). IL-1β, IL-12/p70, IL-2, TNF-α, and IL-6 ELISAs, rGM-CSF, anti-IL-17, anti–IFN-γ, anti–IL-22, and all secondary Abs were purchased from BioLegend.

Generation of bone marrow-derived DCs and bone marrow-derived macrophages

FLT3 ligand (FLT3L)-derived bone marrow (BM) DCs were generated by incubating freshly prepared BM cells for 8 d in IMDM medium supplemented with 100 ng/ml FLT3L, respectively. CD11b+ cells were then purified by magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany). Macrophages were generated from BM cells using L929 cell-conditioned medium (30%).

RNA preparation

CD11b+ fractions of FLT3L-generated BM DCs (3 to 4 × 10^6) were stimulated for 4 h with 100 or 1 μg/ml curdlan in presence or absence of 5 mg/ml GM-CSF. One untreated control group and a GM-CSF–stimulated group were included (total of six groups). Three independent samples for each group were prepared. Total RNA was extracted using the Pure Link RNA Isolation Kit (Invitrogen) and used for cRNA preparation according to each group were prepared. Total RNA was extracted using the Pure Link RNA Isolation Kit (Invitrogen) and used for cRNA preparation according to the manufacturer’s instructions (Affymetrix, Santa Clara, CA).

Array hybridization and analysis

Biotinylated cRNA was hybridized to the Affymetrix GeneChip Mouse Genome 430 2.0 arrays (Affymetrix). Microarray analysis was performed using R/Bioconductor (15). Probe set intensity signals were obtained by a GC robust multiarray average background adjustment, normalized by a quantile method (16), and, to discard the genes with the lowest variability, filtered by an interquartile method with an interquartile range of 0.25. A statistically significant set of differentially expressed genes was defined by both a linear model (17), setting the adjusted p value of the false discovery rate to 0.05 (18), and a log2(Ratio) > 2 among each compared conditions, respectively, represented on the y- and x-axis in a Volcano plot. To describe the overall response of each treatment versus untreated DCs, a hierarchical clustering was performed through the MultiExperiment Viewer software (19). Using an Euclidean distance as a dissimilarity measure and an average linkage as linkage method, each heat map’s colored bar represents the log2(Ratio) between each signal and the median signal of the probe set. Microarray data have been deposited to Gene Expression Omnibus with the accession number GSE32986 for unstimulated, GM-CSF, curdlan (1 and 100 μg/ml), and curdlan/GM-CSF–stimulated FLT3L–derived BM DCs, respectively (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32986).

Flow cytometry analysis

Cells were distributed into 96-well plates and stimulated overnight with a different concentration of curdlan in the presence or absence of GM-CSF (5 ng/ml). Cells were washed and double stained with allophycocyanin-labeled anti-CD11b and PE-labeled anti-CD80, anti-CD86, anti-CD40, anti-CD70, and anti–dectin-1, respectively. Mean fluorescence intensity was assessed by flow cytometry (FACSCalibur; BD Biosciences).

In vitro Th polarization

Naïve T cells were isolated by magnetic cell separation following the manufacturer’s instruction (Miltenyi Biotec). A total of 10^6 cells were plated into a 96-well anti–CD3/CD28 precoated wells together with 2 × 10^4 DCs, which were 24 h stimulated with curdlan alone or in the presence of GM-CSF and 1 ng/ml TGF-β. Four days later, cell supernatant was analyzed for released IL-17, IL-22, and IFN-γ by ELISA. Cells were collected, stained with anti-CD4, subsequently fixed with 2% paraformaldehyde, and permeabilized with 0.5% saponin. Cells were then stained with anti–IL-17, anti–IFN-γ, or anti–IL-22 and analyzed by flow cytometry (FACSCalibur; BD Biosciences).

Cytokine measurements

Cells were distributed into 96-well plates and stimulated overnight with curdlan in absence or in absence of GM-CSF. The next day, cell supernatants were analyzed for IL-12, IL-6, TNF-α, IL-1β, and IL-2 by ELISA following the manufacturer’s instructions. In the case of IL-1β, an additional 1-h incubation with 5 mM ATP was included.

Detection of Syk and MEK 1/2 phosphorylation

Phosphorylated Syk was measured by flow cytometry after 5 min stimulation, respectively. Untreated CD11c−CD11b+ FLT3L–generated DCs or DCs treated with 5 ng/ml GM-CSF, 100 μg/ml curdlan, or a curdlan/GM-CSF mix were fixed and permeabilized with 0.1% saponin solution. Cells were stained with rabbit anti–phospho-Syk (Tyr557/558, Cell Signaling Technology) followed by goat anti-rabbit FITC (Southern Biotechnology Associates). MEK1/2 cells were permeabilized with cold 100% methanol for 10 min and stained for 30 min with anti–phospho-MEK1/2 (Ser217/223, Cell Signaling Technology).

Intracellular detection of pro–IL-1β, NLRP3, caspase-1, and caspase-11

CD11b+ FLT3L–derived DCs incubated overnight untreated or with 5 ng/ml GM-CSF and 100 μg/ml curdlan, or the combination of 5 ng/ml GM-CSF and 100 μg/ml curdlan, respectively. For detection of the pro–IL-1β form, caspase-1, caspase-11, and NLRP3, cells were lysed in buffer containing 1% Triton X-100 supplemented with complete protease inhibitor mixture (Roche, Basel, Switzerland). Lysates were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The blots were developed by chemiluminescence according to the manufacturer’s instructions (PerkinElmer, Waltham, MA).

Western blot

For biochemical analysis, purified CD11c−CD11b+ FLT3L–derived DCs (3 to 4 × 10^6) were seeded in 24-well plates and cultured overnight in FLT3L–containing medium before stimulation with curdlan (100 μg/ml) and GM-CSF (5 ng/ml) alone or in combination. Because biochemical detection of MEK phosphorylation demands a large number of cells, L929–derived BM macrophage cells were used instead of purified CD11b+ DCs. Like CD11b+ DCs, L929–derived BM macrophages express GM-CSF as well as detectin-1. Upon treatment (5 min for STAT5 and Jak2, 15 min for MEK1/2, 20 min for ERK, p38, and JNK, and 2 h for IκBα), cells were lysed, for 10 min in a buffer containing 50 mM Tris (pH 7.5), 0.2 M Sucrose, 0.1 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, Roche protease inhibitor mixture, 0.1% 2-ME, and 1% Triton X-100. Cell extracts were quantified by protein assay (Bio-Rad). Fixed amounts of total protein (20 μg for nonphosphorylated proteins or 40 μg for phosphorylated proteins) were resolved by SDS-PAGE and electroblotted to nitrocellulose membranes. The following Abs were used to visualize total or phosphorylated IκBα, STAT5, Jak2, MEK1/2, p38, ERK, and JNK: mouse anti-tubulin, goat anti-ERK, and rabbit anti–phospho-ERK (Santa Cruz Biotechnology); rabbit anti–IκB-α, mouse anti–phospho-IκBα, rabbit anti-JNK, rabbit anti-
p38, rabbit anti-phospho-JAK2, rabbit anti–phospho-STAT5, rabbit anti–phospho-p38, rabbit anti-MEK1/2, and anti–phospho-MEK1/2 (Cell Signaling Technology); and rabbit anti–phospho-JNK (Invitrogen). The blots were developed by chemiluminescence as described above.

**Nuclear translocation of NF-κB subunits**

To measure nuclear NF-κB subunit p65, p50, p52, RelB, and c-Rel levels, FLTL3-derived CD11b+ DCs were kept untreated or stimulated for 20 h with 5 ng/ml GM-CSF; 100 μg/ml curdlan, or the combination of 5 ng/ml GM-CSF and 100 μg/ml curdlan, respectively. NF-κB DNA binding activity of p65, p50, p52, and RelB was determined using a DNA-binding ELISA kit (TransAM NF-κB transcription factor kit; Active Motif, Carlsbad, CA) using equal amounts of nuclear extracts. c-Rel and USF-2 as an internal standard were monitored by Western blotting.

**MEK1/2 inhibition**

A total of 5 × 10^5 purified FLTL3-derived CD11b+ DCs were seeded into 96-well flat-bottom plates in 200 μl complete medium and pretreated with 10 and 1 μM MEK1/2 inhibitor U0126 (Sigma-Aldrich). One hour later, different stimuli and their combinations were added to the cells (5 ng/ml GM-CSF, 100 μg/ml curdlan, or combination of curdlan/GM-CSF). After overnight incubation, release of TNF-α, IL-1β, and IL-6 was measured by ELISA. In the case of IL-1β, cells were additionally stimulated for 1 h with ATP before the collection of the supernatant.

**Results**

**Gene expression profile in FLTL3-derived CD11b+ DCs upon stimulation with curdlan alone or in combination with GM-CSF**

DNA microarray analysis was performed to investigate the differential effect of curdlan versus GM-CSF and their combinations on DC physiology. At first, we analyzed the gene expression profile induced at early time points upon DC stimulation. Therefore, purified FLTL3-derived CD11b+ DCs were processed for analysis after a short 4-h stimulation with two concentrations of curdlan (1 and 100 μg/ml), alone or in combination with GM-CSF (5 ng/ml). As shown in the Volcano plot, the obtained gene expression analysis revealed clear differences in the transcriptome upon distinct treatments (Fig. 1). A large number of genes were upregulated by >2-fold; in particular, 100 μg/ml curdlan combined with GM-CSF modulated the largest number (871), followed by 1 μg/ml curdlan combined with GM-CSF (571). When 100 and 1 μg/ml curdlan were tested alone, clearly smaller number of genes was upregulated (394 and 63, respectively), whereas GM-CSF treatment alone revealed an upregulation of 400 genes. Clearly, GM-CSF signaling had strong synergistic and diversifying effect on transcriptome induced in conventional FLTL3-derived BM DCs by curdlan.

A summary of genes differentially expressed or modulated in maturing DC compared with nonstimulated cells are listed in Table I. The highest upregulated genes (>10-fold) in the early phase of DC activation in response to the combination of curdlan/GM-CSF include several proinflammatory cytokines (e.g., IL-1α, IL-1β, TNF-α, IL-6, IL-12p35, and IL-2), many chemokines (e.g., CXCL1, CCL17, CXCL3, CCL12), and several costimulatory molecules (e.g., CD80, CD86, CD40, and CD70). Furthermore, a series of C-type lectins, such as Clec5a (MDL-1), Clec4e (Mincl), and Clec4n (dectin-2), recently identified as signaling fungal pattern recognition receptors (PRRs), were differentially modulated. Furthermore, pentraxin 3, a potent antifungal molecule, was also differently expressed in DCs in response to the combination GM-CSF and curdlan. Furthermore, our results show a GM-CSF–mediated increase of TLR2 (Table I), a PRR described to collaborate with dectin-1 for optimal induction of proinflammatory cytokine and chemokine response (20, 21). These results clearly demonstrate that curdlan and GM-CSF can potently act in synergy inducing in DCs an inflammatory signature, which converts immature DCs to potent effector DCs.

**Curdlan and GM-CSF enhance the expression of costimulatory molecules and synergistically boost secretion of proinflammatory cytokines in conventional CD11c^+CD11b^+ DCs**

Purified FLTL3-derived CD11c^+CD11b^+ DCs were analyzed by flow cytometry for expression pattern of CD80, CD86, CD40, and CD70 after 24 h stimulation with 100 μg/ml curdlan alone or in combination with GM-CSF. Curdlan, GM-CSF, and the combination of both stimuli augmented the expression of all four costimulatory receptors when compared with the expression level on unstimulated DCs. Confirming the gene array data, the combination curdlan/GM-CSF was the most potent stimulus as shown in Fig. 2A.

We next focused on the validation of the upregulated cytokines such as IL-12p70, IL-6, TNF-α, and IL-2. Despite the fact that GM-CSF was capable in upregulating the expression of costimulatory molecules on DCs, the growth factor alone was unable to induce subsequent proinflammatory cytokine release. In fact, IL-12p70, TNF-α, IL-6, or IL-2 could not be detected as shown in Fig. 2B. In contrast, GM-CSF combined with curdlan synergistically augmented the production of IL-12p70, TNF-α, and IL-2 when compared with the secretion induced by curdlan alone. IL-6 secretion was only slightly affected by the curdlan/GM-CSF combination at this late stimulation time point of 48 h (Fig. 2B).

These results show that GM-CSF intensifies and sustains the curdlan-mediated DC activation and that the integration of both signals allows a more effective response to potential invading pathogens than each ligand alone.

**FIGURE 1.** Affymetrix GeneChip platform (Affymetrix) was used for global transcriptional analysis in DCs in response to different concentrations of curdlan (1 and 100 μg/ml), GM-CSF, and their combinations with respect to unstimulated DCs. Four hours upon stimulation, mRNA of triplicates was processed and analyzed. Volcano plots of obtained microarray data of the different experimental groups. On x-axis, the log2 (Ratio) of stimulated DCs versus control DCs (unstimulated), in which green and red lines represent the threshold for |log2(Ratio)|>2. On y-axis, the −log2(p value) resulting from the comparison of the two conditions, in which the blue line represents the threshold for log2(p) > 2. In summary, the x-axis represents an intensity measure of the treatment, whereas the y-axis estimates the reliability of this measure.
GM-CSF synergizes curdlan-induced pro–IL-1β synthesis but does not affect the inflammasome components

An efficient IL-1β secretion depends on an NF-κB–dependent synthesis of pro–IL-1β followed by a required danger signal, like ATP, which mediates the activation of the caspase-1 and subsequently the cytokine release. To clarify the role of GM-CSF in IL-1β synthesis and secretion, DCs were stimulated for 2, 4, and 6 h with curdlan in the absence or in the presence of GM-CSF and IL-1β was assessed at the level of mRNA. As already observed in the transcriptome analysis (Table I), both low (1 μg/ml) and high (100 μg/ml) curdlan concentrations combined with GM-CSF resulted in the strongest increase of IL-1β mRNA when compared with curdlan alone (Fig. 3A). Also at the intracellular protein level, Western blot analysis showed five times higher amounts of the pro–IL-1β form in cells stimulated with the combination of curdlan and GM-CSF as compared with cells treated with curdlan alone (Fig. 3B). Together with pro–IL-1β, we measured the content of the inflammasome components, such as NLRP3, caspase-1, and caspase-11 in untreated and differently stimulated cells. We observed that caspase-1 protein levels were similar in all tested conditions, whereas NLRP3 and caspase-11 levels were increased upon stimulation, but without any obvious difference between curdlan and curdlan/GM-CSF treatments. To test the effect of GM-CSF on the IL-1β production in a danger situation, we stimulated FLT3L-derived DC in the presence of ATP with curdlan alone or in combination with GM-CSF. Single stimuli (GM-CSF or curdlan) did not induce any significant cytokine release, but their combination dramatically enhanced in a dose-dependent manner the secretion of IL-1β (Fig. 3C). We ruled out the role of GM-CSF as a danger signal itself because it always needs to partner with ATP to boost the IL-1β release (data not shown). The absolute requirement of ATP as a danger signal suggests that dectin-1 engagement alone in FLT3L-derived myeloid DCs is unable to trigger a strong reactive oxygen species formation (22), which was described to directly activate the NLRP3 inflammasome in macrophages (23).

Taken together, these results indicate that DCs require GM-CSF for increased pro–IL-1β synthesis in response to curdlan.

Curdlan/GM-CSF–activated DCs strongly support Th1 and Th17 polarization

Recent in vivo and in vitro experiments have shown that dectin-1 agonists, including curdlan, promote Th1 as well as Th17 immunity (12). Therefore, we studied the role of GM-CSF in supporting the formation of these two different types of Th subsets. Following an Ag-independent polarization protocol, we stimulated naive T cells on anti-CD3/CD28 plates in the presence or absence of DCs activated via curdlan and GM-CSF, alone or in combination. When analyzed by flow cytometry, curdlan/GM-CSF–activated DCs were more potent in polarizing both Th1 as well as Th17 Th subsets when analyzing an Ag-independent polarization protocol, we stimulated naive T cells on anti-CD3/CD28 plates in the presence or absence of DCs activated via curdlan and GM-CSF, alone or in combination. When analyzed by flow cytometry, curdlan/GM-CSF–activated DCs were more potent in polarizing both Th1 as well as Th17 Th subsets when compared with DCs that were stimulated with curdlan alone (Fig. 4A, upper panel). Interestingly, the combination of stimuli enhanced also the formation of IL-17/IL-22 double-producing Th cells as well as a single secreting IL-22 subset (24, 25) as shown in Fig. 4A, lower panel. The enhanced production of IFN-γ, IL-17, and IL-22 was not only observed at a single-cell level by intracellular staining, but was also confirmed by ELISA (Fig. 4B).

These data suggest that the observed enhanced production of proinflammatory cytokines upon costimulation of curdlan and GM-CSF by DCs generally support a stronger polarization on Th cells. However, as demonstrated by our gene array, the inability of curdlan/GM-CSF to prime p19 mRNA synthesis as well as to induce secretion of bioactive IL-23 in conventional myeloid DCs (data not shown) indicate that this type of DCs does prime but not sustain the further Th17 differentiation.

GM-CSF augments dectin-1 surface expression in DCs

Next, we analyzed whether the basal level of the known curdlan-specific receptor dectin-1 could be further augmented by different

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Fold induction is represented.
CSF intracellular signals, which leads to this NF-κB activation and as a consequence to a stronger cytokine and chemokine secretion. First, we analyzed the signaling pathway induced by engagement of the GM-CSFR. An equal phosphorylation pattern of STAT5 and JAK2 could be observed in the DCs treated with GM-CSF alone or in combination with curdlan (Fig. 7A), whereas the unstimulated cells as well as curdlan-stimulated cells showed none. We next focused on the dectin-1–mediated NF-κB canonical pathway comprising the kinase Syk as one the most upstream elements. Both curdlan and curdlan/GM-CSF induced rapid and equal Syk phosphorylation in DCs, whereas this was the case for ERK (Fig. 7B). No apparent differences in the phosphorylation of p38 was observed between GM-CSF–induced phosphorylation in DCs, whereas control and GM-CSF–activated cells did not (Fig. 7B), which excludes Syk as an integration point of both signals. Finally, we analyzed the phosphorylation pattern of the MAPKs p38, ERK, and JNK because these kinases are known to regulate expression of genes involved in the innate immune response such as proinflammatory cytokines. Stimulation of CD11b+ FLT3L-derived DCs with GM-CSF–induced phosphorylation of two MAPKs, JNK and ERK, whereas no increase above the basal level of phosphorylation was observed in curdlan-treated DCs. In the case of JNK, the phosphorylation pattern could not be further augmented by cotreatment with curdlan/GM-CSF, whereas this was the case for ERK (Fig. 7C). No apparent differences in the phosphorylation of p38 was observed between

Synergy between curdlan and GM-CSF results in strong IκBα phosphorylation, its rapid degradation, and nuclear translocation of different NF-κB subunits

We next analyzed the effect of our different stimuli on the phosphorylation and degradation of IκBα by stimulating FLT3L-generated CD11b+ DCs for 2 h with curdlan and GM-CSF alone or in combination. As shown in Fig. 6A, coagulation with both stimuli resulted in a slight enhanced IκBα phosphorylation and IκBα degradation when compared with cells stimulated with curdlan or GM-CSF alone. Furthermore, we observed that co-agitation of DCs supported augmented nuclear translocation of different NF-κB subunits. In fact, p50, p52, p65, RelB, as well as c-Rel were clearly detectable in nuclear extracts of DCs cotreated with curdlan and GM-CSF (Fig. 6B), results that are consistent with the observed increased proinflammatory cytokine and chemokine response.

Analysis of GM-CSF– and curdlan-induced intracellular signaling pathways

We next sought to clarify the integration point of curdlan and GM-CSF intracellular signals, which leads to this NF-κB activation and synergistic boost of proinflammatory cytokines. Stimulation of FLT3L-generated CD11c+CD11b+ DCs was cultured with 100 μg/ml curdlan in absence (white bars) or presence of 5 ng/ml GM-CSF (black bars). CD86, CD80, CD40, and CD70 upregulation was visualized by FACS analysis. FLT3L-generated cells were left untreated or stimulated overnight with 100 ng/ml curdlan, 5 ng/ml GM-CSF, or a combination of both, and cell lysates were subsequently prepared and separated on a 10% SDS-PAGE gel. C. IL-1β ELISA after stimulation with different curdlan concentrations in absence (white bars) or presence of 5 ng/ml GM-CSF (black bars). A, IL-1β gene expression was assessed by semiquantitative RT-PCR analysis after 2, 4, and 6 h upon stimulation. All samples were normalized with the internal control gene HPRT. B, Western blot analysis of pro–IL-1β, NLRP3, caspase-1, and caspase-11 in FLT3L-derived CD11b+ DCs. Cells were left untreated or stimulated overnight with 100 μg/ml curdlan, 5 ng/ml GM-CSF, or a combination of both, and cell lysates were subsequently prepared and separated on a 10% SDS-PAGE gel. C. IL-1β ELISA after stimulation with different curdlan concentrations in absence (white bars) or presence of GM-CSF (black bars). Additional 1-h incubation with 5 mM ATP was included after 24 h stimulation. Measured cytokines of triplets are represented in picograms per milliliter, and results are representative of two separate experiments.

FIGURE 2. Curdlan and GM-CSF enhances the expression of costimulatory molecules and synergistically boosts secretion of proinflammatory cytokines. A, FLT3L-generated CD11b+ DCs were cultured for 24 h with 100 μg/ml curdlan in absence (white bars) or presence of 5 ng/ml GM-CSF (black bars). CD86, CD80, CD40, and CD70 upregulation was visualized by FACS analysis. B, FLT3L-generated CD11c+CD11b+ DCs were stimulated with different curdlan concentrations ranging from 300 to 1 μg/ml in absence (white circles) or presence of 5 ng/ml GM-CSF (black circles). IL-12p70, TNF-α, IL-6 (48-h stimulation), and IL-2 (24-h stimulation) were measured by ELISA. Results show nanograms per milliliter released cytokines and are representative of three independent experiments.

FIGURE 3. Pro–IL-1β synthesis is synergistically augmented upon curdlan and GM-CSF costimulation. FLT3L-generated CD11c+CD11b+ DCs were cultured for 2, 4, 6, and 24 h with 100 or 1 μg/ml curdlan in absence (white bars) or presence of 5 ng/ml GM-CSF (black bars). A, IL-1β gene expression was assessed by semiquantitative RT-PCR analysis after 2, 4, and 6 h upon stimulation. All samples were normalized with the internal control gene HPRT. B, Western blot analysis of pro–IL-1β, NLRP3, caspase-1, and caspase-11 in FLT3L-derived CD11b+ DCs. Cells were left untreated or stimulated overnight with 100 μg/ml curdlan, 5 ng/ml GM-CSF, or a combination of both, and cell lysates were subsequently prepared and separated on a 10% SDS-PAGE gel. C. IL-1β ELISA after stimulation with different curdlan concentrations in absence (white bars) or presence of GM-CSF (black bars). Additional 1-h incubation with 5 mM ATP was included after 24 h stimulation. Measured cytokines of triplets are represented in picograms per milliliter, and results are representative of two separate experiments.
the differently stimulated groups, as shown in bottom panel of Fig. 7C.

Due to the observed enhanced phosphorylation of ERK, we analyzed more in detail the MAPK pathway triggered by curdlan and GM-CSF and its significance for cytokine production. The phosphorylation profile of MEK1/2, the upstream activating kinases of ERK, was analyzed in purified FLT3L-derived CD11b+ DCs by flow cytometry and in L929-derived BM macrophages by Western blotting. When compared with untreated control cells, the highest p-MEK1/2 staining was observed in CD11b+ DCs stimulated with curdlan (white bars) alone or combined with GM-CSF (black bars). Values represent triplicates ± SEM and are representative of two independent experiments.

Furthermore, we examined the effect of MEK1/2 inhibitor U0126 on the production of different proinflammatory cytokines such as TNF-α, IL-6, and IL-1β. We found that curdlan/GM-CSF combination induced secretion of cytokines in a classic MEK-dependent way because a dose-dependent inhibition could be observed in the presence of the MEK inhibitor (Fig. 8C).

Taken together, we show a higher ERK pathway activity when DCs were costimulated with the combination of curdlan and GM-CSF, which could explain the increased capability in secreting proinflammatory cytokines upon this treatment.

Discussion

PRRs, including a series of TLR and non-TLR receptors, such as dectin-1, recognize highly conserved microbial and fungal patterns (4, 26–29). Their engagement results in activation of APCs, such as DCs, which efficiently initiate both innate and adaptive immune responses against the pathogen. It became evident during the last years that a simultaneous triggering of different PRRs is required to shape an effective pathogen-specific Th-polarizing program in DCs. Synergy between distinct TOLL/IL-1R (TIR) domain-containing adapter inducing IFN-γ coupled TLRs (TLR3 and TLR4) and endosomal TLRs (TLR7 and TLR9) (30) or between TLRs (e.g., TLR2 and TLR4) and non-TLRs (e.g., dectin-1) (31–36) tailors a pathogen-specific immune response that is ultimately required for the final clearance of the invading microbe or fungus.

Recently, we have extended our earlier studies and investigated the potential immunostimulatory capacity of an old growth factor, GM-CSF, mostly known as a hematopoietic growth factor responsible for the differentiation of BM progenitor cells (8), but lately recognized as a key proinflammatory cytokine during inflammation or in response to infection (37). In this context, we recently identified GM-CSF as a key regulator of IL-1 production (38).

In this study, we explored the capacity of GM-CSF to synergize with the β-glucan curdlan, a well-known and specific dectin-1 agonist with immunomodulatory properties. Both stimuli, when applied together, provide a strong inflammatory signature to DCs, leading to the formation of potent effector DCs. Indeed, not surprisingly, our gene profiling analysis revealed the modulation of many different genes including costimulatory molecules as well as proinflammatory cytokines (e.g., IL-1α, IL-1β, IL-2, IL-6, IL-12p35, and TNF-α) and several different chemokines (e.g., CXCL1, CXCL2, CXCL3, CCL12, CCL17). We also analyzed our gene array data with respect to receptors and molecules involved in fungal recognition and immunity. Our transcriptional analysis also revealed that costimulation of DCs with dectin-1 agonist and GM-CSF leads to an augmented expression of several C-type lectins, including MDL-1, Mincl (39), and dectin-2 (40, 41), as well as pentraxin 3, a potent antifungal molecule (42). As also observed in macrophages (43), GM-CSF modulated the
dectin-1 as well as TLR2 expression in FLT3L-derived DCs, increasing the capability for fungal recognition and ability to initiate an effective antifungal response.

This synergistic DC stimulation results in the generation of strong effector DCs with augmented capacity for fungal recognition and ability to initiate an effective antifungal response. The authors also studied the role of GM-CSF in cellular programming of a proinflammatory dectin-1–mediated response in macrophages. Using both curdlan and glucan micro-particles, the authors showed that macrophages do not mount a significant proinflammatory cytokine response upon dectin-1 engagement, which can be dramatically boosted via GM-CSF costimulation. They speculated about a GM-CSF–mediated alteration of the downstream signaling components of the Syk/ CARD9 and NF-κB pathways, but the exact mechanism of action was not clarified. Goodridge et al. (45) also recently reported the capacity of GM-CSF as well as of IFN-γ to efficiently amplify dectin-1–CARD9–Bcl10 to induce NF-κB activation and TNF-α production. Although dectin-1 signals via CARD9 and BCL10 in GM-CSF–derived BM DCs, this pathway does not activate NF-κB

**FIGURE 6.** Enhanced IκBα phosphorylation, IκBα degradation, and NF-κB nuclear translocation upon costimulation of conventional DCs with curdlan/GM-CSF combination. A, FLT3L-generated CD11b+ DCs were stimulated with GM-CSF, curdlan, and their combination for 2 h and assayed by Western blot for IκBα phosphorylation and IκBα degradation. Tubulin was used as internal control to standardize the samples. B, Nuclear recruitment of NF-κB subunits in FLT3L-derived CD11b+ DCs was analyzed 24 h after stimulation by DNA-binding ELISA (p50, p52, RelB, and p65) and Western blot (c-Rel). USF-2 was used as internal control to standardize the samples. Values represent triplicates ± SEM and were confirmed in two independent experiments.

**FIGURE 7.** Curdlan/GM-CSF costimulation augments activation of ERK. A, Western blot analysis of activated phosphorylated forms of JAK2 and STAT5. Two different curdlan concentrations were tested, referred to as low (1 μg/ml) and high (100 μg/ml), respectively. Tubulin was used as internal control to standardize the samples. B, Flow cytometry analysis of Syk activation upon different combinations of stimulation. Cells were fixed and permeabilized 30 min upon treatment and intracellular staining with rabbit anti–phospho-Syk and FITC-labeled goat anti-rabbit, respectively. C, Western blot analysis of activated phosphorylated forms of MAPK p38, ERK, and JNK. After 20 min stimulation, cells were lysed and immunoblotted for active p38, ERK, and JNK and total p38, ERK, and JNK as internal controls. The results are representative of at least two independent experiments.
IL-1

MEK1/2 in FLT3L CD11b+-derived DCs suppresses TNF-α, IL-6, and IL-1β release in BM-derived macrophages as well as in FLT3L-derived DCs. In our study, we confirm the inability of FLT3L-derived DCs to secrete high amounts of proinflammatory cytokines when stimulated with curdlan alone, and we further demonstrate that these cells require GM-CSF to efficiently activate the NF-κB pathway and induce the subsequent proinflammatory cytokine response as previously reported for the BM-derived macrophages (45).

In this work, to elucidate the molecular mechanisms underlying this observed synergistic effect between the two different stimuli, we focused on the analysis of known signaling pathways initiated upon the curdlan-specific receptor dectin-1 as well as GM-CSFR engagement. No cross-talk between these two different receptors has been described so far. It is well documented that GM-CSF activates the Jak2/STAT5, Ras/Raf/MAPK, and PI3K/Akt pathways through its heterodimeric receptor composed by a major binding unit, GM-CSFRα, and a major signaling unit, GM-CSFRβ2 (8, 46, 47). Curdlan initiates via dectin-1 binding a NF-κB canonical pathway comprising the kinase Syk and the adapter proteins Card9, Bcl10, and Malt1 (12, 48) as well as an NF-κB noncanonical Raf-1-dependent signaling pathway (14). In human immature DCs, both pathways converge at the level of NF-κB activation by translocating NF-κB subunits c-Rel and p65 in the case of the canonical pathway or the RelB-p52 subunit in the case of the noncanonical pathway (14). We found that curdlan combined with GM-CSF enhanced dramatically the nuclear translocation of all NF-κB subunits tested, including p50, p52, p65, RelB, and c-Rel.

We next focused on JAK-2/STAT5 and, in particular, on the MAPK signaling pathway because the latest was described to be triggered by GM-CSF as well as by dectin-1 agonists (12, 49–51). As expected, we could not see any differences in the GM-CSF–induced STAT5/JAK2 pathway regardless the presence or absence of curdlan. In the case of the canonical pathway of dectin-1 signaling, we also could not observe any difference of Syk phosphorylation when GM-CSF was coapplied to curdlan. However, one of the MAPKs, ERK, showed a clearly augmented phosphorylation pattern in response to both stimuli when compared in response to single stimuli. The same enhanced phosphorylation was observed for the upstream activating kinases MEK1/2. In contrast, the phosphorylation of other two MAPKs, JNK and p38, were not further enhanced by the combination of the stimuli. In disagreement with other published data (12, 51), we could not detect any significant ERK, JNK, and p38 phosphorylation differences upon stimulation of CD11b+ FLT3L-derived DCs with curdlan alone. Indeed, the observed MAPK phosphorylation in previous studies might be explained by the use of different type of DCs in those biochemical experiments. In fact, curdlan-dependent MAPK phosphorylation could be obtained only when GM-CSF–derived BM DCs were used and/or GM-CSF was included in the culture medium during the stimulation period (12, 51). In our experiment, we used naïve conventional CD11b+ FLT3L-derived DCs that express GM-CSFR and are reactive against the growth factor, which was not used for their derivation.

ERK signaling cascade is not only restricted to the regulation of cellular processes, such as proliferation, differentiation, and oncogenic differentiation, but also in recent years, it has become clear that it also controls proinflammatory cytokine production. ERK, once phosphorylated, can directly activate transcription factors upon translocation into the nucleus or, in contrast, phosphorylate a variety of cytoplasmic targets. Several MAPK kinase kinases, such as MEKK1, MEKK2, and MEKK3, have been identified to be able to stimulate both pathways and induce the subse-quent proinflammatory cytokine response as previously reported. In this study, we further show that inhibition of MEK1/2 efficiently downregulates cytokine expression and reduces the ability of DCs to promote Th1 and Th17 responses. In this study, we further show that inhibition of MEK1/2 suppressed clearly IL-1β as well as TNF-α and IL-6 upon curdlan and GM-CSF costimulation.

In summary, recognition of fungal pathogens involves a coordinated interplay of multiple receptors including several TLRs as well as C-type lectins. In this study, we have shown that a growth factor, GM-CSF, can provide in the presence of curdlan a potent inflammatory signature to conventional DCs, which ensures a robust cytokine and chemokine response and subsequent Th cell polarization. Our results strongly suggest that inflammation caused by fungal invasion is requisite for priming and tailoring an effective host innate and adaptive response. Our
findings might be relevant for the design of novel adjuvant formulations, which are more effective in priming a strong anti-fungal inflammatory response.

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