Adenovirus Vector-Induced Innate Inflammatory Mediators, MAPK Signaling, As Well As Adaptive Immune Responses Are Dependent upon Both TLR2 and TLR9 In Vivo

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*J Immunol* 2008; 181:2134-2144; doi: 10.4049/jimmunol.181.3.2134

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Adenovirus (Ad) vectors are promising candidates for both gene transfer and vaccine applications. In this study, we investigated the role of TLR2 in innate and adaptive immune responses to Ad and/or the transgene it expresses following systemic injection. We found that Ad directly activates ERK1/2 in vivo, but that initiation of ERK1/2 activation is primarily a MyD88/TLR2-independent, but Kupffer cell-dependent, event. The complexity of Ad-induced innate immune responses was confirmed when we also found that both TLR2 and MyD88 functions are required for the sustained activation of ERK1/2. Although we found that the initial activation of NF-κB by Ads is dependent upon MyD88, but independent of TLR2 in (non-Kupffer cells) the liver, TLR2 significantly influenced the Ad-induced late phase NF-κB activation. These very rapid responses were positively correlated with subsequent innate immune responses to the Ad vector, as our results confirmed that the induction of several cytokines and chemokines, and the expression of innate immune response genes following Ad injection were TLR2 dependent in vivo. The requirement of TLR2 in Ad-induced innate responses also correlated with significantly altered adaptive immune responses. For example, our results demonstrate that the generation of Ad-neutralizing Abs, and anti-transgene-specific Abs elicited subsequent to Ad vector treatments, are both dependent upon TLR2 functionality. Finally, we found that several Ad-induced innate immune responses are dependent on both TLR2 and TLR9. Therefore, this study confirms that several (but not all) Ad-induced innate and adaptive immune responses are TLR dependent.


The role of TLRs in innate immune responses to pathogens in general has been an intense area of investigation (reviewed in Ref. 1). We have recently found that several important innate and primary adaptive immune responses generated following systemic administration of adenoviral vectors are partially mediated both in vitro and in vivo by the TLR adaptor proteins MyD88 and TRIF (2, 3). Furthermore, several recent investigations have identified the endosomally localized TLR9 as a partial mediator of Ad-induced innate responses (4–6). However, it is clear that TLR9 does not account for many Ad-induced, MyD88-dependent responses that we have previously reported. This indicates an additional mechanism may exist whereby multiple MyD88-dependent “Ad sensors” are required for the full elaboration of Ad-induced innate and adaptive immune responses in vivo. Furthermore, it is yet to be determined whether MyD88-dependent Ad-induced host gene transcriptional responses, as well as humoral and cellular adaptive immune responses generated against the virus or the transgene it expresses TIR domain-containing adapter inducing IFN-β (TRIF) are dependent upon TLRs (2).

TLR2 is most commonly known as the pathogen-associated motif pattern receptor for peptidoglycan and zymosan moieties present in Gram-positive bacteria or fungi, respectively. Recent studies have also shown that this receptor is a key mediator in responses to dsDNA viruses from the Herpesviridae family, including murine and human CMV as well as herpes simplex virus 1 (7–9). Most TLR2 responses are mediated via signaling through the intracellular adaptor MyD88. Because we have previously noted that many Ad-induced innate and adaptive immune responses appear to be partially, or fully, dependent upon MyD88 functionality, and that only a few of these responses appear to be dependent upon TLR9, we investigated a possible role for TLR2 in the innate and primary humoral response to Ad vectors. The results
presented in this study indicate that TLR2 is significantly involved in liver-specific MAPK activation, immune-specific gene expression, and plasma cytokine and chemokine inductions following administration of Ads. These TLR2-dependent innate immune responses correlate with TLR2-dependent generation of robust humoral responses against the virus capsid as well as the transgene expressed by Ad vectors.

### Materials and Methods

#### Ad vector production and characterization

The recombinant adenoviral vector, [E1-Δ]AdLacZ, is a vector which carries a CMV-LacZ transgene expression cassette which replaces the Ad E1 region of the Ad genome, and was constructed and grown to high titers on human 293 cells as previously described (10). Purification consisted of harvesting infected cell lysates, DNa and RNase treatment, and cesium chloride density gradient bandings per the method of Ng et al. (11). The purified vector preparation was extensively dialyzed against 10 mM Tris chloride density gradient bandings per the method of Ng et al. (11). The purified vector preparation was extensively dialyzed against 10 mM Tris (pH 8.0), and was stored in 1% sucrose, PBS at −80°C. The vector preparation was determined to be free of replication competent Ad by PCR using E1-specific primers and titered by SDS-disruption and OD260 spectrophotometry essentially as previously described (2, 12). The titer was further evaluated by in vitro transduction of 293 cells and enumeration of bacterial β-galactosidase staining cells as previously described. The viral particle (vp)/bacterial β-galactosidase transducing unit titer was ~15.1 (data not shown) (13, 14).

#### Animal procedures

All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee. Adult C57BL/6 and B6.129-Tlr2tm1lkj/F mice were purchased from The Jackson Laboratory. MyD88-KO mice were provided by Dr. S. Akira (Osaka University, Japan). Virus treatment of animals (2–4 mo in age) consisted of i.v. injection (via the retro-orbital sinus) of 200 µl of a PBS solution (pH 7.4), containing either 1.5 × 1010 or 7.5 × 1010 total virus particles of [E1-Δ]AdLacZ as previously described (2). Oligodeoxynucleotide (ODN)-2088 (InvivoGen) was resuspended in sterile PBS to a final concentration of 1 µg/ml. A total of 100 µg was mixed with virus before injection. Plasma and tissue samples were obtained and processed at the indicated times postinfection as previously described (2). Clodronate was a gift of Roche Diagnostics. It was encapsulated in liposomes as previously described (15). A total of 100 µl of the suspension of clodronate liposomes was injected i.v. 48 h before virus injection.

#### Cytokine and chemokine analysis

A mouse 7-plex multiplex-based assay was used to determine the indicated cytokine/chemokine plasma concentrations per the manufacturer’s instructions (Bio-Rad) via Luminex 100 technology essentially as previously described (19). To quantitatively assess LacZ activity, snap-frozen samples were homogenized and LacZ activity was quantified using a β-galactosidase activity detection kit (Strategene) as previously described (19).

#### β-galactosidase enzyme activity measurement/in situ X-gal staining

Sections of snap-frozen liver tissue obtained from sacrificed animals were rapidly embedded in OCT fluid, frozen, and subsequently sectioned at a thickness of 7 µm. Tissue sections were in situ stained for bacterial β-galactosidase (LacZ) expression using X-Gal substrate, as previously described (14). To quantitatively assess LacZ activity, snap-frozen samples were homogenized and LacZ activity was quantified using a β-galactosidase activity detection kit (Strategene) as previously described (19).

#### Ad genome copy number per diploid genome

To assess the number of Ad genome copies per cell, tissues were snap-frozen in liquid nitrogen and then crushed to a fine powder using a mortar and pestle. DNA was extracted from frozen mouse tissue as previously described (20). Copy numbers were assessed using real-time PCR-based quantification. PCR were performed on a ABI 7900HT Fast Real-Time PCR System using SYBR Green PCR Mastermix as described for qRT-PCR technique. Primers generated against the Ad5 Hexon gene have been previously described (18). As an internal control for ensuring adequate DNA amplification, liver DNA was quantified using primers spanning the GAPDH gene. Standard curves were run in duplicate and consisted of six half-log dilutions using total genomic DNA. Melting curve analysis confirmed the quality and specificity of the PCR (data not shown).

#### Bone marrow-derived macrophages (BMDMs)

BMDMs were harvested and cultured as previously described (21). Briefly, bone marrow cells were harvested from both the tibiae and femurs of mice and RBC were lysed. Cells were washed and plated in DMEM containing 10% FBS and 30% filtered conditioned medium from confluent cultures of L929 mouse fibroblasts. Cells were incubated overnight, passed into tissue-culture plates with fresh medium (DMEM/10% FBS/30% filtered conditioned medium/plasma/virus mixture was applied to cells and incubated for 4 days. Control samples were incubated with either virus alone or complete medium alone. CellTiter 96 AQueous One solution (Promega) was added to each dilution and incubated at room temperature for 4 h before application of 20,000 vp/cell Ads-LacZ. Media was collected 24 h after stimulation and analyzed using Bio-Rad 23-plex mouse cytokine reagents per the manufacturer’s protocol. Data presented are results from one experiment, but are representative of four independent repeats.

#### Neutralizing Ab assay

A total of 2 × 105 HEK293 cells were seeded in microwells in 125 µl of complete medium (DMEM, 10% FBS, penicillin streptomycin fungizone). Cells were cultured overnight in a 37°C, 5% CO2 incubator. Plasma was heat inactivated for 60 min at 56°C and brought to room temperature. Dilutions were made as indicated in complete medium in a total volume of 100 µl for each well. A total of 1.3 × 105 vp (~650 vp/cell) was next added to each dilution and incubated at room temperature for 1 h; 100 µl of medium/plasma/virus mixture was applied to cells and incubated for 4 days. Control samples were incubated with either virus alone or complete medium alone. CellTiter 96 AQueous One Solution (Promega) was added to each well and incubated for 2 h in a 37°C, 5% CO2 incubator. A total of...
FIGURE 1. Role of MyD88 and TLR2 in Ad vector-induced ERK phosphorylation and IκBα degradation. Wild-type C57BL/6, TLR2, and MyD88 KO mice were injected i.v. with adenoviral vectors (1.5 × 10^{11} vp/mouse). The livers were collected at the indicated time points, snap-frozen, and lysates were extracted as described in “Materials and Methods.” A, p-ERK1/2 and ERK2; B, IκBα and tubulin levels were determined by Western blot analysis using LI-COR Odyssey. Quantification was performed after normalizing the p-ERK levels to ERK2 and IκBα levels to tubulin, to control for loading. Statistics were completed using one-way ANOVA followed by Tukey’s post-hoc test; *p < 0.05; **p < 0.01; ***p < 0.001.

Ab-titering assay

ELISA-based titering experiments were essentially completed as previously described (22). Briefly, 5 × 10^6 vp/well or 2 μg of β-galactosidase protein/well was (each diluted in PBS) used to coat wells of a 96-well plate overnight at 4°C. Plates were washed with PBS-Tween 20 (0.05%) solution, and blocking buffer (3% BSA in PBS) was added to each well and incubated for 1 h at room temperature. For titering of total IgG Abs, plasma was diluted in blocking buffer as indicated, added to the wells, and incubated at RT for 1 h. Wells were washed using PBS-Tween 20 (0.05%) and HRP-conjugated rabbit anti-mouse Ab (Bio-Rad) was added at a 1/4000 dilution in PBS-Tween 20. Tetramethylbenzidine (Sigma-Aldrich) substrate was added to each well, and the reaction was stopped with 1 N phosphoric acid. Plates were read at 450 nm in a microplate spectrophotometer. Subisotype titering was completed using a hybridoma subisotypic kit (Calbiochem) using plasma at a dilution of 1/200.

Results

TLR2- and MyD88-dependent MAPK activation in liver tissue

We have previously shown that Ad induction of 1) a comprehensive spectrum of cytokines and chemokines; 2) the dysregulation of the expression of a significant number of host genes, as well as 3) humoral and adaptive immune responses to the Ad capsid or the transgene it encodes are dependent upon the TLR adaptor protein MyD88 (2, 3). Furthermore, recent studies have shown that TLR9, a MyD88 using receptor, is required for the induction of several subportions of the Ad-specific innate response (4–6, 24). However, because a significant MyD88-dependent innate response persists in TLR9-KO mice subsequent to i.v. Ad vector challenge, we evaluated the possibility that Ad interactions with TLR2 may also figure prominently in MyD88-dependent responses.

The exposure of Ad to multiple cell types in vitro results in a rapid induction of signaling pathways including MAPK pathways in general, as well as the NF-κB pathway (21, 25). Activation of these pathways has been linked to the Ad induction of cytokines and chemokines in vitro as well as Ad induction of inflammatory gene expression in vivo (25). However, direct in vivo evidence for MAPK pathway activation induced by Ads has not yet been demonstrated. In the present study, we determined the activation kinetics of a classical representative of MAPK pathways, ERK1/2 (using p-ERK1/2 Abs), as well as the degradation of IκBα (as a surrogate for NF-κB activation) in liver tissues up to 60 min after i.v. injection of 1.5 × 10^{11} vp of Ad/mouse, a dose we have used in previous studies to examine liver transcriptome responses (see Fig. 2 and Refs. 2 and 12). Liver tissue was collected at 15, 30, 45, and 60 min after Ad injection, and levels of p-ERK1/2 and IκBα were determined by Western blot analysis. In wild-type mice, Ad injection caused a rapid degradation of IκBα (~60–70% reduction, p < 0.05) and a rapid increase in p-ERK1/2 levels in liver tissue, both returning to baseline levels 60 min after Ad injection (Fig. 1, A and B, respectively). In contrast to the signaling kinetics in wild-type mice, we observed altered Ad-induced signaling kinetics in TLR2-KO mice, indicating a role for TLR2 in the late phases of signal transduction. More specifically, although the initial phase of IκBα degradation and ERK1/2 activation induced by Ad injection into TLR2-KO mice were similar to wild-type mice, both of these events returned to baseline more quickly in TLR2-KO mice. That is, both IκBα and p-ERK1/2 levels returned to baseline by 45 min after Ad injection in TLR2-KO compared with 60 min after Ad injection into wild-type mice (Fig. 1, A and B, respectively). In contrast to the Ad-injected TLR2-KO mice, degradation of IκBα was completely abolished in MyD88-KO mice following Ad injection (Fig. 1A). These results suggest that other MyD88-dependent mechanisms (TLRs) may be important for the initial phases of IκBα degradation, and by inference, NF-κB activation. The rapid “ reappearance” of IκBα in TLR2-KO mice suggests that TLR2 may be necessary for sustained NF-κB activation but not for the initial activation. Interestingly, the kinetics of ERK1/2 activation initiated by Ad injections into MyD88-KO mice was identical with that noted in Ad-injected TLR2-KO mice (Fig. 1B). We also quantified Ad

150 μl of medium from each well was removed into a clean microtiter plate and read at 492 in a microplate spectrophotometer. Blank subtracted OD_{492} values are reported.

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genomes using qPCR to verify that equal numbers of viral particles were injected into all animals at 30 min postinjection (data not shown).

Taken together, these data suggest that while sustained Ikb degradation after Ad injection is dependent upon TLR2 in conjunction with other MyD88-dependent pathways, the sustained activation of ERK1/2 appears to be solely mediated by the TLR2-MyD88 pathway. Furthermore, our study provides direct evidence that both ERK1/2 and NF-kB are activated by Ads in the murine liver, and that both TLR2 and MyD88 are in part required for Ad to maximally activate signaling through these pathways.

Innate immune gene expression following i.v. Ad injection requires TLR2 signaling

We have previously characterized the Ad induction of multiple liver genes after i.v. Ad injections, as well as characterized the MyD88-dependent host transcriptome responses evoked in the murine liver subsequent to high-level Ad transduction of hepatocytes using microarray-based technologies (2, 12). Furthermore, we previously revealed that an overrepresentation of NF-kB consensus sequences were present in the promoters of many Ad-dysregulated genes (3). To determine whether the expression of a subset of these genes was also dependent on TLR2 in vivo, we characterized the expression of various innate immune response genes in the livers of Ad-injected animals at both 1 and 6 h following i.v. injection of either 7.5 \times 10^{10} vp/mouse or 1.5 \times 10^{11} vp/mouse and compared these expression profiles to identically injected MyD88-deficient and wild-type C57BL/6 animals (Fig. 2). Genes in this panel include TLRs (TLR1, 2, 3, 6, 9), TLR adaptor and signaling proteins (TRIF, MyD88, TBK1), as well negative regulators of cytokine signaling (SOCS2, 3) and MAPK activation (MKP2). We also evaluated the induction of NOD-like receptors (NOD1, 2) and IFN response factor (IRF3, 7) transcripts. Following injection of 7.5 \times 10^{10} vp, we observed a dramatic induction of TLR2 (200-fold), TLR3 (40-fold), TLR6 (15-fold), and TLR9 (10-fold) expression in the liver tissues. Furthermore, these analyses revealed the requirement of TLR2 signaling in the expression of TLRs 6 and 9 as well as itself (p < 0.05). This indicates a positive feedback induction of TLR2 gene expression subsequent to Ad injection, and that the general induction of TLR-related genes following Ad injection is TLR2 dependent. In addition, we found that at 6 hours postinjection (hpi), Ad induction of NOD1 and NOD2 gene expression was also dependent on TLR2 (p < 0.05). TLR1, SOCS2, MKP2, and IRF3 were not statistically induced at this dose. In all of these cases of Ad-induced gene dysregulation, we observed an equal or more dramatic requirement for functional MyD88 protein, indicating that both TLR2 and MyD88 are required for Ad induction of host inflammatory gene responses. Although the Ad-induced expression of TLR3 and MyD88 were lower in MyD88-deficient animals, their induced expression was not dependent on TLR2 at this dose.

Following injection of 1.5 \times 10^{11} vp/mouse, Ad induction of TLR2, TBK1, and TLR6 was slightly, yet significantly, lower in TLR2-injected animals compared with Ad-injected C57BL/6 mice.
at 1 hpi ($p < 0.05$). Furthermore, at this dose of virus, we observed significantly reduced levels of TLR2, 3, 6, MKP2, and NOD2 gene expression in Ad-injected TLR2-deficient mice compared with identically injected C57BL/6 mice at 6 hpi ($p < 0.05$). The Ad-induced expression of MyD88, TBK1, SOCS2, and NOD1 were dependent on functional MyD88 protein, but independent of TLR2. Curiously, Ad induction of TLR9 was significantly higher in MyD88-deficient animals compared with either C57BL/6 mice or TLR2-deficient mice ($p < 0.01$). Although similar results were not observed in mice injected with lower doses of virus, these data suggest that in response to high doses of virus, TLR9 expression is induced in a MyD88-independent manner, and may indicate that the expression of this receptor is negatively regulated by MyD88. Interestingly, although IRF3 expression was not significantly induced by Ads in these studies, IRF7 expression was slightly dependent upon functional MyD88 protein expression at the high viral dose used in this study ($p < 0.05$). Because IRF proteins directly influence the induction of type I IFNs we also evaluated the presence of IFN-α and IFN-β transcripts. Surprisingly, there was not a detectable increase in these gene products at either 1 or 6 hpi (data not shown). In general, these results show that TLR2 is required for the full induction of many Ad-induced innate immune response genes, and that this induction also appears to be MyD88 dependent. Because we continue to see Ad induction of many of these genes in MyD88-deficient mice, MyD88-independent mechanisms also play a significant role in Ad-induced expression of immune response genes in the murine liver.

As negative regulators of MAPK proteins (e.g., p-ERK1/2, p-JNK, p38), the expression of MKP proteins is induced shortly after MAPK activation stimuli (26). Of particular interest, we observed a major defect in MKP-2 gene induction in the liver of both MyD88 and TLR2-deficient mice at 1 hpi following injection of $1.5 \times 10^{11}$ vp, relative to Ad injections of normal mice. Of note, we also evaluated the expression of additional members of the MKP family of genes including MKP1 and PAC1. In these cases, we did not observe a statistical induction in mRNA expression at any time point, or any dose tested (data not shown). However, the 15-fold induction of MKP-2 observed in Ad-injected wild-type animals indicates rapid induction of...
MAPK pathways, as well as the importance of TLR2 and MyD88 in the regulation of MAPK pathways in response to i.v.-delivered Ad.

Ad-induced cytokine responses are dependent on TLR2 and TLR9

It has been shown that TLR9 is a critical receptor in mediating the induction of IL-12 and IL-6 cytokines elicited following i.v. Ad delivery. In a similar vein, we used TLR2-KO mice to assess the role of this receptor in mediating the induction of plasma cytokine and chemokine levels following i.v. injection of $7 \times 10^{10}$ vp/mouse. In an attempt to determine the relative roles of TLR2 and TLR9 in the induction of plasma cytokines, we simultaneously injected a TLR9 antagonist, ODN-2088 (ODN), with Ad to inhibit TLR9 signaling in both C57BL/6 wild-type and TLR2-KO mice. Because MyD88 is required for both TLR9 and TLR2 signaling, we also compared these results to identically injected MyD88-KO mice (Fig. 3). At 1 h following i.v. injection, there were significantly lower circulating levels of IL-6 and MCP-1 in wild-type mice simultaneously injected with Ad and ODN. We did not observe a role for TLR2 at this time point. Furthermore, we did not observe a significant role for either TLR2 or TLR9 in the induction of KC or MIP-1β at any time point tested, although the induction of these factors was dependent on MyD88. Also, at 1 hpi there was a small, but significant role for TLR9 in the induction of G-CSF.

At 8 hpi, we observed significantly reduced levels of IL-12(p40), MCP-1, and RANTES in C57BL/6 mice simultaneously injected with Ad and ODN, relative to C57BL/6 mice injected with virus alone ($p < 0.01$). Similarly, we detected significantly lower levels of plasma MCP-1 and RANTES in TLR2-KO mice at this time point. When TLR2-KO mice were simultaneously injected with Ad and ODN, we observed significantly lower levels of MCP-1 when compared with either C57BL/6 mice injected with Ad, Ad/ODN, or Ad-injected TLR2-KO mice. However, complete reduction was not observed, as significantly lower levels of MCP-1 were found in Ad injected MyD88-KO mice. RANTES levels were also significantly lower in Ad/ODN-injected TLR2-KO mice compared with Ad-injected C57BL/6 mice; however, these levels were not statistically different from C57BL/6 mice injected with Ad/ODN, nor were they different from Ad-injected MyD88-KO mice, suggesting that although TLR2 partially mediates Ad induction of RANTES, inhibition of TLR9 is sufficient to reduce the levels of this protein to the level observed in MyD88-KO mice. We were unable to evaluate the role of TLR9 in the induction of G-CSF because it appeared that the injection of ODN stimulated an increase in plasma levels of this chemokine. However, we did determine that Ad-induced G-CSF levels were not dependent on functional TLR2. Replicate studies completed in TLR2-KO mice at 6 hpi yielded similar results (data not shown). An additional set of mice was also injected with a higher dose ($1.5 \times 10^{11}$ vp/mouse) of Ad-LacZ. We were not able to detect a significant role for TLR2 in the rapid increase in any cytokine or chemokine induced by Ad injection at this higher dose, suggesting there is a threshold of viral particles required to induce these cytokines in a TLR2-independent fashion (data not shown). Possibly, TLR2 augments Ad recognition at modest concentrations, but is not required at higher concentration.

Although the induction of MIP-1β and KC by Ads was confirmed to be dependent upon MyD88 functionality, we did not detect a role for either TLR2 or TLR9 in the induction of these innate response factors. In light of this, it is clear that in addition to TLR2 and TLR9, alternative MyD88-dependent immune receptors exist that are responsible for the induction of these important inflammatory mediators in response to Ad.

**FIGURE 4.** Transduction efficiency and levels of viral genomes are independent of TLRs. Livers from mice injected with $7 \times 10^{10}$ vp/mouse were harvested at 24 hpi. A, In situ β-galactosidase staining of sectioned livers at 24 hpi ($\times 200$ magnification). Seven-micrometer liver sections were cut from livers preserved in OCT compound, and stained with X-gal. The picture shown is representative of three independent mice. B, Quantification of β-galactosidase expression in Ad-LacZ-injected C57BL/6 (n = 4), TLR2-KO (n = 7), and MyD88-KO (n = 4) mice. No statistical difference was observed. C, qPCR was used to quantify Ad5-LacZ genomes in livers harvested from C57BL/6 (n = 3), TLR2-KO (n = 4), and MyD88-KO (n = 4) mice at 24 hpi. No statistical differences were observed. In all cases, bars represent mean ± SD.

It is possible that the altered responses observed in both TLR2-KO and MyD88-KO mice are a result of decreased liver transduction and/or transgene expression in TLR2- and MyD88-deficient mice vs their identically injected C57BL/6 counterparts. To eliminate this possibility, we used both histological and biochemical approaches to evaluate liver transduction (Fig. 4). In cryosectioned liver samples, we did not observe a difference in transgene expression in livers derived from either TLR2-KO or MyD88-KO mice compared with C57BL/6 mice. Furthermore, we also evaluated the abundance of viral genomes in liver tissue by way of qPCR. We did not detect statistically different levels of viral genomes (on a per cell basis) in Ad-injected TLR2 or MyD88-deficient mice as compared with Ad-injected C57BL/6 mice. Furthermore, plasma levels of ALT measured at 24 hpi did not indicate severe liver toxicity in any strain tested (data not shown).

**TLR-dependent and -independent induction of macrophage-derived innate responses and Kuffer cells**

Macrophages make up a large population of cells responsible for cytokine and chemokine responses as well as Ag presentation in
I extract as described in "Materials and Methods." p-ERK1/2, ERK2, and were collected at the indicated time points, snap-frozen, and lysates were levels to ERK2 to control for loading; bars represent the mean Cytokines and chemokines in the cell medium were detected using a bead-based mouse cytokine ELISA method. Bars represent the mean responses. A, BMDMs were exposed to 20,000 vp/cell Ad5-LacZ for 24 h. Cytokines and chemokines in the cell medium were detected using a bead-based mouse cytokine ELISA method. Bars represent the mean ± SD from three technical replicates. The figures represent one of four experimental repeats (n = 4 independent mice from each genotype) with consistent results. ** Statistical difference in values compared with the C57BL/6 mouse derived cells using a homoscedastic t test (p < 0.01). B, Fluorescence microscopy using F4/80-specific Ab was completed to verify effective elimination of Kupffer cells 48 h post-clodronate liposome administration. C, Wild-type C57BL/6 mice were injected i.v. with clodronate liposomes 48 h before i.v. Ad delivery (7.5 × 10^10 vp/mouse). The livers were collected at the indicated time points, snap-frozen, and lysates were extracted as described in "Materials and Methods." p-ERK1/2, ERK2, and IxBa levels were determined by Western blot analysis using LI-COR Odyssey. Quantification was performed by normalizing the p-ERK and IxBa levels to ERK2 to control for loading; n = 3. D, Plasma from mock or the context of a pathogenic infection. In this way, macrophages impact the development of adaptive immune responses by exposing T cells to Ag, and secreting regulatory cytokines and chemokines that influence T cell development (e.g., Th1, Th2 responses). Although murine macrophages are relatively resistant to Ad-mediated gene transduction, it has been shown that murine BMDMs are activated following Ad exposure (21). Although one study provides evidence for MyD88 required signaling in Ad exposed BMDMs, the requirement of TLR2 in this activation has not been elucidated (21). To address this, we measured the induction of cytokines and chemokines in the medium of BMDMs harvested from C57BL/6, TLR2-KO, and MyD88-KO mice 24 h following the exposure of 20,000 vp/cell of Ad (Fig. 5A). Interestingly, we detected only a partial role for both TLR2 and MyD88 in the induction of MCP-1 secretion from Ad exposed BMDMs. However, levels of secreted MCP-1 did not differ between TLR2-KO- and MyD88-KO-derived macrophages. This is consistent with our in vivo analyses and indicates that MCP-1 is secreted from macrophages in a TLR2-MyD88-dependent pathway. We did not detect a role for TLR2 in Ad-induced MIP-1β secretion in vitro, although in vitro MIP-1β induction was partially dependent on MyD88. Interestingly, we did not detect a significant difference in the secretion of any other cytokine tested, indicating that a more complex interaction of macrophages and other cell types exists in vivo that cannot be recapitulated in vitro. In an attempt to correlate in vitro cytokine induction with signaling, we examined p-ERK1/2 and IxBa levels in BMDMs at 3, 10, 20, 30, 60, and 90 min following Ad exposure in vitro. Surprisingly, we did not detect IxBa degradation or ERK1/2 activation at any time point tested in these cells (data not shown).

Kupffer cells are resident macrophages found within the liver sinusoids, and have been shown to interact with Ad within 10 min of i.v. injection. Because we have established a role for TLR signaling in the activation of both ERK1/2 and NF-κB in vivo, we asked whether Kupffer cells are mediating these responses to Ad. To address this question, we depleted Kupffer cells from mice liver before Ad injection using clodronate-laden liposomes. Immunofluorescence analysis of mouse liver tissue 48 h after clodronate liposome injection confirmed undetectable Kupffer cell staining using an Ab directed toward the macrophage-specific surface protein F4/80 (Fig. 5B). Although p-ERK1/2 levels were slightly higher in Kupffer cell-depleted liver tissue, we observed significantly less increases in Ad-induced p-ERK1/2 within 15 min post i.v. injection of 7.5 × 10^10 vp of Ad5-LacZ in Kupffer cell-depleted mice (Fig. 5C). However, we did not observe a significant difference in the Ad induction of IxBa degradation in these same mice. These data indicate that in the absence of liver-resident macrophages (i.e., Kupffer cells), cells of the murine liver retain their ability to up-regulate NF-κB activity in response to Ad. These results suggest that both Kupffer cells and non-Kupffer cells are important in specific Ad-induced signaling; Kupffer cells for ERK activation and non-Kupffer cells for NF-κB activation.

It has been previously demonstrated that portions of the Ad-induced innate immune response are reduced in macrophage-depleted mice (27–29). In this study, we expand upon these observations and show that Ad induction of both KC and MCP-1 are also reduced at both 1 hpi, and 6 hpi in macrophage-depleted mice (Fig. 5D). Furthermore, plasma levels of RANTES and IL-12(p40) virus-injected animals was harvested at 6 hpi, and cytokines and chemokines were analyzed using the multiplex bead-based ELISA analysis; n = 3 for each group. Bars represent mean ± SD. A homoscedastic t test was used to determine statistical differences between untreated and animals pretreated with clodronate liposomes; *, p < 0.05; **, p < 0.01.

FIGURE 5. The role of macrophages in Ad-induced innate immune responses. A, BMDMs were exposed to 20,000 vp/cell Ad5-LacZ for 24 h. Cytokines and chemokines in the cell medium were detected using a bead-based mouse cytokine ELISA method. Bars represent the mean ± SD from three technical replicates. The figures represent one of four experimental repeats (n = 4 independent mice from each genotype) with consistent results. ** Statistical difference in values compared with the C57BL/6 mouse derived cells using a homoscedastic t test (p < 0.01). B, Fluorescence microscopy using F4/80-specific Ab was completed to verify effective elimination of Kupffer cells 48 h post-clodronate liposome administration. C, Wild-type C57BL/6 mice were injected i.v. with clodronate liposomes 48 h before i.v. Ad delivery (7.5 × 10^10 vp/mouse). The livers were collected at the indicated time points, snap-frozen, and lysates were extracted as described in "Materials and Methods." p-ERK1/2, ERK2, and IxBa levels were determined by Western blot analysis using LI-COR Odyssey. Quantification was performed by normalizing the p-ERK and IxBa levels to ERK2 to control for loading; n = 3. D, Plasma from mock or the context of a pathogenic infection. In this way, macrophages impact the development of adaptive immune responses by exposing T cells to Ag, and secreting regulatory cytokines and chemokines that influence T cell development (e.g., Th1, Th2 responses). Although murine macrophages are relatively resistant to Ad-mediated gene transduction, it has been shown that murine BMDMs are activated following Ad exposure (21). Although one study provides evidence for MyD88 required signaling in Ad exposed BMDMs, the requirement of TLR2 in this activation has not been elucidated (21). To address this, we measured the induction of cytokines and chemokines in the medium of BMDMs harvested from C57BL/6, TLR2-KO, and MyD88-KO mice 24 h following the exposure of 20,000 vp/cell of Ad (Fig. 5A). Interestingly, we detected only a partial role for both TLR2 and MyD88 in the induction of MCP-1 secretion from Ad exposed BMDMs. However, levels of secreted MCP-1 did not differ between TLR2-KO- and MyD88-KO-derived macrophages. This is consistent with our in vivo analyses and indicates that MCP-1 is secreted from macrophages in a TLR2-MyD88-dependent pathway. We did not detect a role for TLR2 in Ad-induced MIP-1β secretion in vitro, although in vitro MIP-1β induction was partially dependent on MyD88. Interestingly, we did not detect a significant difference in the secretion of any other cytokine tested, indicating that a more complex interaction of macrophages and other cell types exists in vivo that cannot be recapitulated in vitro. In an attempt to correlate in vitro cytokine induction with signaling, we examined p-ERK1/2 and IxBa levels in BMDMs at 3, 10, 20, 30, 60, and 90 min following Ad exposure in vitro. Surprisingly, we did not detect IxBa degradation or ERK1/2 activation at any time point tested in these cells (data not shown).

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were significantly lower at 6 hpi in macrophage-depleted mice as compared with Ad-injected control mice. Of note, although we observed reduced levels of G-CSF, MIP-1 \( \beta \)/H9252, and IL-6 in clodronate liposome-treated mice compared with control mice, these levels did not reach statistical significance (data not shown). This contrasts with observations in MyD88-KO mice where the same inflammatory mediators were not significantly induced by Ad (Fig. 3 and Ref. 2). This underscores the importance of MyD88-mediated Ad sensing by “non-Kupffer” cells of the liver and/or cells derived from other tissues in vivo.

**Ad-neutralizing Ab titers and transgene-specific Ab responses are dependent on TLR2**

We have previously shown that Ad-specific primary humoral responses generated after i.v. injection are dependent upon MyD88 expression in mice (2). Similarly, it has been shown that depletion of Kupffer cells results in reduced generation of transgene-specific Abs, at least in the short term (27, 30). To determine whether TLR2 mediates these responses, we evaluated the neutralizing Ab titers in plasma derived from C57BL/6 animals vs TLR2-KO animals (\( p < 0.05 \)). ##, A statistical difference between control cells (cells not incubated with virus) compared with cells exposed to virus mixed with C57BL/6 plasma (\( p < 0.01 \)). B, At day 23, plasma was analyzed for anti-Ad capsid-specific total IgG Abs at the appropriate dilutions. Bars represent mean \( \pm \) SD.

**FIGURE 6.** Ad-neutralizing Ab titers are dependent on TLR2. C57BL/6 (\( n = 3 \)) and TLR2-KO (\( n = 3 \)) were injected with \( 7.5 \times 10^{10} \) vp/mouse of Ad5-LacZ. A, Plasma samples were analyzed for neutralizing Abs at days 10 and 23 postinjection at three different dilutions of plasma (see Materials and Methods). ****, A statistical difference, as measured by a homoscedastic \( t \) test, in neutralizing Abs in plasma derived from C57BL/6 animals vs TLR2-KO animals (\( p < 0.05 \)). ##, A statistical difference between control cells (cells not incubated with virus) compared with cells exposed to virus mixed with C57BL/6 plasma (\( p < 0.01 \)). B, At day 23, plasma was analyzed for anti-Ad capsid-specific total IgG Abs at the appropriate dilutions. Bars represent mean \( \pm \) SD.

C, Anti-Ad-specific Abs were evaluated at 2, 5, 10, 23, and 77 days postinjection. Data points represent mean \( \pm \) SD. *, **, A statistical difference between C57BL/6 and TLR2-KO mice (\( p < 0.05 \), \( p < 0.01 \), respectively).

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**A**

**Ad neutralizing antibody 10dpi**

**B**

**Anti Ad-capsid IgG**

**C**

**IgM**

<table>
<thead>
<tr>
<th>IgG1</th>
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**D**

**Antibody Levels**

**E**

**Antibody Levels**

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**F**

**Antibody Levels**

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We also assessed the humoral response specific to the Ad-encoded transgene (in this case β-gal). Like Ad-capsid-specific responses, we detected a robust β-Gal specific IgG response 23 days following i.v. injection of $7.5 \times 10^{10}$ vp of Ad5-LacZ into wild-type mice (Fig. 7A). However, we did not detect an altered response in TLR2-deficient mice. To evaluate whether differences in various Ab subtypes existed, we assayed mouse plasma for levels of transgene-specific subtypes (Fig. 7B). In this case, we found significantly lower levels of anti-LacZ-specific IgM, IgG2b, IgG3, and IgA Ab subtypes. These data indicate that although total anti-transgene IgG levels appear unaltered, TLR2 is required for efficient production of multiple Ab subtypes generated against an Ad5-delivered transgene.

**Discussion**

The use of viral vectors in general, and Ad-derived vectors specifically, remains one of the most promising tools in gene therapy and vaccine trials to date. However, the innate and adaptive immune responses generated by mammalian organisms against Ad have proven to be complex. Conversely, these immune responses have made these vectors more attractive to vaccine biologists, as the virus can theoretically be used as both an Ag-specific delivery vector as well as an adjuvant to stimulate adaptive humoral and cellular responses to the encoded Ag. In light of this, it has become increasingly important to understand the mechanisms by which immunity against Ad occurs within the host. The use of murine models with deficiencies in various innate immune signaling pathways, specifically TLR pathways, has allowed virology purists, and both gene therapy and vaccine biologists, to more accurately study the roles of these pathways in both innate and adaptive immune defenses generated by pathogens and viruses.

The role for TLR signaling in Ad-generated innate immune responses has recently been implicated using MyD88 as well as TLR9-deficient mouse models. Specifically, we have previously shown that Ad induction of cellular inflammatory gene expression in vitro, ~50% of the Ad-induced transcriptome in vivo, as well as the elaboration of numerous cytokines and chemokines by Ads in vivo are dependent upon functional MyD88 protein (2, 3). Furthermore, it was recently shown that TLR9 is required for helper-dependent Ad (HD-Ad) induced systemic IL-6, and IL-12 secretion in vivo (4). Importantly, because innate responses in these mice were blunted, but not as reduced as what we had previously reported in Ad-injected MyD88-KO mice, these studies suggested to us that the TLR9-derived signaling in response to Ads is but one of the various innate immune response pathways required for the full elaboration of these MyD88-dependent phenotypes. For these reasons, we investigated the role for TLR2 in the immune responses generated by delivery of Ad vectors.

Activation of MAPK signaling has been shown to be a critical event in immune signaling. Using pharmacological inhibitors, Tibbles et al. (25) indirectly showed that both p38 and ERK activation are important mediators of Ad-induced innate immune responses in vitro and in vivo. In this study, we provide direct evidence for the rapid induction of p-ERK1/2 in the murine liver following i.v. Ad injection. Furthermore, we found that the initial induction is MyD88 and TLR2 independent. However, sustained activation of ERK1/2 requires a TLR2-MyD88-independent mechanism. These nuances of the role of TLRs or TLR adaptors are not unfounded in TLR pathway signaling. For example, in LPS-induced TLR4 signaling, MyD88 is required for the early phase induction of NF-κB while TRIF is required for late-phase induction (31, 32). It is possible that a similar system exists in Ad-induced signaling whereby a MyD88-independent mechanism is required for the immediate induction of p-ERK1/2 whereas a TLR2-MyD88

![FIGURE 7](http://www.jimmunol.org/)
interaction is required for late-phase activation. In support of this, a recent publication suggests that induction of p-ERK1/2 in response to coxsackievirus B3 is mediated through a CAR-dependent (a MyD88-independent receptor) mechanism (33). Because pharmacological inhibition of various MAPK pathways in vivo alters the expression of proinflammatory genes, it is possible that altered MAPK-signaling kinetics in TLR2 and MyD88-KO mice may partially account for the reduced circulating cytokine and chemokine levels, as well as the reduced anti-Ad-neutralizing and anti-transgene Abs that we also observed subsequent to Ad injection in these mice.

It has been shown that NF-κB, a key transcription factor in modulating a vast array of immune responses, is activated by Ad infection of multiple cell types in vitro and liver tissue in vivo (21, 27, 34). In this analysis, we illustrate the kinetics of the rapid Ad-mediated induction of NF-κB in vivo. Our results implicate MyD88 as a key component in the rapid and sustained induction of NF-κB in mouse liver tissue following Ad administration. Furthermore, we show that late-phase NF-κB activity in response to Ad injection is dependent on functional TLR2 protein expression, and that liver macrophages (Kupffer cells) are not necessary for this response to occur. These data confirm a role for TLR2 in these responses, and also implicate a mechanism by which a minor reduction of cytokines is observed in TLR2 mice (compared with the global reduction found in MyD88 mice) in response to systemic Ad injection. It should be noted, however, that only mouse liver tissue was subjected to these analyses, and Ad interactions with other tissues may also play roles in these responses.

As a global modulator of immune response pathways, it seemed logical that immune regulatory genes, such as TLRs themselves, would be regulated in a TLR-NF-κB pathway. In fact, through microarray analysis we found that the promoter regions of a large proportion of Ad-dysregulated genes contained NF-κB-binding sites, and in a subsequent study, showed that many of these genes were induced through a MyD88-dependent mechanism in vivo (2, 3). In this study, we found that the induction of many of these genes (TLRs and NODs) also required TLR2. The ability of Ad-based vectors to dysregulate the expression of TLR and non-TLR genes underscores its potential usefulness in numerous vaccine applications, and also identifies possible areas of future intervention that may make these responses more robust.

We also show that Ad-induced increases in plasma levels of two chemokines are dependent on TLR2. These results have been summarized in Table I. Specifically, we found that the increase in MCP-1 and RANTES 8 hpi of Ad is TLR2 dependent, while Ad-induced KC and MCP-1 at 1 hpi, and IL-6, IL-12(p40), G-CSF, and MIP-1β at 8 hpi are TLR2 independent, yet MyD88 dependent. Interestingly, we also observed a critical role for TLR9 in Ad-induced IL-6 and MCP-1 levels at 1 hpi, an observation that has not previously been reported. We also observed additive roles for TLR9 and TLR2 in the induction of MCP-1 at 8 hpi. It is possible that upon Ad injection, TLR9 is required for the initial induction of MCP-1, whereas TLR2 is required for late-phase sustenance of MCP-1, paralleling our results observed in NF-κB and MAPK activation analyses. These results support studies completed in TLR9-KO mice, studies in which no significant role for TLR9 was found for MCP-1 elaboration at 6 hpi of an HD-Ad, unfortunately the induction of MCP-1 by the HD-Ad at 1 hpi was not evaluated (4).

The induction of RANTES at 8 hpi was also found to be dependent on both TLR2 and TLR9, although inhibition of TLR9 signaling resulted in RANTES levels equaling those found in Ad-injected, MyD88-KO mice. Importantly, in this report, we show that Ad induction of KC and MIP-1β is independent on both TLR2 and TLR9 at both 1 and 8 hpi. Taken together, these studies suggest that although both TLR2 and TLR9 play significant roles in certain aspects of the innate immune response to Ad, additional MyD88-dependent sensors are also active in these pathways.

As first-line immune defenders, macrophages are a main source of the high levels of cytokines and chemokines observed following pathogenic infections. Interestingly, in cultures of BMDMs, we did not observe a required role for MyD88 or TLR2 in the induction of many Ad-induced cytokines evaluated in this study. This is in contrast to recent reports indicating a role for MyD88 in Ad induction of IL-6 in this cell type (21). We did, however, reveal a TLR2-MyD88-dependent induction of MCP-1, and a MyD88-dependent induction of MIP-1β. Because our in vitro analyses fail to completely match our in vivo data, the results suggest that Ad interactions with other cell types (e.g., dendritic cells, macrophages, endothelial cells, and blood factors) may also impact levels of systemic cytokines and chemokines found in vivo. We expand upon this hypothesis by characterizing the signaling events occurring within the murine liver devoid of Kupffer cells. Intriguingly, we did not see a significant difference in Ad-induced IkBα degradation underscoring a potential role for hepatocytes, or endothelial cells in response to systemically delivered Ad. However, we did observe a required role for Kupffer cells in the induction of ERK phosphorylation in the liver. Therefore, the initiation of ERK activity in the liver is uniquely Kupffer cell dependent, yet TLR independent. Furthermore, we found that a number of Ad-induced cytokines and chemokines were elaborated in a Kupffer cell-dependent manner, correlating these inductions with ERK activation. It is clear that MAPK pathway activation is a critical determinant in the heightened immune response after Ad injection, further delineating the mechanism by which both NF-κB and ERK are activated by Ad will clear the way for potential areas of targeted intervention.

Generation of neutralizing Ab is a major concern in all viral-mediated gene transfer strategies, as repeated injection strategies are hampered by rapid neutralization of virus in non-naive hosts. Importantly, we found a substantial role for TLR2-mediated signaling in the generation of neutralizing Ab to the virus at both 10 and 23 days post-i.v. injection. Furthermore, we also found decreased anti-transgene-specific Ab responses in TLR2-deficient mice i.v. injected with Ad, relative to identically injected normal mice. Thus, the use of TLR2 antagonists in a gene therapy application may attenuate the neutralizing Ab response to Ad vectors. Consequently, it may be possible to overcome major limitations of the usage of Ads in gene transfer strategies, namely the inability to

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<tr>
<th>Table 1. Requirement of TLR9, TLR2, and MyD88 for Ad-induced cytok chemokines</th>
<th>IL-6</th>
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<sup>a</sup> Ad-induced KC and MIP-1β require MyD88, but are independent of both TLR9 and TLR2. IL-12(p40) and RANTES at 1 hpi and KC at 8 hpi are not elevated over mock-injected mice.

<sup>b</sup> Induction of MCP-1 at 8 hpi depends on both TLR2 and TLR9 and is additive.

<sup>c</sup> Induction of RANTES at 8 hpi depends on both TLR2 and TLR9, but is not additive. NA, not applicable; ND, not detected.
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