Involvement of Toll-like Receptor (TLR) 2 and TLR4 in Cell Activation by Mannuronic Acid Polymers*

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The alginate capsule produced by the human pathogen Pseudomonas aeruginosa is composed mainly of mannuronic acid polymers (poly-M) that have immunostimulating properties. Poly-M shares with lipopolysaccharide the ability to stimulate cytokine production from human monocytes in a CD14-dependent manner. In the present study we examined the role of Toll-like receptor (TLR) 2 and TLR4 in responses to poly-M. Blocking antibodies to TLR2 and TLR4 partly inhibited tumor necrosis factor production induced by poly-M in human monocytes, and further inhibition was obtained by combining the antibodies. By transiently transfecting HEK293 cells, we found that membrane CD14 together with either TLR2 or TLR4/MD-2 could mediate activation by poly-M. Transfection of HEK293 cells with TLR2 and fluorescently labeled TLR4 followed by co-patching of TLR2 with an antibody revealed no association of these molecules on the plasma membrane. However, macrophages from the Tlr4 knockout C3H/HeJ mice and TLR4 knockout mice were completely non-responsive to poly-M, whereas the tumor necrosis factor release from TLR2 knockout macrophages was half of that seen with wild type cells. Taken together the results suggest that both TLR2 and TLR4 are involved in cell activation by poly-M and that TLR4 may be required in primary murine macrophages.

Alginate is a family of linear co-polymers of 1–4-linked β-D-mannuronic acid (M) and α-L-guluronic acid with highly variable composition and sequence structure depending on the source from which it is isolated (for review, see Ref. 1). In nature, alginate is found mainly as the structure substance of marine brown seaweed but also as an exopolysaccharide produced by bacteria, like the opportunistic pathogen Pseudomonas aeruginosa that colonizes patients with cystic fibrosis (2) and may cause severe infections in critically ill patients.

Our group has previously shown that alginates are able to stimulate monocytes to cytokine production (3). The potency increases with the content of M residues and molecular size, and polymers isolated from mucoid pseudomonads (poly-M, 92–96% M) are the most active (3). However, attaching oligomeric M blocks of low activity to particles enhances their TNF-inducing potency by 2–4 log units, suggesting that optimal immunostimulating activity is determined from the polymer conformation (4, 5). Given prophylactically, poly-M has also been shown to protect mice from lethal Escherichia coli infection* and x-irradiation (6) and to stimulate murine hematopoiesis (6).

Like lipopolysaccharide (LPS) from Gram-negative bacteria (7), poly-M induces TNF production from human monocytes by binding to the surface receptor CD14 (8), and the response is enhanced by serum as a source of LPS-binding protein and soluble CD14 (5, 7, 9, 10). CD14 lacks an intracellular part (11) and probably mediates cell activation by interaction with other signal transducing molecules. Ten human Toll-like receptors (TLRs) have been cloned, and some of them have been shown to be microbial signal transducers (for review, see Ref. 12). The LPS hyporesponsiveness of C3H/HeJ and C57Bl/10ScCr mice is due to a dominant negative point mutation and recessive null mutation, respectively, in Tlr4 (13). Furthermore, TLR4 knockout (TLR4–/–) mice are non-responsive to LPS, whereas TLR2–/– mice respond normally (14, 15). In addition to TLR4, a soluble protein, MD-2, associates with TLR4 and is needed for efficient LPS signaling (16). Two uronic acid-containing polysaccharides are also found to induce cell activation through TLR4, namely the Gram-positive Micrococcus luteus teichuronic acids (17) and the fungal Cryptococcus neoformans glucuronoxymannnan (18). TLR2 recognizes a range of different structures such as several Gram-positive bacterial components (15, 19–21); yeast zymosan (21); mycobacterial lipoarabinomannan (22); and lipoproteins and lipopeptides from spirochetes, mycobacteria, and mycoplasma (20, 23, 24). The present study was undertaken to examine whether TLR2 or TLR4 is involved in signaling cytokine production induced by poly-M.

** Experimental Procedures

Reagents—Poly-M (–350 kDa, 92% M) was isolated from mucoid P. aeruginosa, and the batch was the same as that previously used (4, 5).

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The abbreviations used are: M, β-D-mannuronic acid; poly-M, mannuronic acid polymers; TLR, Toll-like receptor; LPS, lipopolysaccharide; TNF, tumor necrosis factor; ELAM, endothelial leukocyte adhesion molecule; MALP, macrophage-activating lipopeptide; mAb, monoclonal antibody; IL, interleukin; FCS, fetal calf serum; HEK, human embryonic kidney; DMEM, Dulbecco’s modified Eagle’s medium; YFP, yellow fluorescent protein.

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2 T. Espevik and G. Skjåk-Bræk, unpublished results.
The endotoxin content was 6 ng/mg as measured by the Limulus amebocyte lysate assay (Chromogenix AB, Mölndal, Sweden). LPS O26:B6 from E. coli, Re595 LPS from Salmonella minnesota and polymyxin B were purchased from Sigma. Phenol-extracted protein-free LPS from E. coli K235 (25) was kindly provided by Dr. S. N. Vogel (Bethesda, MD). Synthetic hexapeptide 47L from polymyxin B was a kind gift from Dr. G. A. Finlay (New York, NY) by gradient centrifugation with Lymphoprep as described by the manufacturer (Amersham Biosciences). Adherent human monocytes (105/dish) were purchased from the manufacturer (San Francisco, CA). Recombinant human TNF (specific activity, 1–108 units/mg) was purchased from Genzyme Pharmaceuticals (Cambridge, MA).

**Stimulation and Culture Conditions of Peripheral Blood Mononuclear Cells and Cell Lines—**Peripheral blood mononuclear cells were isolated from human A+ buffy coats (The Bloodbank, RIT, Trondheim, Norway) by gradient centrifugation with Lymphoprep as described by the manufacturer (Amersham Biosciences). Adherent human monocytes (1–105 monocytes/well in 24-well dishes) were stimulated twice in Hanks’ balanced salt solution (Invitrogen) and pretreated with a 10 μg/ml concentration of the indicated mAbs or with 1 μg/ml synthetic lipopeptide (MAA) derived from mycoplasmula (a kind gift from the manufacturer (Ortho-McNeil, Raritan, NJ) with 0.5 μg/ml TNF (specific activity, 7.6 × 106 units/mg) supplied by Genentech Inc. (South San Francisco, CA), and recombinant human IL-6 (specific activity, >1–106 units/mg) was purchased from Genzyme Pharmaceuticals (Cambridge, MA).

**Peripheral Macrophages—**Peripheral macrophages from C3H/HeN and C3H/HeJ mice (Hbl–Bliss, Basel, Switzerland) were isolated from peritoneal lavage fluid by F. Chan and M. Lenardo (National Institutes of Health, Bethesda, MD). Macrophages were adhered (5 × 104 cells/well) on 24-well plates for 24 h, washed, and incubated with 300 μl of RPMI, 10% FCS in the presence of stimulants for 24 h until assayed for TNF activity in the WEHI 164 clone 13 bioassay as described previously (27). Results from one representative experiment are presented as mean ± S.D. of triplicate TNF measurements.

**Results—**Poly-M Fails to Induce IL-6 Production from CD14-transfected U373 Cells—The U373 astrocytoma cell line does not express CD14, but the cells are responsive to LPS in the presence of serum as a source of soluble CD14 (9). We have shown that although poly-M shares with LPS the ability to stimulate monocyte TNF production in a CD14-dependent manner (8) and that serum or soluble CD14 enhances the response (10), serum or soluble CD14 cannot support poly-M-induced IL-6 production from U373 cells (8). To examine whether membrane CD14 is necessary for activation by poly-M, mock-transfected and CD14-transfected U373 cells were compared for responses to Poly-M and poly-M (Fig. 1A).

In the presence of 1% human serum, LPS induced dose-dependent IL-6 production from U373/CD14 cells that was 1–15 log units higher than that in non-transfected cells. Poly-M failed to activate U373 cells even when the cells were transfected with CD14 (Fig. 1A). U373 cells did express endogenous TLR4, TLR6, and MD-2 but not TLR2 (reverse transcription–PCR data not shown). We next transiently transfected U373/
CD14 cells with an ELAM-luciferase reporter plasmid together with combinations of TLR2, MD-2, and TLR4 and analyzed NF-κB activation by poly-M (Fig. 1B). Expression of TLR2 made U373/CD14 cells responsive to the TLR2-TLR6 ligand MALP-2 (24), confirming a functional TLR2. Similarly, overexpression of MD-2 and TLR4 resulted in increased NF-κB activation induced by LPS. However, none of the receptor combinations were sufficient for poly-M to induce NF-κB translocation in U373 cells. Thus, the results in Fig. 1B demonstrate that the inability of poly-M to activate U373 cells was not due to the absence of TLR2 or low level expression of TLR4 or MD-2, suggesting that U373 cells lack other components needed for responses to poly-M. Altogether these results demonstrate that LPS and poly-M have different requirements for inducing cell activation.

**TLR2 and TLR4 Are Involved in Signaling TNF Production from Human Monocytes in Response to Poly-M**—CD14 is involved in both LPS- and poly-M-induced monocyte TNF production (7, 8), but LPS signal transduction occurs through TLR4 in primary cells such as monocytes/macrophages (13–15). Blocking mAbs were used to examine the possible involvement of TLR2 and TLR4 in mediating poly-M-induced TNF production from monocytes. As shown in Fig. 2A, mAbs to CD14 (3C10), TLR2 (TL2.1), and TLR4 (HTA125) all inhibited TNF production induced by poly-M, although with different efficiencies. From several experiments, inhibition by TL2.1 and HTA125 was 60–95% and 40–90%, respectively, but combinations of TL2.1 and HTA125 yielded greater inhibition than either mAb alone (Fig. 2A and not shown). mAbs to CD14 and TLR4, but not to TLR2, blocked LPS-induced activation of monocytes (Fig. 2B), and the control mAb, 6H8, did not influence stimulation by either LPS or poly-M (Fig. 2). Thus, poly-M and LPS share the involvement of CD14 and TLR4, but in addition poly-M uses TLR2 for inducing monocyte release of TNF.

The activity of LPS is dependent on the nature and number of acyl chains in the lipid A part (30), and some lipid A analogs antagonize LPS activation of human cells (30). In other species, some of these molecules are agonists, and it has been demonstrated that TLR4 is responsible for the species-specific recognition of lipid A structures (31). As seen in Fig. 2C, synthetic lipid IVA (compound 406) inhibited both LPS- and poly-M-induced TNF production, supporting the results obtained with blocking mAbs that poly-M is recognized by TLR4. The stimulation of monocytes by poly-M was not due to contaminating LPS since polymyxin B, which binds to the lipid A part of LPS, inhibited activation by LPS and not by poly-M (Fig. 2D).

**Poly-M Can Use Either CD14/TLR2 or CD14/TLR4/MD-2 to Activate HEK293 Cells**—We next sought to delineate the minimum receptor requirement of poly-M-induced cell activation. HEK293 cells lacking CD14, TLR2, TLR4, and MD-2 (data not shown) were transiently transfected with combinations of these receptors together with an ELAM-luciferase reporter plasmid and analyzed for NF-κB activation by poly-M (Fig. 3). HEK293 cells transfected with TLR2, TLR4, and/or MD-2 were unresponsive to poly-M unless membrane CD14 was expressed, indicating that, in contrast to LPS, soluble CD14 cannot substitute for the membrane-bound form in cell activation by poly-M (data not shown). HEK293 cells co-transfected with CD14 and TLR2 responded to poly-M with NF-κB activation, and MD-2 was not required for signaling (Fig. 3). The same pattern was observed for MALP-2. In contrast, expression of CD14 and TLR4 was not sufficient to mediate poly-M responses, and additional co-transfection with MD-2 was needed.
for poly-M to induce translocation of NF-κB (Fig. 3). Although we did not adjust for transfection efficiency, no obvious improvement of poly-M-induced NF-κB activation was obtained by co-transfection with TLR2 and TLR4/MD-2. MD-2 was also required together with TLR4 to signal LPS activation in HEK293 cells. These results indicate that poly-M can use either CD14/TLR2 or CD14/TLR4/MD-2 for signal transduction. 

TLR2 and TLR4 Are Not Preassociated on the Plasma Membrane of HEK293 Cells—Since poly-M was found to signal through both TLR2 and TLR4 with CD14 or CD14/MD-2 as co-receptors, respectively, we found it important to examine whether TLR2 and TLR4 co-localized on the plasma membrane in live cells. To perform these studies we engineered a TLR4 construct with YFP on the C terminus of the molecule. The fluorescent TLR4 was functionally active when transfected into HEK293 cells.4 TLR2 or CD14 was transiently transfected into HEK293 cells stably expressing YFP-TLR4 and patched with antibodies to TLR2 (TL2.1) and CD14 (3C10), respectively. As seen in Fig. 4, A–C, TLR4 did not accumulate in TLR2 patches, indicating that TLR2 and TLR4 are not preassociated on the plasma membrane of HEK293 cells. Incubation with poly-M to induce receptor complex formation or co-transfecting CD14 and MD-2 together with TLR2 did not change this pattern (data not shown). In contrast, patching of CD14 resulted in pronounced co-localization of TLR4 (Fig. 4, D–F), suggesting a close association between these two membrane molecules. Similar results were obtained when cells expressing YPP-TLR6 were transfected with TLR2 and patched with TL2.1 (data not shown).

Macrophages from Tlr4 Mutant C3H/HeJ and TLR4+/+ Mice Do Not Respond to Poly-M, whereas TLR2−/− Cells Are Partially Responsive—The importance of TLR4 in mediating LPS signaling was revealed by the finding that the genetic defect in LPS-hyporesponsive C3H/HeJ mice is a P712H missense mutation.4 E. Latz, A. Visintin, E. Lien, K. Fitzgerald, B. G. Monks, E. Kurt-Jones, D. Golenbock, and T. Espevik, unpublished data.
macrophages were unresponsive even to 200 ng/ml poly-M. Macrophages from HeN mice responded to poly-M by producing TNF, further indicating that TLR4 may be necessary for induction of TNF production by poly-M. The TNF level produced by TLR2 homozygous mice was reduced more than 50% compared with that of wild type cells (Fig. 5A, right). Similar results were obtained with LPS, although the HeN macrophages were less sensitive to LPS than to poly-M (Fig. 5A, left). A TLR2 ligand, lipohexapeptide 47L from Rhodobacter capsulatus, induced comparable amounts of TNF production in HeN and HeJ macrophages (Fig. 5A, middle). These results confirm that HeJ mice express functional TLR2 and further indicate that TLR4 may be necessary for induction of TNF production by poly-M.

To directly assess the relative importance of TLR2 and TLR4 in mediating activation by poly-M, we compared poly-M-induced TNF release from wild type, TLR4−/−, and TLR2−/− knockout mice. Poly-M triggered a dose-dependent TNF release from wild type macrophages that was completely absent in TLR4−/− cells (Fig. 5B), confirming the results obtained with HeJ macrophages (Fig. 5A). Macrophages from TLR2−/− mice showed a reduced but significant response to poly-M. The TNF level produced by TLR2−/− cells was ~50% compared with that of wild type cells (Fig. 5B). LPS-induced TNF production was comparable from wild type and TLR2−/− cells but absent in TLR4−/− cells. Moreover, MALP-2 activated macrophages from wild type and TLR4−/− mice but not from TLR2−/− mice, altogether confirming that the function of TLR2 and TLR4 was normal in TLR4- and TLR2-deficient cells, respectively. Thus, although TLR2 participates in poly-M-induced TNF release, these results suggest that TLR4, and not TLR2, is required for poly-M responses in primary murine macrophages.

**DISCUSSION**

Medzhitov and Janeway (33) have proposed that innate immune cells express pattern recognition receptors that discriminate between self and non-self by recognition of conserved pathogen-associated molecular patterns. Several of the pattern recognition receptors, like CD14 (34) and the TLRs (12, 33), recognize microbial components with no apparent structural similarity. Poly-M was the first CD14 ligand different from LPS to be described (8). Here we show that poly-M is also recognized by TLR2 and TLR4. The finding that poly-M could not activate U373 cells implies that these cells lack a component(s) different from CD14, TLR2, TLR4, and MD-2 that is needed for responses to poly-M, but not to LPS, and that is expressed in monocytes/macrophages and HEK293 cells. This result supports a model in which specific host responses are mediated by combinations of molecules rather than by single pattern recognition receptors.

TLR4 is shown to mediate species-specific recognition of lipid A structural analogs (31), and we found that synthetic tetraacyl lipid A, also known as lipid IVA or compound 406, inhibited both LPS- and poly-M-induced TNF production at low concentrations of antagonist. In a previous report we found that the synthetic pentaacyl analog of *Rhodobacter capsulatus* lipid A, B975, inhibited activation of human monocytes induced by LPS, but not by poly-M, in serum-free conditions (5). The reason for this discrepancy is not known but may be related to differences in the ability to inhibit signaling through TLR4, especially in the absence of serum (35).

Various investigators have pointed to the problem of purifying bacterial components to homogeneity (36), and several data in the present and previous works confirm that the results obtained with poly-M are not due to contaminating LPS. First, the U373/CD14 cells were sensitive to very low concentrations of LPS (less than 0.1 ng/ml, not shown) but completely insensitive to poly-M. Second, polymyxin B, which binds to lipid A and blocks LPS responses, did not affect activation of human monocytes with poly-M (Fig. 1D and Ref. 3). Third, the TNF-inducing capabilities of high molecular poly-M is greatly reduced by hydrolytic or enzymatic breakdown but restored or even enhanced when the resultant low molecular chains are attached to particles (4, 5). Finally, the lack of response to poly-M in TLR4−/− cells rule out possible contamination with lipoproteins or lipopeptides.

The first sets of experiments with human monocytes indicated that CD14, TLR2, and TLR4 are involved in mediating cell activation by poly-M. To further address the relative contribution and importance of TLR2 and TLR4, we used two other experimental systems: gain-of-function studies in the human cell line HEK293 and loss-of-function studies in TLR-defective murine macrophages. The results from HEK293 cells suggested that two separate signaling pathways exist for poly-M as expression of either CD14/TLR2 or CD14/TLR4/MD-2 was sufficient for NF-κB translocation. TLR4 is proposed to form homodimers (21) that together with MD-2 could mediate poly-M activation. As suggested by Ozinsky et al. (37) TLR2 does not signal as homodimers but induces cytokine production in heteromeric complexes with TLR1 or TLR6 for instance (37–40). Co-patching experiments of YFP-TLR4-expressing HEK293 cells transfected with TLR2 or CD14 revealed that CD14, but not TLR2, was preassociated with TLR4 on the plasma membrane. However, we cannot exclude that TLR2 and
TLR4 may interact in other cells, such as primary macrophages. The results from TLR2- and TLR4-deficient mouse peritoneal macrophages clearly indicated that TLR4, and not TLR2, is required for cytokine induction by poly-M. Nevertheless, the reduced TNF response in TLR2-deficient cells argues for a role of TLR2, possibly together with TLR4. The reason for this divergence is unclear but may be due to differences in cell systems and experimental setup. We suggest that primary cells may better mirror the responses that can occur in vivo. Common observations in all cell systems, both primary cells and cell lines, lead us to the conclusion that the induction of cell activation by poly-M involves CD14, TLR2, and TLR4, and we can hypothesize a mechanism where TLR2 and TLR4 both participate in mediating an optimal response to poly-M. In addition to its cytokine-inducing effect, antitumor activity has been demonstrated for algal high M alginates (47). Furthermore, poly-M protects mice from lethal x-irradiation and bacterial infections without imparting toxicity (6). Thus, a possible exploitation of poly-M could be as a general immunostimulator for protection against disease.

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