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Aryl hydrocarbon receptor protects against bacterial infection by promoting macrophage survival and reactive oxygen species production

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Aryl hydrocarbon receptor (AhR) is crucial for various immune responses. The relationship between AhR and infection with the intracellular bacteria *Listeria monocytogenes* (LM) is poorly understood. Here, we show that in response to LM infection, AhR is required for bacterial clearance by promoting macrophage survival and reactive oxygen species (ROS) production. AhR-deficient mice were more susceptible to listeriosis, and AhR deficiency enhances bacterial growth *in vivo* and *in vitro*. On the other hand, pro-inflammatory cytokines were increased in AhR-deficient macrophages infected with LM despite enhanced susceptibility to LM infection in AhR-deficient mice. Subsequent studies demonstrate that AhR protects against macrophage cell death induced by LM infection through the induction of the antiapoptotic factor, the apoptosis inhibitor of macrophages, which promotes macrophage survival in the setting of LM infection. Furthermore, AhR promotes ROS production for bacterial clearance. Our results demonstrate that AhR is essential to the resistance against LM infection as it promotes macrophage survival and ROS production. This suggests that the activation of AhR by its ligands may be an effective strategy against listeriosis.

Keywords: apoptosis inhibitor of macrophages, dioxin receptor, innate immunity, *Listeria monocytogenes*

Introduction

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that belongs to the basic-helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) family (1–3). Upon binding with a ligand, AhR undergoes a conformational change, translocates to the nucleus and dimerizes with the AhR nuclear translocator (ARNT). Within the nucleus, the AhR/ARNT heterodimer binds to a specific sequence, designated as the xenobiotic responsive element (XRE), which causes a variety of toxicological and pharmacological effects (4–6). It has been reported that AhR serves not only as a transcriptional factor but also as a ligand-dependent E3 ubiquitin ligase (7). While AhR induces the transcription of many target genes, such as genes for xenobiotic-metabolizing enzymes (including the cytochrome p450 superfamily members, CYP1A1, CYP1A2 and CYP1B1), it also regulates selective target protein degradation such as estrogen receptor α. This indicates that AhR has a dual function in controlling intracellular protein levels.

Recently, various mechanisms of AhR have been demonstrated to explain its immunoregulatory potency. AhR is critical for the differentiation of IL-17-producing helper T cells (T_h17 cells) and the ability of T_h17 cells to produce IL-22 (8–10). In addition, AhR plays a role in regulating the differentiation of Foxp3 (+) T_{reg} cells and IL-10-producing type I regulatory T cells (T_r1 cells) (8, 11). AhR also has different effects on non-T-cell lineages. For instance, AhR negatively regulates LPS-induced inflammatory responses in macrophages and immunogenicity in dendritic cells (12, 13). More recently, it has been reported that AhR participates in the development of gut innate lymphoid cells producing IL-22 (ILC22 cells) (14). These reports indicate that AhR is critical for various immune systems; however, little is known regarding how AhR regulates the immune responses in the setting of microbial infections.

Listeria monocytogenes (LM) is a Gram-positive, facultative intracellular bacterium that can cause severe illness in humans and animals and is widely studied as an intracellular pathogen model. After LM infection, pro-inflammatory cytokines such as type II interferon (IFN-γ), TNF-α and IL-6 promote macrophage activation and produce anti-microbial mediators (15, 16), whereas the anti-inflammatory cytokine IL-10 abolishes innate resistance to LM (17). It has recently been reported that IL-23 induces IL-17A production from γδ T cells during LM infection and that these two cytokines are necessary for resistance

against systemic LM infection (18). On the other hand, although IL-23 can also induce IL-22 production during LM infection, IL-22 is not required for bacterial clearance or tissue protection (19). LM is typically engulfed by macrophages and then contained in a membrane-bound vacuole called a phagosome. Because macrophages play a critical role in the uptake and killing of LM, it is important to understand the precise mechanisms that control macrophage survival during LM infection. One recently reported example of such a mechanism involves a nuclear receptor, namely, the liver X receptor (LXR) (20).

Despite considerable progress in the understanding of the AhR-mediated regulation of immune responses, the role of AhR in bacterial infections has not been clearly demonstrated. In this study, we show that AhR is induced in macrophages infected with LM. Mice with an AhR deficiency due to AhR knockout (AhR KO mice) showed high susceptibility to LM infection, despite increased levels of inflammatory cytokines in macrophages after LM infection. The susceptibility to LM infection was improved by AhR ligands. AhR contributed to macrophage survival during LM infection by inducing the apoptosis inhibitor of macrophages (AIM). Furthermore, AhR promoted the production of reactive oxygen species (ROS) during LM infection, resulting in enhanced bacterial clearance. Taken together, we demonstrate that AhR plays an important role in optimal innate immunoprotection against microbial infection through the induction of AIM and ROS.

Methods

Mice and bacteria

C57BL/6 wild-type (WT) mice were obtained from CLEA Japan Inc., Tokyo, Japan. AhR KO mice (C57BL/6 background) have been described previously (8). All the mice were maintained under specific pathogen-free conditions. A streptomycin-resistant LM strain 10403s was obtained from T. Chakraborty (Justus-Liebig University Giessen, Giessen, Germany). LM was cultured in brain–heart infusion (BHI) broth with 50 μg/ml streptomycin. Heat-killed LM (HKLM) was prepared by incubation of mid-log bacteria at 80°C for 3 h followed by three washes with sterile PBS.

Cell culture and infection of cells with LM

Peritoneal macrophages were prepared as previously described (12). The thioglycolate-elicited peritoneal macrophages and a mouse macrophage cell line (RAW cells) were cultured in RPMI 1640 with 10% FCS, 100 μg/ml streptomycin and 100 units/ml penicillin G. RAW cells were stably transfected with AhR cDNAs as described previously (12). Stable transfected RAW mutant lines (RAW/Neo, RAW/AhR) were maintained in the presence of 500 μg/ml G418. Peritoneal macrophages and RAW cells were infected with the indicated dose of LM or HKLM, and 100 μg/ml gentamicin was added at 1 h post-infection to kill all extracellular bacteria. TLR2 signaling was inhibited by using anti-mTLR2-IgG antibody (1 μg/ml, InvivoGen).

In vivo experiments

Six-week-old AhR KO mice and littermate WT mice were infected i.p. with the indicated dose of LM. Organs and peritoneal macrophages were lysed/homogenized with lysis buffer solution (sterile water containing 0.2% Triton X-100). Organ/cell lysates were diluted *ad libitum* and plated onto BHI agar plates containing 50 μg/ml streptomycin. After incubation at 37°C, the colony-forming unit (CFU) per organ or macrophages were counted.

Cytokine and AIM ELISA

The cells were infected with the indicated dose of LM for 24 h. Mouse IL-6, TNF-α and IL-10 from either the supernatant or the serum were measured by ELISA, according to the manufacturer's instructions (R&D Systems). Mouse AIM from the supernatant was measured by ELISA, according to the manufacturer's instructions (CycLex).

Western blot analysis

Peritoneal macrophages and RAW cells were infected with the indicated dose of LM for the indicated times. Cells were lysed with a lysis buffer [1% NP-40, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM Na_2VO_4, 0.5 mM dithiothreitol, 1/100 protease inhibitor cocktail] and then subjected to SDS–PAGE. Whole cell lysates were analyzed with western blotting using anti-AhR (BIOMOL International) or anti-cleaved Caspase-3 (Cell Signaling).

Luciferase assay

RAW cells were transfected with 1 μg of the reporter plasmid and, in cotransfection experiments, with 0.1 μg of pRL-TK for use as an internal control reporter. Cells were infected with LM at a multiplicity of infection (MOI) of 1 for 12 h and lysed with luciferase lysis reagent (Promega). Luciferase activity was determined with a commercial Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. Relative light units of Firefly luciferase activity were normalized with Renilla luciferase activity.

Cell death assays

Peritoneal macrophages and RAW cells were infected with the indicated dose of LM for 24 h. For the lactate dehydrogenase (LDH) release assay, culture supernatant was collected after infection and cell death was quantified using a cytotoxicity detection kit according to the manufacturer's instructions (Roche). For the cell imaging assay, cells were stained with a LIVE/DEAD Cell Imaging kit, according to the manufacturer's instructions (Molecular Probes) and imaged on Keyence BZ-9000 to determine whether they were live (green) or dead (red). Using an MEBCYTO Apoptosis Kit (MBL), cells were washed in PBS and re-suspended in 100 μl of binding buffer. Cells were then incubated with 10 μl of annexin V-FITC for 15 min at room temperature in the dark, followed by the addition of 400 μl of binding buffer and analysis using a BD FACSCanto II. For the cytotoxicity assay, cells were seeded 24 h before the assay in 96-well plates at a density of 2×10^5 cells per well. Cells were treated with the indicated dose of pyocyanin in the presence or absence of AIM. After treatment for 24 h, cell viability was assessed with a Cell Counting Kit (Dojin Laboratories, Kumamoto, Japan).

ROS detection

RAW/Neo and RAW/AhR cells were infected with LM at an MOI of 1 for 12 h. Cells were stained with a ROS detection kit, according to the manufacturer's instructions (Enzo Life Sciences) and analyzed using flow cytometry.

Invasion assays

Invasion assays were performed in 24-well plates using the gentamicin survival assays. RAW cells were infected with LM at an MOI of 1 for the indicated times, and for 1 h in the presence of gentamicin (100 μg/ml). Cells were lysed/homogenized with lysis buffer solution (sterile water containing 0.2% Triton X-100). Cell lysates were diluted *ad libitum* and plated onto a BHI agar plate containing 50 μg/ml streptomycin. After incubation at 37°C, the CFU per organ or macrophages were counted. For fluorescence analysis, LM was labeled with FITC. RAW cells were infected with LM–FITC at an MOI of 1 for 24 h and washed twice with PBS. Cells were fixed with 3.7% formaldehyde in PBS for 10 min and permeabilized with 0.1% NP-40 in PBS for 5 min. Phalloidin-Alexa594 was added in the cells and then imaged on Keyence BZ-8000.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed according to Upstate Biotechnology's protocol. In brief, macrophages were infected with LM at an MOI of 1 for 24 h, and then fixed with formaldehyde for 10 min. The cells were lysed, sheared by sonication and incubated overnight with anti-AhR (BIOMOL International) followed by incubation with protein A-agarose saturated with salmon sperm DNA (Upstate Biotechnology). Precipitated DNA was analyzed with quantitative PCR (35 cycles) using primers 5′-TTGGAGAAAACGATTGTTAG-3′ and 5′-AAGGGCATGGAAAGCTGTCA-3′ for the AHRE-II site in the AIM promoter.

RT–PCR and quantitative real-time PCR

Total RNA was prepared using RNeasy (Qiagen), and cDNA was prepared as described in elsewhere (8). Quantitative real-time PCR was performed using the primers in combination with SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) by a CFX384 real-time PCR detection system (Bio-Rad). The expression level of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was evaluated as an internal control. The specific primers for quantitative real-time PCR were as follows: $p40^{phox}$, sense 5′-GCCGCTATCGCCAGTTCTAC-3′ and anti-sense 5′-GCAGGCTCAGGAGGTTCTTC-3′; G3PDH, sense 5′-AACTTTGGCATTGTGGAAGG-3′ and anti-sense 5′-GGATGCAGGGATGATGTTCT-3′.

Statistical analysis

Student's *t*-test was used to analyze data for significant differences. Values of $P < 0.05$ were regarded as significant.

Results

AhR KO mice are highly susceptible to LM infection

Although Shi *et al*. (21) have previously shown that AhR deficiency enhanced the bacterial burden, they did not investigate the mortality of AhR KO mice after LM infection. To clearly demonstrate the role of AhR in host-protective responses *in vivo*, we first treated AhR KO mice and littermate WT mice with various doses of LM. All AhR KO mice died within 3 and 5 days of infection with 5×10^5 and 1×10^5 CFU, respectively (Fig. 1A), which indicates that AhR KO mice are highly susceptible to LM infection compared with WT mice. Next, AhR KO and WT mice were i.p. infected with 1×10^5 CFU of LM, and the bacterial burdens in the spleen and liver were measured after 2 days. LM counts in each organ of AhR KO mice were higher than those in WT mice (Fig. 1B), which is consistent with the previous report (21).

We examined the effect of AhR ligands on LM infection in WT mice. It has been reported that AhR ligands, such as the prototypic, environmental AhR agonist, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCCD), or the putative, endogenous ligand 6-formylindolo[3,2-b]carbazole (FICZ), affect various immune responses, including T_h17, Treg and T_r1 cell differentiation (8–10). WT mice were infected with 1×10^6 CFU of LM in the presence or absence of FICZ. As shown in Fig. 1(C), the administration of FICZ drastically reduced the mortality induced by higher LM infection, whereas no effect of FICZ administration was observed in AhR KO mice (Fig. 1D). Thus, AhR activation by FICZ protects against LM infection.

AhR deficiency results in the hyperactivation of macrophages during LM infection in vitro

AhR is induced by various stimuli in various types of immune cells (22). To determine whether AhR is induced in macrophages infected with LM, peritoneal macrophages were challenged with LM, and AhR expression was measured by western blotting. AhR protein was induced in peritoneal macrophages infected with LM (Fig. 2A). Next, we examined the effect of AhR on the production of cytokines during LM infection. As shown in Fig. 2(B), IL-6 and TNF-α were induced after LM infection, and those levels were significantly higher in AhR KO peritoneal macrophages than in WT cells. In contrast, the production of anti-inflammatory cytokine IL-10 was reduced in LM-infected AhR KO macrophages (Fig. 2C).

To directly address the role of AhR in macrophage responses to LM infection, we established a mouse macrophage-like cell line (RAW cells) that constitutively expressed AhR (RAW/AhR). With RAW/Neo cells functioning as a control, RAW/AhR cells were infected with LM and the production of pro-inflammatory cytokines was then examined by ELISA. We found that IL-6 and TNF-α production was severely reduced in AhR-over-expressing RAW cells compared with that in RAW/Neo cells during LM infection (Fig. 2D). LM contains multiple TLR ligands (23), suggesting that pro-inflammatory cytokine production by LM infection is dominantly induced through NF-κB activation. We next investigated whether AhR regulates NF-κB activation using the Dual-Luciferase Reporter Assay System. As expected, AhR inhibited NF-κB-dependent luciferase activity in LM-infected RAW cells (Fig. 2E), which is consistent with the previous finding that AhR suppresses LPS signaling in macrophages

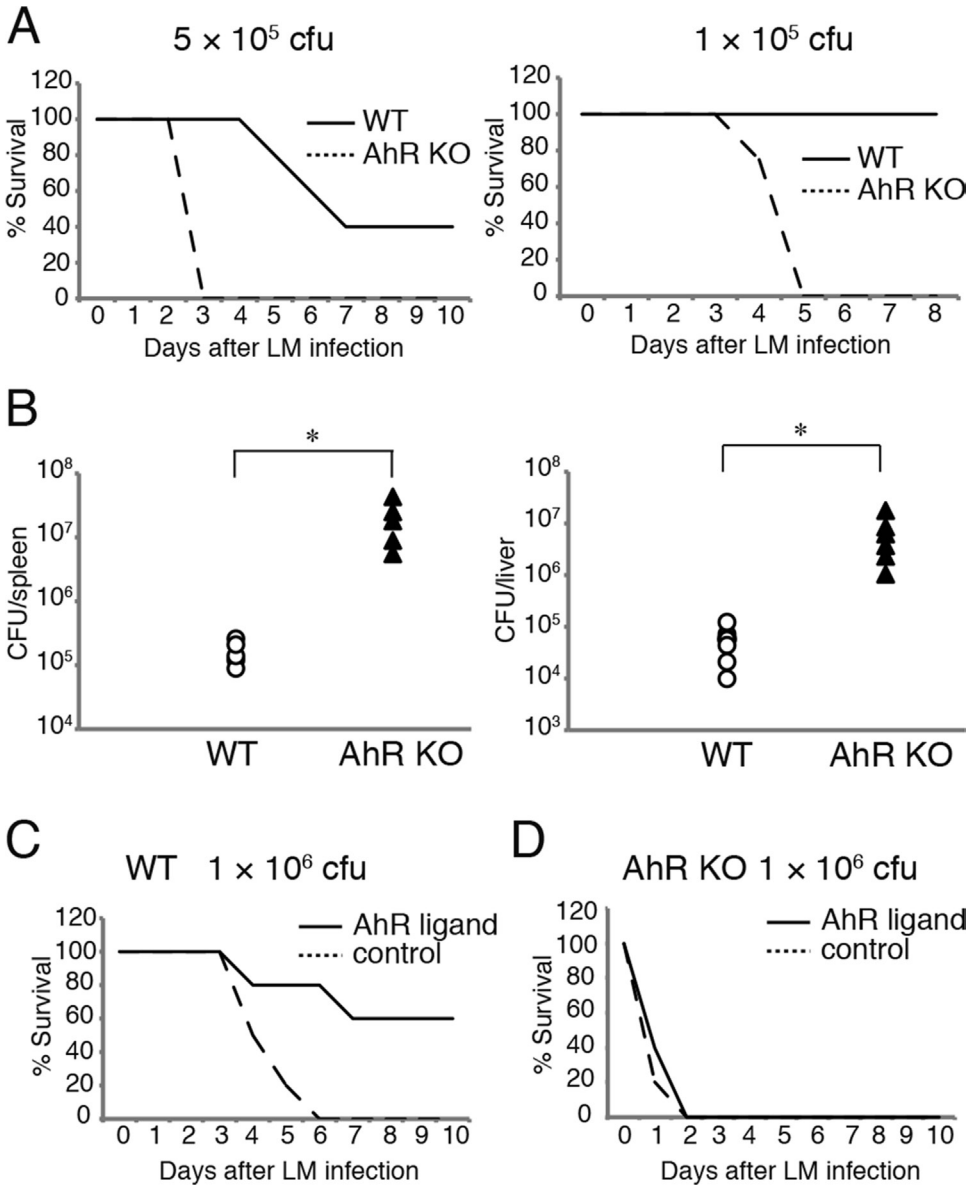

Fig. 1. Enhanced mortality in AhR KO mice after LM infection. Six- to eight-week-old AhR KO mice and littermate WT mice were infected i.p. with 5×10^5 CFU, 1×10^5 CFU (A and B) or 1×10^6 CFU (C and D) of LM. (A) Lethality was observed over 8 days after LM treatment. Data are representative of three independent experiments. (B) Bacterial load in spleen and liver was determined at 2 days post-infection. Data are representative of three independent experiments (*$P < 0.05$). (C and D) WT mice and AhR KO mice were infected i.p. with 1×10^6 CFU of LM. FICZ (100 μg/kg) was injected i.p. daily. Lethality was observed over 10 days after LM treatment with or without FICZ. Data are representative of three independent experiments.

(12). Collectively, although AhR has a protective role against LM infection, AhR negatively regulates pro-inflammatory responses induced by LM infection.

AhR regulates TLR2-dependent and -independent innate immune responses during LM infection

It has been shown that TLR2 signaling induced by LM lipoproteins is critical for the host immune response (24). We next investigated the role of AhR in TLR2-mediated innate immune responses induced by LM infection. Peritoneal macrophages from WT and AhR KO mice were challenged with low and high doses of LM in the presence or absence of TLR2-neutralizing antibody. When infected at a low dose (MOI = 1) of LM, the blockade of TLR2 signaling with the neutralizing antibody robustly prevented the production of IL-6 in both WT and AhR KO macrophages (Fig. 3A). However, we found that the TLR2-neutralizing antibody could not suppress IL-6 production induced by LM infection at a high dose (MOI = 10). Moreover, the levels of IL-6 in AhR KO macrophages remained higher than those in the control cells (Fig. 3B). These results indicate that AhR regulates not only TLR2-mediated immune responses but also other innate

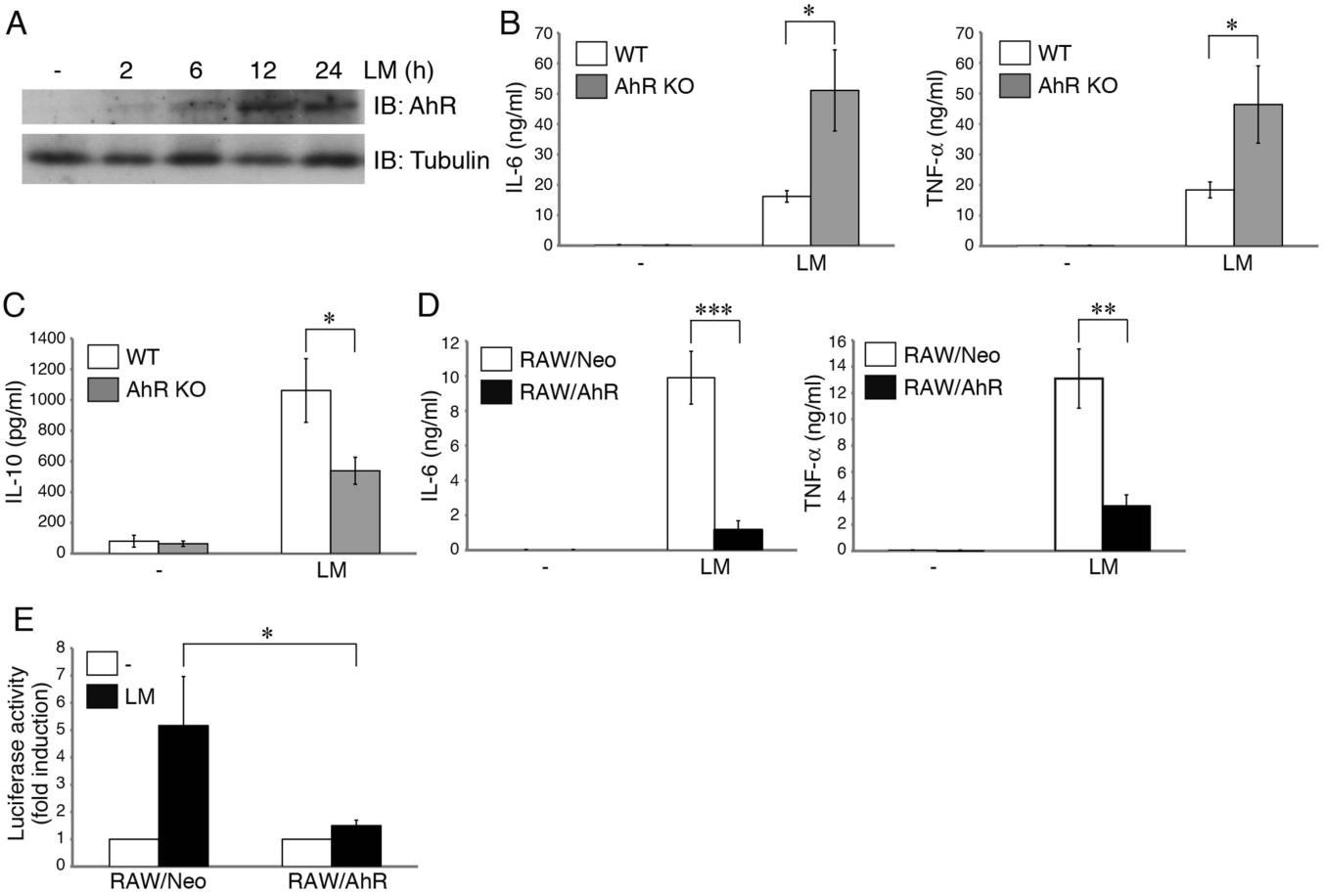

Fig. 2. Enhanced pro-inflammatory responses in AhR-deficient macrophages after LM infection. (A) Peritoneal macrophages were infected with LM for 24 h. The cells were lysed and subjected to immunoblotting analysis for the expression of AhR and tubulin. IB denotes immunoblot. (B and C) WT and AhR-deficient macrophages were infected with LM at an MOI of 1. Supernatant was collected 24 h after infection and the production of IL-6, TNF-α (B) and IL-10 (C) was measured by ELISA. Data show means ± SEM (*$P < 0.05$). (D) RAW/Neo and RAW/AhR cells were infected with LM at an MOI of 1. Supernatant was collected 24 h after infection and the production of IL-6 and TNF-α was measured by ELISA. Data show means ± SEM (**$P < 0.005$; ***$P < 0.001$). (E) RAW/Neo and RAW/AhR cells were transiently transfected with κB-luciferase reporter plasmid. Six hours after transfection, cells were infected with LM for a further 12 h. The luciferase assay and quantitation were performed as described in Methods. Data show means ± SEM (*$P < 0.05$). All data are representative of at least three separate experiments.

immune signaling during LM infection, which is dependent on the dose of LM. On the other hand, when stimulated with HKLM, the blockade of TLR2 signaling could completely inhibit the production of IL-6 in both WT and AhR KO macrophages irrespective of the HKLM dose (Fig. 2C and D), indicating that AhR dominantly regulates TLR2 signaling in the macrophages stimulated with HKLM. Consistent with our data, it has been reported that live LM contains ligand(s) for both TLR2 and other innate immune signaling, and that HKLM only contains ligand(s) capable of stimulating responses through TLR2 (25).

Next, we examined whether AhR can actually regulate various innate immune responses. WT and AhR KO macrophages were stimulated with Pam3CSK4 (TLR2-TLR1 ligand) and PGN-SA (TLR2 ligand), and then pro- and anti-inflammatory cytokines were measured. As expected, the cytokine levels of IL-6 and TNF-α were significantly increased in AhR KO macrophages (Supplementary Figure 1A and B, available at *International Immunology* Online), whereas IL-10 production was reduced in AhR KO macrophages (Supplementary Figure 1C, available at *International Immunology* Online). We have shown that AhR suppresses LPS-dependent signaling (12). These data indicate that AhR negatively regulates TLR1-, TLR2- and TLR4-mediated innate immune responses. Nucleotide-binding oligomerization domain 2 (NOD2) is identified as a general sensor for both Gram-positive and -negative bacteria (26). To explore the function of AhR in NOD2 signaling, we analyzed IL-6 production in macrophages stimulated with muramyl dipeptide (MDP), which is known to be a NOD2 ligand. Induction of IL-6 by MDP was inhibited in the macrophages expressing AhR (Supplementary Figure 1D and E, available at *International Immunology* Online). These results demonstrate that AhR can regulate various innate immune responses stimulated not only by TLR ligands but also by a NOD ligand. Taken together, AhR is required for protection against LM infection, although it suppresses pro-inflammatory cytokine production *via* various innate immune signaling pathways during LM infection.

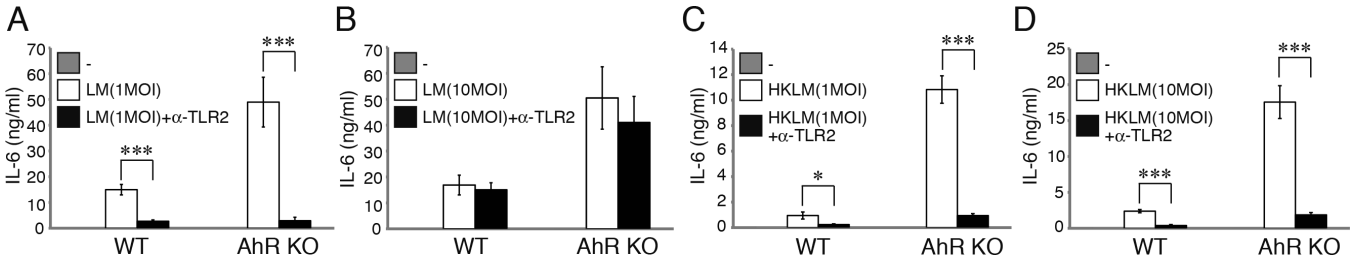

Fig. 3. AhR suppresses LM-induced pro-inflammatory cytokine production by regulating both TLR2 and other innate immune signaling. WT and AhR-deficient macrophages were infected with the indicated dose of LM (A and B) or HKLM (C and D) in the presence or absence of anti-TLR2-neutralizing antibody. IL-6 production was measured by ELISA at 24 h after LM or HKLM infection. Data show means ± SEM (***$P < 0.001$). Data show means ± SEM (***$P < 0.001$). All data are representative of at least three separate experiments.

AhR promotes bacterial clearance through enhancing ROS production

To address the effector functions of AhR in protection against LM infection, we next infected RAW/Neo and RAW/AhR cells with LM and measured the bacterial growth. There was no difference in the number of LM between these cell lines at 1, 3 and 6 h after LM infection, indicating that AhR has no effect on the initial entry of LM into macrophages (Fig. 4A). However, the intracellular growth of LM was significantly reduced 12–24 h after infection in RAW/AhR cells as compared with RAW/Neo cells (Fig. 4A). Furthermore, we found that AhR deficiency failed to inhibit the bacterial growth in the peritoneal macrophages (Fig. 4A). Cell imaging analysis also revealed increased LM growth in RAW/Neo cells compared with RAW/AhR cells (Fig. 4B). Thus, macrophages without AhR are more permissive for LM growth than AhR-expressing cells are.

Because ROS is well known as an anti-microbial reagent (27) and 3-methylcholantren (3-MC), one of the AhR ligands, increases ROS production through inducing the gene expression of $p40^{phox}$, a member of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits, in an AhR-dependent manner in livers and a cultured hepatocyte, Hepa-1 (28), we investigated whether AhR regulates ROS production in macrophages infected with LM. RAW/Neo and RAW/AhR cells or WT and AhR-deficient macrophages were infected with LM for 12 h, and then ROS production was detected using flow cytometry. As shown in Fig. 4(C), AhR enhanced the generation of ROS in RAW cells and peritoneal macrophages during LM infection. To clarify if AhR promotes bacterial clearance dependently on ROS, RAW/Neo and RAW/AhR cells were infected with LM in the presence or absence of *N*-acetyl cysteine (NAC), a well-known inhibitor of ROS. Consistent with Fig. 4(A), AhR suppressed bacterial growth; however, NAC canceled its inhibitory effect in RAW/AhR cells (Fig. 4D). Furthermore, we confirmed that AhR promotes the gene expression of $p40^{phox}$, a member of the NADPH oxidase subunits, in macrophages infected with LM (Fig. 4E). These data indicate that AhR controls bacterial growth through promoting ROS production.

IL-17A produced by γδ T cells has been reported to play a critical role in innate immunity against LM infection (29). To investigate whether AhR is involved in the induction of IL-17A-producing γδ T cells, we challenged WT and AhR KO mice with LM and analyzed splenic IL-17A-producing γδ T cells using flow cytometry. There was no difference in the IL-17A-producing γδ T-cell development between WT and AhR KO mice (Supplementary Figure 2, available at *International Immunology* Online), indicating that increased LM growth in macrophages without AhR is not due to impaired IL-17A-producing γδ T-cell development.

AhR promotes macrophage survival in the setting of LM infection

Given that macrophages play a critical role in bacterial clearance, it is important to control macrophage survival during LM infection. We first investigated the number of macrophages in WT and AhR KO mice during LM infection *in vivo*. We infected WT and AhR KO mice i.p. with 1×10^5 CFU of LM, collected peritoneal macrophages at 3 days and then counted the number of peritoneal macrophages from WT and AhR KO mice. While there was no difference in the number of macrophages between WT and AhR KO mice under steady-state conditions, the number of macrophages from AhR KO mice was significantly decreased compared with that from WT mice after LM infection (Fig. 5A). In addition, we measured the bacterial burdens in the same number of macrophages (1×10^6) from WT and AhR KO mice. The bacterial burdens in AhR-deficient macrophages were drastically higher than those in WT cells (Fig. 5B). These data led us to hypothesize that AhR may exert an inhibitory effect on cell death during LM infection, resulting in reduced bacterial growth in macrophages.

To test this hypothesis, we first examined whether AhR regulates macrophage cell death induced by LM infection. Cell imaging analysis revealed an increased rate of cell death in AhR KO macrophages and RAW/Neo cells compared with WT and RAW/AhR cells, respectively (Fig. 5C). In agreement with these results, flow cytometry using annexin V antibody and propidium iodide showed increased cell death in RAW/Neo cells compared with that seen in RAW/AhR cells during LM infection (Fig. 5D). Furthermore, we confirmed the reduction of host cell death in LM-infected RAW/AhR cells compared with RAW/Neo cells by quantifying the level of LDH released into the supernatant of LM-infected macrophages (Fig. 5E). The AhR-mediated regulation of apoptosis was also confirmed by caspase-3 activation. Caspase-3 activation was promoted in AhR-deficient macrophages, whereas it was suppressed in RAW/AhR cells during LM infection (Fig. 5F). These data establish that AhR is

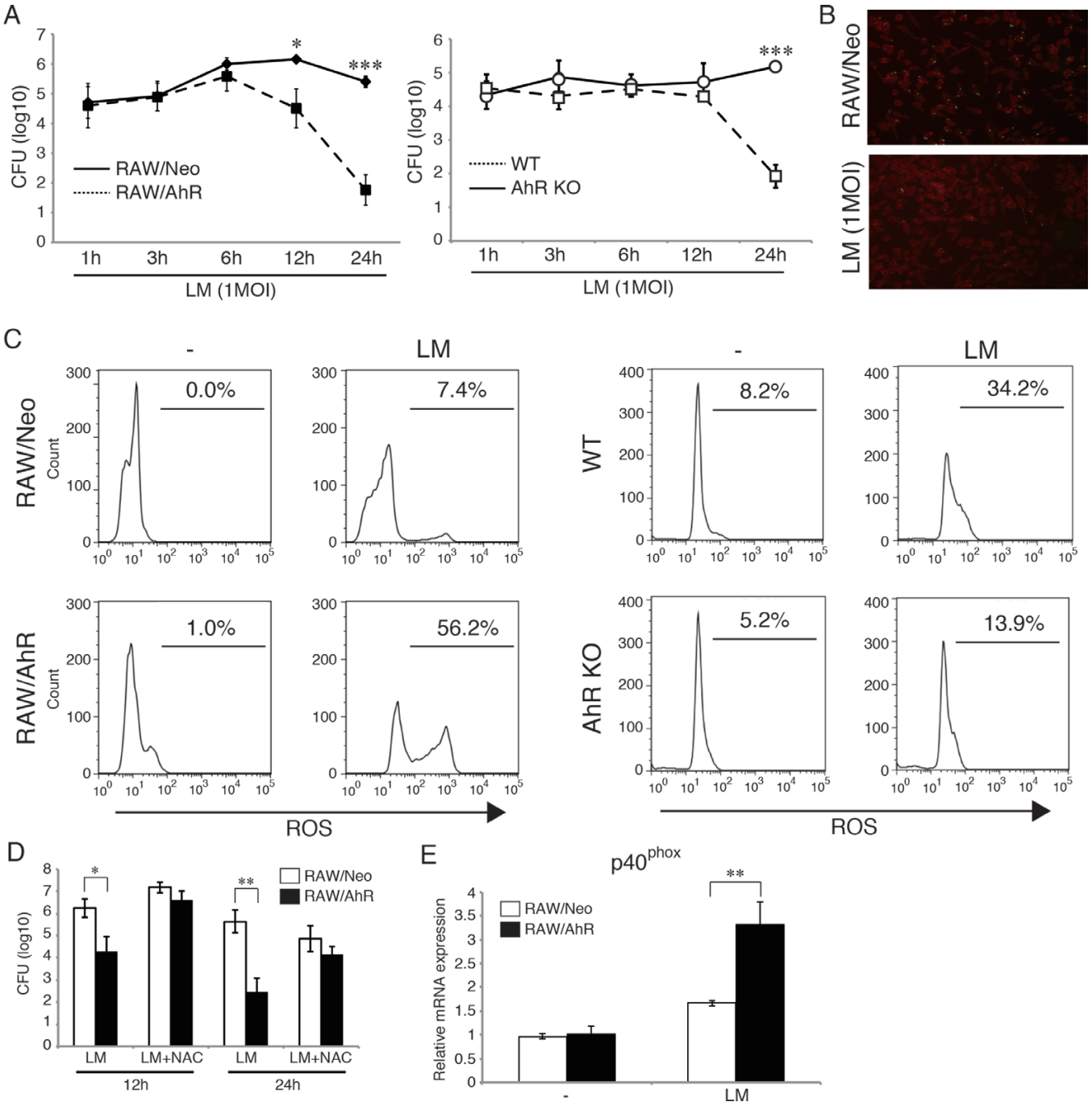

Fig. 4. ROS-dependent suppression of bacterial growth in AhR-expressing macrophages. (A) RAW/Neo and RAW/AhR cells or WT and AhR-deficient macrophages were infected with LM at an MOI of 1. Bacterial load was determined at 1-, 3-, 6-, 12- and 24-h post-infection (p.i.). Data show means ± SEM (*$P < 0.05$; ***$P < 0.001$). (B) RAW/Neo and RAW/AhR cells were infected with FITC-labeled LM at an MOI of 1. FITC-labeled LM and polymerized actin (phalloidin) were analyzed by fluorescence microscopy 24 h after infection. (C) RAW/Neo and RAW/AhR cells or WT and AhR-deficient macrophages were infected with LM at an MOI of 1. ROS production was measured by flow cytometry 12 h after infection. (D and E) RAW/Neo and RAW/AhR cells were infected with LM at an MOI of 1. (D) The bacterial load was determined 12- and 24-h p.i. Data show means ± SEM (*$P < 0.05$; **$P < 0.005$). (E) $p40^{phox}$ mRNA was detected by quantitative real-time PCR 12 h after infection. Data show means ± SEM (**$P < 0.005$). All data are representative of at least three separate experiments.

critical for macrophage survival in the setting of LM infection. It is possible that macrophage apoptosis could be indirectly induced by TNF-α during LM infection. However, there was no difference in the induction of apoptosis by TNF-α between RAW/Neo and RAW/AhR cells (Supplementary Figure 3A and B, available at *International Immunology* Online). Thus, AhR regulates macrophage cell death *via* a mechanism that is independent of inflammatory cytokines.

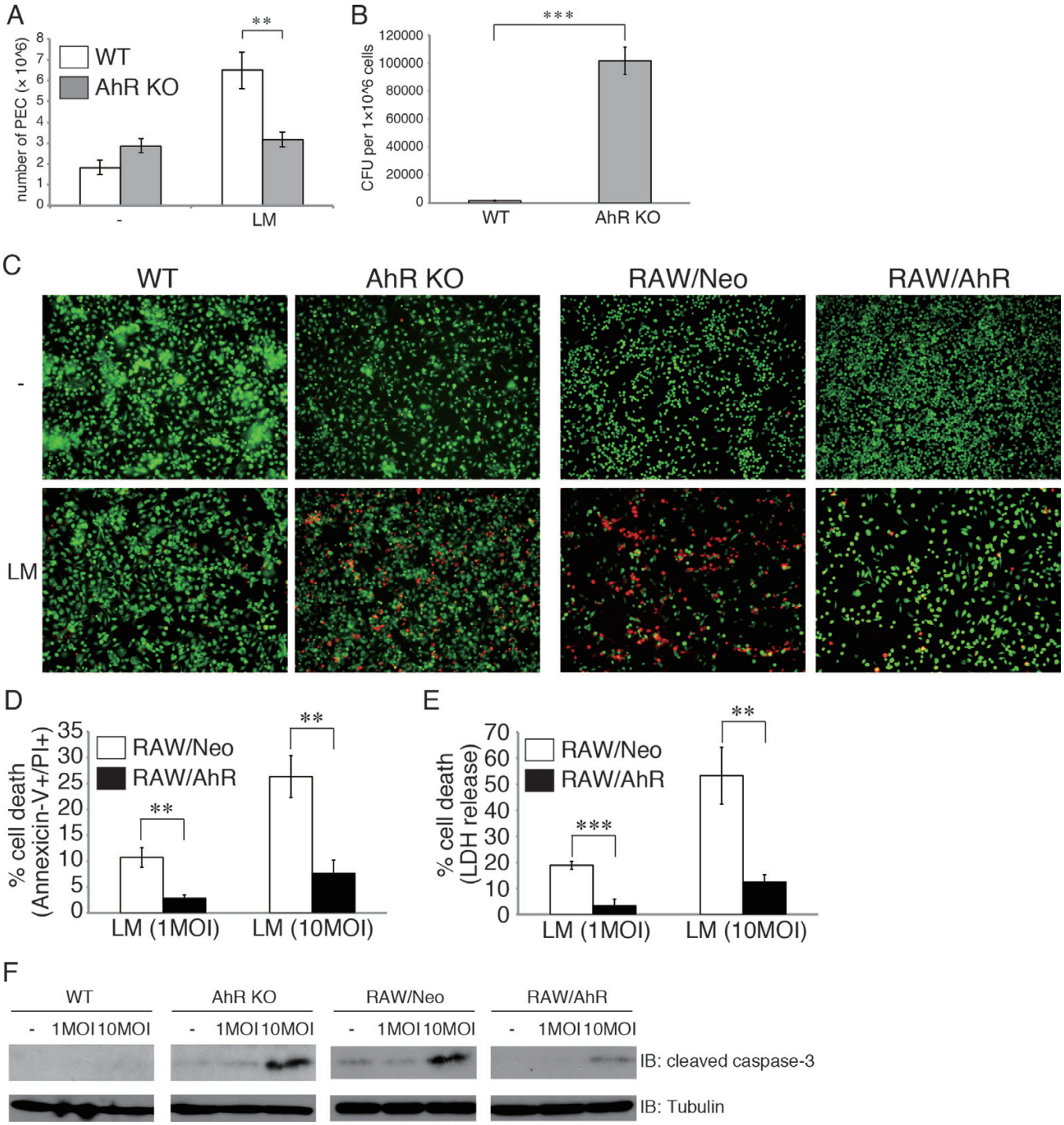

Fig. 5. AhR participates in protecting against macrophage cell death induced by LM infection. (A and B) Six- to eight-week-old AhR KO mice and littermate WT mice were infected i.p. with 1×10^5 CFU of LM. (A) Peritoneal macrophages from both mice were elicited and the number of cells was counted 3 days after LM infection (**$P < 0.005$). (B) Bacterial burdens in the same number of macrophages (1×10^6) elicited from WT and AhR KO mice were determined at 3 days post-infection (p.i.) (***$P < 0.001$). (C) WT and AhR-deficient macrophages or RAW/Neo and RAW/AhR cells were infected with LM at an MOI of 10. At 24-h p.i., cells were stained with a LIVE/DEAD Cell Imaging kit and live (green) and dead (red) cells were discriminated. (D and E) RAW/Neo and RAW/AhR cells were infected with the indicated doses of LM for 24 h. (D) Cells were stained with propidium iodide (PI) and mAb to annexin V. Data show means ± SEM (**$P < 0.005$). (E) Supernatant was collected for the measurement of cell death by quantifying LDH release. Data show means ± SEM (**$P < 0.005$; ***$P < 0.001$). (F) WT and AhR-deficient macrophages or RAW/Neo and RAW/AhR cells were infected with the indicated doses of LM for 24 h. The cells were lysed and subjected to immunoblotting analysis for the detection of cleaved caspase-3 and tubulin. IB denotes immunoblot. All data are representative of at least three separate experiments.

Induction of AIM by AhR is critical for the inhibition of macrophage cell death in LM infection

To demonstrate the biological basis by which AhR protects against macrophage cell death induced by LM infection, we investigated the potential targets for AhR that are known to play important roles in antiapoptotic function. It has previously been demonstrated that LXR-induced AIM expression is important for macrophage survival (20). To determine if AhR can control the expression of AIM during LM infection, we examined its expression in WT and AhR-deficient macrophages or RAW/Neo and RAW/AhR cells infected with LM. We found that AIM is robustly induced in AhR-expressing cells (Fig. 6A) and that the administration of AIM reduces LM-induced cell death in AhR KO macrophages (Fig. 6B).

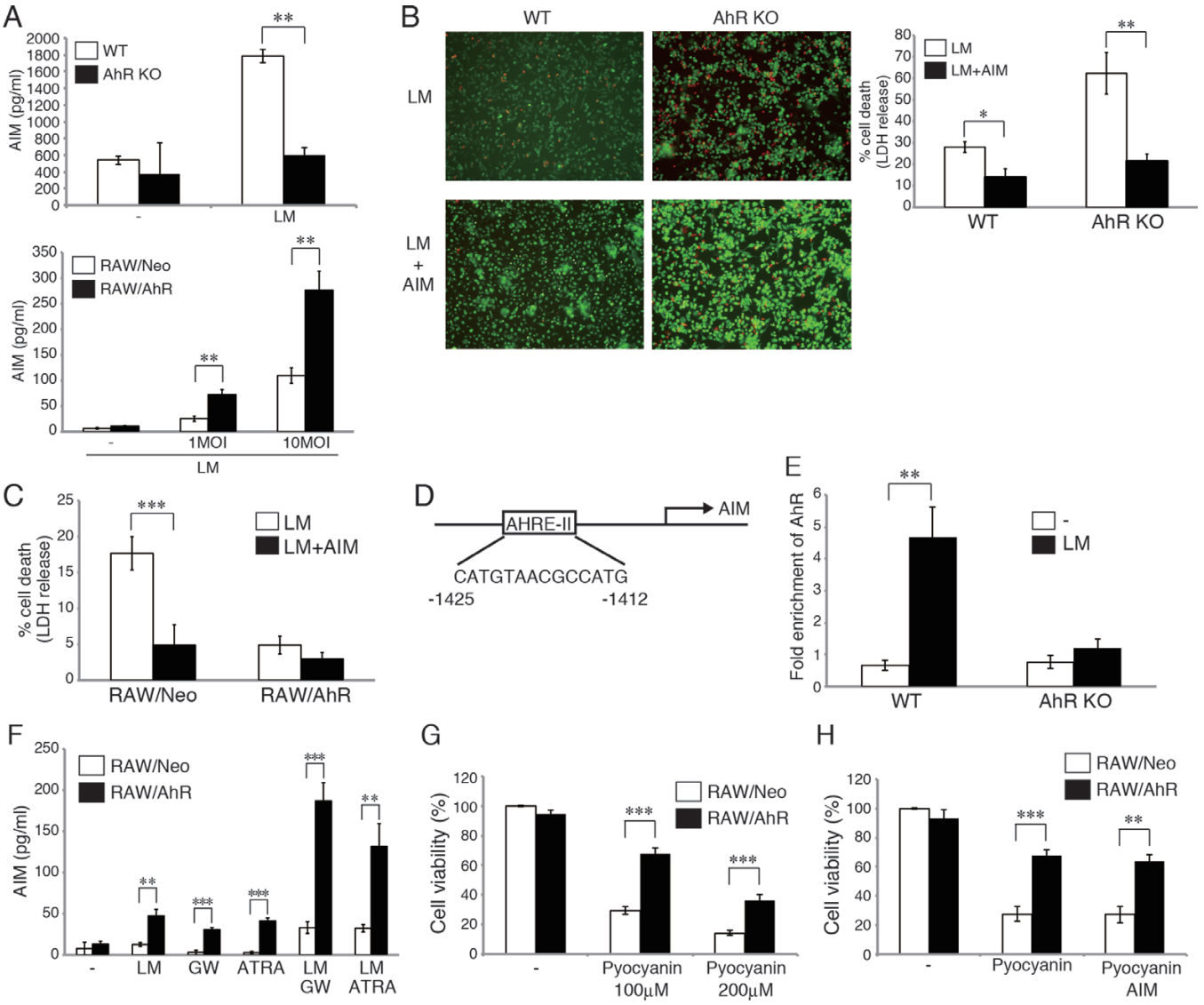

Fig. 6. AhR is required for the induction of AIM to prevent macrophage cell death induced by LM. (A) WT and AhR-deficient macrophages or RAW/Neo and RAW/AhR cells were infected with LM at an MOI of 1 and 10 for 24 h. Supernatant was collected and AIM production was measured by ELISA. Data show means ± SEM (**$P < 0.005$). (B) WT and AhR-deficient macrophages were infected with LM at an MOI of 10 (Cell Imaging) or 20 (LDH release) in the presence or absence of AIM. At 24-h post-infection, cells were stained with a LIVE/DEAD Cell Imaging kit and live (green) and dead (red) cells were discriminated. Supernatant was collected for measurement of cell death by quantifying LDH release. Data show means ± SEM (*$P < 0.05$; **$P < 0.005$). (C) RAW/Neo and RAW/AhR cells were infected with LM at an MOI of 1 in the presence or absence of AIM for 24 h. Supernatant was collected for measurement of cell death by quantifying LDH release. Data show means ± SEM (***$P < 0.001$). (D) Alignment of AHRE-II from the AIM promoter. (E) WT and AhR-deficient macrophages were infected with LM at an MOI of 1 for 24 h, and the ChIP assay was performed using anti-AhR. Purified DNA fragments were amplified using primers specific for the AHRE-II region in the AIM promoter. Data show means ± SEM (**$P < 0.005$). (F) RAW/Neo and RAW/AhR cells were treated with LXR ligand (GW3965) and/or RAR ligand (ATRA) and/or infected with LM at an MOI of 1 for 24 h. Supernatant was collected and AIM production was measured by ELISA. Data show means ± SEM (**$P < 0.005$; ***$P < 0.001$). (G and H) RAW/Neo and RAW/AhR cells were treated with the indicated doses of pyocyanin for 24 h in the presence or absence of AIM. Cell viability was analyzed by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay.

Similarly, AIM reduced LM-induced cell death in RAW/Neo cells (Fig. 6C). B-cell lymphoma 2 (Bcl2) is known to be an antiapoptotic protein (30). Therefore, we compared its expression level between WT and AhR KO macrophages after LM infection. In contrast to AIM expression, AhR had no effect on the expression of Bcl2 (Supplementary Figure 4, available at *International Immunology* Online).

Next, we investigated whether the AIM gene is a direct target of AhR. It has been reported that there are several binding motifs for AhR (31). We identified a putative AHRE-II element upstream –1425 of the start site in the AIM promoter (Fig. 6D). ChIP assay confirmed that AhR is recruited to the AHRE-II motif of AIM after LM infection (Fig. 6E). These data suggest that AIM is directly induced by AhR, which contributes to the protection against cell death in macrophages infected with LM. GW3965, an LXR ligand, has an additional effect on AIM expression during LM infection (20). To test whether AhR participates in LM-induced AIM induction together with LXR signaling, we treated RAW/Neo and RAW/AhR cells with LM in the presence or absence of GW3965. Interestingly, AIM induction was drastically increased in RAW/AhR cells treated with GW3965 compared with that in RAW/AhR cells without GW3965 or RAW/Neo cells treated with GW3965 (Fig. 6F). Surprisingly, similar phenomena were also observed in the treatment with retinoic acid receptor (RAR) ligand (Fig. 6F). These results suggest that AhR may cooperate with LXR and RAR signaling to control the expression of AIM in the setting of LM infection.

We demonstrated that AhR promotes ROS production in macrophages infected with LM in Fig. 4(C). Because it is known that ROS acts as signaling molecules to trigger apoptosis (32), we next investigated whether AhR inhibits ROS-induced cell death in macrophages. RAW/Neo and RAW/AhR cells were stimulated with a ROS inducer (pyocyanin) and the cell viability was examined. Similarly to the results observed with LM infection, AhR also inhibited ROS-induced cell death (Fig. 6G). Whereas AIM reduced LM-induced cell death, ROS-induced cell death was not rescued by the administration of AIM (Fig. 6H), indicating that AhR suppresses ROS-induced cell death independently of AIM. Taken together, AhR has a protective role in both LM-induced and ROS-induced cell death *via* AIM-dependent and -independent mechanisms, respectively.

Discussion

AhR is classically known as a transcription factor that is activated by endogenous and environmental ligands. Recent studies have established that AhR is crucial for various immune responses such as T_h17 cell differentiation, T_{reg} cell development and innate immune responses in macrophages (8–12). In addition, several studies have reported that AhR ligands, including dioxin, smoking and the UV-light-generated metabolite FICZ, are associated with autoimmune diseases such as rheumatoid arthritis, psoriasis, systemic lupus erythematosus and multiple sclerosis (33–37). Certain individuals are susceptible to developing autoimmune diseases and severe pathology in bacterial infection in which there are various genetic and environmental factors. However, the physiological relevance of environmental factors has not been established. Here, we demonstrated that AhR controls both macrophage survival and pathogen clearance during LM infection. These results establish that AhR—a factor in the environmental response—plays an important role in the host resistance against bacterial infection.

Previous work has shown that AhR KO mice are hyperresponsive to LPS and that AhR regulates innate immune responses (12). In this study, we demonstrate that AhR KO mice are highly susceptible to LM infection, although AhR KO macrophages produced increased levels of pro-inflammatory cytokines, including IL-6 and TNF-α, during LM infection. On the other hand, a study by Shi *et al.* (21) showed that there is no difference in pro-inflammatory cytokine production between AhR KO and AhR heterozygous mice during LM infection. However, they did not compare the levels of cytokines between AhR KO mice and littermate WT mice, which may have caused the discrepancy between our and their results. AhR negatively regulates LPS-induced pro-inflammatory cytokine production by interacting with NF-κB and inhibiting its activation (12). In addition, the induction of many pro-inflammatory cytokines is suppressed by AhR activation in *Streptococcus pneumonia*-infected mice (38). These observations are supportive of our data presented here. Along with TLR signaling, NOD-like receptors (NLRs) such as NOD1 and NOD2 contribute to host defense against microbial pathogens (39, 40). As reported herein, AhR can regulate both TLR and NLR signaling in macrophages infected with LM. Thus, although our results reveal AhR as a negative regulator of inflammatory signaling to protect the host from LM infection, AhR KO mice are more susceptible to listeriosis. These results suggest that AhR may have a unique function in protecting against LM infection.

One of the striking findings of this study is that AhR protects against macrophage cell death induced by LM infection (Fig. 7). To our knowledge, it has not been previously recognized that AhR is critical for host cell survival during bacterial infection. We found that AhR deficiency accelerates macrophage cell death dependent on the activation of caspase-3. Interestingly, the number of peritoneal macrophages from AhR KO mice was less than the number from WT mice after LM infection, indicating that AhR is efficient for *in vivo* host macrophage survival. It is important to control the host cell death properly in LM infection. Macrophage cell death is an important mechanism for the down-regulation of inflammatory responses to prevent sepsis, whereas it has been reported that decreased macrophage apoptosis shows more resistance to LM infection (41). In this study, it has been demonstrated that AhR enhances the anti-microbial activity through inhibiting macrophage cell death.

The key question arising from our results was how AhR protects against macrophage cell death during LM infection. AIM is directly induced by LXR signaling, and it negatively regulates apoptosis in macrophages during bacterial infection (20, 42). In this report, we have demonstrated a direct link between AhR and AIM in preventing LM-induced cell death (Fig. 7). AhR directly induces the expression of AIM by binding to AHRE-II in the AIM promoter. Moreover, the addition of AIM in macrophages without AhR promotes macrophage survival to a similar level in the cells expressing AhR, indicating that the enhanced susceptibility of AhR KO mice and macrophages results, at least in part, from loss of AIM expression

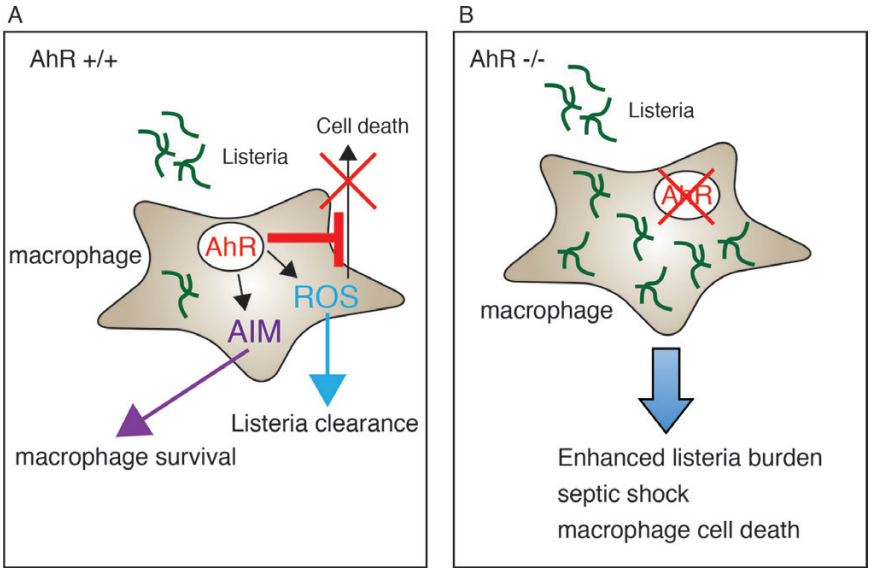

Fig. 7. Model for protective functions of AhR in macrophages against LM infection. (A) In WT macrophages, AhR is induced after LM infection. While AhR suppresses LM-induced pro-inflammatory cytokine production, AhR induces both AIM and ROS during LM infection. AIM and ROS induced by AhR results in macrophage survival and LM clearance, respectively. Additionally, AhR suppresses ROS-induced cell death. These AhR functions enhance host resistance against LM infection. (B) In AhR-deficient macrophages, the induction of AIM and ROS was impaired, whereas pro-inflammatory cytokine production is augmented, resulting in macrophage cell death, enhanced bacterial burden and septic shock.

during LM infection. Surprisingly, AhR enhanced LM-induced AIM in the presence of LXR or RAR ligands. Although we have not explored the interaction between AhR and nuclear receptors such as LXR and RXR, it is possible that AhR cooperates with them in the induction of AIM during LM infection.

There is much evidence that ROS production plays an important role in inhibiting bacterial propagation and killing bacteria, which is regulated by the NADPH oxidase. The NADPH oxidase contains some *phox* subunits including $p91^{phox}$ and $p22^{phox}$ and several cytoplasmic components: $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$. By exogenous stimulation, $p40^{phox}$ is induced and translocated from the cytoplasm to the membrane and associated with $p91^{phox}$ and $p22^{phox}$, leading to ROS formation. The other important finding of this study is that AhR induces the expression of $p40^{phox}$ and promotes ROS generation in macrophages infected with LM (Fig. 7). AhR has conflicting functions in controlling ROS production. While AhR promotes ROS production through inducing the gene expression of $p40^{phox}$ (28), it is involved in the prevention of ROS generation through functional cross-interaction between AhR and Nrf2 (43, 44). Thus, the role of AhR in ROS generation is multiple. In this study, we demonstrated that AhR induces the expression of $p40^{phox}$ during LM infection, which may cause the increase of ROS levels through enhancing the NADPH activity. In fact, AhR contributes to the bacterial clearance through enhancing the production of ROS. Further studies will be needed to determine the role of AhR in the generation of ROS during various bacterial infections.

In summary, our study highlights that AhR is a critical factor for the confinement and clearance of LM *in vitro* and *in vivo* by protecting against macrophage cell death and promoting ROS production (Fig. 7). Additionally, our results suggest that appropriate ligand-activated AhR may bring about the optimal treatment for listeriosis. Although the mechanism by which AhR ligands mediate the resistance to LM infection requires further investigation, an important goal of this study has been to define the relationship between AhR ligands as environmental factors and the severe pathology in bacterial infection.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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