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The Tumor Suppressor ARF Regulates Innate Immune Responses in Mice

Paqui G. Través,*1 Raquel López-Fontal,†,1 Alfonso Luque,†,‡ and Sonsoles Hortelano†,§

The innate immune system is the first line of defense against invading organisms, and TLRs are the main sensors of microbial components, initiating signaling pathways that induce the production of proinflammatory cytokines and type I IFNs. An antiviral action for the tumor suppressor alternative reading frame (ARF) has been reported; however, the precise role of ARF in innate immunity is unknown. In this study, we show that ARF plays an important role in regulation of inflammatory responses. In peritoneal macrophages and bone marrow-derived macrophages from ARF-deficient animals, the induction of proinflammatory cytokines and chemokines by TLR ligands was severely impaired. The altered responses of ARF−/− cells to TLR ligands result from aberrant activation of intracellular signaling molecules including MAPKs, IkBo degradation, and NF-kB activation. Additionally, animals lacking ARF were resistant to LPS-induced endotoxic shock. This impaired activation of inflammation in ARF−/− mice was not restricted to TLRs, as it was also shown in response to non-TLR signaling pathways. Thus, ARF−/− mice were also unable to trigger a proper inflammatory response in experimental peritonitis or in 12-O-tetradecanoylphorbol-13-acetate–induced edema. Overexpression of ARF, but not its downstream target p53, rescued the ARF-deficient phenotype, increasing TLR4 levels and restoring inflammatory reaction. An increase in the E2F1 protein levels observed in ARF−/− macrophages at basal condition and after LPS stimulation may be involved in the impaired response in this system, as E2F1 has been described as an inflammatory suppressor. These results indicate that tumor suppressor ARF is a new regulator of inflammatory cell signaling. The Journal of Immunology, 2011, 187: 6527–6538.

Innate immunity constitutes the first line of host defense in multicellular organisms. Its functions include the monitoring of tissue homeostasis, protection against invading or infectious pathogens, elimination of damaged cells, and the prevention of tumor development. An important aspect of the anti-tumor function of the immune system is the rapid elimination of pathogens and the resolution of inflammation to prevent the persistence of an inflammatory environment that can promote tumorigenesis. TLRs recognize specific microbial pathogen-associated molecular patterns and play critical roles in host defense against invading pathogens. Upon ligand binding, TLRs activate complex signaling networks that induce the production of proinflammatory cytokines, the acquisition of a phagocytic phenotype, and the initiation of subsequent innate effector mechanisms that can enhance host anti-tumor responses (1). Different TLRs sense and transmit signals by detecting different pathogen components. For example, TLR2, TLR3, TLR4, and TLR9 are respectively activated by lipoproteins/peptidoglycans (2), viral RNA, LPS (3), and bacterial DNA (4). Activated TLRs initiate a plethora of signaling cascades via specific adapter proteins such as MyD88, MyD88-adaptor–like (also known as TIR domain-containing adapter protein), TIR domain-containing adapter inducing IFN-β (TRIF), and TRIF-related adapter molecule (5). All TLRs except TLR3 recruit the adapter molecule MyD88 through the TIR domain, triggering the so-called MyD88-dependent pathway. TLR3 activates a MyD88-independent pathway that signals through TRIF, whereas TLR4 signals through the MyD88 and the TRIF pathways (5). Stimulation of TLRs leads to activation of the NF-kB, MAPK, and IFN regulatory factor signaling pathways (6). These signals are essential for the classical outcome of TLR activation: the orchestration of host innate and adaptive immune responses.

An unexpected involvement in innate immunity has been reported for the tumor suppressor alternative reading frame (ARF), which has been shown to play a role in the surveillance of viral infection (7). ARF is among the most important oncogenic stress sensors in mammalian cells, often being mutated in cancer (8). The INK4a/ARF locus encodes two unrelated proteins, p16INK4a and p19ARF (p14ARF in humans), that respectively regulate the activity of two tumor suppressors, Rb and p53 (9, 10). ARF is expressed at relatively low levels in the tissues of young humans and rodents but is induced in aging tissues and in response to abnormal proliferative signals. These signals include prolonged in vitro culturing (11) and the inappropriate expression of proliferative oncogenes including...
activated Ras, c-myc, E2F, E1A, and v-Abl (9, 12–14). The tumor-suppressor action of ARF derives from its interaction with Mdm2, which inhibits Mdm2-mediated proteasomal degradation of p53 and thus results in activation of the p53 pathway (12, 15–18). However, ARF functions are not confined to the ARF–Mdm2–p53 pathway. Indeed, several p53-independent actions of ARF have recently been described (19), including antiviral response (7). Several reports have identified E2F1 as a potential mediator of ARF functions independently of p53 (20–22). Thus, ARF may target E2F1 for its degradation, thereby inhibiting E2F1-dependent transcription (20–22). The transcription factor E2F1 is one of the key proteins in the regulation of the G1/S phase transition, acting as a critical regulator of cell survival and proliferation (23). In addition, E2F1 has been shown to regulate a wide range of genes in response to inflammatory stimulation of macrophages and to contribute to T cell activation in response to pathogens, suggesting that E2F1 may act as an anti-inflammatory or immunosuppressive transcription factor (24–28).

The precise role of ARF in innate immunity is not fully understood. In this study, we examined the role of ARF in inflammatory processes. Our results show that ARF is required for TLR-induced cytokine and chemokine production and efficient NF-κB and MAPK signaling. Thus, ARF-deficient mice are resistant to LPS-induced endotoxic shock. The impaired activation of inflammatory responses in ARF−/− cells was also seen in response to independent-TLR pathways as those elicited by 12-O-tetradecanoylphorbol acetate (TPA) and thioglycolate. We have also observed an increase in the E2F1 protein levels in ARF−/− macrophages at basal condition and after LPS stimulation. These data suggest that E2F1 may be a potential mediator of the ARF functions in regulation of inflammatory processes. Overall, these findings indicate that the tumor suppressor ARF plays an unexpected role as a regulator of inflammatory cell signaling.

Materials and Methods

Reagents

Ultrapure 0111:B4 LPS from *Escherichia coli*, purified *Staphylococcus aureus* lipoteichoic acid (LTA), polynonisin-polycytidylic acid (poly(I:C)), and CpG-DNA were purchased from InvivoGen (San Diego, CA). The mouse RT² Profiler PCR Inflammatory Cytokines and Receptors Array, the RT² First Strand Kit, and SYBR Green/Fluorescein qPCR Master Mix were from SABiosciences (Frederick, MD). TRZol reagent was from Invitrogen (Carlsbad, CA). TPA was from Sigma-Aldrich (St. Louis, MO). Difco thioglycolate broth was from Becton-Dickinson (Franklin Lakes, NJ). Bio-Plex cytokine assay was from Bio-Rad (Hercules, CA). Abs to total MAPK and phosphorylated forms (p38, JNK-SAPK, and ERK) were from Cell Signalling Technology (Beverly, MA). Anti-p19ARF, anti-iNOS, and anti–COX-2 were from Abcam (Cambridge, U.K.), anti-β-Actin, anti-p65, anti-p53, anti-p21, and anti-E2F1, and anti–β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-polypyrrolidone tract-binding protein-associated splicing factor (anti-PSF) was from Sigma. Western blot reagents (polyvinylidene difluoride membranes and ECL kit) were from GE Healthcare (Pittsburgh, PA). CytomaxCount counting beads were purchased from DakoCytomation (Carpinteria, CA). The Mouse Macrophage Nucleofector Kit was from Amaxa (Walkersville, MD).

Animals

All procedures involving animals were carried out in accordance with European Union guidelines and Declaration of Helsinki principles for the handling and use of laboratory animals. Studies were performed on 12-wk-old ARF+/- (wild type; WT), ARF+/+ (transgenic [TG]), and ARF−/− (knockout) mice on the C57BL/6J genetic background. Peritoneal macrophages and BMDMs were untreated or stimulated with LPS (200 ng/ml), LTA (10 μg/ml), poly(I:C) (30 μg/ml), and CpG-DNA (8 μg/ml) for different times.

Cytokine assay

Cytokine production by cultured macrophages was quantified by the Bio-Plex cytokine assay following the supplier’s instructions (Bio-Rad Laboratories). Serum TNF-α and IL-6 were measured with ELISA kits from R&D Systems according to the manufacturer’s instructions.

Determination of NO synthesis

NO was measured by the Griess reaction as previously described (29). Briefly, NO release was determined spectrophotometrically as the accumulation of nitrite in the medium. Absorbance at 548 nm was compared with standard NaNO2 solutions.

RNA analysis and quantitative PCR

Total RNA was isolated from cells and mouse tissues with TRZol reagent (Invitrogen). Quantitative PCR (SYBR Green) analysis was performed with an ABI 7900 sequence analyzer as described (29). Each sample was run in duplicate, and all samples were analyzed in replicate for the expression of the housekeeping gene 36B4 (acidic ribosomal phosphoprotein P0), which was used as an endogenous control for normalization of the expression level of target genes. Fold induction was determined from mean replicate values. Primer sequences are available on request.

RT² PCR array

Expression of inflammatory genes was evaluated with the mouse RT² Profiler PCR Inflammatory Cytokines and Receptors Array (SABiosciences). Two micrograms of RNA were used for cDNA synthesis with the RT² First Strand Kit (SABiosciences). The RT² Profiler array was probed according to the manufacturer’s protocol using the Profiler PCR Array System and SYBR Green/Fluorescein qPCR Master Mix (SA-Biosciences) in an ABI 7900 sequence analyzer (Applied Biosystems). Gene expression was compared with the dedicated Web-based software package (http://www.superarray.com/prcr/arrayanalysis.php), which automatically performs all ΔΔCt based fold-change calculations from the specific uploaded raw threshold cycle data. Microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database and are accessible through GEO Series accession number GSE32446 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32446).

Cytosolic and nuclear extracts

Cells were washed twice with ice-cold buffer A (10 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF) containing 120 mM NaCl and protease inhibitor mixture (Sigma) and scraped off the plate. Cells were lysed at 4°C with 0.2 ml buffer A supplemented with 0.5% Nonidet P-40 and under continuous shaking. After centrifugation, the supernatant was stored at −80°C (cytosolic extract), and the pellets were resuspended in 50 μl buffer A supplemented with 20% glycerol–0.4 M KCl and gently shaken for 30 min at 4°C. Nuclear protein extracts were obtained by centrifugation at 13,000 × g for 15 min, and the supernatant was stored at −80°C. Protein content was assayed with the Bio-Rad protein reagent. All cell fractionation steps were carried out at 4°C.

Western blot analysis

Western blots of cell extracts were prepared and incubated with the appropriate primary and secondary Abs (Supplemental Table I). After treatment with 100 mM 2-mercaptoethanol, 2% SDS in TBS, and heating at 80°C for 30 min, blots were sequentially reprobed with Abs. Blots were developed with ECL, according to the manufacturer’s instructions (GE Healthcare). β-Actin was used as a loading control for whole-cell and cytosolic extracts, and anti-PSF was used as a loading control for nuclear extracts.
Coimmunoprecipitation studies

For coimmunoprecipitation, cell nuclear extracts (25 μg) were brought to a final volume of 250 μl with buffer containing 10 mM PBS, 50 mM KCl, 0.05 mM EDTA, 2.5 mM MgCl₂, 8.5% glycerol, 1 mM DTT, 0.1% Triton X-100, and BSA 2% for 6 h at 4°C and incubated with 4 μg anti-p65. Immunocomplexes were captured by incubating the samples with 50 μl protein A–agarose suspension (Santa Cruz Biotechnology) overnight at 4°C on a rocker platform. Agarose beads were collected by centrifugation and washed three times with PBS containing protease inhibitors. After microcentrifugation, the pellet was resuspended with 25 μl SDS-PAGE sample buffer and boiled for 5 min at 100°C. The resultant supernatant was subjected to electrophoresis on 10% SDS-PAGE and immunoblotted with the corresponding Abs.

NF-κB (p65) transcription factor binding activity

The NF-κB (p65) transcription factor binding activity in macrophage nuclear extracts was measured with the NF-κB (p65) Transcription Factor Assay Kit according to the manufacturer’s instructions (Rockland, Gilbertsville, PA).

Macrophage transfection

Peritoneal macrophages were transiently transfected using Nucleofector II (Amaxa) according to the manufacturer’s instructions. Briefly, pCMVSPORT mouse TRP53 cDNA expression vector (Open Biosystems), the pLPC mouse p19ARF cDNA expression vector (a gift from M. Serrano, Centro Nacional de Investigaciones Oncológicas, Madrid, Spain), and empty vector control (pcDNA; 1 μg DNA) were delivered to macrophages (10⁶) using Amoxa Mouse Monocyte Nucleofector kit (VPA-1009). Transfection efficiency using protocol Y001 (78.5%) was determined using the proprietary pmaxGFP vector provided by Amaxa and flow cytometric analysis of 50,000 cells.

Thioglycolate-induced neutrophil influx

Mice were injected i.p. with 2.5 ml sterile thioglycolate (3% w/v). Peritoneal exudates were collected in cold PBS at various times after challenge.

Flow cytometry analysis of TLR expression

The expression of TLR4 and TLR2 in peritoneal macrophages from WT and ARF⁻/⁻ mice was determined by flow cytometry. One hundred microliters of a suspension containing 1 × 10⁶ peritoneal macrophages were incubated at 4°C for 30 min with 0.4 μg anti-mouse TLR4–PE or anti-mouse TLR2–FITC (clone UT41 and 6C2, respectively; eBioscience, San Diego, CA) in combination with 0.4 μg anti-mouse F4/80–PE–Cy5 (clone BM8; eBioscience). After washing, cells were analyzed by use of a FACSCalibur (Becton Dickinson Biosciences, San Jose, CA). Macrophages were gated on the basis of forward and side scatter and were >95% positive for F4/80. Expression of PE-labeled TLR4 and FITC-labeled TLR2 on the gated population (1 × 10⁶ cells) was analyzed using CellQuest software (Becton Dickinson).

Analysis of leukocyte populations by flow cytometry

Total leukocytes in peritoneal lavage were counted by flow cytometry using Cytocounts number-calibrated microbeads (DakoCytomation). Analysis of leukocyte populations was performed by flow cytometry after staining with 0.2 μg anti-mouse F4/80–FITC (clone BM8; eBioscience) and 0.2 μg anti-mouse Ly-6G(Gr-1)–PE–Cy7 (clone RB6-8C5; eBioscience) to detect monocytes/macrophages and neutrophils/granulocytes, respectively. Events were recorded with a FACSCalibur (Becton Dickinson Biosciences).

Peripheral blood analysis

Blood was obtained by retro-orbital venous plexus sampling in polypropylene tubes containing EDTA. Complete blood counts were determined using an automated cell counter (Abacus).

Table I. Inflammatory gene expression in LPS-elicited WT and ARF⁻/⁻ macrophages

<table>
<thead>
<tr>
<th>Chemokines</th>
<th>Cytokines</th>
<th>Other Genes</th>
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<tbody>
<tr>
<td>Symbol</td>
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<td>ARF⁻/⁻</td>
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<tr>
<td>Ccl1</td>
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<td>Ccl2</td>
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Peritoneal macrophages from WT and ARF⁻/⁻ mice were stimulated with LPS (200 ng/ml, 4 h), and expression of inflammatory genes was analyzed by RT² PCR analysis.
Mouse ear edema and myeloperoxidase activity

TPA (2.5 μg) dissolved in 20 μl acetone was applied in 10-μl volumes to both the inner and outer surfaces of the right ear of mice (20–25 g). Animals were sacrificed after 4 h, and equal-sized samples of both ears were homogenized in 750 μl saline and centrifuged at 10,000 × g for 15 min at 4°C. Myeloperoxidase activity was measured in aliquots of supernatants by a modification of the method of Suzuki et al. (32). The reaction mixture contained 50 μl supernatant, 150 μl PBS, 20 μl 0.22 M NaH₂PO₄ (pH 5.4), 20 μl 0.026% (v/v) H₂O₂, and 20 μl 18 mM tertamethylbenzidine in 8% (v/v) aqueous dimethylformamide. After 10-min reaction at 37°C, 30 μl sodium acetate (1.46 M, pH 3) was added, and the absorbance at 620 nm was read with a microtiter plate reader.

LPS-induced endotoxic shock

Mice were challenged by i.p. injection of 10 mg/kg LPS, and survival was monitored for 6 d. Serum was collected at 2 h after injection.

Statistical analysis

Data shown are means ± SD (n = 3). Statistical significance was estimated by Student t test for unpaired observations, using the InStart program (GraphPad Software). Differences in survival values were compared by Fisher exact test. Differences were considered significant at p < 0.05.

Results

Induction of proinflammatory cytokines in response to TLR ligands is impaired in ARF−/− mice

Macrophages are a major cellular component of all inflammatory situations, generating proinflammatory cytokines such as TNF-α, IL-1, and IL-6 that are central to the initiation and maintenance of inflammation (33). To determine whether ARF plays a role in inflammatory gene expression, we used an 84-gene RT² PCR array to examine the expression of inflammation-associated genes in WT and ARF-deficient macrophages treated with the TLR4 ligand LPS. Genes whose expression in WT cells was increased 2-fold or more upon LPS stimulation were classed as LPS-inducible genes. By this criterion, 56 LPS-inducible genes were identified (Table I). Of these genes, 51 were expressed at a lower level in LPS-treated ARF−/− cells, including classical inflammatory genes such as TNF-α and IL-1β (Table I, Fig. 1A), and 4 genes were expressed at a similar level. In contrast, the anti-inflammatory cytokine IL-10 was highly upregulated in ARF−/− cells (Fig. 1A). Consistent with the RT² PCR array results, the culture supernatants of ARF−/− LPS-treated thioglycolate-elicited macrophages showed a weaker accumulation of TNF-α and IL-1β than WT counterparts (Fig. 1B, 1C), and similar differences in cytokine production were observed after LPS stimulation of BMDMs for 24 h (Fig. 1D).

ARF deficiency also impaired cytokine production induced by ligands for TLR2 (LTA), TLR3 [poly(I:C)], and TLR9 (CpG-DNA) in peritoneal macrophages and BMDMs (Fig. 2A, Supplemental Fig. 1). To investigate the mechanisms by which ARF participates in inflammatory pathways, we next examined the activation of the proinflammatory enzymes iNOS and COX-2. Treatment of WT macrophages with poly(I:C) or LPS induced the release of NO (Fig. 2B). As with cytokine production, poly(I:C)- and LPS-induced increases in NO production were significantly lower in macrophages from ARF−/− mice (Fig. 2B), and this was paralleled by a weaker induction of macrophase iNOS protein expression in ARF−/− mice (Fig. 2C). A similar impairment was observed in the induction of COX-2 protein (Fig. 2C). Quantitative PCR analysis revealed that the induction of iNOS and COX-2 was impaired at the mRNA level (Fig. 2D), suggesting a role of ARF on the transcriptional activation of these inflammatory genes. LPS or poly(I:C) challenged ARF−/− macrophages also showed impaired gene induction of the proinflammatory cytokine IL-6 (Fig. 2E). These results indicate that ARF plays an important role in TLR-mediated production of inflammatory cytokines and inflammatory mediators.

ARF deficiency decreases NF-κB and MAPK activation in LPS-stimulated macrophages

TLR engagement culminates in the induction of various inflammatory cytokines and inflammatory mediators, such as IL-6, iNOS, and COX-2, whose gene promoters contain binding sites for NF-κB and transcription factors activated by MAPK cascades (ERK, JNK, and p38 MAPKs). Examination of LPS-treated macrophages showed that iκB degradation, NF-κB (p65) nuclear translocation, and MAPK phosphorylation were all impaired in ARF−/− macrophages (Fig. 3A). Moreover, LPS-induced p65 DNA binding activity was markedly lower in ARF−/− macrophages than in WT cells (Fig. 3B). These data thus suggest that ARF has a critical role in the activation of NF-κB and MAPK signaling pathways induced by LPS.

TLR expression is impaired in ARF−/− macrophages

We next explored the possibility that TLR ligands could regulate ARF expression. In mice, the INK4a/ARF locus is expressed at
very low levels and is generally detectable only in old mice (13, 34, 35). We therefore used quantitative PCR to detect INK4a and ARF mRNA. LPS or poly(I:C) stimulation had no effect on the levels of ARF mRNA in WT macrophages (Fig. 4A). However, as expected by previous data (7, 36), IFN-γ treatment of peritoneal macrophages from WT animals induced mRNA and protein expression of ARF (Fig. 4B and 4C, respectively). ARF−/− cells were used as control. In addition, the absence of ARF was not associated with a compensatory increment of INK4a expression after LPS treatment (Fig. 4D). These results suggest that the function of ARF in the inflammatory response is not due to direct regulation by LPS or poly(I:C).

To address whether the decreased inflammatory response of ARF−/− macrophages is due to altered TLR expression, we analyzed TLR expression by quantitative PCR and flow cytometry. The basal mRNA expression of TLR2 and TLR4 was significantly lower in ARF−/− macrophages compared with WT mice (Supplemental Fig. 2A, 2B) with no differences in the number of cells expressing these receptors (Supplemental Fig. 2C). However, TLR2 and TLR4 expression in spleen is unaffected, indicating that this regulation is tissue specific (Supplemental Fig. 2A, 2B). Indeed, splenocyte responses to LPS are the same in WT and ARF−/− mice showing similar release of TNF-α and IL-6 (Supplemental Fig. 3).

Overexpression of ARF rescues the inflammatory defects of ARF−/− macrophages

To demonstrate that the signaling defects in ARF−/− cells are in fact due to the absence of ARF, we transiently transfected ARF−/− macrophages with an ARF expression plasmid. ARF−/− macrophages versus WT macrophages (TLR4: 80.2 versus 91.3%; TLR2: 77.2 versus 90.9%) (Fig. 4F). Consistent with previous reports (3, 37), stimulation with LPS reduced the mRNA expression of TLR4 in WT macrophages (Fig. 4G); however, there was no significant reduction in TLR4 mRNA levels in LPS-stimulated ARF−/− cells. LPS-induced expression of TLR2 mRNA was also impaired in ARF−/− macrophages (Fig. 4G). Additionally, in vivo analysis showed that TLR2 and TLR4 expression is lower in liver and heart tissue of ARF−/− mice compared with that in WT (Supplemental Fig. 2A, 2B) with no differences in the number of cells expressing these receptors (Supplemental Fig. 2C). However, TLR2 and TLR4 expression in spleen is unaffected, indicating that this regulation is tissue specific (Supplemental Fig. 2A, 2B). Indeed, splenocyte responses to LPS are the same in WT and ARF−/− mice showing similar release of TNF-α and IL-6 (Supplemental Fig. 3).
FIGURE 4. ARF positively regulates TLR expression in macrophages. A. Quantitative PCR analysis of ARF mRNA expression in WT macrophages stimulated with LPS or poly(I:C) for the indicated times. B. Peritoneal macrophages from WT and ARF−/− mice were stimulated with IFN-γ (100 ng/ml) for 4 h, and p19ARF mRNA expression was detected by Western blot. C. ARF mRNA expression was determined by quantitative PCR. D. Peritoneal macrophages from WT and ARF−/− mice were stimulated with LPS, and mRNA expression of p16INK4a was monitored by quantitative PCR. E. Quantitative PCR of the basal expression of TLR2, TLR3, TLR4, and TLR9 in WT and ARF−/− macrophages. F. Representative flow cytometry histograms showing the expression of TLR4 and TLR2 on peritoneal macrophages from WT and ARF−/− mice. Filled histograms, WT macrophages; open histograms, ARF−/− macrophages. G. Quantitative PCR analysis of TLR2 and TLR4 mRNA expression in WT and ARF−/− macrophages stimulated with LPS for the indicated times. Data are means ± SD (n = 4), *p < 0.05, **p < 0.01 (with respect to WT mice); *p < 0.05, **p < 0.01 (with respect to control).

reconstituted with exogenous ARF expressed high levels of ARF (Fig. 5A) and expressed WT levels of TLR4 mRNA (Fig. 5B). In addition, ARF reversed the impaired iNOS activation, increasing LPS-stimulated iNOS induction above that obtained in WT cells (Fig. 5C). To confirm whether TLR4 expression in the ARF-deficient cells is the main mechanism involved in the regulation of inflammation by ARF, we transfected ARF-deficient macrophages with pcDNA-TLR4 and analyze NO release and iNOS activation after LPS stimulation. As expected by our previous data, TLR-transfected ARF−/− macrophages exhibited an increase in NO release and iNOS activation after LPS treatment (Fig. 5D). However, they did not reach the levels observed in WT macrophages, indicating that although regulation of TLR4 expression by ARF is an important mechanism in inflammation, other signaling pathways—probably non-TLR dependent—may be involved.

To corroborate these findings further, we analyzed the inflammatory response in macrophages from transgenic mice overexpressing ARF. These animals showed an enhanced inflammatory response to TLR activation, with clear upregulation of key inflammatory cytokines and mediators including TNF-α, IL-6, COX-2, and iNOS after stimulation with different TLR ligands (Fig. 5E–H).

Regulation of TLR expression by ARF is not mediated by p53

The primary target of ARF signaling is the p53 transcription factor. A recent report describes that p53 positively affects the TLR3 promoter activity (38). To test the possible involvement of p53 in ARF-regulated TLR gene expression, we first determined its expression in LPS-activated WT and ARF−/− cells. LPS upregulated the expression of p53 in both cell types to a similar extent (Fig. 6A). Overexpression of p53 in ARF−/− cells (Fig. 6B) did not appreciably influence TLR4 mRNA expression (Fig. 6C). Moreover, consistent with previous findings (39), p53 overexpression not only did not augment iNOS induction, but in fact decreased it (Fig. 6D). These results indicate that p53 does not contribute to ARF-mediated regulation of macrophage TLR expression. It has also been shown that p21 (a well-known p53 target) potentiates inflammatory response (40, 41). To analyze the potential role of this protein on the effects of ARF on TLR4 signaling, we have determined p21 mRNA and protein expression in LPS-stimulated WT and ARF−/− cells. As observed in Fig. 6E, LPS-stimulated WT and ARF−/− cells also showed a similar transient induction in the expression of p21. These data seem to indicate that p21 does not contribute to ARF-mediated regulation of macrophage TLR expression.

E2F1 is increased in ARF−/− macrophages

E2F1 has recently been described as a repressor of NF-κB activity (24, 28, 42). Additionally, this transcription factor is also modulated by ARF (20–22). To test the hypothesis that E2F1 might be involved in the regulation of inflammatory responses by ARF, we investigated mRNA and protein levels of E2F1 in WT and ARF−/− macrophages after LPS stimulation. We found that ARF−/− macrophages express higher levels of E2F1 than those of WT cells at basal state and after LPS stimulation (Fig. 6F). The repressive function of E2F1 on NF-κB activity is related to its ability to bind p65 in competition with p50, inhibiting the formation of functional NF-κB (p65/p50) (43). The finding of an overexpression of E2F1 in ARF−/− macrophages led us to examine the physical interaction between the p65 subunit of NF-κB and E2F1. As shown in Fig. 6G, communoprecipitation studies revealed that LPS enhanced the interaction of p65 and E2F1 in ARF−/− macrophages. Taken together, these results suggest that E2F1 might be implicated in the
regulation of inflammation by ARF as proposed in the model shown in Fig. 6H.

ARF is required for neutrophil and monocyte infiltration into the peritoneal cavity

Acute inflammation is characterized by rapid recruitment of leukocytes from the periphery to the site of inflammation. To study the physiological role of ARF, we used the thioglycolate-induced peritonitis model to elicit the endogenous inflammatory program in WT and ARF−/− mice. Inflammatory challenge led to intense recruitment of leukocytes into the peritoneal cavity of WT mice at 6 and 24 h after thioglycolate administration (Fig. 7A) and 48 h (data not shown). In contrast, leukocyte counts were significantly lower in the peritoneal lavage fluid of ARF−/− mice (Fig. 7A). Flow cytometry analysis of leukocyte subpopulations 24 h after thioglycolate administration showed a significant inhibition of the migration of granulocytes (73%), lymphocytes (38%), and monocytes (57%) into the inflamed peritoneum of ARF−/− mice (Fig. 7B). However, basal leukocyte counts in the peritoneal cavity and in peripheral blood did not differ between WT and ARF-deficient mice (Fig. 7A, 7C), indicating that the lower thioglycolate response is due to impaired entry into the peritoneal cavity after induction of inflammation and not to an overt leukocyte defect. Indeed, analysis of circulating blood in ARF-deficient mice confirmed a typical leukocyte composition, with 5–6% monocytes, 70–80% lymphocytes, and 10–20% granulocytes, and normal hematocrit and platelet counts (Fig. 7C, Table II). Spleen and liver mass in ARF−/− mice was also normal (Fig. 7D).

TPA-induced inflammation is attenuated in ARF−/− skin

TPA is an effective inducer of inflammation in the skin. We measured the extent of TPA-induced skin edema in ARF−/− mice in ear swelling assays. TPA-induced ear thickening was 52% less in ARF−/− mice than that in WT (Fig. 8A). Moreover, myeloperoxidase activity, an index of granulocyte infiltration, in ARF−/− mice was 15% of that detected in WT mice (Fig. 8B). Analysis of H&E-stained sections showed extensive leukocyte infiltration in TPA-treated WT ears, but much lower infiltration and less swelling in ARF−/− ears (Fig. 8C). These results indicate that TPA-induced inflammation is attenuated in ARF−/− mice and are consistent with the in vitro data described earlier, suggesting a key role for this tumor suppressor in the augmentation of inflammatory responses.

ARF−/− mice are resistant to LPS-induced endotoxic shock

To understand better the physiological relevance of ARF in the regulation of inflammatory responses, we used the LPS-induced endotoxic shock model to determine the effect of ARF on macrophage activation in vivo. After administration of LPS, survival was monitored. All WT mice died by 72 h after injection with 10 mg/kg LPS; in contrast, ~80% of ARF−/− mice survived to this stage (Fig. 8D). Consistent with the lethality curves, serum from
WT mice accumulated high levels of proinflammatory cytokines (TNF-α and IL-6), whereas cytokine levels were significantly lower in the serum of ARF/2/2 mice (Fig. 8E). The serum levels of alanine aminotransferase and aspartate aminotransferase were also significantly lower in ARF/2/2 mice, providing evidence that ARF deficiency protects against LPS-induced organ damage (Fig. 8F).

**Discussion**

The innate immune system is the first line of defense against invading organisms, and TLRs are the main sensors of microbial components, initiating signaling pathways that induce the production of proinflammatory cytokines and type I IFNs. The tumor suppressor ARF has recently been implicated in antiviral defense (7), but its exact role in the regulation of innate immune responses was unknown. In this report, we have characterized the physiological functions of ARF in innate immune responses. ARF deficiency had profound effects on the function of activated macrophages, most obviously the reduced production of proinflammatory mediators; for example, the lowered expression of inflammatory cytokines (TNF-α, IL-1β) and iNOS and COX-2 in ARF/2/2 cells after activation via TLR4. This impaired activation of inflammatory responses in ARF/2/2 cells was moreover not restricted to TLR4 signaling but also seen in response to other TLR ligands such as TLR2, TLR3, and TLR9 ligands and non-TLR signaling pathways such as those elicited by TPA and thioglycolate.

**FIGURE 6.** E2F1 but not p53 is involved in ARF-mediated regulation of TLR expression. A, Western blot showing p53 protein expression in WT and ARF/2/2 macrophages stimulated with LPS. n.s., non-specific band. B, Western blot showing expression of p53 in ARF/2/2 macrophages transfected with p53 expression plasmid. Control cells were transfected with empty vector (pLPC) or p19ARF expression plasmid. C, Quantitative PCR of TLR4 mRNA expression in LPS-stimulated WT, ARF/2/2, and p53-transfected ARF/2/2 macrophages. D, Quantitative PCR of iNOS mRNA expression in LPS-stimulated WT, ARF/2/2, and p53-transfected ARF/2/2 macrophages. None, no stimulus. E, Quantitative PCR determination of p21 mRNA expression in LPS-stimulated macrophages and Western blot analysis showing p21 protein expression in WT and ARF/2/2 macrophages stimulated with LPS. F, Western blot analysis showing E2F1 protein expression and quantitative PCR determination of E2F1 mRNA expression in WT and ARF/2/2 macrophages stimulated with LPS. G, Protein extracts of WT and ARF/2/2 macrophages stimulated with LPS were immunoprecipitated using an anti-p65 Ab and then subjected to SDS-PAGE and immunoblotted with an anti-E2F1 Ab. H, Proposed model for the regulation of inflammatory response by ARF. In normal cells, stimulation of TLRs or TPA and thioglycolate treatment leads to activation of the NF-κB signaling pathway. NF-κB translocates to the nucleus followed by the binding of NF-κB onto its target genes. E2F1–ARF interaction results in destabilization of E2F1 protein and activation of NF-κB. In the absence of ARF, E2F1 is overexpressed. Excessive E2F1 inhibits NF-κB activity by binding to p65 and transcriptional expression of TLRs. Data are means ± SD of three independent experiments carried out in triplicate. Western blot in A, B, and E–G show representative experiments of three. *p < 0.05, **p < 0.01 (with respect to WT mice).
Full induction of target genes upon TLR stimulation requires a combination of downstream signaling events, including transcriptional activation via NF-kB factors and activity of the MAPK proteins (44). The impaired signaling via both of these pathways in ARF-deficient macrophages indicates that ARF is required for certain aspects of NF-kB and MAPK modulation. The decreased IkBα degradation, p65 translocation, and NF-kB DNA binding activity in ARF−/− macrophages is consistent with the reported inhibition of IkB phosphorylation in ARF-deficient macrophages stimulated with vesicular stomatitis virus (7). A similar situation has been observed with the tumor suppressor Rb (45). An important question is whether this reduced activation of NF-kB and MAPK pathways in ARF-deficient cells is linked to the regulation of ARF by LPS. Several reports have described the activation of ARF upon the expression of viral proteins (46, 47), viral infection (7), or type I IFN treatment (Ref. 36, Fig. 4C). However, we did not detect changes in ARF mRNA expression after LPS or poly(I:C) treatment, indicating that the substantial reduction in TLR-mediated cytokine production is not related to transcriptional regulation of ARF. A critical role has recently been described for p53 in the regulation of TLR3 expression, establishing a new relationship between tumor suppressors and TLRs (38). Given that TLRs mediate recognition of viral and bacterial products, we examined the possible regulation of TLR by ARF. Positive modulation of TLR2 and TLR4 by ARF is supported by the down-regulation of their basal expression in ARF−/− macrophages, the loss of LPS regulation in ARF-deficient cells, and the restoration of TLR4 expression upon forced expression of ARF in ARF-deficient cells. Importantly, the regulation of TLR expression affected the downstream induction of cytokines and inflammatory mediators, as evidenced by the enhanced expression and activity of iNOS upon transient expression of ARF in deficient cells and the exacerbated inflammatory response (iNOS, COX-2, and TNF-α production) in ARF TG mice stimulated with TLR ligands. Nevertheless, modulation of TLR4 expression by ARF seems not to be the only mechanism involved in the regulation of inflammation by ARF, as demonstrated by the results obtained in TLR4-transfected ARF−/− macrophages. These cells exhibited an increase in NO release and iNOS activation after LPS treatment, but they did not reach the levels observed in WT macrophages, indicating that other signaling pathways may be involved.

Whether ARF regulates TLR directly or through other downstream targets is not clear. One of the principal mechanisms of action of ARF is p53. ARF localizes mainly in nucleoli where it interacts with Mdm2 (15, 48), inhibiting its E3 ligase activity (49), which results in the stabilization and activation of p53. However, LPS induced similar levels of p53 and its transcriptional target p21 in WT and ARF-deficient cells, indicating that ARF regulation of inflammatory responses is independent of p53 status. This conclusion is supported by the finding that p53 overexpression did not increase TLR4 levels in ARF-deficient cells and in fact reduced iNOS expression, in line with the known repressive role of p53 on a number of NF-kB regulated genes (50). Indeed, although ARF actions were initially linked to oncogenic stress-induced p53 stabilization (51), several p53-independent actions of ARF have recently been described (19), including antiviral actions (7). Furthermore, the p53 pathway has been shown to have anti-inflammatory actions; for example, p53-deficient mice show a more pronounced activation of cellular mediators of innate immunity (50, 52). The opposite effects of the p53 pathway and ARF on inflammatory responses further support a p53-independent mechanism for ARF-regulated immune modulation. Additionally to the p53 pathway, several reports have described a role for p21 in the regulation of inflammatory processes. Thus, in peritoneal macrophages from p21−/− mice, a lower expression of cytokines such as IL-1β, MIP-1, and MIP-2 has been observed (40). Moreover, targeted disruption of p21 was associated with decreased NF-kB activation in glial cells in response to LPS (41). Regarding the possible role of p21 in the regulation of inflammatory response by ARF, we have observed a similar transient induction of p21 mRNA and protein expression in LPS-stimulated WT and ARF−/− cells. These data seem to indicate that p21 does

Table II. Hematological analysis of WT and ARF−/− mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>ARF−/−</th>
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<tr>
<td>WBCs, × 10⁶/µl</td>
<td>8.60 ± 2.05</td>
<td>8.94 ± 2.24</td>
</tr>
<tr>
<td>Lymphocytes, × 10⁶/µl</td>
<td>6.91 ± 1.76</td>
<td>7.05 ± 1.42</td>
</tr>
<tr>
<td>Monocytes, × 10⁶/µl</td>
<td>0.50 ± 0.47</td>
<td>0.58 ± 0.25</td>
</tr>
<tr>
<td>Granulocytes, × 10⁶/µl</td>
<td>0.76 ± 0.38</td>
<td>0.58 ± 0.3</td>
</tr>
<tr>
<td>RBCs, × 10⁶/µl</td>
<td>10.56 ± 0.79</td>
<td>11.79 ± 0.58</td>
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<tr>
<td>Hemoglobin, g/dl</td>
<td>16.00 ± 1.47</td>
<td>17.40 ± 0.94</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>47.03 ± 3.33</td>
<td>48.55 ± 1.95</td>
</tr>
<tr>
<td>Platelets, × 10⁹/µl</td>
<td>696 ± 128</td>
<td>689 ± 24.75</td>
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Complete blood cell counts of peripheral blood from WT (n = 10) and ARF−/− (n = 10) mice. Data are means ± SD.
FIGURE 8. ARF deficiency reduces the skin inflammatory response to TPA and decreases susceptibility to LPS-induced endotoxemic shock. A, Ears of male mice were treated with TPA or acetone vehicle, and animals were killed after 4 h. Percentage induced edema was estimated as the weight difference between TPA- and vehicle-treated ears. B, Myeloperoxidase (MPO) activity in TPA-treated ears. C, H&E staining of corresponding sections of vehicle- or TPA-treated ears. D, Survival plots for WT (n = 7) and ARF−/− (n = 7) mice injected i.p. with 10 mg/kg LPS. E, TNF-α and IL-6 concentrations in serum of WT and ARF−/− mice, determined 2 h after LPS injection. F, Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined 24 h after LPS-induced endotoxemic shock in WT and ARF−/− mice (n = 7). Results are means ± SD of three independent experiments; C shows a representative experiment of three. *p < 0.05, **p < 0.01 (with respect to the TPA condition; A, B) or *p < 0.05, **p < 0.01, ***p < 0.001 (with respect to the WT condition; E, F).

not contribute to ARF-mediated regulation of macrophage TLR expression. Indeed, according to our results, no differences on basal TLR4 expression have been observed between WT and p21−/− microglia, and a similar decrease in TLR4 mRNA expression was detected in both types of cells after LPS stimulation (41), corroborating that regulation of TLR4 expression is independent of p21.

The transcription factor E2F1 is one of the key proteins in the regulation of the G1/S phase transition, acting as a critical regulator of cell survival and proliferation (23). Recent studies have described that ARF may target E2F1 for its degradation, thereby inhibiting E2F1-dependent transcription (20–22). Thus, overexpression of E2F1 in ARF−/− macrophages at basal condition.

E2F1 protein levels in ARF−/− mice (54). In keeping with this fact, we have observed an increase in the expression of ARF significantly inhibits E2F1-dependent transcription (20–22). Thus, overexpression of ARF significantly inhibits E2F1-dependent activation (53). The inhibition of E2F1 transcriptional activity by ARF has been directly attributed to the interaction of ARF with E2F1 through forming ARF–E2F1/partner-DNA supercomplexes (53) and provides a mechanism for p53-independent ARF activity. Consistent with this view, it has been observed that loss of ARF expression is frequently associated with elevated levels of E2F1 (54). In keeping with this fact, we have observed an increase in the E2F1 protein levels in ARF−/− macrophages at basal condition. This raises the question of whether ARF-suppressed E2F1 transcription activity must be linked to inflammatory regulation. Recently, E2F1 has been shown to regulate a wide range of genes in response to inflammatory stimulation of macrophages and to contribute to T cell activation in response to pathogens, suggesting that E2F1 may act as an anti-inflammatory or immunosuppressive transcription factor. In this regard, E2F1 has previously been shown to suppress TNF-α–induced activation of human aortic endothelial cells by inhibiting NF-κB activation and translocation (24). Moreover, E2F1 acts as a suppressor of dendritic cell maturation, inhibiting the activation of several major signaling pathways, including ERK1/2, Akt, and NF-κB (28). Finally, mice lacking E2F1 are reported to be more susceptible to the development of autoimmune syndromes (25–27).

Supporting the role of E2F1 as an inflammatory suppressor in ARF−/− macrophages, LPS treatment of ARF−/− cells upregulates E2F1 and enhances physical interaction between the p65 subunit of NF-κB and E2F1, inhibiting the formation of functional NF-κB (p65/p50) (43). Thus, our data suggest that the E2F1–ARF interaction results not only in the destabilization of E2F1 protein but also in the activation of important signaling pathways (NF-κB, MAPKs, etc.) involved in regulation of inflammatory processes. In this regard, overexpression of E2F1 in ARF−/− macrophages and

FIGURE 9. Putative E2F binding sites are located in TLRL2 and TLRL4 promoters. Nucleotide sequences of the mouse TLR2 gene 5′ upstream region from −1620 to −1501 and of the mouse TLR4 gene 5′ upstream regions from −3000 to −2401 and from −600 to −541 are shown (from Ensembl release 63, June 2011, © Wellcome Trust Sanger Institute and European Bioinformatics Institute). The predicted E2F binding sites are in boxes (TRANSFAC threshold score 85%).
subsequent inactivation of NF-κB and MAPKs pathways may explain the attenuation of inflammation in the non-TLR signaling models. Indeed, in the TPA-induced model, it has been described that topical application of TPA results in a marked increase in the phosphorylated form of MAPKs and NF-κB (55–57), whereas i.p. injection of thioglycollate elicits a robust influx of neutrophils into the peritoneal cavity through chemokines such as CXCL1, CXCL2, and CXCL8 whose expression is also regulated by NF-κB signaling. Indeed, the attenuation of thioglycollate-induced peritoneal neutrophil migration in ARF−/− mice cannot be due merely to an overt leukocyte defect, as analysis of peripheral blood showed no differences in the blood profiles of WT and ARF−/− mice, and there were also no differences in the weight of hematopoietic organs. This agrees with previous reports showing that ARF deficiency does not compromise hematopoietic differentiation (58).

However, it is not clear how ARF can influence TLR transcription. One hypothetical way might be via its possible interaction with E2F1. Using the transcription factor binding site database TRANSFAC, we performed a computational (in silico) detection of transcription factor binding sites in upstream regulatory regions of the mouse TLR genes (TLR2, TLR4). A putative consensus E2F binding site (TTTGGCCC at −1559/−1552 bp) was identified within the TLR2 promoter regions, whereas three putative consensus E2F binding site (TTTGACGC at −589) were identified within the TLR4 promoter regions (Fig. 9). Sequence alignment between the consensus E2F binding site TTT (C/G)(C/G)CGC (59) and the predicted E2F binding sites shows high identity with a threshold score of 85%. The identification of the E2F consensus binding site in the TLR2 and TLR4 promoters suggests that members of the E2F family may regulate TLR transcriptional expression as it has previously been described for other transcription factors such as IFN regulatory factor-3 (60).

Based on these data, we propose a hypothetic model to explain the role of ARF in the regulation of inflammatory response (Fig. 6H). Stimulation of TLRs or TPA and thioglycolate treatment leads to activation of the NF-κB signaling pathway. NF-κB translocates to the nucleus followed by the binding of NF-κB onto its target genes. In normal cells, E2F1–ARF interaction results in destabilization of E2F1 protein and activation of NF-κB. In contrast, in the absence of ARF, E2F1 is overexpressed. Although NF-κB translocates to the nucleus, excessive E2F1 inhibits its activity by binding to p65, thereby suppressing NF-κB–dependent genes (NOS-2, COX-2, chemokines, etc.). Moreover, excessive E2F1 may inhibit transcriptional expression of TLRs as suggested by the identification of the E2F consensus binding site in TLR promoters.

Overall, the data presented here provide evidence that ARF is a new regulator of innate immunity. The effect of ARF on inflammatory responses was initially shown for TLR4 activation, but our findings suggest that it is applicable to several TLRs and non-TLR signaling pathways as confirmed by the results from the in vivo models. We describe results suggesting a potential mechanism by which ARF interacts with E2F1, regulating NF-κB signaling pathways and thereby inflammatory response. Future efforts to delineate the signaling pathways and mechanisms involved will help us better understand the link between tumor suppressors, TLRs, and inflammation.

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