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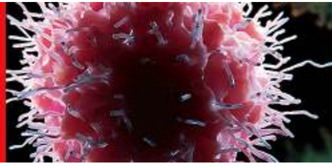

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J Immunol 2001; 166:574-581; ;
doi: 10.4049/jimmunol.166.1.574
http://www.jimmunol.org/content/166/1/574

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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CD11b/CD18 Acts in Concert with CD14 and Toll-Like Receptor (TLR) 4 to Elicit Full Lipopolysaccharide and Taxol-Inducible Gene Expression[1,2,3]

Pin-Yu Perera,* Tanya N. Mayadas,† Osamu Takeuchi,‡ Shizuo Akira,‡ Meirav Zaks-Zilberman,* Sanna M. Goyert,§ and Stefanie N. Vogel[4]*

Overproduction of inflammatory mediators by macrophages in response to Gram-negative LPS has been implicated in septic shock. Recent reports indicate that three membrane-associated proteins, CD14, CD11b/CD18, and Toll-like receptor (TLR) 4, may serve as LPS recognition and/or signaling receptors in murine macrophages. Therefore, the relative contribution of these proteins in the induction of cyclooxygenase 2 (COX-2), IL-12 p35, IL-12 p40, TNF-α, IFN-inducible protein (IP)-10, and IFN consensus sequence binding protein (ICSBP) genes in response to LPS or the LPS-mimetic, Taxol, was examined using macrophages derived from mice deficient for these membrane-associated proteins. The panel of genes selected reflects diverse macrophage effector functions that contribute to the pathogenesis of septic shock. Induction of the entire panel of genes in response to low concentrations of LPS or Taxol requires the participation of both CD14 and TLR4, whereas high concentrations of LPS or Taxol elicit the expression of a subset of LPS-inducible genes in the absence of CD14. In contrast, for optimal induction of COX-2, IL-12 p35, and IL-12 p40 genes by low concentrations of LPS or by all concentrations of Taxol, CD11b/CD18 was also required. Mitigated induction of COX-2, IL-12 p35, and IL-12 p40 gene expression by CD11b/CD18-deficient macrophages correlated with a marked inhibition of NF-κB nuclear translocation and mitogen-activated protein kinase (MAPK) activation in response to Taxol and of NF-κB nuclear translocation in response to LPS. These findings suggest that for expression of a full repertoire of LPS-/Taxol-inducible genes, CD14, TLR4, and CD11b/CD18 must be coordinately engaged to deliver optimal signaling to the macrophage. *The Journal of Immunology,* **2001, 166: 574–581.**

The continuous exposure of a host to microorganisms in the environment has led to the development of an immune system that protects the host against deleterious effects caused by these pathogens (1). Among the common organisms encountered by the host, Gram-negative bacteria constitute an important group as a result of host exposure to resident commensals in the gastrointestinal tract and their ubiquitous occurrence in the environment (2). The adverse effects caused by Gram-negative bacteria are a result of an overt inflammatory response by host cells, primarily monocytes/macrophages, to LPS. LPS is an integral component of the outer membrane of Gram-negative bacteria that results in the production of a proinflammatory cytokine cascade and other inflammatory mediators (reviewed in Ref. 3).

Over the years, many LPS-binding proteins have been identified on monocytes/macrophages and other LPS-responsive cell types (reviewed in Refs. 4 and 5). The identification of CD14, a GPI-anchored protein, as a major cell-associated, LPS binding protein, represents a seminal observation in the understanding of LPS signaling (6, 7). However, as CD14 lacks a transmembrane region and is incapable of signaling (8), transmembrane proteins with the potential for intracellular signaling were sought as CD14 coreceptors. Recently, several members of the highly conserved family of Toll-like receptor (TLR)[5] proteins have been characterized as cosignaling molecules for CD14 (reviewed in Ref. 9). Briefly, the evidence that TLR4 is an LPS signaling molecule was first suggested by the finding that a constitutively active form of TLR4 resulted in activation of NF-κB, IL-8 production, and activation of costimulatory molecules (10). These data were complimented by the findings that cells derived from LPS-hyporesponsive C3H/HeJ and C57BL/10ScCR mice exhibit missense and deletion mutations, respectively, in the *tlr4* gene (11, 12). Interestingly, F_1 progeny, hemizygous for the defective allele carried by the C3H/HeJ mice (e.g., derived from a cross of C3H/HeJ mice with a radiation-induced mutant that carries a ~9-kb deletion that encompasses *tlr4*), respond to LPS in vivo to produce significant levels of TNF-α (13), suggesting the possibility that other signaling molecules may be used in vivo, and may depend on the particular genetic background

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Received for publication June 9, 2000. Accepted for publication October 9, 2000.

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[1] This work was supported by National Institutes of Health Grant AI-18797 (to S.N.V.), ROI HL65095 (to T.M.N.), and AI-23859 (to S.M.G.).

[2] The opinions or assertions contained within are the private views of the authors and should not be construed as official or necessarily reflecting the views of Uniformed Services University of the Health Sciences or Department of Defense.

[3] Research was conducted according to the principles set forth in *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animal Resources, National Research Council Publication 85-23 (National Institutes of Health).

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[5] Abbreviations used in this paper: TLR, Toll-like receptor; COX-2, cyclooxygenase 2; IP-10, IFN-inducible protein-10; ICSBP, IFN consensus sequence binding protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase; HPRT, hypoxanthine-guanine phosphoribosyltransferase; CHO, Chinese hamster ovary.

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used. In addition to TLR4, TLR2 has also been implicated in LPS-mediated signaling both in transfected cell lines, where CD14 co-expression was found to synergize for LPS-induced NF-κB-mediated signaling (14–16), and in human PBMCs, as evidenced by the efficacy of an anti-TLR2 mAb to block LPS-induced IL-12 mRNA expression almost completely (17). However, analysis of mice with targeted disruptions in their *tlr*2 or *tlr*4 genes revealed that TLR4 knockouts, but not TLR2 knockouts, are LPS unresponsive, whereas TLR2 appears to be essential for the response to other non-LPS bacterial cell wall products (18, 19). One possible explanation for the discrepancies related to TLR2 use by LPS can be found in recent work by Hirschfeld et al. (20). In these experiments, commercial LPS preparations, which were active on both TLR2 and TLR4 transfectants, were repurified to remove highly bioactive, endotoxin-associated proteins. Following repurification, the preparations were active only on TLR4 transfectants, suggesting that TLR2 transfectants are very sensitive to the contaminants within these commercial LPS preparations. Lastly, oligomerization among different TLR molecules may create unique LPS signaling receptors.

Taxol, a plant diterpene, shares a number of macrophage stimulatory functions with LPS including the ability to discriminate between LPS-responsive and hyporesponsive strains of mice (21–26). Due to its superior chemistry, Taxol analogs with highly reactive substituents have been used as a novel approach to identify LPS-binding and signaling molecules. Using a photoaffinity Taxol analog to cross-link mouse macrophage membranes, Taxol was shown to bind to the common CD18 subunit of heterodimeric β_2 integrins (27), a family of proteins that has been shown previously to bind LPS (28). Of the three β_2 integrins expressed on leukocytes, macrophages predominantly express CD11b/CD18 (also called Mac-1 or CR3).

In this study, the importance of CD11b/CD18 in LPS- and Taxol-induced expression of cyclooxygenase 2 (COX-2), IL-12 p35, TNF-α, IP-10, and IFN consensus sequence binding protein (ICSBP) genes, as well as in the translocation of NF-κB to the nucleus and activation of mitogen-activated protein kinase (MAPK), was evaluated in macrophages derived from CD11b/CD18-deficient and normal mice. This panel of genes was selected for analysis based on their high levels of expression in normal macrophages in response to LPS and for the capacity of their products to mediate diverse macrophage effector functions in the host that contribute to septic shock. For example, COX-2 is an enzyme that regulates the production of prostaglandins, mainly PGE_2, by macrophages and is responsible for the generation of fever and pain during inflammation (29, 30). COX-2 enzymatic activity also results in the generation of PGI_2, which induces vasodilatation and contributes to hypotension during septic shock (31). IL-12 has been implicated in the production of cell-mediated immune responses against a variety of pathogens, in addition to its lethal role in LPS-induced shock in mice (32, 33). TNF-α is a proinflammatory cytokine commonly associated with septic shock (Ref. 34; reviewed in Ref. 35). IP-10 is a CXC chemokine that regulates monocyte, lymphocyte, and NK cell trafficking to sites of inflammation and thereby participates in host defense and inflammation (36, 37). ICSBP is a transcription factor that is critical for the expression of IL-12 p40 (38). The extent of the requirement for coreceptors CD14 and TLR4 leading to the activation of these same genes was also examined.

We report that at low concentrations of LPS or Taxol, CD14 and TLR4 were obligatory for gene expression, but many of the genes examined were CD14 independent at high concentrations. Certain genes, e.g., IP-10 and ICSBP, are extremely CD14 dependent, even at the highest concentrations of LPS and Taxol tested. For optimal induction of COX-2, and both IL-12 p35 and p40 genes, which encode heterodimeric bioactive IL-12 p70, all three receptors, CD11b/CD18, CD14, and TLR4, are required. These results support a model in which the coordinated interaction of LPS/Taxol with CD14 and/or CD11b/CD18 results in the stabilization of a multimeric receptor complex that includes TLR4, and that CD14 and CD11b/CD18 contribute differentially to the activation of signaling pathways for optimal inflammatory gene expression.

Materials and Methods

Macrophage culture conditions

The generation and breeding of all knockout mice used in this study have been reported previously (18, 39, 40). Mice with a targeted mutation in CD11b, which gives rise to mice that express a paucity of CD11b/CD18 (Mac-1) on the surface of their macrophages (40), and background-matched control wild-type mice were bred at Longwood Medical Research Center (Boston, MA). CD14-deficient mice, backcrossed onto a C57BL/6 background, were bred at North Shore University Hospital (Manhasset, NY). TLR4-deficient mice were bred at Osaka University (Osaka, Japan). C57BL/10ScNCR mice were purchased from the National Cancer Institute (Frederick, MD). C57BL/6J (controls for CD14-deficient mice) and C57BL/10J mice (controls for C57BL/10ScNCR mice) were purchased from The Jackson Laboratory (Bar Harbor, ME). Four days after the injection of 3 ml/mouse of 3% sterile fluid thioglycollate i.p., peritoneal exudate macrophages were extracted by lavage with 0.9% saline, pelleted by centrifugation, and resuspended in RPMI 1640 containing 2% FCS, 2 mM glutamine, 30 mM HEPES, 0.3% $NaHCO_3$, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For generation of RNA and cytoplasmic extracts, 6.5×10^6 cells were plated onto each well of a six-well tissue culture plate, incubated overnight, and treated as indicated in a total volume of 2.6 ml.

Reagents

Highly purified, phenol-water-extracted *Escherichia coli* K235 LPS (<0.008% protein) was prepared according to the method of McIntire et al. (41). Taxol was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (National Institutes of Health, Bethesda, MD). Taxol was solubilized in DMSO to form a 20-mM stock, and working dilutions of Taxol were prepared by dilution of the stock with supplemented RPMI 1640. Rabbit polyclonal anti-phospho-extracellular signal-related kinase (ERK)-1/2 and anti-phospho-c-Jun N-terminal kinase (JNK)-1/2 Abs were purchased from Promega (Madison, WI). Rabbit polyclonal anti-phospho-p38 MAPK Ab was purchased from New England Biolabs (Beverly, MA). Rabbit polyclonal anti-COX-2 Ab was obtained from Cayman Chemical (Ann Arbor, MI). Rabbit polyclonal anti-IκB-α/MAD-3 and anti-p38 (total-p38) Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-ERK-1 and -ERK-2 Ab (total ERK) was purchased from Zymed (South San Francisco, CA).

Isolation of total cellular RNA and RT-PCR

Total cellular RNA was extracted and reverse transcribed as detailed elsewhere (23). PCR amplifications were performed on the resultant cDNA for the gene of interest as described previously. The sequences of the specific primers and probes used in the detection of IL-12 p35, IP-10, ICSBP, and hypoxanthine-guanine phosphoribosyltransferase (HPRT) have been detailed elsewhere (23, 42–44). The expression of HPRT was included as a housekeeping gene to control for differences in cDNA for each treatment during the amplification reaction. The sequences of the specific primers used in the detection of COX-2 and IL-12 p40 are as follows: COX-2 sense, 5′-GCAAATCCTTGCTGTTCCAATC-3′; COX-2 antisense, 5′-GGAGAAGGCTTCCCAGCTTTTG-3′; IL-12 p40 sense, 5′-ATCGTTT TGCTGGTGTCTCC-3′; IL-12 p40 antisense, 5′-AGTCCCTTTGGT CCAGTGTG-3′. PCR amplification products were electrophoresed on a 1% agarose gel and blotted overnight onto a Nytran membrane. The DNA was then UV cross-linked onto the membrane and baked at 80°C for 2 h. The amplified PCR products were detected by gene-specific oligonucleotide probes labeled with the Amersham 3-oligolabeling and detection systems (Amersham, Buckinghamshire, U.K.). The sequences of the gene-specific probes used in the detection of COX-2 and IL-12 p40 are as follows: COX-2 probe, 5′-CCCACTTCAAGGGAGTCTGGAACA-3′; IL-12 p40 probes, 5′-CGTGCTCATGGCTGGTGCAAA-3′ and 5′-AGCAGTAGCAGTTCCCCTGA-3′.

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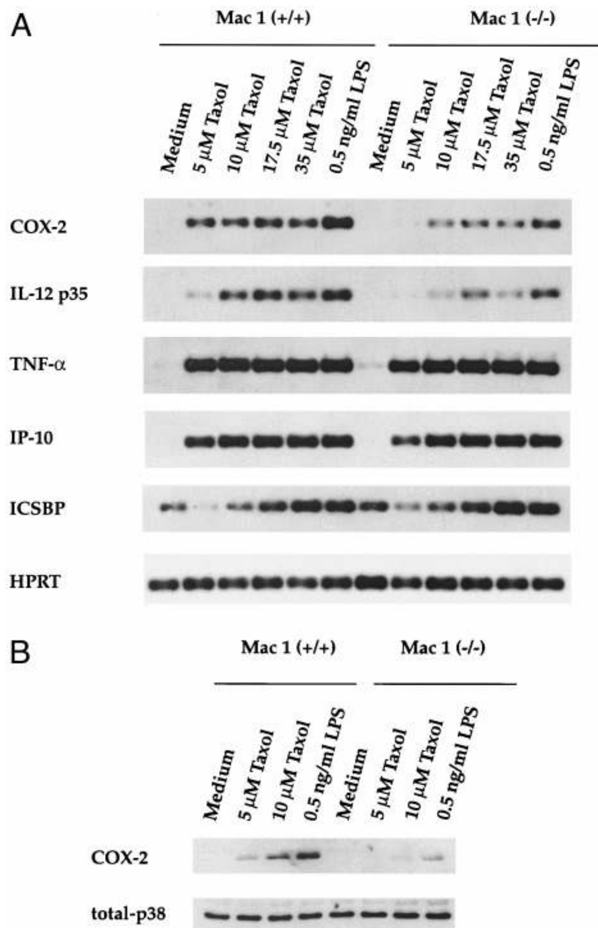

FIGURE 1. *A*, Analysis of gene expression in CD11b/CD18-deficient and control macrophages treated with LPS and Taxol by RT-PCR. Thioglycollate-elicited peritoneal macrophages derived from CD11b/CD18-deficient mice and control wild type-mice were treated with medium alone or indicated concentrations of Taxol or LPS for 4 h. Total cellular RNA was generated, reverse transcribed, amplified, and detected as described in *Materials and Methods*. These results are representative of three separate experiments performed. *B*, Detection of COX-2 production in CD11b/CD18-deficient and control macrophages treated with LPS and Taxol. Macrophages from CD11b/CD18-deficient and control mice were treated with medium, Taxol, or LPS for 7–8 h. Cytoplasmic extracts were generated and immunoblotted for COX-2. Following COX-2 detection, membrane was stripped and reblotted with an anti-p38 Ab that detects total p38 as a loading control. The data is representative of two separate experiments performed.

SDS-PAGE and Western analysis

Cytoplasmic extracts of macrophages were prepared following treatment with either LPS or Taxol for the required times as described elsewhere (45). Proteins were then resolved on 10% SDS-polyacrylamide gels, transferred onto polyvinylidene fluoride membranes, and immunoblotted with specific Abs raised against the proteins of interest. Specific proteins were then detected by enhanced chemiluminescence. Certain membranes were reblotted after removal of the first Ab or stained with Ponceau S (Sigma Diagnostics, St. Louis, MO) to ensure equal protein loading in all lanes.

DNA-protein interactions and EMSA

Nuclear extracts of macrophages were generated following treatment with either LPS or Taxol, and specific DNA-protein interactions were determined with ^{32}P-labeled, double-stranded oligonucleotides that contain a canonical NF-κB site as described previously (25).

Table I. *IL-12 p70 secretion by Mac-1$^{+/+}$ and Mac-1$^{-/-}$ macrophages treated with LPS or Taxol*[a]

Treatment	Mac-1$^{+/+}$	Mac-1$^{-/-}$
Medium	<15[b]	<15
Taxol, 10 μM	257 ± 20	<15
Taxol, 17.5 μM	799 ± 14	145 ± 11
Taxol, 35 μM	1425 ± 45	342 ± 21
LPS, 0.5 ng/ml	232 ± 4	<15
LPS, 1 ng/ml	432 ± 42	26 ± 8

[a] Twenty-four-hour culture supernatants of 2.4 × 10^5 Mac-1$^{+/+}$ and Mac-1$^{-/-}$ macrophages treated with medium, LPS, or Taxol were assayed for IL-12 p70 in an ELISA. IL-12 p70 is expressed as pg/ml.

[b] The data are the mean of duplicate samples within the same experiment ± SD and are representative of four similar experiments performed.

Results

Differential gene expression in CD11b/CD18-deficient and wild-type macrophages in response to LPS and Taxol

We previously reported that CD11b/CD18-deficient macrophages treated with LPS or Taxol exhibited a striking deficiency in their capacity to elicit IL-12 p40 gene expression (27). To delineate further the role of CD11b/CD18 in the mediation of LPS- and Taxol-induced gene expression in macrophages, macrophages from CD11b/CD18-deficient and control wild-type mice were treated with LPS or Taxol, and the inducibility of COX-2, IL-12 p35, TNF-α, IP-10, ICSBP, and HPRT steady-state mRNA was assessed by RT-PCR. As indicated in Fig. 1*A*, the induction of COX-2 and IL-12 p35 mRNA was also diminished in CD11b/CD18-deficient macrophages at all concentrations of Taxol and LPS examined, compared with macrophages derived from background-matched, wild-type mice. In contrast, the levels of TNF-α, IP-10, and ICSBP mRNA were induced comparably in macrophages derived from wild-type and CD11b/CD18-deficient mice. These results suggest that CD11b/CD18 plays a role in the induction of COX-2, IL-12 p35, and IL-12 p40 genes by both Taxol and LPS, whereas induction of TNF-α, IP-10, and ICSBP genes is CD11b/CD18-independent.

Impaired IL-12 p70 and COX-2 production in CD11b/CD18-deficient macrophages in response to both LPS and Taxol

We have previously shown that anti-Mac-1 Ab (which is directed against the CD11b subunit) blocks LPS- and Taxol-induced IL-12 p70 protein production in normally LPS-responsive control macrophages (27). These results were further extended by the finding that CD11b/CD18-deficient macrophages produced lower levels of immunoreactive IL-12 p70 in response to both LPS and Taxol than control macrophages (Table I), consistent with the finding that both IL-12 p40 and p35 gene expression were diminished in LPS/Taxol-stimulated, CD11b/CD18-deficient macrophages. To determine whether the decrease in COX-2 mRNA observed in CD11b/CD18-deficient macrophages would also result in a corresponding decrease in COX-2 protein levels, macrophages from CD11b/CD18-deficient and control wild-type mice were treated with LPS or Taxol. Cytoplasmic extracts were generated and then subjected to SDS-PAGE and Western analysis for COX-2 protein. As shown in Fig. 1*B*, there was considerably less COX-2 protein produced by CD11b/CD18-deficient macrophages when compared with control macrophages in response to LPS and Taxol, indicating the importance of CD11b/CD18 in both COX-2 mRNA and protein production. The equivalence of total p38 protein detected on the same blot indicates that this difference cannot be attributed to differences in protein loading.

Decreased NF-κB nuclear translocation in CD11b/CD18-deficient macrophages in response to LPS and Taxol

Previous reports have demonstrated a critical role for the transcription factor NF-κB in LPS-induced COX-2, and IL-12 p40 mRNA in macrophages (46, 47). Arguments for and against the role of NF-κB in the induction of LPS-induced TNF-α have also been presented (48, 49). Therefore, the activation of NF-κB, as evidenced by its translocation to the nucleus, in response to LPS and Taxol, were examined in CD11b/CD18-deficient and wild-type macrophages. Macrophages were treated with LPS or Taxol, nuclear extracts were generated, and EMSA for NF-κB was performed. Nuclear translocation of NF-κB was markedly less in CD11b/CD18-deficient macrophages when compared with control macrophages in response

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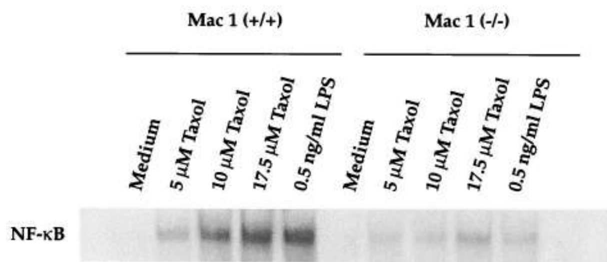

FIGURE 2. Activation and nuclear translocation of NF-κB from CD11b/CD18-deficient and control mice treated with Taxol and LPS. Macrophages from CD11b/CD18-deficient and control mice were treated with medium or the indicated concentrations of Taxol or LPS for 1 h. Nuclear extracts were generated, and EMSAs were performed as described in *Materials and Methods*. The data is representative of two separate experiments performed.

to both LPS and Taxol (Fig. 2), a finding that was confirmed by a concomitant mitigation of IκB-α degradation in cytoplasmic extracts (data not shown). Therefore, the observed diminution of COX-2 and IL-12 p40 gene expression in LPS- or Taxol-stimulated CD11b/CD18-deficient macrophages may result from dysregulated signaling leading to diminished translocation of NF-κB to the nucleus.

Differential phosphorylation of p38, JNK, and ERK MAPKs in CD11b/CD18-deficient macrophages treated with LPS or Taxol

A recent study by Lu et al. (50) reported that LPS stimulation of macrophages from MAPK kinase 3-deficient mice resulted in defective IL-12 p40 and IL-12 p35 mRNA, and IL-12 protein production, indicating a requirement for p38 MAPK in the production of IL-12. In another study, Scherle et al. (51) implicated a role for ERK-1 in the generation of COX-2 mRNA in human monocytes in response to LPS, whereas Moos et al. (52) reported that Taxol-induced COX-2 was also p38 dependent. Therefore, we investigated the possibility that the diminished levels of COX-2, IL-12 p35, and IL-12 p40 mRNA in CD11b/CD18-deficient macrophages in response to LPS or Taxol might be related to a decrease in MAPK phosphorylation. Macrophages from both CD11b/CD18-deficient and wild-type mice were treated with LPS or Taxol, and cytoplasmic extracts were analyzed for the presence of phosphorylated forms of MAPKs, e.g., p38, JNK-1, JNK-2, ERK-1, and ERK-2. As illustrated in Fig. 3*A*, there was marked reduction in the levels of phosphorylation of all isoforms of the three subfamilies MAPKs examined in the CD11b/CD18-deficient macrophages in response to Taxol, in comparison to wild-type macrophages. The observed decrease in phosphorylation of p38 and ERK-1/2 in response to Taxol in CD11b/CD18-deficient macrophages was seen at all time points examined (Fig. 3*B*). These results suggest that activation of MAPKs may play a role in the induction of COX-2, IL-12 p35, and IL-12 p40 mRNA in response to Taxol as a result of its interaction with CD11b/CD18. Interestingly, only a slight decrease in phosphorylated p38 was observed in CD11b/CD18-deficient macrophages in response to LPS when compared with wild-type macrophages, and no detectable alteration in levels of phospho-JNK or phospho-ERK were detected in LPS-stimulated CD11b/CD18-deficient macrophages.

LPS- and Taxol-mediated COX-2, IL-12 p35, IL-12 p40, and ICSBP gene expression exhibit differing CD14 dependencies

That steady-state levels of COX-2, IL-12 p35, and IL-12 p40 genes were diminished, but not totally ablated, in CD11b/CD18-deficient macrophages upon LPS and Taxol treatment, suggests that CD11b/CD18 acts in concert with other LPS receptors to elicit optimal gene expression. Previous studies have indicated an interaction between GPI-linked proteins and CD11b/CD18, and more recently, the transient association of CD11b/CD18 with CD14 following addition of LPS to cells was demonstrated by fluorescence resonance energy transfer microscopy (Ref. 53; reviewed in Ref. 54). Although a major role for CD14 as a receptor for LPS-induced gene expression has been well documented (reviewed in Ref. 55), previous studies have demonstrated a strong CD14 dependence at low concentrations of LPS, but a lesser CD14 dependence for Taxol in the induction of TNF-α, IL-1β, and IP-10 genes in macrophages derived from CD14 knockout mice (26). Therefore, the involvement of CD14 as a coreceptor in the expression of COX-2, IL-12 p35, IL-12 p40, and ICSBP genes by LPS and Taxol was next examined in CD14-deficient and control macrophages. The results in

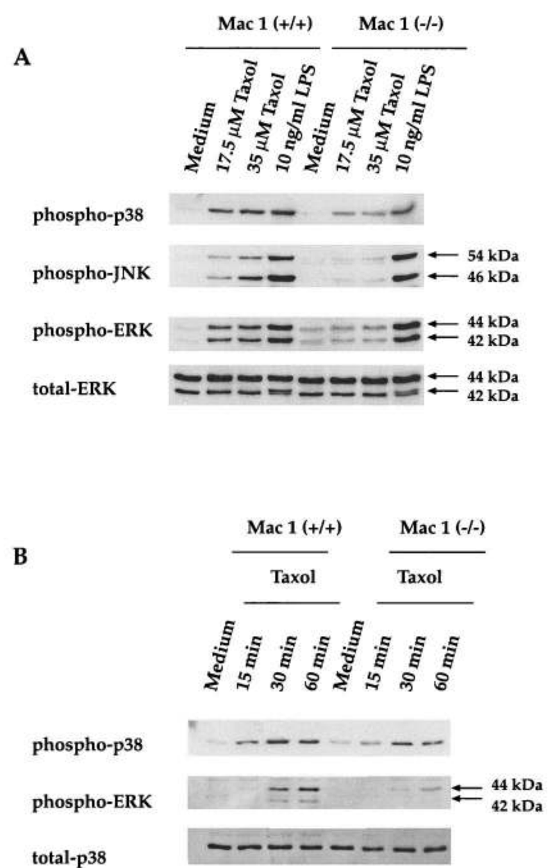

FIGURE 3. Detection of phosphorylated MAPK in CD11b/CD18-deficient and control macrophages treated with LPS and Taxol. *A*, Macrophages from CD11b/CD18-deficient and control mice were treated with medium, indicated concentrations of Taxol, or LPS for 15 min. Cytoplasmic extracts were generated, subjected to SDS-PAGE, and immunoblotted for phospho-specific-p38, -JNKs, and -ERKs as described in *Materials and Methods*. Following phospho-JNK detection, membrane was stripped and reprobed with an anti-ERK Ab that detects total ERKs as a loading control. The data is representative of two separate experiments performed. *B*, Macrophages from CD11b/CD18-deficient and control wild-type mice were treated with medium or 10 μM Taxol for the indicated times. Cytoplasmic extracts were generated, subjected to SDS-PAGE, and immunoblotted for phospho-p38 and phospho-ERKs as described in *Materials and Methods*. Following phospho-ERK detection, membrane was stripped and reblotted with an anti-p38 Ab that detects total p38 as a loading control.

Fig. 4 illustrate that in the absence of membrane-associated CD14 there is no detectable COX-2, IL-12 p35, and IL-12 p40 gene expression in response to low concentrations of LPS, whereas the induction of these same genes is CD14 independent at high concentrations of LPS. In addition, mitigated mRNA expression for these genes was observed at low concentrations of Taxol. These results indicate that, like TNF-α and IL-1β (26), CD14 is essential for the induction of COX-2, IL-12 p35, and IL-12 p40 genes by low concentrations of LPS and Taxol, but not at high concentrations. In contrast, induction of ICSBP mRNA was CD14 dependent even at the highest concentrations of LPS and Taxol (Fig. 4), similar to the pattern of gene expression described previously for IP-10 (26).

Diminished phosphorylation of p38, JNK, and ERK MAPKs in CD14-deficient macrophages treated with LPS or Taxol

We have previously reported that CD14-deficient macrophages treated with low and high concentrations of LPS induced nuclear translocation of negligible amounts of the proinflammatory transcription factor NF-κB in comparison with control macrophages (26). Therefore, activation of proinflammatory intracellular signaling cascades, the MAPKs, was also examined. Macrophages from both CD14-deficient and control mice were treated with LPS or Taxol, and cytoplasmic extracts were analyzed for the

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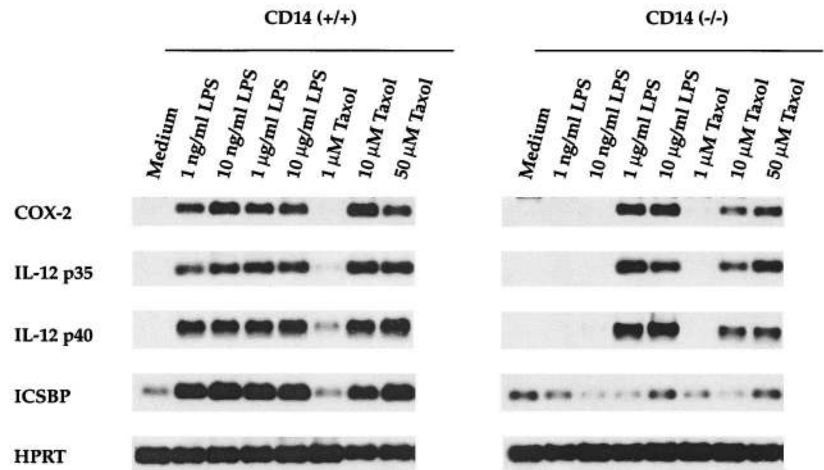

FIGURE 4. RT-PCR analysis of LPS- and Taxol-induced gene expression in macrophages derived from CD14-deficient and control mice. Macrophages from CD14-deficient and control mice were treated with medium or the indicated concentrations of Taxol or LPS for 4 h. Total cellular RNA was generated, reverse transcribed, amplified, and detected as described in *Materials and Methods*. The data are representative of two separate experiments performed.

presence of phosphorylated forms of MAPKs: p38, JNK-1, JNK-2, ERK-1, and ERK-2. As shown in Fig. 5, CD14-deficient macrophages treated with low and high concentrations of LPS or Taxol induced very little phosphorylation of all isoforms of the three subfamilies of MAPKs when compared with control macrophages.

LPS- and Taxol-mediated induction of COX-2, IL-12 p35, IL-12 p40, TNF-α, IP-10, and ICSBP is TLR4 dependent

The importance of TLR4 in LPS-mediated signaling was recently established by the identification of TLR4 as the product of the *Lps* gene (11, 12). Previous studies from our laboratory have also shown that TLR4-defective C3H/HeJ macrophages are completely refractory to high concentrations of highly purified LPS, as measured at the level of gene expression or MAPK activation (56). To examine further the role of TLR4 in LPS- and Taxol-mediated expression of COX-2, IL-12 p35, IL-12 p40, TNF-α, IP-10, and ICSBP genes, the expression of these genes was next examined in C57BL/10ScNCR and in TLR4 knockout macrophages. The C57BL/10ScNCR strain is the predecessor of the LPS-unresponsive C57BL/10ScCR strain (57) and also carries a deletion mutation in the *tlr4* gene (12). As illustrated in Fig. 6*A*, none of the genes examined was induced in the C57BL/10ScNCR macrophages when compared with control C57BL/10J macrophages in response to LPS or Taxol, indicating an absolute requirement for TLR4 for the induction of the entire panel of genes by both LPS and Taxol. These results were further confirmed in macrophages derived from TLR4 knockout mice (Fig. 6*B*).

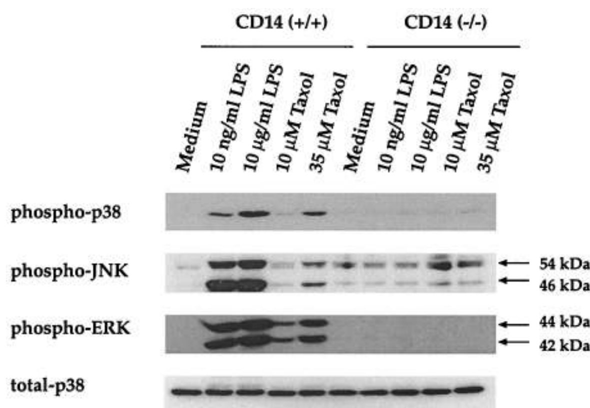

FIGURE 5. Detection of phosphorylated MAPK in CD14-deficient and control macrophages treated with LPS or Taxol. Macrophages from CD14-deficient and control mice were treated with medium and indicated concentrations of LPS or Taxol for 15 min. Cytoplasmic extracts were generated, subjected to SDS-PAGE, and immunoblotted for phospho-specific-p38, -JNKs, and -ERKs as described in *Materials and Methods*. The data is representative of two separate experiments performed.

Discussion

The CD11/CD18 β_2 integrins are a family of obligate heterodimeric glycoproteins expressed on leukocytes as a 95-kDa CD18 β subunit noncovalently associated with one of three α subunits, e.g., CD11a (LFA-1), CD11b (Mac-1, CR3), or CD11c (CR4) (reviewed in Ref. 54). Although all three types of β_2 integrins are detectable on macrophages, macrophages express CD11b/CD18 heterodimers predominantly. The CD11/CD18 integrins were originally identified as LPS receptors in studies that demonstrated binding of Gram-negative bacteria and LPS-coated erythrocytes to these receptors on human macrophages (28). Subsequent studies performed on monocytes and macrophages derived from CD18-deficient patients found normal levels of TNF-α and IL-1β and led to the conclusion that CD18 is not essential for cellular responses to LPS (58). Murine CD18 was more recently identified as a Taxol binding and signaling receptor by using a photoaffinity-labeled Taxol analog as a probe (27). The data in this report indicate that although CD11b/CD18 may not be necessary for the LPS- or Taxol-inducible expression of cytokines such as TNF-α, which is typically used to quantify cellular responses to LPS, CD11b/CD18 plays a significant role in the optimal production of COX-2, IL-12 p35, and IL-12 p40 genes. The correlative findings that both MAPK and NF-κB activation are dysregulated in CD11b/CD18 (Mac-1)-deficient macrophages strengthen the importance of these pathways in the optimal induction of a subset of LPS-inducible genes. As summarized in Table II, our results indicate that for optimal expression of COX-2, IL-12 p35, and IL-12 p40 gene expression in response to LPS or Taxol, the participation of all three receptors, CD11b/CD18, CD14, and TLR4, is required. In contrast, induction of TNF-α mRNA requires only the presence of both CD14 and TLR4 at low levels of LPS or Taxol, whereas the induction of ICSBP and IP-10 mRNA requires both CD14 and TLR4 even at higher concentrations of LPS and Taxol. Although a physical interaction between CD14 and TLR4 in response to LPS has not yet been reported, a recent study by Yang et al. (16) demonstrated an association between transiently expressed CD14 and TLR2 in the absence of LPS and, upon LPS stimulation, in 293 cells. A transient association of CD14 with CD11b/CD18 has also been reported in response to LPS in neutrophils (53). Interestingly, of all the genes evaluated, ICSBP was found to be most CD14 dependent, as evidenced by its failure to be induced in CD14 knockout macrophages by extremely high concentrations of either Taxol or LPS, a finding previously observed for IP-10 (26). Therefore, there must be additional signals required

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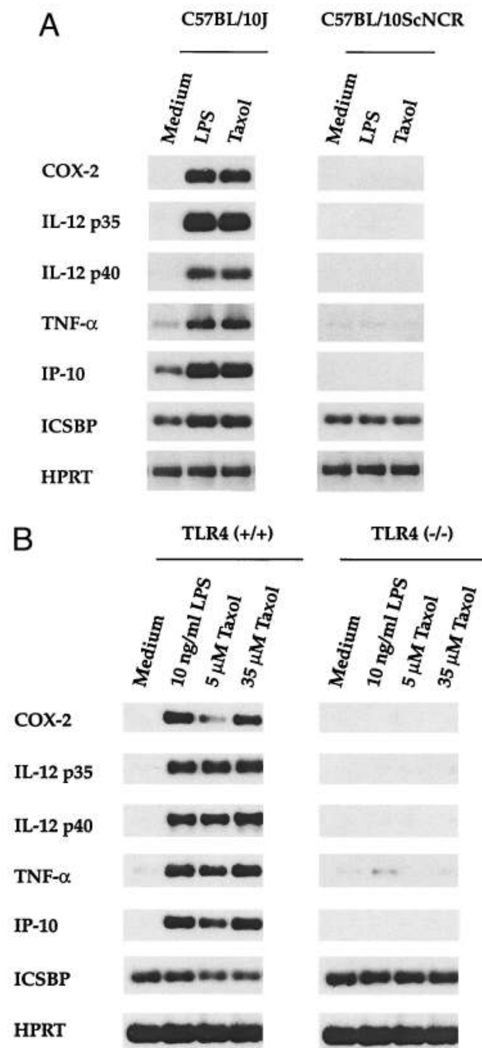

FIGURE 6. *A*, RT-PCR analysis of LPS- and Taxol-induced gene expression in macrophages derived from C57BL/10ScNCR and control mice. Macrophages from C57BL/10ScNCR and control mice were treated with medium, 35 μM Taxol, or 100 ng/ml LPS for 4 h. Total cellular RNA was generated, reverse transcribed, amplified, and detected as described in *Materials and Methods*. *B*, RT-PCR analysis of Taxol- and LPS-induced gene expression in macrophages derived from TLR4-deficient and control mice. Macrophages from TLR4-deficient and control littermate mice were treated with medium, Taxol (5 and 35 μM), or LPS (10 ng/ml) for 4 h. Total cellular RNA was generated, reverse transcribed, amplified, and detected as described in *Materials and Methods*.

for these two genes that are only generated in the presence of CD14. This also implies that TLR4 is necessary, but not sufficient, to induce the full panel of LPS-induced genes.

Despite the lack of ICSBP mRNA induction by high concentrations of LPS or Taxol in CD14-deficient macrophages, normal induction of IL-12 p40, an ICSBP-dependent gene, was observed. Similarly, although the constitutive expression of ICSBP mRNA was decreased upon the addition of a low concentration of Taxol in CD11b/CD18-deficient and control macrophages, detectable levels of IL-12 p40 mRNA were induced. These results imply the presence of an ICSBP-independent mechanism for the induction of IL-12 p40. These observations are consistent with those of Scharton-Kersten et al. (59), who reported that the ratio of IL-12 p40 mRNA levels in $ICSBP^{-/-}$ vs $ICSBP^{+/+}$ macrophages in response to LPS was 35%.

Table II. *Summary of LPS and Taxol dependency on TLR4, CD14, and CD11b/CD18 for induction of gene expression*[a]

Gene	TLR4	CD14 Low [LPS/Taxol]	CD14 High [LPS/Taxol]	CD11b/CD18
TNF-α	+	+	−	−
IL-12 p35	+	+	−	+
IL-12 p40	+	+	−	+[c]
COX-2	+	+	−	+
IP-10	+	+	+[b]	−
ICSBP	+	+	+	−

[a] +, Indicates dependency on the indicated gene; −, indicates independence.
[b] Taken from Ref. 26.
[c] Taken from Ref. 27.

Because cellular CD14 lacks signaling transmembrane and cytoplasmic regions and both GPI-anchored and integral membrane forms of CD14 mediate identical cellular responses to LPS (7, 8), it is likely that CD14 functions by binding LPS with high affinity and interacts with signal transducing receptors. Such a signaling role has been ascribed to several members of the TLR family because treatment of monocytic cells and cells engineered to express TLR2 and TLR4 with LPS have been reported to result in the activation of NF-κB by mechanisms that involve shared components of the IL-1 receptor signaling pathway, including MyD88, IRAK kinases, and TNFR-associated factor 6 (15, 16, 60). The importance of this pathway in LPS signaling has been corroborated by the absence of both IL-1 receptor-associated kinase and NF-κB activation in TLR4-deficient mice in response to LPS and, conversely, LPS unresponsiveness in mice with targeted mutations in MyD88 and TNFR-associated factor 6 genes (19, 61, 62). In contrast to high affinity binding of LPS to CD14 (6), Yang et al. (14) reported the binding of LPS to TLR2 to be of low affinity. Further confirmation of a signaling role for TLR4 was recently provided by Kawasaki et al. (63) who reported that although the cytoplasmic region of the TLR4 was essential for LPS- and Taxol-induced NF-κB activation, there was no demonstrable binding of LPS or Taxol to either TLR4 or to its obligatory cofactor, MD-2. Although our data support a role for CD14 in Taxol-mediated responses in murine macrophages, the mechanism is unclear. Similar to the aforementioned studies with TLR4, we were unable to demonstrate

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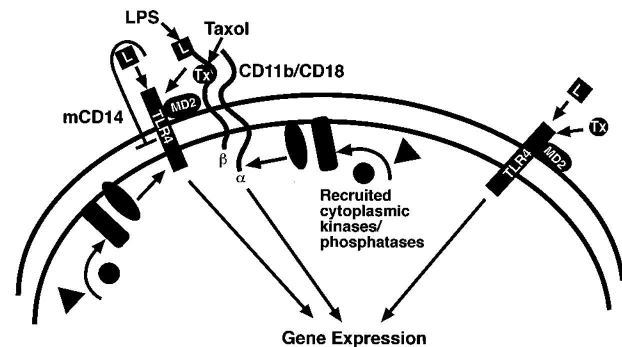

FIGURE 7. Proposed model for the interaction of CD14, TLR4, and CD11b/CD18 in LPS- and Taxol-induced signaling of murine macrophages. Interaction of all three molecules following ligand engagement is proposed to deliver a coordinated set of intracellular signals that results in the induction of the complete panel of LPS/Taxol-inducible genes. In the absence of either CD14 or CD11b/CD18, engagement of TLR4 by LPS/Taxol is postulated to deliver some, but not all, of the signals required for optimal gene expression, resulting in subset dependence described in Table II.

binding of a photoaffinity Taxol analog to CD14 using either purified soluble CD14 or isolated macrophage membrane preparations (27). It is more likely that CD14 somehow facilitates an interaction between Taxol-bound CD11b/CD18 and TLR4 that is necessary for induction of signaling leading to NF-κB translocation. However, signaling through TLR4 is necessary, but not sufficient, to elicit the entire panel of LPS-/Taxol-inducible genes. Additional studies (reviewed in Ref. 64) suggest that the physical interaction of β_2 integrins with GPI-linked proteins enables signal transduction upon binding of ligand to the GPI-linked protein through the activation of the G protein Rho, and the assemblage of an intracellular platform that includes protein kinase C, protein tyrosine kinases, and MAP kinases, components implicated in LPS signaling (reviewed in Ref. 64). Thus, the observations that LPS-induced NF-κB translocation (26) and MAPK activation are strongly depressed in both CD14 knockout macrophages as well as in CD11b/CD18-deficient macrophages (Fig. 2), and are eliminated in TLR4 knockout (19) and TLR4-defective C3H/HeJ macrophages (22, 25, 56), suggest that these three receptors work in concert to elicit optimal gene expression.

The model proposed in Fig. 7 illustrates this hypothetical interaction after ligand binding. Consistent with the reported interaction of CD14 and CD11b/CD18 following LPS treatment of cells (53), the LPS receptor complex, like the TCR complex, is undoubtedly comprised of additional signaling elements that are recruited to the complex upon appropriate ligand engagement. Among the other potential molecules that are likely to be recruited and participate in optimal signaling for gene expression, small G proteins (reviewed in Ref. 65), heat shock protein 90 (66), and others have been implicated in LPS- and/or Taxol-induced macrophage activation. The role of CD11b/CD18 might be analogous to that of CD14, where CD11b/CD18 functions to bind Taxol (via CD18) and LPS, whereas associated TLR4 molecules function as signal transducing receptors. Direct evidence for a ligand binding role for CD11b/CD18 in LPS signaling was first provided by Ingalls et al. (67) who determined that Chinese hamster ovary (CHO) cell transfectants that expressed mutant forms of CD11b/CD18 receptors with largely truncated cytoplasmic domains were still capable of eliciting an NF-κB-mediated response to LPS. Thus, it is likely that other receptors present on CHO cells, such as TLR4, are responsible for the signal transmission because CHO cells lack functional TLR2 molecules (68). The fact that the presence of CD11b/CD18 cannot fully compensate for the absence of CD14 in primary macrophages, as evidenced by the qualitative and quantitative differences in gene expression exhibited by macrophages from CD14-deficient mice, particularly at low concentrations of LPS, suggests that the affinities of the two receptors for LPS may differ significantly. It is also noteworthy that at high concentrations of LPS there is markedly reduced activation of signaling molecules, NF-κB (26), and MAPKs in CD14-deficient macrophages despite high levels of gene expression, suggesting that there is compensation by other receptors like CD11b/CD18 or TLRs.

Collectively, the data from this study indicate that the interaction of macrophages with LPS or Taxol leads to the formation of multimeric receptor complexes that elicit complex patterns of signaling, which, in turn, dictate which genes are activated. It is likely that these individual receptors are brought together following ligand binding and that each contributes toward the production of general signaling molecules, such that the overall effect of these individual receptor associations determines the threshold, strength, and the specificity of each response and the genes induced.

Acknowledgments

We thank Liyanage P. Perera, Karen M. Kopydlowski, Cindy A. Salkowski, and Jorge C. Blanco for advice and discussion.

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