Differential roles of Toll-like receptors in the elicitation of proinflammatory responses by macrophages

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Abstract

***Background*—Mammalian Toll-like receptor (TLR) proteins are pattern recognition receptors for a diverse array of bacterial and viral products. Gram negative bacterial lipopolysaccharide (LPS) activates cells through TLR4, whereas the mycobacterial cell wall glycolipids, lipoarabinomannan (LAM) and mannosylated phosphatidylinositol (PIM), activate cells through TLR2. Furthermore, short term culture filtrates of *M tuberculosis* bacilli contain a TLR2 agonist activity, termed soluble tuberculosis factor (STF), that appears to be PIM. It was recently shown that stimulation of RAW264.7 murine macrophages by LPS, LAM, STF, and PIM rapidly activated NF-κB, AP1, and MAP kinases.**

***Results*—This study shows that signalling by TLR2 and TLR4 also activates the protein kinase Akt, a downstream target of phosphatidylinositol-3'-kinase (PI-3-K). This finding suggests that activation of PI-3-K represents an additional signalling pathway induced by engagement of TLR2 and TLR4. Subsequently, the functional responses induced by the different TLR agonists were compared. LPS, the mycobacterial glycolipids, and the OspC lipoprotein (a TLR2 agonist) all induced macrophages to secrete tumour necrosis factor α (TNFα), whereas only LPS could induce nitric oxide (NO) secretion. Human alveolar macrophages also exhibited a distinct pattern of cellular response after stimulation with TLR2 and TLR4 agonists. Specifically, LPS induced TNFα, MIP-1β, and RANTES production in these cells, whereas the TLR2 agonists induced only MIP-1β production.**

***Conclusion*—Together, these data show that different TLR proteins mediate the activation of distinct cellular responses, despite their shared ability to activate NF-κB, AP1, MAP kinases, and PI-3-K.**

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Mammalian Toll-like receptor (TLR) proteins are a new family of proteins that share sequence similarity with the *Drosophila* Toll receptor proteins.[1] Ten TLR proteins have been identified in the mammalian genome, though the functions of only five TLR proteins (TLR2, TLR4, TLR5, TLR6, and TLR9) have been studied in detail (reviewed by Means *et al*[2] and Imler and Hoffman[3]). TLR4 appears to be unique among the TLR proteins in that it uses two additional proteins, CD14 and MD-2, as co-receptors. Cells that express TLR4, but lack these co-receptors fail to be activated by most TLR4 agonists.[4–6] The intracellular domain of all TLR proteins is highly conserved, and shares substantial sequence similarity with both the interleukin (IL) 1 and IL18 receptors.[7] This conserved intracellular domain has been termed the Toll interleukin receptor domain and it mediates the signal transducing capacities of the TLR and IL1 receptor proteins. Not unexpectedly, the Toll interleukin receptor domains of the TLR, IL1, and IL18 receptors activate similar signal transduction cascades. These cascades sequentially activate the adapter protein MyD88, one of several IL1 receptor associated kinases, TRAF-6, and ultimately, the IκB kinase complex. Phosphorylation, ubiquitination, and proteolytic degradation of the inhibitory protein IκB allows NF-κB to translocate to the nucleus. In addition to NF-κB nuclear translocation, several protein kinases are also activated by the TLR signalling cascade. These include MAP kinases (ERK, p38, JNK) and phosphatidylinositol-3'-kinase (PI-3-K). TLR signal transduction leads to the expression of several proteins that have important roles in the innate immune response to pathogens. These proteins include proinflammatory cytokines, chemokines, costimulatory proteins (for example, B7.1), and inducible nitric oxide synthase (iNOS).

A great deal of effort has been focused on the identification of TLR agonists. To date, agonists have only been definitively identified for four TLR proteins. Several bacterial cell wall products have been shown to function as TLR2 agonists. These agonists include bacterial lipoproteins,[8–10] peptidoglycan,[11] mycobacterial glycolipids,[12 13] and lipopolysaccharide (LPS) from both *Leptospira interrogans*[14] and *Prophyromonas gingivalis*.[15] Bacterial TLR4 agonists include LPS from Gram negative bacteria[16 17] and lipoteichoic acid.[18] Interestingly, the plant product taxol, the respiratory syncytial virus coat protein F, and mammalian heat shock protein 60 (HSP60) have also been reported to be TLR4 agonists.[6 19 20] In the last case, mammalian HSP60 may serve as an endogenous TLR4 agonist when it is released from damaged or dying cells. Bacterial DNA that contains unmethylated CpG dinucleotide sequences (CpG DNA) has been reported to activate cells through TLR9,[21] and bacterial flagellin has been identified as an agonist for

TLR5.[22] Despite the plethora of potential TLR agonists that have been reported, there has been no clear demonstration of direct binding of any of these agonists to the TLR protein itself. Nevertheless, TLR proteins seem to be able to sense the presence of various bacterial and viral cell wall products, bacterial DNA, and perhaps endogenous "danger signals" released by damaged or dying cells.

Two recently published studies have shown that TLR2 and TLR4 agonists differ in their abilities to induce cytokine and nitric oxide (NO) production by murine macrophages in vitro.[13 15] Despite these distinct patterns of cellular responses, engagement of both TLR2 and TLR4 led to the activation of NF-κB, AP1, and MAP kinases.[13] Thus the biochemical basis for these distinct functional responses has yet to be determined. Here we have further explored the signal transduction pathways activated by TLR2 and TLR4 in an attempt to characterise the biochemical basis for differential activation of cellular responses by distinct TLR agonists.

Materials and methods

CELLS, CELL LINES, AND REAGENTS

The RAW264.7 murine macrophage (TIB-71) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and were cultured as previously described.[12] TLR2−/− mice were provided by Dr Shuzio Akira (University of Osaka Medical School) and have been described previously.[18] Primary peritoneal macrophages were prepared from these mice using thioglycollate elicitation, as previously described.[12] Macrophages from C57BL/6 mice (Jackson Laboratories, Bar Harbor ME) were used as control cells. All medium components contained <10 pg/ml final concentration of LPS as measured by Limulus amoebocyte lysate kit (BioWhittaker, Walkersville, MD). Primary human alveolar macrophages were obtained from healthy volunteers by bronchoalveolar lavage, performed according to a protocol recommended by the American Thoracic Society and approved by the Institutional Review Board. Alveolar macrophages were cultured for three days before use, as previously described.[23]

LPS (purified from *E coli* 055:B5) was purchased from Sigma (St Louis, MO) and repurified by the method of Hirschfeld *et al*.[24] Mycobacterial arabinose-capped lipoarabinomannan (LAM), purified from a rapidly growing avirulent *Mycobacterium* species (AraLAM), and phosphatidylinositol dimannoside (PIM) were provided by Dr John Belisle (Colorado State University). The lipoprotein OspC, from *Borrelia burgdorferi*, was provided by Dr Justin Radolf (University of Connecticut Health Science Centre). The levels of contaminating LPS in the LAM and PIM preparations were determined by a quantitative Limulus lysate assay (BioWhittaker), and were <1 pg/ml final concentration in all experiments. Soluble tuberculosis factor (STF) was prepared from cultures of the *M tuberculosis* strain H37Ra (ATCC). Bacterial cultures were grown in LPS-free Middlebrook 7H9 medium supplemented with Tween 80, glycerol and OADC (Difco, Detroit, MI) at 37°C in LPS-free flasks under biosafety level 3 conditions to mid-logarithmic phase (OD at 620 nm = 0.4). Bacteria were then removed by centrifugation, followed by two rounds of filtration through a 0.22 μm membrane. This short term culture filtrate was digested with proteinase K (100 μg/ml) for 18 hours at 56°C, and then used as a source of crude STF.

MEASUREMENT OF TNFα, CHEMOKINE, AND NO LEVELS

Tumour necrosis factor α (TNFα), MIP-1β, and RANTES levels in culture supernatants were determined by specific enzyme linked immunosorbent assays (ELISAs; Quantikine ELISA kits, R&D Systems, Minneapolis MN), as recommended by the manufacturer. The limit of sensitivity for these assays was always <10 pg/ml. Levels of NO catabolite nitrite were measured in the culture supernatants by the Greiss reagent assay, as previously described.[25] The limit of sensitivity of this assay was always <3 μmol/l. All assays were performed in triplicate, and data are expressed as mean values (SEM). The data were subsequently analysed using analysis of variance to determine statistical significance.

WESTERN BLOT ANALYSIS

Whole cell lysates were prepared from unstimulated RAW264.7 macrophages and from cells stimulated with different TLR agonists for various times, as we have previously described.[13] Aliquots containing 20–100 μg of total protein per lane were fractionated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then electrophoretically transferred to nitrocellulose membranes (Bio-Rad). Transferred proteins were detected using specific antisera against the phosphorylated and non-phosphorylated forms of Akt and the p38 MAP kinase (New England Biolabs, Beverly, MA), according to the manufacturer's instructions. Membranes were developed using a donkey antirabbit antiserum linked to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ), and then visualised with an enhanced chemiluminesence reagent (CL-HRP substrate system, Pierce Corp, Rockford, IL).

Results

MYCOBACTERIAL GLYCOLIPIDS FAIL TO ACTIVATE TLR2−/− MACROPHAGES

Previously, we showed that the mycobacterial glycolipids LAM and PIM, as well crude STF, could activate a CHO cell reporter line that overexpressed TLR2 and not TLR4.[12 13] This finding did not exclude the possibility that these TLR agonists might also activate cells through additional TLR proteins. Thus we used TLR2−/− macrophages to determine whether TLR2 was the sole receptor necessary for macrophage activation by these mycobacterial TLR agonists. Peritoneal exudate macrophages were prepared from normal and TLR2−/− mice and, then stimulated in vitro with LPS, LAM, PIM, and STF. Culture supernatants were collected 18 hours later and

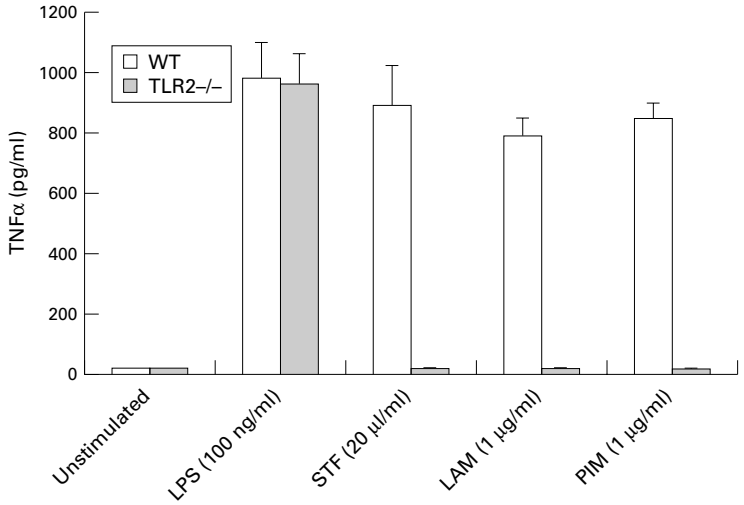

Figure 1 TLR2 is required for macrophage activation by mycobacterial glycolipids. Adherent peritoneal macrophages elicited by thioglycollate were collected from TLR2−/− and C57BL/6 (WT) mice and stimulated for 24 hours with LPS (100 ng/ml), STF (20 µl/ml), LAM (1 µg/ml), and PIM (1 µg/ml). Supernatants were collected and concentrations of secreted TNFα were determined by ELISA. Assays were performed in triplicate and repeated on three separate occasions. A single representative experiment is shown and data are expressed as mean values (SEM). LPS = lipopolysaccharide; STF = soluble tuberculosis factor; LAM = lipoarabinomannan; PIM = phosphatidylinositol dimannoside.

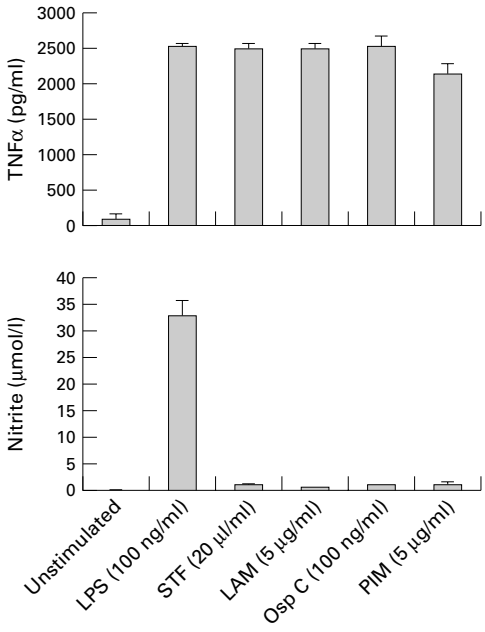

Figure 2 TLR2 agonists fail to induce nitric oxide production. LPS and the mycobacterial glycolipids were analysed for their abilities to induce TNFα and nitric oxide (NO) production in the murine macrophage-like cell line RAW264.7. NO levels were indirectly determined by measuring the levels of the stable NO catabolite nitrite in the culture supernatants of stimulated macrophages. Cells were stimulated for 24 hours with LPS (100 ng/ml), STF (20 µl/ml), LAM (5 µg/ml), lipoprotein OspC (100 ng/ml), and PIM (5 µg/ml). Supernatants were collected and analysed for the presence of nitrite using the Greiss assay, and for TNFα by ELISA. Assays were performed in triplicate and repeated on three separate occasions. A single representative experiment is shown and data are expressed as mean values (SEM). LPS = lipopolysaccharide; STF = soluble tuberculosis factor; LAM = lipoarabinomannan; PIM = phosphatidylinositol dimannoside.

assayed for the presence of TNFα by ELISA. Figure 1 shows that all the normal macrophages secreted TNFα after stimulation with LPS, LAM, PIM, and STF. In contrast, only LPS activated the TLR2−/− macrophages to secrete TNFα. This finding showed that TLR2 is necessary for activation of primary murine macrophages by the mycobacterial glycolipids.

DIFFERENT TLR AGONISTS INDUCE DISTINCT CELLULAR RESPONSES IN MACROPHAGES

We subsequently sought to compare the cellular responses induced by these various TLR2 and TLR4 agonists. RAW264.7 murine macrophages were stimulated with LPS, LAM, and STF for 18 hours, and culture supernatants were then collected. The levels of secreted TNFα and nitrite, a stable catabolite of NO, were measured by ELISA and the Greiss reagent assay, respectively. Figure 2 shows that LPS induced RAW264.7 macrophages to secrete TNFα and NO. In contrast, LAM, PIM, and STF could only induce TNFα secretion. Similar results were obtained with the lipoprotein TLR2 agonist OspC. The latter finding demonstrates that a TLR2 agonist that is structurally distinct from the mycobacterial glycolipid TLR2 agonists also lacks the ability to induce NO production in macrophages. Together, these studies show that the cellular responses of macrophages induced by stimulation with different TLR2 and TLR4 agonists are qualitatively distinct.

We subsequently compared the responses of primary human alveolar macrophages to TLR2 and TLR4 agonists. Because LPS does not activate NO production by these cells, we measured the release of TNFα and the chemokines MIP-1β and RANTES after stimulation with either LPS or STF for 48 hours. Table 1 shows that LPS induced human alveolar macrophages to secrete TNFα, MIP-1β, and RANTES. In contrast, STF induced MIP-1β secretion, but not significant amounts of TNFα and RANTES. These experiments were performed using alveolar macrophages from five different donors and a similar trend was seen in all cases. Data from a single representative experiment, performed in triplicate, are shown in table 1. Both LPS and STF could also induce MIP-1α secretion by the macrophages (data not shown). Also, stimulation of the alveolar macrophages with PIM resulted in the same pattern of responses as those seen when cells were stimulated with STF (data not shown). It should be noted that although the levels of LPS and STF used in these experiments induced similar levels of TNFα secretion in the murine RAW264.7 cells (fig 2), the human alveolar macrophages were markedly hyporesponsive to STF with respect to TNFα secretion. Our data show that human alveolar macrophages, like the murine RAW264.7 cell line, also elicited distinct responses to the different TLR agonists. Unlike the RAW264.7 cells, the human alveolar macrophages did not secrete TNFα after stimulation with STF. This was not owing to non-responsiveness of the human cells to STF because significant amounts of MIP-1α and MIP-1β were secreted under the same conditions. Although the pattern of cytokine responses induced in the

Table 1 Response of human alveolar macrophages to Toll-like receptor agonists. Results are shown as mean (SD) cytokine response in pg/ml

Cytokine response	*Unstimulated*	*STF (20 μl/ml)*	*LPS (100 ng/ml)*
TNFα	ND	12 (16)	1180 (49)
MIP-1β	ND	5170 (90)	67186 (848)
RANTES	ND	ND	19447 (458)

ND = none detected.

alveolar macrophages by TLR2 and TLR4 agonists differed from the pattern seen in the murine macrophages, our data provide another example of differential cellular responsiveness to distinct TLR agonists.

TLR DEPENDENT ACTIVATION OF THE PROTEIN KINASE AKT

We previously reported that both LPS and the mycobacterial glycolipids activated the MAP kinase ERK, though the glycolipids were relatively poor inducers of JNK.[13] Despite the relative inability of the glycolipids to activate JNK, these TLR2 agonists could still activate downstream JNK dependent responses. Specifically, the glycolipids could activate AP1 nuclear translocation and the activation of an AP1 dependent promoter. Similar results were obtained for NF-κB nuclear translocation and NF-κB dependent transcription. Therefore, the ability of LPS and the mycobacterial glycolipids to induce similar AP1 and NF-κB dependent responses could not explain how distinct cellular responses were induced by stimulation of macrophages with different TLR2 and TLR4 agonists. We therefore sought to determine whether Akt (also known as protein kinase B) was differentially activated by the TLR2 and TLR4 agonists. Akt activation is dependent on the activation of two upstream kinases, PDK and PI-3-K, and is often used as an indication of cellular activation via the PI-3-K signalling cascade. RAW264.7 macrophages were stimulated for 10 minutes with either LPS or PIM. Cells were then harvested, cell lysates were prepared and analysed by western blotting using antibodies that specifically recognise phosphorylated and non-phosphorylated forms of Akt. In parallel, lysates were also analysed by western blotting using antibodies that specifically recognise phosphorylated and non-phosphorylated forms of the p38 MAP kinase. Figure 3 shows that LPS and PIM were equally capable of activating Akt, demonstrating that signalling by both TLR2 and TLR4 could lead to the activation of this protein kinase. This activation could be prevented if the macrophages were stimulated in the presence of the PI-3-K inhibitor LY294002, consistent with Akt activation being a downstream consequence of PI-3-K activation. The specificity of the inhibitor was demonstrated by the observation that activation of the p38 MAP kinase in cells stimulated with LAM and PIM was not blocked by LY294002 (fig 3). Thus PI-3-K activation appears to be a specific consequence of macrophage activation by TLR2 and TLR4, and this event leads to the subsequent activation of Akt.

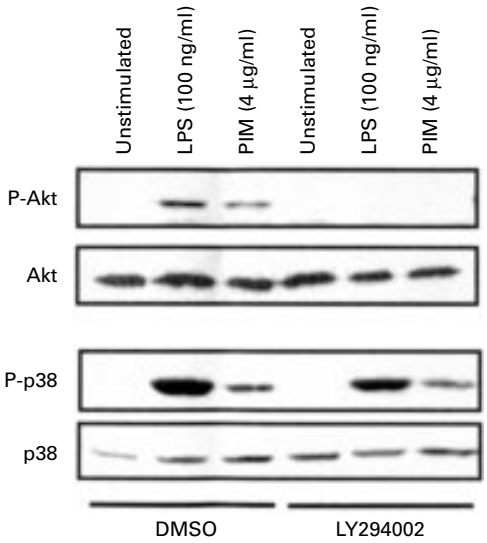

Figure 3 Both TLR2 and TLR4 agonists induce Akt activation in a PI-3-K dependent manner. RAW264.7 cells were pretreated for one hour with either the PI-3-K inhibitor LY294002 (25 μM) or vehicle (DMSO, 6.5 μl/ml), and then stimulated with either LPS (100 ng/ml) or PIM (4 μg/ml), for 30 minutes. After stimulation, whole cell lysates were prepared, fractionated by SDS-PAGE (50 μg lysate/lane), and transferred to nitrocellulose membranes. The membranes were then probed with antibodies against the phosphorylated forms of Akt and the p38 MAP kinase. Duplicate membranes were also probed with antibodies specific for the non-phosphorylated forms of Akt and p38. Bound primary antibodies were detected using secondary antibodies conjugated to HRP. LPS = lipopolysaccharide; PIM = phosphatidylinositol dimannoside.

TREATMENT OF MACROPHAGES WITH IFNγ CONFERS ON TLR2 AGONISTS THE ABILITY TO INDUCE NO PRODUCTION

The data presented above suggest that several intracellular signals are similarly activated by both TLR2 and TLR4 agonists. However, the inability of TLR2 agonists to induce all of the responses evoked by LPS suggests that these agonists do not activate a signal necessary to elicit some responses (for example, NO production in RAW264.7 cells and TNFα production in alveolar macrophages). We therefore sought to determine whether interferon γ (IFNγ), a well known macrophage activating factor, could supply the additional signal necessary to elicit these responses in cells stimulated with TLR2 agonists. RAW264.7 cells were pretreated with murine IFNγ for one hour, then stimulated with LPS, STF, or LAM. In parallel, cells that did not receive IFNγ were also stimulated with the TLR agonists. After 18 hours, the culture supernatants were recovered and assayed for nitrite, an indirect indicator of NO production. Figure 4 shows that only LPS induced NO production by macrophages that were not pretreated with IFNγ, consistent with the data presented in fig 2. In contrast, cells that were pretreated with IFNγ acquired the ability to produce large amounts of NO after stimulation with STF and LAM. This finding suggests that IFNγ may provide the "missing signal" that is given upon engagement of TLR4, but not TLR2. Alternatively, IFNγ may provide a different signal that can functionally substitute for the missing signal provided by TLR4 activation.

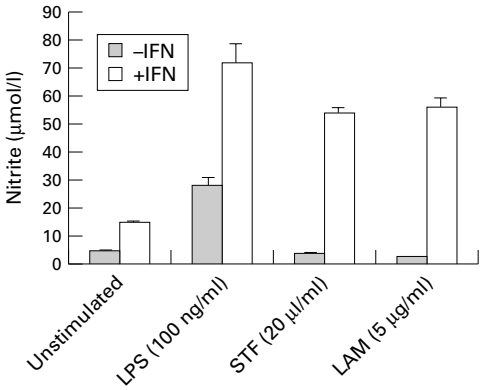

Figure 4 Interferon gamma (IFNγ) confers on TLR2 agonists the ability to induce NO production. RAW264.7 macrophages were pretreated for one hour with 10 ng/ml of murine IFNγ and then stimulated for 24 hours with LPS (100 ng/ml), STF (20 μl/ml), or LAM (5 μg/ml). Supernatants were collected and analysed for NO using the Greiss assay. Assays were performed in triplicate and repeated on three separate occasions. A single representative experiment is shown and data are expressed as mean values (SEM). LPS = lipopolysaccharide; STF = soluble tuberculosis factor; LAM = lipoarabinomannan.

Discussion

Numerous studies have reported that engagement of TLR proteins leads to the activation of NF-κB and MAP kinases. These activation events are elicited upon engagement of TLR2, TLR4, and TLR9 by their distinct agonists,[9 12 21] suggesting that they are shared responses that use a common signal transduction pathway. This pathway is likely to be mediated by MyD88, and the downstream signalling components IL1 receptor associated kinases and TRAF6 (reviewed by Means *et al*[2] and O'Neill and Greene[7]). Published studies have shown that engagement of TLR4, but not TLR2 and TLR9, can also lead to the activation of an MyD88 independent pathway and then to NF-κB and MAP kinase activation.[26 27] Together, these reports show that TLR proteins use both shared and unique signal transduction pathways. Here we have extended these findings by identifying a cellular response that is induced by different TLR agonists, and a distinct response that is restricted to specific TLR proteins. Specifically, both TLR2 and TLR4 agonists induce TNFα production in RAW264.7 murine macrophages, whereas only a TLR4 agonist (LPS) induced NO production by these cells. In human alveolar macrophages, LPS induced both TNFα and chemokine secretion. In contrast, the TLR2 agonists induced MIP-1β secretion, but failed to induce TNFα and RANTES secretion. Thus using two distinct macrophage populations, we have shown that TLR2 and TLR4 agonists induce different patterns of cellular responses. Lastly, we found that treatment of macrophages with exogenous IFNγ can further alter the pattern of cellular responses to TLR2 agonists, resulting in responses that are similar to those induced by TLR4 agonists.

Like the murine RAW264.7 cell line, human alveolar macrophages also displayed different responses in vitro when stimulated with TLR2 and TLR4 agonists. The inability of mycobacterial TLR2 agonists to induce TNFα secretion in the human alveolar macrophages is not a characteristic of all human macrophages, because human macrophages isolated from blood have been reported to secrete TNFα in response to these agonists,[28] but instead probably represents a difference in functional responsiveness between alveolar and non-alveolar macrophages. Qualitative differences in responses of alveolar and non-alveolar macrophages to the TLR4 agonist LPS have previously been described.[29] Also, preliminary studies have shown that primary murine alveolar macrophages also failed to secrete TNFα after stimulation in vitro with LAM, a TLR2 agonist (Ryan LK, Vermeulen MW, Fenton MJ, unpublished observations). Furthermore, we previously reported that regulation of TNFα gene expression is controlled by distinct mechanisms in alveolar and non-alveolar macrophages.[30] Thus it is these mechanistic differences that are likely to determine whether a particular macrophage population has the ability to respond to TLR2 agonists by producing TNFα.

The mechanism that underlies the differential patterns of cytokine and NO production induced by the TLR2 and TLR4 agonists remains unclear as both TLR2 and TLR4 agonists are equally capable of activating p38 and Akt (this study), as well as ERK, NF-κB, and AP1.[13] Furthermore, the mycobacterial glycolipids seem to be relatively poor inducers of JNK activation, though JNK dependent downstream events (for example, AP1 DNA binding activity and trans-activation function) were activated equally well by both LPS and the glycolipids.[13] Nevertheless, the most likely explanation is that TLR4 engagement leads to a full complement of intracellular signals that are necessary for cytokine and NO production. We propose that engagement of TLR2 fails to induce all of the different signal transduction pathways necessary for these cellular responses. The nature of this/these missing signal(s) remains unclear, though treatment of macrophages with IFNγ seems to provide such a signal and confers on TLR2 agonists the ability to induce NO production. This finding raises the possibilities that either (*a*) LPS directly activates a signal that can also be induced by treatment of macrophages with exogenous IFNγ, or (*b*) that LPS induces the production of a cytokine through an autocrine/paracrine mechanism in a manner similar to exogenous IFNγ. Although it is also possible that both LPS and exogenous IFNγ induce distinct, but functionally redundant, signals we believe this is a less likely possibility. Because IFNγ is not secreted by LPS stimulated macrophages, the missing signal cannot be provided by IFNγ itself. Instead, we propose that LPS-induced type I IFNs (IFNα/β) may provide this missing signal. This possibility is supported by the recent demonstration that iNOS mRNA expression was markedly reduced in LPS stimulated macrophages from type I IFN receptor knockout (IFNα/βR−/−) mice.[31] Furthermore, these investigators showed that neutralising antibodies against type I IFN could also block LPS-induced

iNOS mRNA expression in wild-type macrophages. Because engagement of the IFNα/βR and the IFNγR induces similar intracellular signalling events, specifically activation of the transcription factors ISGF3 and STAT1, it is likely that addition of exogenous IFNγ to macrophages mimics responses that would normally be provided by endogenous IFNα/β. Studies are currently underway in our laboratory to determine whether TLR2 agonists fail to induce type I IFN production, and whether supplementation of macrophage cultures with IFNα/β can restore the missing signal and functional responses not induced by TLR2 agonists alone.

Earlier studies had reported that inhibitors of PI-3-K substantially blocked LPS-induced NO production, and that this reduction was partially attributable to impaired LPS-induced IFNβ secretion.[32] As discussed above, an absence of type I IFN secretion in cells activated by TLR2 may account for the missing signal, but this is not a consequence of the absence of PI-3-K activation. We have shown here that activation of macrophages by both TLR2 and TLR4 leads to the activation of Akt. Akt activation, in turn, was shown to be downstream of PI-3-K activation because it could be blocked by the PI-3-K inhibitor, LY294002. This is consistent with earlier observations in LPS stimulated macrophages using the PI-3-K inhibitor, wortmannin.[33] Thus PI-3-K activation is likely to occur as a consequence of cellular activation by TLR2 and TLR4. Furthermore, Arbibe *et al* recently showed that TLR2 was physically associated with the p85 regulatory subunit of PI-3-K.[34] Although it has not been reported to date, it is likely that TLR4 also physically associates with PI-3-K. Irrespective of the potential role of PI-3-K in mediating the expression of type I IFN in LPS stimulated macrophages, our data can clearly exclude PI-3-K and Akt as candidates for the missing signal in cells activated by TLR2.

In summary, our data demonstrate that different TLR agonists elicit distinct cellular responses from macrophages in vitro. Similar findings were reported by Hirschfeld *et al* who used primary murine peritoneal macrophages to show that LPS from *Prophyromonas gingivalis*, a TLR2 agonist, induced a subset of the cellular responses induced by Gram negative bacterial LPS (a TLR4 agonist).[15] Some of the genes that were not activated by LPS and by the mycobacterial glycolipids (IP-10 and iNOS) were also not induced by *E coli* LPS in macrophages from IFNα/βR−/− and STAT1−/− mice.[31] This is consistent with our hypothesis that LPS-induced type I IFN production, and subsequent activation of STAT1 through engagement of the IFNα/βR, represents a common regulatory pathway that controls the expression of some LPS-inducible genes. How these findings might relate to the different functions of TLR2 and TLR4 agonists in vivo remains unclear. Although most bacterial pathogens can activate cells through TLR2, only *M tuberculosis* and Gram negative bacteria possess significant TLR4 agonist activity.[35] Some Gram positive and treponemal bacterial products have also been reported to be TLR4 agonists,[18 36] but the contributions of these molecules to TLR4 dependent activation of cells by the intact micro-organism remains unknown. Thus it is likely that the host response to these various bacteria may be partially defined by the ability of these organisms to differentially activate TLR2 and TLR4. Even if the relative TLR2 and TLR4 activating capacities of various bacteria could be measured in vivo, it is also possible that endogenous TLR agonists might contribute to the total TLR dependent responses. For example, HSP60 is a potential endogenous TLR4 agonist,[20] and release of this protein upon infection (or by dying cells) could induce a local TLR4 dependent proinflammatory response that further alters the pattern of cellular responses induced by the pathogen alone. Thus, clearly, TLR proteins have important roles in vivo, though their relative contributions to inflammation and innate immunity remain to be determined.

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