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Mannose-Containing Molecular Patterns Are Strong Inducers of Cyclooxygenase-2 Expression and Prostaglandin E₂ Production in Human Macrophages

Nieves Fernández,* Sara Alonso,* Isela Valera,* Ana González Vigo,* Marta Renedo,* Luz Barbolla,† and Mariano Sánchez Crespo†‡

The induction of cyclooxygenase-2 (COX-2) and the production of PGE₂ in response to pathogen-associated molecular patterns decorated with mannose moieties were studied in human monocytes and monocyte-derived macrophages (MDM). Saccharomyces cerevisiae mannan was a robust agonist, suggesting the involvement of the mannose receptor (MR). MR expression increased along the macrophage differentiation route, as judged from both its surface display assessed by flow cytometry and the ability of MDM to ingest mannosylated BSA. Treatment with mannose-BSA, a weak agonist of the MR containing a lower ratio of attached sugar compared with pure polysaccharides, before the addition of mannan inhibited COX-2 expression, whereas this was not observed when agonists other than mannan and zymosan were used. HeLa cells, which were found to express MR mRNA, showed a significant induction of COX-2 expression upon mannan challenge. Conversely, mannann did not induce COX-2 expression in HEK293 cells, which express the mRNA encoding Endo180, a parent receptor pertaining to the MR family, but not the MR itself. These data indicate that mannann is a strong inducer of COX-2 expression in human MDM, most likely by acting through the MR route. Because COX-2 products can be both proinflammatory and immunomodulatory, these results disclose a signaling route triggered by mannose-decorated pathogen-associated molecular patterns, which can be involved in both the response to pathogens and the maintenance of homeostasis. The Journal of Immunology, 2005, 174: 8154–8162.

Immune immunity is a mechanism of host defense widely conserved, the capacity of which to recognize pathogens depends on germline-encoded molecules called pattern recognition receptors (PRR). Unlike the receptors of the adaptive immune system, PRR do not undergo somatic mutation by recombination-mediated rearrangement and recognize conserved microbial structures shared by large groups of pathogens, which are collectively called pathogen-associated molecular patterns (PAMP). LPS from Gram-negative bacteria and branched sugars with β-glucan and α-mannose moieties from different microorganisms are archetypal examples of PAMP. Among PRR, the mannose receptor (MR), first described by Stahl et al. (1), has been the object of detailed scrutiny. This receptor recognizes glycosylated molecules with termin-

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3 Abbreviations used in this paper: PRR, pattern recognition receptor; AU, arbitrary unit; C3, the third component of the complement system; C3bi-IC, immune complex bound to C3bi; COX-2, cyclooxygenase-2; CR3, complement receptor 3; DC-SIGN, dendritic cell-specific ICAM-grabbing nonintegrin; DC-SIGNR, DC-SIGN-related; IC, immune complex; MDM, monocyte-derived macrophage; MR, mannose receptor; MUC3-3, mucin 3; PAMP, pattern-associated molecular pattern; PMN, polymorphonuclear leukocyte; sPLA₂, secreted phospholipase A₂.

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Some members of the MR family have been associated with signal transduction events, for instance, the secreted phospholipase A₂, M-type receptor (14, 15), but few reports have addressed this functional capacity for the MR itself. In fact, the function of the MR has currently been related to the capture, internalization, and presentation of mannosylated Ags (16) bypassing the oxidative burst in human macrophages (17) as well as to the activation of an anti-inflammatory and immunosuppressive program in monocyte-derived dendritic cells (18). This agrees with the finding of normal defense against Candida and Pneumocystis infection in animals with targeted disruption of the MR gene (19, 20). Conversely, the synthesis of proinflammatory cytokines (21) in infection (23) in the context of HIV infection (4).

In a previous study we addressed the effect of mannose- and β-glucan-containing polysaccharides on the release of arachidonic acid by human monocytes (24), which might be of some relevance in the inflammatory response in view of the wide scope of physiological actions of eicosanoids. In the present study we addressed the effect of mannose-containing polysaccharide on the expression of cyclooxygenase-2 (COX-2), the inducible isoform of cyclooxygenase that converts arachidonic acid into the unstable PGG₂. PGG₂ is subsequently reduced to PGH₂ and serves as a substrate for the production of other PGs, such as PGE₂ and PGD₂, through the action of isomerases and synthases. The effect of mannose is more potent than that elicited by similar concentrations of zymosan particles and seems to be mediated by the MR. The observed response cannot be accounted for by LPS contamination, is normal human serum as previously described (24). Coating of C3bi to zymosan was conducted by incubation with normal human serum, followed by extensive washing with PBS. The characteristics of the oligonucleotide primers used in PCRs for the detection of dectin-1 mRNA (26), DC-SIGN (27), DC-SIGN-related (DC-SIGNR) (28), Endo180 (29), TLR-2 (30), MR (31), and sPLA₂ M-type receptor (32) are shown in Table I. Hemagglutinin-tagged cDNA of human TLR-2 and the mutation corresponding to the dominant negative TLR4 (P712H substitution) in C3H/HeJ mice (33) cloned into the expression plasmid pReCMV (Invitrogen Life Technologies) were provided by Dr. M. Rehli (University of Regensburg, Regensburg, Germany).

**Cell culture**

THP-1 cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine and 10% heat-inactivated FBS. Human monocytes were isolated fromuffy coats of healthy volunteer donors by centrifugation onto Ficoll cushions and adherence to plastic dishes for 2 h. At the end of this period, nonadhered cells were removed by extensive washing. Differentiation of monocytes into macrophages was conducted by culture of adherent monocytes in the presence of 5% human serum for 2 wk in Primaria six-well dishes (BD Biosciences), in the absence of exogenous cytokines. HEK293 cells and HeLa cells were transiently transfected using the calcium phosphate method.

**Assays for endo/phagocytosis and flow cytometry**

Cells were incubated for different times at 37°C with FITC-labeled mannosylated BSA and subsequently washed and resuspended in 500 μl of PBS supplemented with 1 mM EDTA for analysis by flow cytometry in a FACScan cytofluorometer (BD Biosciences). Parallel controls were performed at 4°C to block endocytic uptake of the particles. The surface display of both CD16 and CD206/mannose receptor was determined by indirect immunofluorescence with mouse anti-human CD206 and CD16 IgG1 mAb, followed by washing with PBS and incubation with goat anti-mouse IgG1 mAb, followed by washing with PBS and incubation with goat anti-mouse IgG1 mAb.

**Materials and Methods**

**Reagents**

Zymosan, soluble β-glucan from seaweed (laminarin, ~8 kDa), soluble α-mannan from Saccharomyces cerevisiae, and porcine mucin 3 (MUC-3), a mucin from the gastrointestinal tract, which is a natural ligand of the MR, were purchased from Sigma-Aldrich. 4-[5-(4-Chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide (SC-236; a COX-2 inhibitor) and 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole (SC-560; a COX-1 inhibitor) were obtained from Calbiochem. Mouse monoclonal anti-human CD206/mannose receptor and CD16/FcRγII were purchased from BD Pharmingen. IgG-OVA equivalence immune complexes (IC) were made according to classical procedures with optimal amounts of Ag using IgG Ab raised in rabbits. To obtain IC bound covalently to C3bi, IC were extensively washed with PBS and incubated with normal human serum as previously described (24). Coating of C3bi to zymosan was conducted by incubation with normal human serum, followed by extensive washing with PBS.

**Table I. Oligonucleotide primers used for the detection of the mRNA encoding for receptors of the innate immune system**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Orientation</th>
<th>Sequence (5’-3’)</th>
<th>GeneBank Accession No.</th>
<th>Ref. No.</th>
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<td>Dectin-1</td>
<td>Forward</td>
<td>TTAGAAATTTGGAGATGGAGAAGA</td>
<td>NM_021155</td>
<td>27</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>AAGTGCGGCGCAGCTACACAGAAGA</td>
<td>NM_021155</td>
<td>27</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Forward</td>
<td>AGGTCCCGAGCTCATAAAGT</td>
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<td>27</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTCTGGAAAGTCCTACACCTCAAG</td>
<td>NM_021155</td>
<td>27</td>
</tr>
<tr>
<td>DC-SIGNR</td>
<td>Forward</td>
<td>CGAACCTCTCTCTCTCTCTATGC</td>
<td>AF245219</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCCTGCAAGCTCATTCCTACCTTG</td>
<td>AF245219</td>
<td>28</td>
</tr>
<tr>
<td>Endo180</td>
<td>Forward</td>
<td>ATTTTGTAGTTGCTCGAGCTG</td>
<td>AF134838</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGCTGAGGACCCATGGTACAGT</td>
<td>AF134838</td>
<td>29</td>
</tr>
<tr>
<td>TLR-2</td>
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<td>GCCAAATCTCTGTATGGAGATGG</td>
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<tr>
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<td>Reverse</td>
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<td>30</td>
</tr>
<tr>
<td>MR</td>
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<td>NM_002438</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACAGAGCAATTTGGTTAAAGC</td>
<td>NM_002438</td>
<td>31</td>
</tr>
<tr>
<td>sPLA₂ M-type</td>
<td>Forward</td>
<td>AAAGAAACCCACCTGAAATGGGCC</td>
<td>U17033</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTCTTTGAAATGGCAATCCACC</td>
<td>U17033</td>
<td>32</td>
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</table>
anti-mouse IgG-FITC conjugate Ab (Sigma-Aldrich; 1/100 dilution) for 30 min at 4°C. Isotype-matched irrelevant Ab was used as a control.

Confocal microscopy
Human monocytes were seeded in 35-mm Primaria culture dishes (BD Biosciences) to allow their differentiation into MDM as described above. Twenty-four hours before being used, cells were serum-starved. At different times after the addition of stimuli, MDM were extensively washed with HBSS to discard background extracellular fluorescence, and the dishes were observed in vivo by confocal microscopy using a Bio-Rad Laser scanning system Radiance 2100 coupled to a Nikon inverted microscope with a thermostatized chamber. The objective was a ×20 and numerical aperture of 0.5. Green fluorescence (fluorescein) was monitored at 488 nm argon excitation using a HQ500 long-bandpass blocking filter. Images were merged using Adobe Photoshop 6.0 software.

Immunoblots of COX-2
The amount of protein in each cell lysate was assayed using the Bradford reagent, and 50 μg of protein from each sample was loaded on each lane of a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes using a semidyey transfer system. The membranes were blocked with dry milk and used for immunoblot using a goat polyclonal antisem (SC-1745) and, in some experiments, rabbit anti-COX-1 Ab (SC-7950) from Santa Cruz Biotechnology. This was followed by incubation with donkey anti-goat IgG-HP-conjugated Ab. Detection was performed using the Amersham Biosciences ECL system. β-Actin immunodetection was used to address the occurrence of similar protein loading across the gels.

RT-PCR assays for dectin-1, Endo180, DC-SIGN, DC-SIGNR, TLR-2, MR, and sPLA2 M-type receptor
Total cellular RNA was extracted by the TRizol method (Invitrogen Life Technologies). First-strand cDNA was synthesized from total RNA by RT reaction. The reaction mixture containing 0.2 mg/ml total RNA, 2.5 μl of H2O, 20 U of RNasin RNase inhibitor, 4 μl of 5× buffer, 2 μl of 0.1 M DTT, 4 μl of 2.5 mM dNTP, 1 μl of 0.1 mM hexanucleotide, and 200 U of Moloney murine leukemia virus reverse transcriptase. The reaction was conducted at 37°C for 60 min in a volume of 20 μl. The cDNA was amplified by PCR in a reaction mixture containing 2 μl of DNA template; 10 μl of H2O; 2.5 μl of 5× buffer; 0.75 μl of 50 mM MgCl2; 1.0 μl of 2.5 mM dNTP; 1.25 μl of each forward and reverse primer of dectin-1, DC-SIGN, DC-SIGNR, TLR-2, MR, and β-actin; and 0.25 μl of 5 U/ml Taq DNA polymerase. The amplification profile for detection included one cycle of initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 30 s, and one cycle of final extension at 72°C for 7 min. The expression of β-actin was used as a control for the assay of a constitutively expressed gene. PCR products were identified by automatic sequencing of the DNA eluted from the agarose gel by excision of the band under UV light, followed by purification using a QIAquick PCR purification kit (Qiagen).

ELISA for PGE2
Quantitation of cellular PGE2 levels was determined using an enzyme immunoassay kit (Amersham Biosciences) according to the manufacturer’s instructions. This kit is based on competition between unlabeled PGE2 in the sample and a fixed amount of labeled PGE2 for a PGE2-specific Ab. The detection limit of this assay is 20 pg/ml. The samples for the assay were collected 24 h after the addition of the stimuli, because this allows the detection of PGE2 produced during a prolonged period by the action of COX enzymes on the arachidonate made available in the deacylation/reacylation cycle.

Results
Induction of COX-2 protein and production of PGE2 by mononuclear phagocytes in response to different stimuli
Incubation of overnight-adhered monocytes with mannan induced a mild expression of COX-2 protein above basal levels, whereas treatment with laminarin, zymosan particles, complement-coated zymosan particles, and preformed IC did not noticeably increase COX-2 above the levels observed in resting adhered monocytes (Fig. 1A, left panel). Because mannan appeared as the most potent stimuli, additional experiments were conducted using different concentrations of this substance. However, a clear dose-dependency of the response was not observed for concentrations >5 mg/ml (Fig. 1A, right panel), most likely because of the possible induction of COX-2 expression by adherence-dependent signals (34), which could impede the correct appraisal of receptor-mediated induction. By contrast, in experiments conducted on monocytes cultured in the presence of human serum for 7 days, there was no expression of COX-2 in resting cells, suggesting that these conditions are most adequate for a fine-tuning assessment of ligand-induced COX-2 expression. In keeping with this view, both mannan and zymosan induced a net expression of COX-2 protein, with mannan clearly behaving as the most potent stimulus (Fig. 1B). MDM obtained after 2 wk of culture showed noticeable induction of COX-2 protein with concentrations of mannan as low as 0.1 mg/ml, 1 mg/ml zymosan, and 5 mg/ml soluble β-glucan laminarin (Fig. 1C). Interestingly, the natural ligand of the mannose receptor, MUC-3, also induced COX-2 expression; this effect was not synergistic with that produced by the combination of MUC-3 and mannan, thereby agreeing with the hypothesis that both molecules act through the same receptor (Fig. 1D). It is noteworthy that COX-1 expression was not influenced by mannan and zymosan treatment (Fig. 1D).
The time course of COX-2 induction was studied in MDM. Maximal induction occurred at 4–7 h with all agonists (Fig. 2A); the soluble β-glucan laminarin was again the stimulus displaying the lowest potency, whereas mannan was the strongest stimulus. The production of PGE₂ showed a good correlation with COX-2 induction, because in keeping with the noticeable basal expression of COX-2 protein, mannan slightly increased PGE₂ production above that observed in resting adhered monocytes, whereas it was a potent stimulus in MDM (Fig. 2B). It should be noted that PGE₂ production was most inhibited by 10 μM SC-236, a highly selective inhibitor of COX-2, the reported IC₅₀ value of which for COX-1 inhibition is 17.8 μM. In contrast, a more limited inhibition was produced by 0.3 μM SC-560, i.e., a concentration significantly higher than its reported IC₅₀ value of 9 nM for COX-1 inhibition (35). THP-1 cells are currently used as an archetypal model for human monocytes, because they display many different PRR even though they do not express CD14 (36) and do not adhere to plastic surfaces in the absence of phorbol ester treatment. On this basis, this cell line was used to obtain additional insight into the results observed in both monocytes and MDM. As shown in Fig. 2C, zymosan was a robust inducer of COX-2; this effect was enhanced by C3 bi-coating, which agrees with previous observations indicating a synergistic effect of complement coating on the proinflammatory effects of zymosan particles, most likely explained by the ability of zymosan-C3bi to concomitantly engage both dectin-1 (see below) and complement receptor 3 (CR3) (24). In contrast, the response to mannan was less remarkable, thus agreeing with the lower expression of MR in THP-1 cells (Fig. 2D) compared with MDM (see below). The possible autocrine effect of PGE₂ on COX-2 induction (37) was addressed by assaying the effect of exogenous PGE₂. However, concentrations of PGE₂ as high as 15 ng/ml failed to induce COX-2 protein expression (data not shown), which most likely indicates that under these experimental conditions, E-prostanoid receptors engaged by PGE₂ are not involved in autocrine COX-2 synthesis.

Expression of PRR in monocytic cells

Because the effect of zymosan (a polymer of β-glucan that can also contain mannan) on myeloid cells has been associated with the engagement of several receptors, namely, dectin-1 (38, 39), DC-SIGNR (40), MR (9, 17, 41), and CR3 (42), and the effect of mannosyl-containing particles has been associated to the engagement of MR, TLR-2 (43–45), and DC-SIGN (11–13), the expression of this set of receptors was assessed in different cell types. As shown in Fig. 3A, the expression of dectin-1 mRNA was observed in all cell types studied at all stages of differentiation; however, semiquantitative PCRs showed the highest levels of expression in both polymorphonuclear leukocyte (PMN) and MDM. In keeping with the previously reported existence of two predominant and six minor transcripts obtained by alternative splicing and small insertions in the human dectin-1 gene (26), a main PCR product of ~558 bp was found in all cell types together with a lower Mr product and other minor products. The expression of Endo180 mRNA in monocytic cells was somewhat similar to that of dectin-1, inasmuch as it increased with monocyte differentiation into macrophages and was also detected in THP-1 cells. By contrast, Endo180 mRNA was not observed in PMN, and only a slight

FIGURE 2. Effects of mannan and zymosan particles on COX-2 expression and PGE₂ production. MDM were incubated in the presence of the indicated additions for the times indicated, then the cell lysates were collected for immunodetection of COX-2 (A). Cell culture medium of both monocytes and MDM treated for 24 h in the presence of the indicated additions was used for the assay of PGE₂ (B). These are typical experiments of three or four performed with identical results. In the case of the PGE₂ assay, results express the mean ± SEM of three independent experiments (*, p < 0.05, SC-236-treated vs vehicle-treated MDM). THP-1 cells were incubated for 6 h in the presence of the various stimuli, then the cell lysates were collected for the immunodetection of COX-2 (C). β-Actin was detected to address the occurrence of similar protein loading across the gels. These data are representative of three experiments with identical results. The surface display of both CD206/MR and CD16/FcγRIII in THP-1 cells assayed by immunofluorescence flow cytometry is shown in D. Zymosan-C3bi indicates zymosan particles coated with C3bi.
expression was observed in adhered monocytes (Fig. 3A). Interestingly, Endo180 mRNA showed a high expression level in both HEK293 cells and HUVEC, thus agreeing with the reported expression of this receptor in endothelial cells (29) (Fig. 3B). Of note, the mRNA of neither DC-SIGNR nor DC-SIGN could be detected in the same set of cell types (not shown). TLR-2 mRNA was detected in blood and THP-1 cells (Fig. 3A). It is noteworthy that THP-1 cells expressed high amounts of sPLA$_2$ M-type receptor (Fig. 3C). As shown in Fig. 3D, maximal expression of MR was observed in MDM, although a weak, but significant, expression was also observed in nonadherent mononuclear cells, THP-1 monocytes, and HeLa cells (Fig. 3, C and D). This point was addressed in additional detail by indirect immunofluorescence flow cytometry. As shown in Fig. 4A, the surface display of MR was barely detectable in monocytes, but it blatantly increased after several days in culture, pari passu with the increasing size of the cells (Fig. 4B). The fluorescence intensity on day 1 was $3.6 \pm 0.3$ (mean $\pm$ SEM; $n = 6$; arbitrary units (AU)) and increased to $6.38 \pm 0.4$ and $39.2 \pm 4$ at 7 and 14 days, respectively. Moreover, FITC-conjugated, mannansylated BSA was readily uptaken by MDM, as assessed by flow cytometric assays (Fig. 5A). Confocal immunofluorescence microscopy showed that under these conditions, the uptake of FITC-conjugated, mannansylated BSA was very rapid, with focal images compatible with capping being detected as early as 2 min after addition of the stimulus, and label reaching the whole cytoplasm by 20 min. FITC-mannansylated BSA uptake was not observed at $4^\circ$C nor in the presence of 10 mg/ml mannan-BSA. Moreover, it was not observed in HEK293 cells after long periods of incubation (Fig. 5B).

The expression of CD16/Fc$\gamma$RIII, a receptor involved in the adaptive humoral immune responses, also increased along the macrophage differentiation route (Fig. 4A), with mean fluorescence intensity values of $0.34 \pm 0.05$, $2 \pm 0.03$, and $4.77 \pm 0.2$ (mean $\pm$ SEM; $n = 6$; AU) on days 1, 7, and 14, respectively, thus indicating that this marker is a good indicator of macrophage differentiation, which is only detected in $\sim$10% of peripheral blood monocytes (46).

**FIGURE 3.** Expression of mRNA encoding for PRR in different cell types. Total mRNA extracted from blood leukocytes and cell lines was extracted and used for RT-PCR amplification with primers for dectin-1 (A), Endo180 (A and B), TLR-2 (A), MR (C and D), and type M sPLA$_2$ receptor (C, lower panel). The expression of $\beta$-actin mRNA is shown as an example of constitutive gene expression (A, lower panel). These data are representative of three experiments with similar results.

**COX-2 induction by mannan is not accounted for by TLR-2 activation nor LPS contamination**

Because the most robust induction of COX-2 by mannan was observed in MDM after 14 days in culture, and the receptors capable of binding terminal mannose residues preferentially expressed in these cells are MR, Endo180, and TLR-2, these receptors were envisaged as being involved in conveying signals for COX-2 induction. Additional experiments were conducted in cell lines that have been used for similar purposes, namely, HEK293 (47) and HeLa cells (48). Unlike blood cells, these cell lines do not express type-M sPLA$_2$ receptor (Fig. 3C) or TLR-2 mRNA (Fig. 6A), but readily express TLR-2 mRNA upon transfection with an expression vector encoding for human TLR-2 (Fig. 6A). However, upon stimulation with mannan, TLR-2-expressing HEK293 cells did not exhibit COX-2 protein expression (data not shown), whereas they produced an 8-fold increase in $\kappa$B-driven transcriptional activity upon transfection with a firefly luciferase-linked $5\times$ NF-$\kappa$B reporter plasmid DNA and stimulation with 10 mg/ml peptidoglycan, i.e., a natural ligand for TLR-2 (49). In contrast, HeLa cells showed a noticeable basal expression of COX-2, which was enhanced by 10 mg/ml mannan (Fig. 6B). This response was not influenced by transfection of empty vector (pRc/CMC) and TLR-2-encoding vector (Fig. 6, C and D, upper panels), although transfection with TLR-2-encoding vector endowed HeLa cells with the ability to respond to the TLR-2 ligand peptidoglycan (Fig. 6, C and D, lower panels). Moreover, transfection of HeLa cells with the TLR-2 dominant negative mutant (P712H) did not abrogate mannan response, whereas these cells did not show COX-2 induction in response to peptidoglycan above the expression level observed in control cells (Fig. 6E). Taken together, these data indicate that a receptor other than TLR-2, most likely the MR, should be involved in mannan-induced COX-2 expression in HeLa cells. As shown in Fig. 7A, polymyxin B blocked the effect of *Escherichia coli* LPS on human MDM, whereas this was not observed after mannan treatment. This finding allows us to rule out a contamination of mannan by LPS. Conversely, mannosyl-BSA treatment
inhibited in a dose-dependent manner the effect of mannan, whereas it did not influence LPS effect (Fig. 7, B and C). Of note, mannose-BSA partially inhibited the zymosan effect (Fig. 7C, right panel), whereas this was not observed on the response to the pure β-glucan laminarin (Fig. 7C, left panel). These findings and the previously observed inhibition of zymosan uptake by mannan (24) are in keeping with previous reports indicating that the MR may be involved in a portion of the biological effects of zymosan, which are purportedly related to its content in mannose moieties (10, 17, 40).

Discussion

Molecular structures containing terminal mannose are unusual components of mammalian tissues; however, they are abundant in the walls of a variety of microorganisms, which is a prime condition for these structures to behave as PAMP. Moreover, host cells express different PRR able to recognize mannose-decorated components. The MR is the archetypal receptor, but DC-SIGN and Endo180 have the ability to recognize mannose moieties, thus making it difficult to ascertain the role displayed by each receptor in physiologically relevant cell systems. Inhibition of proinflammatory cytokines has been related to the engagement of the MR (44) and DC-SIGN (12, 13), whereas the proinflammatory effects of mycobacterial lipomannans depend on their ability to interact with TLR-2 (43–45), which is reminiscent of the ability of this receptor to interact with β-glucans (50), although most recent studies have disclosed a more complex paradigm in which dectin-1 behaves as the recognition receptor (38, 39) and TLR-2 acts as a signal integrator targeting intracellular molecules into a signaling complex involving MyD88, IL-1R-associated kinase, and TNFR-associated factor-6 (51).

Our first approach to address this interplay of receptors was to analyze the pattern of receptors expressed in monocytic cells and its correlation with the cell response along the route to macrophage differentiation. We observed a reduced surface display of the MR on human monocytes, which agrees with previous reports (16), and...
an increased expression of this receptor along the macrophage differentiation route, pari passu with the expression of CD16/FcγRIII, a receptor implicated in adaptive immunity that is a well-known marker of macrophage differentiation. In contrast, we have not observed the expression of DC-SIGN at any point in the differentiation process, which is in keeping with the reported pattern of expression of this receptor in cell microdomains during the development of human monocyte-derived dendritic cells (52). Furthermore, we have not detected the expression of DC-SIGN mRNA at any time of macrophage differentiation. This finding and the unique activation of DC-SIGN by β-glucan make it unlikely that this receptor is involved in the response to α-mannan moieties. With regard to dectin-1, our data agree with the preferential involvement of this receptor in the response to β-glucan particles, inasmuch as the response to zymosan is clearly observed in THP-1 cells, which definitely express dectin-1 mRNA, and is enhanced by C3bi coating, thus indicating a potentiation of the response by the simultaneous engagement of CR3. The involvement of MR in mediation of the COX-2 induction produced by mannans is suggested by several factors: 1) the correlation of the magnitude of the response with the extent of MR expression, as assessed by both RT-PCR and flow cytometry; 2) the inhibition by mannose-BSA, a ligand for the MR that is engulfed upon receptor binding and leads to noticeable endocytosis, but behaves as a weak agonist because it contains a lower ratio of attached sugar compared with pure polysaccharides (16); and 3) the similar effect of a natural ligand of the MR (MUC-3) together with the lack of synergism of mannans and MUC-3, thus pointing to an effect of both ligands on the same receptor. With regard to the possible involvement of Endo180, initial studies have stressed its preferential binding by N-acetylglucosamine, suggesting the existence of a different array of ligands for this receptor and the MR (29). Even though this idea has recently been modified by showing that under certain circumstances mannose oligosaccharides can bind to the receptor in a Ca$^{2+}$-dependent manner (53), this has been observed for Ca$^{2+}$-dependent mannans and LPS in the presence of various concentrations of polymyxin B. After 6 h, cell lysates were collected and used for the immunodetection of COX-2 (A). The effect of the preincubation of MDM with mannosylated BSA on the induction of COX-2 by various stimuli is shown in B and C. These are representative of three independent experiments.

Regardless of the receptors involved in mannann effects, our data show that the soluble mannose polysaccharide mannann from S. cerevisiae as well as the natural ligand of the mannose receptor MUC-3 produce both COX-2 induction and PGE2 release in human macrophages, which, in combination with our previous description of the release of arachidonic acid by mannans, links this polysaccharide to the eicosanoid cascade (24). This finding raises several questions of functional relevance, because the MR might limit inflammation by counterbalancing signals from TLR and other PRR (54), and PGE2 down-regulates inflammation and dendritic cell migration via E-prostanoid receptor 2 (55). Interestingly, microarray analysis of gene expression in human dendritic cells stimulated with mannann has shown a significant enhancement of COX-2 expression in the context of an outstanding overlap of the

**FIGURE 6.** Effect of ectopic expression of TLR-2 on mannan-induced COX-2 expression. The expression of TLR-2 mRNA in THP-1 monocytes and HEK293 and HeLa cells in both the absence and the presence of transfection with a vector encoding human TLR-2 sequence is shown in A. The effect of various agonists on the expression of COX-2 in HeLa cells is shown in nontransfected cells (B) or after transfection of both a mock vector (pRc/CMV; C) and TLR-2 vector (D) 6 h after addition of stimuli. The effect of transfection of HeLa cells with both a TLR-2 vector and a dominant negative TLR-2 construct on the response to mannan and peptidoglycan is shown in E. These data are representative experiments of three with similar results. The expression of β-actin is shown as an example of constitutive gene expression.

**FIGURE 7.** Effects of polymyxin B and mannose-BSA on the induction of COX-2 produced by different stimuli. MDM were incubated with mannan and LPS in the presence and the absence of various concentrations of polymyxin B. After 6 h, cell lysates were collected and used for the immunodetection of COX-2 (A). The effect of the preincubation of PMNs with mannosylated BSA on the induction of COX-2 by various stimuli is shown in B and C. These are representative of three independent experiments.
monia. In contrast, studies of monocyte-derived dendritic cells (61), and it has been suggested that reduced MR (25).

orated molecules and might also influence the expression of the paracrine immunomodulatory route associated with PG production (18). The possibility that cell-specific signaling pathways might be coupled to the MR, thus yielding different patterns of response, seems to be of pathophysiologic relevance. In this connection, MR is to date the only innate PRR known to be significantly up-regulated in a typical chronic inflammatory condition such as nasal polyposis, thus suggesting a central role for MR in the pathophysiology of this disease (62).

In summary, our data have shown a strong capacity of ligands displaying terminal mannoses to induce COX-2 in human macrophages. This capacity increases along the differentiation of these cells into the macrophage stage and best correlates with the expression of MR. These findings disclose the existence of a paracrine immunomodulatory route associated with PG production, which is associated with the endocytosis of mannose-decorated molecules and might also influence the expression of the MR (25).

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Disclosures

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