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Toll-like receptor–induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens

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Abstract

Toll-like receptor (TLR) signaling in macrophages is required for antipathogen responses, including the biosynthesis of nitric oxide from arginine, and is essential for immunity to *Mycobacterium tuberculosis*, *Toxoplasma gondii* and other intracellular pathogens. Here we report a ‘loophole’ in the TLR pathway that is advantageous to these pathogens. Intracellular pathogens induced expression of the arginine hydrolytic enzyme arginase 1 (Arg1) in mouse macrophages through the TLR

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AUTHOR CONTRIBUTIONS

K.C.E.K., J.E.Q. and A.M.S. did most of the experiments. J.T.P. and R.W.T. did the *T. gondii* experiments. M.H.-T., R.J.B., T.K., U.S., M.-S.K., G.K., E.I.T., I.M.O. and C.B. did the infection and biochemistry experiments. K.A.F. and T.-D.K. contributed key research reagents. P.J.M. created the Arg1 conditional knockout. P.J.M. and A.M.S. bred and backcrossed the mice. T.A.W. and P.J.M. conceived and designed the project and wrote the manuscript.

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pathway. In contrast to diseases dominated by T helper type 2 (T_H2) responses, TLR-mediated Arg1 induction was independent of the T_H2-associated STAT6 pathway. Specific elimination of Arg1 in macrophages favored host survival in *T. gondii* infection and decreased lung bacterial load in tuberculosis infection.

In macrophages, TLRs activate protective immune responses, including recognition of pathogens, activation of antipathogen effector pathways and transition to protective adaptive responses[1]. Classically activated macrophages (CAMs) are important in combating infections caused by intracellular pathogens. A key antipathogen effector of CAMs is nitric oxide (NO), which is required for host control of intracellular infections, including *Mycobacteria* species, *T. gondii*, *Leishmania* species and *Trypanosoma cruzi*[2], and has direct antimicrobial toxicity[3]. TLR and interferon pathways synergistically trigger NO production by transcriptional and post-transcriptional mechanisms that enhance expression of inducible nitric oxide synthase (iNOS), the enzyme responsible for NO production from arginine in macrophages[2,4,5]. As a countermeasure, some pathogens deploy NO scavengers[6] or their own arginases[7,8] or, in the case of *M. tuberculosis*, adapt to NO made by activated macrophages[9] and exclude iNOS from phagosomes[10]. However, mechanisms by which intracellular pathogens induce host responses that reduce or bypass NO remain largely uncharted.

Although a published report has shown that arginase activity is induced in the J774 macrophage-like cell line by infection with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)[11], the isoform of arginase induced, the mechanism of induction and the biological consequences in primary macrophages and whole-animal models remain unknown. Here we report that Arg1 was considerably induced in primary mouse macrophages by mycobacterial infection. Although macrophage Arg1 expression is commonly linked to the hypothesized antiworm functions of alternatively activated macrophages (AAMs)[12,13], we found that Arg1 was induced in CAMs and functioned, in part, to suppress NO production in intracellular infection. In whole-animal models of intracellular infection, mice lacking macrophage Arg1 expression had an advantage in terms of clearance of pathogens or survival.

RESULTS

Mycobacteria induce Arg1 independent of STAT6 pathway

Our first clue that intracellular pathogens influence host pathways to counter NO came from our observation of robust expression of the host gene encoding arginase 1 (*Arg1*), but not *Arg2* (Supplementary Fig. 1 online). Levels of Arg1 protein increased over time and peaked 48 h after infection of primary mouse macrophages with *M. bovis* BCG (Fig. 1a). Although Arg1 is expressed by AAMs in response to infection by extracellular pathogens such nematodes and trematodes[12,14], Arg1 expression has not been generally associated with intracellular infections that critically involve CAM activity. In contrast to infection with intracellular pathogens, the AAM response to extracellular pathogens does not involve iNOS, which is normally regarded as detrimental in AAM-dominated responses[15]. Rather, in AAMs, interleukin 4 (IL)-4 and IL-13 stimulate host Arg1 production through the STAT6 pathway, and AAM-produced Arg1 is hypothesized to be involved in the repair and resolution of worm-induced tissue damage[12,13]. In determining whether the BCG-induced expression of host Arg1 was regulated through the IL-4–IL-13–STAT6 pathway, we initially reasoned that BCG infection might cause macrophages to express IL-4, IL-13 (ref. 16) or another factor that could activate STAT6. We used an assay to transfer supernatants from BCG-infected macrophage cultures to uninfected macrophages, followed by measurement of STAT6 phosphorylation. No factors were detectable in BCG-infected culture supernatants that could activate STAT6 phosphorylation (Supplementary Fig. 2 online). As STAT6 is essential for Arg1 expression in

macrophages stimulated by cytokine(s) that favor AAM development[17], we next infected bone marrow–derived macrophages (BMDMs) isolated from *Stat6*$^{-/-}$ mice. Arg1 was induced by BCG infection to the same extent in *Stat6*$^{-/-}$ and control BMDMs (Fig. 1a), albeit with a slight delay in the *Stat6*$^{-/-}$ mice.

Mycobacteria-induced Arg1 depends on TLR MyD88 pathway

Because BCG-induced Arg1 expression was STAT6 independent, we examined whether a pathogen detection pathway was involved in Arg1 expression. We tested BCG-infected macrophages lacking key components of the interferon pathway (*Irf3*$^{-/-}$ and *Ifnar*$^{-/-}$), the Nod1 and Nod2 pathways[18] (*Ripk2*$^{-/-}$), the inflammasome (*Nlrp3*$^{-/-}$ and *Pycard*$^{-/-}$) and the TLR and IL-1 pathways (*Myd88*$^{-/-}$ and *Il1r*$^{-/-}$) for Arg1 expression. Only the adaptor molecule MyD88 was required for the BCG-mediated induction of Arg1 expression (Fig. 1b and Supplementary Fig. 3 online). However, the MyD88-dependent pathway was most likely to use TLR receptors, as the IL-1 receptor was not required for expression of Arg1 (Fig. 1b). We next confirmed the mRNA findings at the protein level: in BMDMs isolated from *Tlr2*$^{-/-}$ and *Myd88*$^{-/-}$ mice, Arg1 expression after BCG infection was largely dependent on MyD88 and partially dependent on TLR2, regardless of an intact STAT6-dependent Arg1 expression pathway (Fig. 1c). The partial function of TLR2 in Arg1 expression is consistent with the essential function of TLR2 in macrophage-mediated mycobacterial recognition and subsequent downstream cytokine production[19]. Finally, we infected mice intraperitoneally with BCG and collected their spleens 10 d later. Whereas adherent splenocytes isolated from normal mice did not express detectable Arg1, BCG-infected mice had robust Arg1 expression, confirming that mycobacteria can induce Arg1 *in vivo* (Fig. 1d).

The T_H2 cytokine-driven increase in macrophage Arg1 expression is controlled by an enhancer that is ~3 kb upstream of the basal promoter and is active in hepatocytes[20,21]. The *Arg1* enhancer binds STAT6 and other proteins, including the transcription factor C/EBPβ[20,21]. We tested whether the MyD88-dependent pathway for Arg1 expression targets the basal *Arg1* promoter or the enhancer. In a reporter assay[21], BCG induced the expression of the *Arg1* reporter only when the upstream enhancer was present (Fig. 1e). Notably, BCG-mediated induction of *Arg1* reporter activity was independent of the STAT6 binding site in the enhancer that is essential for IL-4- and IL-13-mediated expression of Arg1 (refs. 21,[22]; Supplementary Fig. 4 online).

We next considered that BCG-induced Arg1 expression could be linked to polyamine amounts in infection. Arginases supply substrate (ornithine) to ornithine decarboxylase (encoded by *Odc1*), the rate-limiting enzyme for polyamine synthesis. Studies have shown that *Helicobacter pylori* induces expression of both *Arg2* and *Odc1* as a means to perturb polyamine homeostasis[23,24]. We therefore tested the possibility that *Odc1* mRNA amounts would be increased by BCG infection as a possible mechanism for polyamine sequestration by mycobacteria. In contrast to H. pylori infection, however, *Odc1* mRNA decreased after BCG infection (Supplementary Fig. 5a online) in a MyD88-dependent, STAT6-independent way (Supplementary Fig. 5b). Collectively, our results suggest that BCG-mediated upregulation of Arg1 is unlikely to cause an increased requirement for polyamine production in our *in vitro* culture system.

C/EBPβ is central in mycobacterial-induced Arg1

To determine the mechanisms that link the MyD88 pathway to *Arg1* regulation, we focused on transcription factors that target the *Arg1* enhancer. Given that the BCG-mediated increase in Arg1 expression required the upstream enhancer but not STAT6, we tested the requirement for the C/EBPβ site, which is adjacent to the STAT6 site[21]. We created luciferase reporter lines in RAW macrophages in which the basal promoter (−31/− 2365) and promoter enhancer

(−31/−3810) were cloned into an insulated backbone where the reporter was flanked by duplicated β-globin insulators[25]. An additional reporter line was made in which the C/EBPβ binding site was mutated[21]. After infection with BCG, the enhancer was required for reporter activity, consistent with the transient transfections described above (Fig. 2a and Supplementary Fig. 4). Deletion of the C/EBPβ site ablated BCG-mediated induction of reporter activity.

Because C/EBPβ is an essential component of Arg1 induction by the IL-4–STAT6 pathway[26], we tested whether C/EBPβ is also required for Arg1 expression in mycobacterial infection. Arg1 levels were much lower in BCG-infected *Cebpb*$^{-/-}$ macrophages than in BCG-infected wild-type macrophages (Fig. 2b). We next asked whether C/EBPβ expression is increased in BCG-infected macrophages. RNA blot analysis and a quantitative RT-PCR assay revealed that BCG infection resulted in a four- to five-fold increase in C/EBPβ mRNA in *Myd88*$^{+/+}$ macrophages but not in *Myd88*$^{-/-}$ macrophages (Fig. 2c,d). Thus, BCG induced Arg1 expression in a STAT6-independent way that was dependent on MyD88-mediated induction of C/EBPβ (Supplementary Fig. 4), but both the STAT6 and MyD88 pathways of Arg1 expression require upstream regulatory elements for full activity. These data indicate the existence of two distinct pathways to induce Arg1 expression in macrophages. One previously described pathway is linked to AAM function and regulated by STAT6. Our results identify a second pathway that is independently controlled by MyD88-dependent TLR signaling to C/EBPβ and does not require STAT6.

Deletion of Arg1 in macrophages

Although AAM Arg1 is associated with antihelminth functions[13-15], we considered the findings that pathogen arginases subvert the host's NO-based antimicrobial response[7,8]. We hypothesized that induction of CAM Arg1 cripples CAM antimicrobial activity by hydrolyzing the substrate required for NO production. Such a mechanism would suggest that Arg1 is an essential component of the strategy used by intracellular pathogens to survive inside NO-generating macrophages. To test the physiological importance of macrophage Arg1 expression in intracellular infection, we constructed an *Arg1* conditional knockout allele (*Arg1*$^{flox/flox}$; Supplementary Fig. 6 online) and used it in the LysMcre deleter strain (B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J (*Lysz* is now called *Lyz2*); transgene abbreviated here as *LysMcre*) to generate mice lacking Arg1 in macrophages and neutrophils (*Arg1*$^{flox/flox}$*; LysMcre*; Supplementary Fig. 7 online). We generated another mouse strain lacking Arg1 in all hematopoietic lineage cells using the Tie2cre deleter strain (B6.Cg-*Tg(Tekcre)*1Ywa (*Tie2* is now known as *Tek*); transgene abbreviated here as *Tie2cre*; Fig. 3a). We also produced a complete null mutation of *Arg1* (*Arg1*$^{Δ/Δ}$) that replicated the published conventional Arg1 knockout[27]. The *Arg1*^{Δ} allele was used as a control for absolute Arg1 deficiency (Fig. 3). As Arg1 is predominantly expressed in myeloid but not lymphoid lineage cells[12], the different strains provided parallel systems for testing the function of Arg1 in macrophages *in vivo*. Macrophages isolated from the Tie2cre intercross showed almost complete deletion of Arg1 protein and complete ablation of enzyme activity in all macrophage types tested (Fig. 3a,b). The *Arg1*$^{flox/flox}$*; LysMcre* mice also showed efficient deletion in BMDMs and >80% deletion in postmitotic macrophages (Supplementary Fig. 7).

Macrophage NO production *in vitro* requires Arg1

We first tested whether Arg1 is an essential component of a pathway for arginine depletion that hinders macrophage NO production. We examined NO production through a substrate depletion assay that uses a defined order of cytokine stimulation to induce first Arg1 and then iNOS[28]. Applying this assay to macrophages deficient in Arg1, we found that Arg1 was essential for decreasing NO production by the substrate depletion mechanism (Supplementary Fig. 8 online). Given our finding that host Arg1 expression is stimulated by intracellular pathogens, we next tested whether Arg1 influences NO synthesis in response to

lipopolysaccharide (LPS) or BCG infection. After BCG infection, macrophages lacking Arg1 produced more NO, suggesting that TLR-mediated regulation of Arg1 is a mechanism that inhibits macrophage production of NO (Fig. 3c). After stimulation with LPS, an extracellular TLR4 ligand, macrophages lacking Arg1 similarly produced more NO, suggesting that TLR-mediated regulation of Arg1 is a common mechanism that restricts macrophage production of NO (Fig. 3d).

Loss of Arg1 benefits host in experimental tuberculosis

Because NO is required for immune-mediated eradication of many intracellular pathogens, we next tested the response of *Arg1*$^{flox/flox}$*; Tie2cre* mice lacking macrophage Arg1 to *M. tuberculosis* infection. We first measured Arg1 expression in the lungs of *M. tuberculosis*–infected mice, given that human peripheral blood mononuclear cells from pulmonary tuberculosis patients have elevated arginase activity[29]. Using mice infected with CDC1551, a rapidly growing human isolate of *M. tuberculosis*, we measured the expression of iNOS, Arg1 and Arg2 in the lungs, in parallel with colony counts (Fig. 4a–d). Whereas iNOS and Arg1 mRNA expression increased over the course of infection, Arg2 mRNA amounts did not change during the observation period. Next, we infected mice lacking macrophage Arg1 with *M. tuberculosis* H37Rv through the aerosol route with an inoculum of 200 bacilli. Compared to wild-type littermates, mice lacking macrophage Arg1 had lower bacterial counts in the lungs after the initial bacterial growth period (Fig. 4e). Lower bacterial load was also noted in the spleens of the macrophage Arg1–deficient mice at day 70 after infection (data not shown). Lung granulomas in macrophage Arg1–deficient mice occupied a smaller fraction of the total lung area (Supplementary Fig. 9 online) and were associated with a more pronounced lymphocytic infiltrate than were control mice (Fig. 4f). Collectively, these data indicate that loss of macrophage Arg1 improved host control of *M. tuberculosis* infection.

We next assessed whether loss of Arg1 in macrophages causes elevated NO in tissues associated with mycobacterial infection. Because NO has a half-life of seconds, we used immunohistochemistry to stain nitrotyrosine as an indirect measure of the NO 'footprint'[30]. Compared to infected control sections, we found greater nitrotyrosine staining in liver granulomas from BCG-infected macrophage Arg1-deficient mice and in macrophage-rich areas in the lungs of tuberculosis-infected macrophage Arg1-deficient mice (Fig. 5). We could therefore associate the loss of Arg1 in macrophages to increased NO production in infected tissues where NO is made in abundance. It is likely that elevated NO in the absence of Arg1 is one pathway that contributes to the decrease in colony counts in *M. tuberculosis*–infected *Arg1*$^{flox/flox}$*; Tie2cre* mice.

Arg1 loss increases survival during *T. gondii* infection

Another intracellular pathogen that is controlled by the host NO response is *T. gondii. T gondii* induces NO production from macrophages, which is essential for long-term control of the infection[31,32]. *T. gondii* injects ROP kinases that activate host STAT proteins, including STAT6 (ref. 33). We reasoned that Arg1 might be a target of the ROP kinases through *T. gondii*–mediated 'hijacking' of the STAT6 response. We therefore infected macrophages with *T. gondii* strain ME49 and measured STAT6 phosphorylation and Arg1 expression (Fig. 6a). Arg1 protein expression was rapidly increased by *T. gondii* infection (within 1 h) but was independent of STAT6, as *Stat6*$^{-/-}$ macrophages had an identical response to wild-type cells. STAT6 tyrosine phosphorylation was induced by *T. gondii* as expected, but occurred after the increased expression of Arg1. These data confirm that, like *M. bovis* BCG, *T. gondii* induces Arg1 in a STAT6-independent way.

Given these findings, we next considered that *T. gondii* may have evolved a pathway to use host Arg1 to suppress NO production. In this scenario, mice lacking Arg1 in macrophages

would have a survival advantage over control mice. We infected control (*Arg1*$^{flox/flox}$ or C57BL/6 mice) or *Arg1*$^{flox/flox}$*; LysMcre* mice with ME49 and measured weight and overall appearance of the mice over 7 weeks. Loss of Arg1 expression in macrophages improved host ability to combat infection with ME49. Systemic infection of C57BL/6 or *Arg1*$^{flox/flox}$ control mice with ME49 caused a wasting disease that required the mice to be killed, whereas mice lacking macrophage Arg1 did not lose weight and did not show signs of disease (Fig. 6b). Thus, the observations we made with the mycobacterial infection models can be extended to an apicomplexan intracellular parasite that also requires NO for effective immune control.

Arg1 is not essential for regulating immunity in sepsis

To determine whether the protective effects of macrophage Arg1 deletion are specific to intracellular pathogens or also occur in systemic models of infection and sepsis, we used two models of infection challenge known to systemically increase NO: LPS challenge and *Streptococcus pneumoniae*, which replicates extracellularly[34,35]. We challenged macrophage Arg1–deficient mice with LPS or with a strain of *S. pneumoniae* that spreads throughout the host after intranasal inoculation (Supplementary Fig. 10 online). In multiple experiments, control, *Arg1*$^{flox/flox}$*; LysMcre* and *Arg1*$^{flox/flox}$*; Tie2cre* mice showed identical survival, blood nitrates and systemic bacterial numbers (Supplementary Fig. 10). Thus, macrophage Arg1 has a specific function in host susceptibility to intracellular pathogens and does not seem to affect the response to systemic infection with a Gram-positive pathogen or the response to systemic LPS administration, even though stimulation of Arg1-deficient macrophages with LPS leads to increased NO (Fig. 3d). Our data are therefore consistent with a model in which NO made during sepsis is derived largely from sources other than macrophages[36] and does not seem to be under the control of macrophage Arg1.

DISCUSSION

CAMs are required to control and kill mycobacteria and *T. gondii*, which grow within the macrophages[2]. Infected CAMs are activated by innate recognition pathways, including the TLR and interferon-γ pathways, to make NO. We found that macrophage Arg1 is involved in preventing this NO production, consistent with previous reports that arginases can compete with NO synthases for their common substrate, arginine[28,37-39]. Our data suggest that in CAMs, where NO is thought to be involved in directly killing pathogens, successful chronic infections are associated with pathogen-induced Arg1 expression, which in turn keeps NO production in check. Indeed, the absence of Arg1 was associated with increased macrophage NO production and was linked to enhanced control of mycobacteria and *T. gondii*. An implication of this finding is that transient interruption of macrophage Arg1 function by competitive inhibitors[40] may augment the ability of the immune system to control or eliminate intracellular pathogens like toxoplasma and mycobacteria, which establish long-term latent infections.

The products of arginase catalysis are urea and ornithine. It remains to be determined whether obligate intracellular pathogens such as *M. tuberculosis* hijack the Arg1 pathway not only to suppress NO production but also to supply substrates for growth and survival, as has been proposed for *Leishmania* species, whose growth depends in part on host polyamines derived from ornithine[41]. *M. tuberculosis* strains made genetically deficient in urease are being tested for their ability to act as an attenuated live vaccine[42]. The function of *M. tuberculosis* urease is unknown, but the enzyme presumably uses urea as a substrate for production of compounds needed for survival within macrophages.

Our data provide a possible rationale for the previous finding that *T. gondii* injects ROP kinases that activate the host's STAT proteins[33]. It seems likely that *T. gondii*–activated STAT6 induces Arg1 by bypassing the IL-4 or IL-13 receptors and directly activating the *Arg1*

enhancer. Other toxoplasma-induced mechanisms are probably involved in Arg1 expression, as we found rapid *T. gondii*–induced Arg1 expression in macrophages derived from STAT6-deficient mice. *T. gondii* therefore uses multiple strategies to induce macrophage Arg1, potentially as a means to regulate exposure to NO. *T. gondii* induces a robust host inflammatory response that is considered essential to the parasite's ability to establish chronic infections. NO production from iNOS is crucial for host control of the chronic phase of toxoplasmosis[31,32]. This is consistent with our finding that Arg1 is advantageous to the pathogen in the CAM-dependent response to *T. gondii* infection. Although we attribute the elevated macrophage NO in the absence of Arg1 as a major mechanism for host survival in *T. gondii* infection, it is possible that macrophage Arg1 has additional functions that the parasite could use as a survival strategy.

Unlike CAMs, AAMs are a distinct macrophage population that arise in polarized T_H2 responses and are not the cellular host for pathogens. AAMs are thought to be involved in immune responses associated with asthma, worm infections and pathological scenarios involving T_H2 cytokines[12-15]. In AAMs, T_H2-driven immune responses, driven by IL-4 or IL-13, induce Arg1 expression and other markers of AAM activity, such as the mannose receptor, chitinases and metalloproteinases[43]. AAM expression of Arg1 absolutely requires STAT6 (ref. 17), and we and others have shown that IL-4- and IL-13- mediated expression of macrophage Arg1 requires direct binding of STAT6 to an upstream enhancer element in the −3 kb region of the *Arg1* gene[20,21]. However, AAMs are not known to require NO for antihelminth immunity, and AAMs do not express much iNOS. Thus, the functions of Arg1 in AAMs remain unknown.

We found that distinct mechanisms regulate Arg1 expression in different types of infections. AAMs require the functions of both STAT6 and C/EBPβ but are independent of MyD88. In contrast, expression of Arg1 induced by mycobacteria is independent of the STAT6 pathway but depends on C/EBPβ and MyD88. These data are consistent with studies documenting the induction of Arg1 expression by LPS[35,44]. We speculate that the direct or indirect activation of C/EBPβ by LPS is most likely to be responsible for Arg1 induction in LPS-stimulated macrophages. As we found no obvious protective or pathogenic function for macrophage Arg1 in acute experimental LPS challenge, TLR-induced Arg1 has a more specific function for intracellular pathogens that require NO for their control.

Not all intracellular pathogens are killed by the NO pathway. NO has no obligate function in the clearance of chlamydia[45], which have evolved to parasitize various cell types in diverse anatomical niches including the eye, lungs, and genital tract. Notably, MyD88-dependent Arg1 expression was found in total lung homogenates in the early phase of *Chlamydia pneumoniae* infection[46]. The role of Arg1 in infections where NO is not essentially required for killing remains to be determined.

Our studies raise the issue of why an antipathogen response would include a component that favors intracellular pathogens. We speculate that the TLR-mediated induction of macrophage Arg1 has positive antimicrobial effector functions against other types pathogens detected by the TLR system.

METHODS

Mice

We generated a conditional *Arg1* allele, complete *Arg1* knockout alleles and crosses to the *LysMcre* and *Tie2cre* deleter strains as described in Supplementary Methods online. Backcrossing strategies are described in Supplementary Methods. All mice in this study were used according to protocols approved by the Institutional Animal Care and Use Committees

at St. Jude Children's Research Hospital, the National Institute of Allergy and Infectious Diseases, Colorado State University and University of Freiburg.

Macrophage isolation

Bone marrow–derived macrophages, peritoneal inflammatory macrophages and macrophages differentiated from the livers of embryonic day 14 mice were isolated and cultured as described[47].

LPS and infectious challenges

Age-matched mice were challenged with *Escherichia coli* LPS as described in ref. [48]). Mice were challenged with *S. pneumoniae* D39X strain, modified for *in vivo* luciferase expression and imaged with the Xenogen IVS system as described[49]. Mice (6–8 weeks of age) were challenged intraperitoneally with *M. bovis* BCG Pasteur strain. For *M. tuberculosis* challenges, mice were infected with 200 colony-forming units of H37Rv as described[50]. Two mice were killed on day 1 to measure bacterial amounts in the lungs at the initial infection. Thereafter, four or five mice of each genotype were killed at the times indicated in Figure 6 for histological evaluation of the lungs and bacterial counts in lung and spleen homogenates. *T. gondii* challenges were done and evaluated as described[31].

Enzymatic assays

Griess assays for nitrite accumulation and arginase assays were done as described[17].

Immunoblotting and immunohistochemistry

Immunoblotting was done as described[47]. We used chicken polyclonal antibody to Arg1 (1:2,000; gift from S. Morris), rabbit polyclonal antibody to iNOS (1:5,000; Chemicon International), mouse monoclonal antibody to iNOS (1: 1,000; sc-7271 from Santa Cruz Biotechnology or gift from C. Nathan) and mouse monoclonal antibody to Grb2 (1:2,000; Signal Transduction Laboratories). Immunohistochemistry for nitrotyrosine was done with rabbit polyclonal antibody to nitrotyrosine (Upstate Biochemicals).

In vitro macrophage BCG co-cultures

BMDMs were plated in 12-well plates and allowed to adhere overnight. Cells were washed with DMEM containing 10% FCS and incubated in a final volume of 1 ml. BCG cultures containing ~1 ml packed bacteria were washed in PBS and sonicated in 10 ml to reduce clumping and then diluted across a range of concentrations so that the approximate infection ratio was 100, 10 or 1 bacteria per macrophage. Infection cultures were maintained for 72–96 h, with time points for protein, RNA or NO analysis.

Statistics

We used a two-sided Student *t* test for the *in vivo M. tuberculosis* infection study. Survival curves were analyzed by nonparametric Kaplan-Meier statistics embedded in the Prism software package. $P < 0.05$ was considered significant in both tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature 2007;449:819–826. [PubMed: 17943118]
2. Bogdan C. Nitric oxide and the immune response. Nat. Immunol 2001;2:907–916. [PubMed: 11577346]
3. Chan J, Xing Y, Magliozzo RS, Bloom BR. Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. J. Exp. Med 1992;175:1111–1122. [PubMed: 1552282]
4. Thoma-Uszynski S, et al. Induction of direct antimicrobial activity through mammalian toll-like receptors. Science 2001;291:1544–1547. [PubMed: 11222859]
5. Brightbill HD, et al. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science 1999;285:732–736. [PubMed: 10426995]
6. Poole RK. Nitric oxide and nitrosative stress tolerance in bacteria. Biochem. Soc. Trans 2005;33:176–180. [PubMed: 15667299]
7. Gobert AP, et al. *Helicobacter pylori* arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. Proc. Natl. Acad. Sci. USA 2001;98:13844–13849. [PubMed: 11717441]
8. Gaur U, et al. An effect of parasite-encoded arginase on the outcome of murine cutaneous leishmaniasis. J. Immunol 2007;179:8446–8453. [PubMed: 18056391]
9. Schnappinger D, et al. Transcriptional adaptation of Mycobacterium tuberculosis within macrophages: insights into the phagosomal environment. J. Exp. Med 2003;198:693–704. [PubMed: 12953091]
10. Davis AS, et al. Mechanism of inducible nitric oxide synthase exclusion from mycobacterial phagosomes. PLoS Pathog 2007;3:e186. [PubMed: 18069890]
11. Talaue MT, et al. Arginine homeostasis in J774.1 macrophages in the context of Mycobacterium bovis BCG infection. J. Bacteriol 2006;188:4830–4840. [PubMed: 16788192]
12. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat. Rev. Immunol 2005;5:641–654. [PubMed: 16056256]
13. Gordon S. Alternative activation of macrophages. Nat. Rev. Immunol 2003;3:23–35. [PubMed: 12511873]
14. Anthony RM, Rutitzky LI, Urban JF Jr, Stadecker MJ, Gause WC. Protective immune mechanisms in helminth infection. Nat. Rev. Immunol 2007;7:975–987. [PubMed: 18007680]
15. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. Nat. Rev. Immunol 2004;4:583–594. [PubMed: 15286725]
16. Kim EY, et al. Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease. Nat. Med 2008;14:633–640. [PubMed: 18488036]
17. Rutschman R, et al. Cutting edge: STAT6-dependent substrate depletion regulates nitric oxide production. J. Immunol 2001;166:2173–2177. [PubMed: 11160269]
18. Park JH, et al. RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs. J. Immunol 2007;178:2380–2386. [PubMed: 17277144]
19. Reiling N, et al. Cutting edge: Toll-like receptor (TLR)2- and TLR4- mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. J. Immunol 2002;169:3480–3484. [PubMed: 12244136]

20. Gray MJ, Poljakovic M, Kepka-Lenhart D, Morris SM Jr. Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBPbeta. Gene 2005;353:98–106. [PubMed: 15922518]
21. Pauleau AL, et al. Enhancer-mediated control of macrophage-specific arginase I expression. J. Immunol 2004;172:7565–7573. [PubMed: 15187136]
22. Vats D, et al. Oxidative metabolism and PGC-1beta attenuate macrophage-mediated inflammation. Cell Metab 2006;4:13–24. [PubMed: 16814729]
23. Bussiere FI, et al. Spermine causes loss of innate immune response to *Helicobacter pylori* by inhibition of inducible nitric-oxide synthase translation. J. Biol. Chem 2005;280:2409–2412. [PubMed: 15548540]
24. Gobert AP, et al. *Helicobacter pylori* induces macrophage apoptosis by activation of arginase II. J. Immunol 2002;168:4692–4700. [PubMed: 11971019]
25. Zhou L, et al. An inducible enhancer required for Il12b promoter activity in an insulated chromatin environment. Mol. Cell. Biol 2007;27:2698–2712. [PubMed: 17242186]
26. Albina JE, et al. Macrophage arginase regulation by CCAAT/enhancer-binding protein beta. Shock 2005;23:168–172. [PubMed: 15665733]
27. Iyer RK, et al. Mouse model for human arginase deficiency. Mol. Cell. Biol 2002;22:4491–4498. [PubMed: 12052859]
28. Modolell M, Corraliza IM, Link F, Soler G, Eichmann K. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. Eur. J. Immunol 1995;25:1101–1104. [PubMed: 7537672]
29. Zea AH, et al. Decreased expression of CD3zeta and nuclear transcription factor kappa B in patients with pulmonary tuberculosis: potential mechanisms and reversibility with treatment. J. Infect. Dis 2006;194:1385–1393. [PubMed: 17054067]
30. Choi HS, Rai PR, Chu HW, Cool C, Chan ED. Analysis of nitric oxide synthase and nitrotyrosine expression in human pulmonary tuberculosis. Am. J. Respir. Crit. Care Med 2002;166:178–186. [PubMed: 12119230]
31. Scharton-Kersten TM, Yap G, Magram J, Sher A. Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen Toxoplasma gondii. J. Exp. Med 1997;185:1261–1273. [PubMed: 9104813]
32. Schluter D, et al. Inhibition of inducible nitric oxide synthase exacerbates chronic cerebral toxoplasmosis in Toxoplasma gondii-susceptible C57BL/6 mice but does not reactivate the latent disease in T. gondii-resistant BALB/c mice. J. Immunol 1999;162:3512–3518. [PubMed: 10092808]
33. Saeij JP, et al. Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. Nature 2007;445:324–327. [PubMed: 17183270]
34. Kerr AR, Wei XQ, Andrew PW, Mitchell TJ. Nitric oxide exerts distinct effects in local and systemic infections with Streptococcus pneumoniae. Microb. Pathog 2004;36:303–310. [PubMed: 15120156]
35. Sonoki T, et al. Coinduction of nitric-oxide synthase and arginase I in cultured rat peritoneal macrophages and rat tissues in vivo by lipopolysaccharide. J. Biol. Chem 1997;272:3689–3693. [PubMed: 9013624]
36. Karaghiosoff M, et al. Central role for type I interferons and Tyk2 in lipopolysaccharide-induced endotoxin shock. Nat. Immunol 2003;4:471–477. [PubMed: 12679810]
37. Munder M, Eichmann K, Modolell M. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4+ T cells correlates with Th1/Th2 phenotype. J. Immunol 1998;160:5347–5354. [PubMed: 9605134]
38. Munder M, et al. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. J. Immunol 1999;163:3771–3777. [PubMed: 10490974]
39. El-Gayar S, Thuring-Nahler H, Pfeilschifter J, Rollinghoff M, Bogdan C. Translational control of inducible nitric oxide synthase by IL-13 and arginine availability in inflammatory macrophages. J. Immunol 2003;171:4561–4568. [PubMed: 14568929]
40. Di Costanzo L, et al. Crystal structure of human arginase I at 1.29-A resolution and exploration of inhibition in the immune response. Proc. Natl. Acad. Sci. USA 2005;102:13058–13063. [PubMed: 16141327]

41. Wanasen N, Soong L. L-arginine metabolism and its impact on host immunity against Leishmania infection. Immunol. Res 2008;41:15–25. [PubMed: 18040886]

42. Grode L, et al. Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guerin mutants that secrete listeriolysin. J. Clin. Invest 2005;115:2472–2479. [PubMed: 16110326]

43. Murray PJ. The JAK-STAT signaling pathway: input and output integration. J. Immunol 2007;178:2623–2629. [PubMed: 17312100]

44. Ryan JL, Yohe WB, Morrison DC. Stimulation of peritoneal cell arginase by bacterial lipopolysaccharides. Am. J. Pathol 1980;99:451–462. [PubMed: 6990772]

45. Igietseme JU, et al. Chlamydial infection in inducible nitric oxide synthase knockout mice. Infect. Immun 1998;66:1282–1286. [PubMed: 9529043]

46. Rodriguez N, et al. MyD88-dependent changes in the pulmonary transcriptome after infection with Chlamydia pneumoniae. Physiol Genomics 2007;30:134–145. [PubMed: 17374847]

47. Lang R, et al. SOCS3 regulates the plasticity of gp130 signaling. Nat. Immunol 2003;4:546–550. [PubMed: 12754506]

48. Gingras S, Parganas E, de Pauw A, Ihle JN, Murray PJ. Reexamination of the role of suppressor of cytokine signaling 1 (SOCS1) in the regulation of toll-like receptor signaling. J. Biol. Chem 2004;279:54702–54707. [PubMed: 15491990]

49. Orihuela CJ, Gao G, Francis KP, Yu J, Tuomanen EI. Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. J. Infect. Dis 2004;190:1661–1669. [PubMed: 15478073]

50. Cooper AM, Pearl JE, Brooks JV, Ehlers S, Orme IM. Expression of the nitric oxide synthase 2 gene is not essential for early control of Mycobacterium tuberculosis in the murine lung. Infect. Immun 2000;68:6879–6882. [PubMed: 11083808]

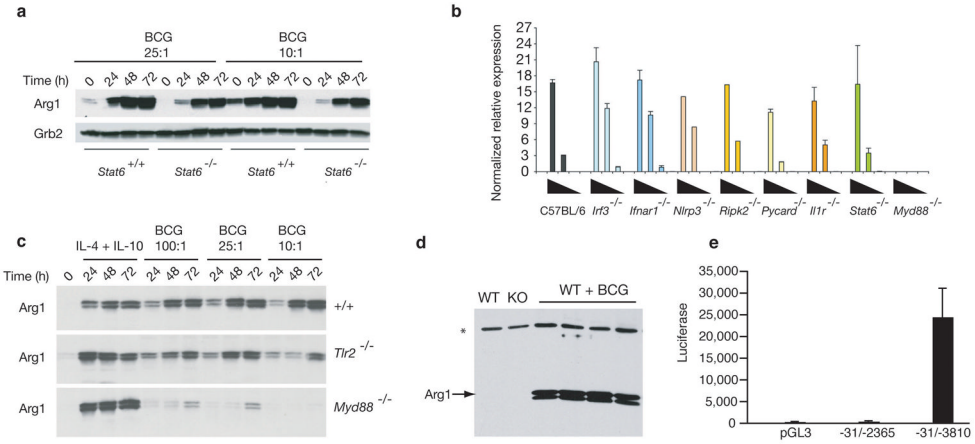

Figure 1.

Arg1 expression in *in vitro* mycobacterial infection is regulated by a STAT6-independent, Myd88-dependent pathway. (**a**) Immunoblot of lysates of BMDMs from matched control ($Stat6^{+/+}$) or $Stat6^{-/-}$ mice (129, C57BL/6 mixed background) infected with *M. bovis* BCG. At the times indicated, lysates were prepared and Arg1 expression was measured. Grb2 was used as a loading control. Data are representative of four experiments. (**b**) Quantitative RT-PCR for Arg1 mRNA expressed in BMDMs from mice with targeted mutations in key steps of the interferon production and response pathway ($Irf3^{-/-}$ and $Ifnar1^{-/-}$), the inflammasome and Nod1-Nod2 pathways ($Nlrp3^{-/-}$, $Pycard^{-/-}$ and $Ripk2^{-/-}$) and the TLR and IL-1 signaling pathway ($Myd88^{-/-}$ and $Il1r^{-/-}$) after infection with BCG at various multiplicities of infection (indicated by black triangles). Arg1 mRNA was measured at 24, 48 and 72 h after infection. Data from the 48-h time point are shown. Additional studies to test the MyD88 dependence of Arg1 expression are shown in Supplementary Figure 2. (**c**) Immunoblot of lysates from BMDMs from control C57BL/6 (+/+) and $Tlr2^{-/-}$ or $Myd88^{-/-}$ mice (both on a C57BL/6 background) infected with BCG or stimulated with cytokines as shown. Arg1 was detected as in **a**. Equal loading was confirmed by Grb2 immunoblotting (omitted for clarity). Data in **b** and **c** are representative of two independent experiments. (**d**) Immunoblot for Arg1 in adherent splenocytes lysates from mice infected intraperitoneally with BCG (1×10^7 colony-forming units) for 10 d. Controls include uninfected mice (WT) or mice lacking Arg1 in macrophages as a control for antibody specificity (KO). Lysates from four individual infected mice are shown. Asterisk indicates a cross-reactive band used as a loading control. (**e**) Luciferase assay on RAW macrophages transfected with luciferase reporter plasmids[21] (control, pGL3; −31/−2365 in pGL3 containing the −2.3 kb region from the start site; −31/−3810 containing the −4 kb region, including the 5′ enhancer) and infected with BCG (25:1 ratio of bacteria to macrophages). Luciferase activity was recorded 48 h after infection. Data are representative of four independent experiments. Error bars indicate means ± s.d.

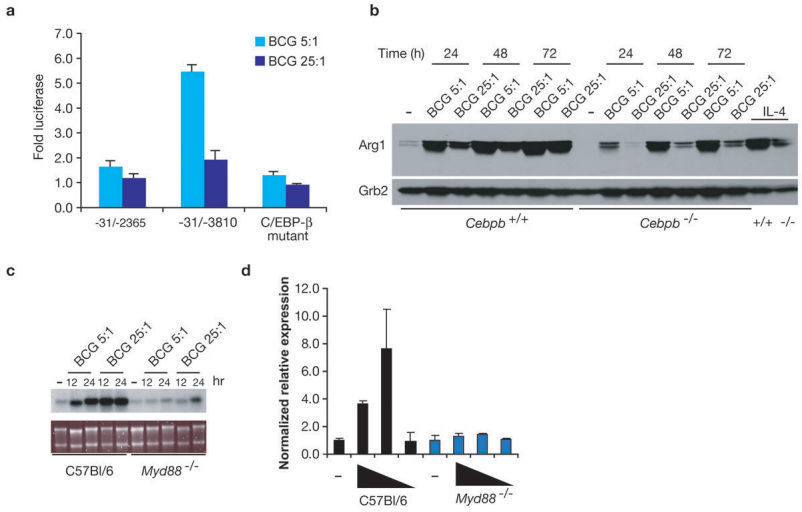

Figure 2.

Central function for the MyD88-C/EBPβ pathway in *M. bovis* BCG–mediated Arg1 expression. (**a**) RAW macrophages were stably transfected with insulated luciferase reporter plasmids and infected in triplicate with BCG at the ratios shown. Luciferase activity was recorded 72 h after infection and expressed as fold activity above the baseline uninfected cultures processed in parallel. Data are representative of three independent *in vitro* infection studies. (**b**) Immunoblot for Arg1 in lysates from BMDMs from control (C57BL/6) and *Cebpb*$^{-/-}$ mice infected with BCG at various ratios or stimulated with IL-4. Equal loading was confirmed by Grb2 immunoblotting. (**c,d**) RNA blot analysis (**c**) and quantitative RT-PCR (**d**) of C/EBPβ mRNA 48 h after infection of control (C57BL/6) and *Myd88*$^{-/-}$ mice with BCG. Data in **c** and **d** are representative of three independent experiments. Error bars indicate means ± s.d.

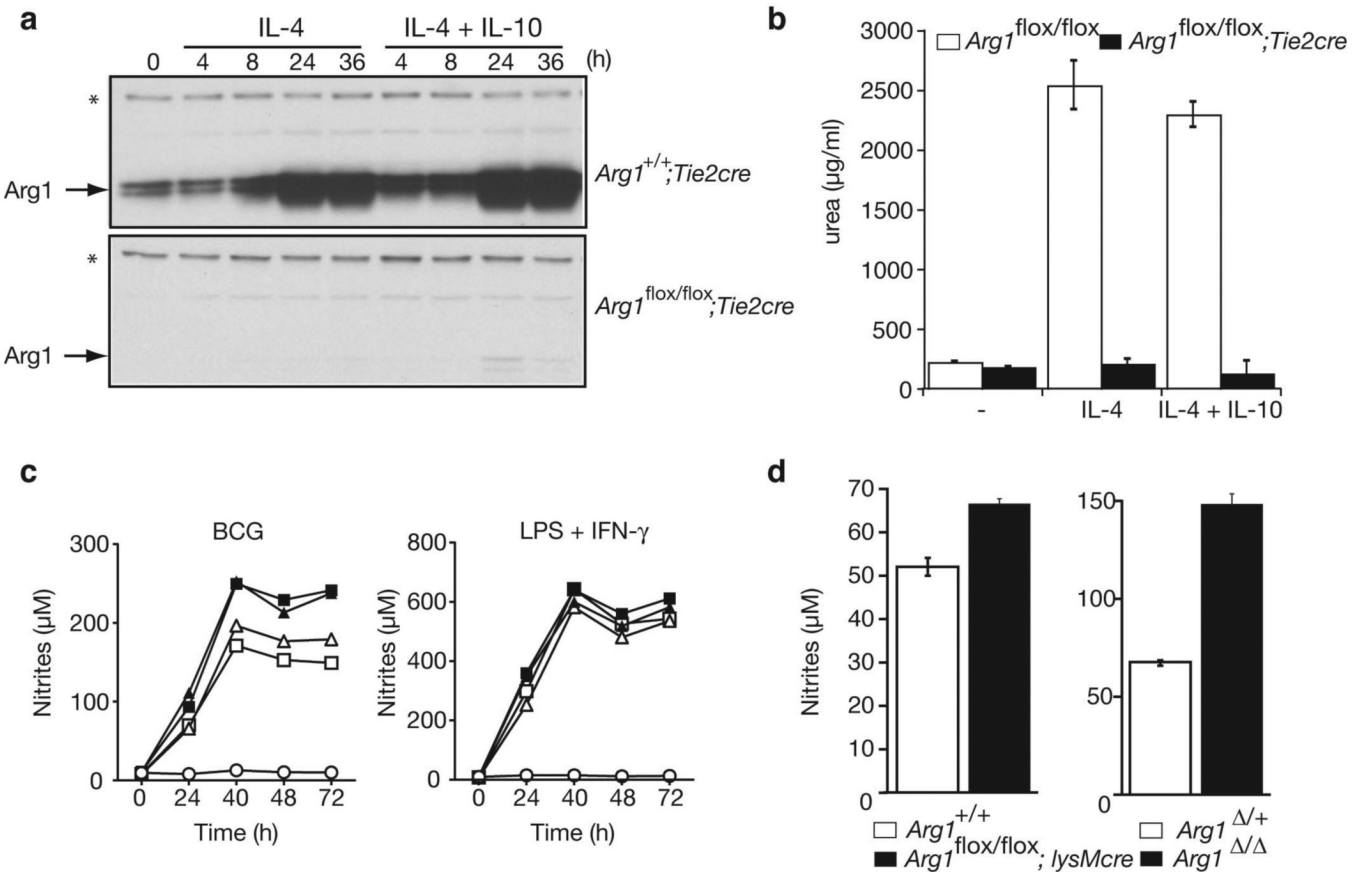

Figure 3.

Specific deletion of Arg1 in macrophages increases NO production. (**a**) Immunoblot of inflammatory peritoneal-derived macrophages from *Arg1*$^{flox/flox}$*; Tie2cre* or control mice isolated and stimulated to express Arg1 with IL-4 or IL-4 and IL-10. Asterisks indicate a background band that served as a loading control. (**b**) Arginase enzyme activity (mean ± s.d., $n = 3$ samples) detected by urea production in peritoneal-derived macrophages from *Arg1*$^{flox/flox}$*; Tie2cre* mice after 20 h of stimulation with IL-4 (10 ng ml^{-1}) or IL-4 and IL-10 (10 ng ml^{-1} each). (**c**) NO production from BMDMs from individual control mice ($n = 2$; open square and triangles), *Arg1*$^{flox/flox}$*; Tie2-cre* mice ($n = 2$; filled squares and triangles) or *Nos2*$^{-/-}$ mice (open circles) infected with *M. bovis* BCG (left) or stimulated with LPS + interferon-γ (right). Data are means from triplicate wells where each mouse is presented individually. Data are representative of four independent *in vitro* infection experiments. (**d**) NO production from BMDMs isolated from the strains indicated and stimulated with LPS (100 ng ml^{-1}) for 16 h. Data are representative of four individual experiments with *Arg1*$^{flox/flox}$*; LysMcre* mice or two experiments with *Arg1*$^{\Delta\Delta}$ fetal liver–derived macrophages.

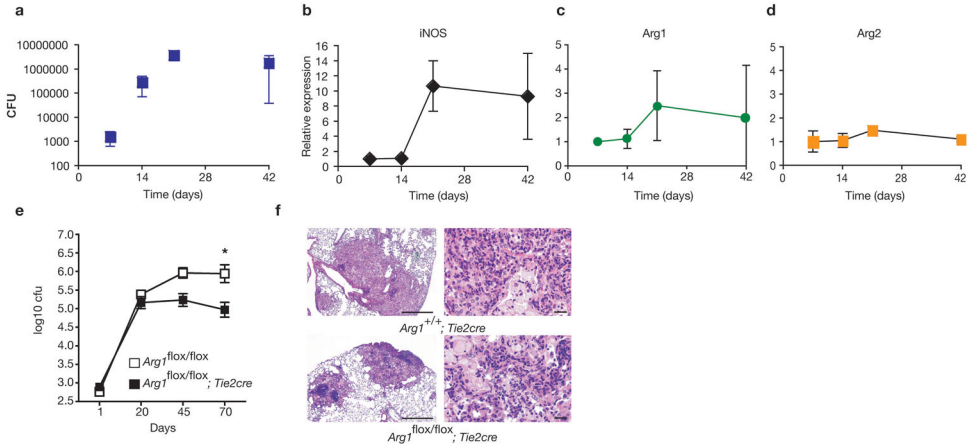

Figure 4.

Arg1 deficiency in macrophages increases clearance of *M. tuberculosis*. (**a**) Colony counts (log_{10} scale) in the lungs of three or four mice per time point after infection with CDC1551. CFU, colony-forming units. (**b–d**) Quantitative RT-PCR analysis of iNOS, Arg1 and Arg2 in the lungs of CDC1551-infected mice. Data are means of normalized relative expression ± s.d. (**e**) *Arg1*$^{flox/flox}$*; Tie2cre* mice (filled squares) or control mice (open squares) were infected with 200 colony-forming units of *M. tuberculosis* H37Rv on day 0. Bacterial loads in the lungs (n = 4–5 per group; mean ± s.d. on log_{10} scale) were measured at the times indicated. $P < 0.05$ on day 70. (**f**) Lung histology at low magnification (left; scale bar, 1 mm) or high magnification (right; scale bar, 100 μm).

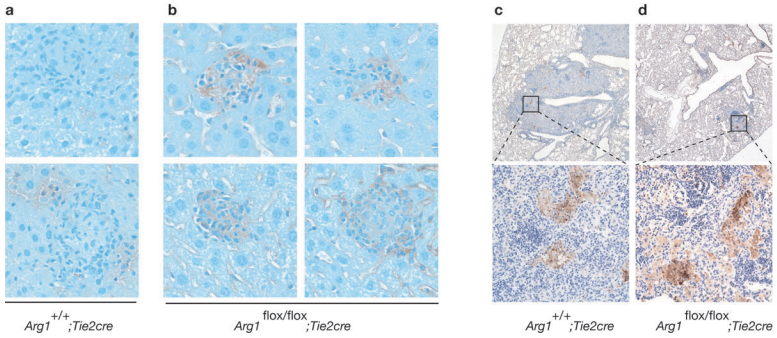

Figure 5.

Nitrotyrosine staining of mycobacterial tissues. (**a,b**) Granulomas from *M. bovis* BCG–infected livers 10 weeks after infection. Brown areas are positive for nitrotyrosine. Slides were counterstained with hematoxylin to indicate the lack of background in the liver parenchyma. Slides were photographed through a 40× objective. Granuloma staining is representative of ~50 granulomas examined in three separate mice per genotype. (**c,d**) Nitrotyrosine staining of lungs from *M. tuberculosis* H37Rv–infected mice. Lung sections from infected mice were stained with antibody to nitrotyrosine. Upper panels were photographed through a 2× objective; lower panels show boxed areas through a 40× objective.

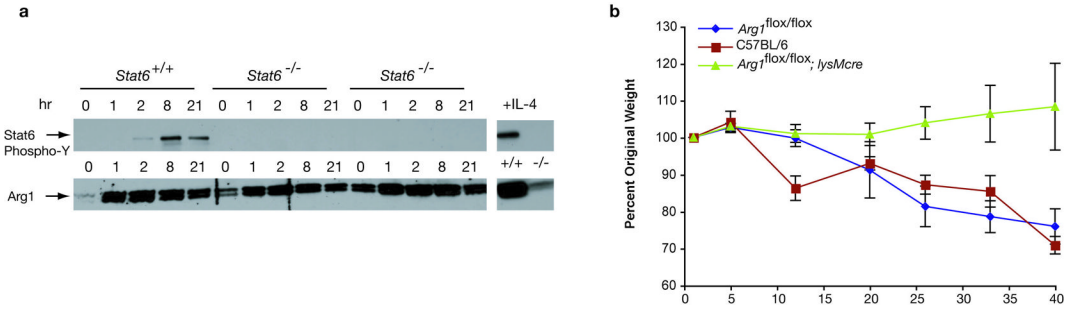

Figure 6.

Macrophage Arg1 is a susceptibility factor in experimental toxoplasmosis. (**a**) Immunoblot of tyrosine-phosphorylated (phospho-Y) STAT6 or Arg1 in lysates of BMDMs from *Stat6*$^{+/+}$ or *Stat6*$^{-/-}$ mice infected with ME49 tachyzoites. Lysates were prepared at the indicated times after infection. (**b**) *Arg1*$^{flox/flox}$*; LysMcre* (n = 5) mice and control *Arg1*$^{flox/flox}$ mice (n = 4) were infected with *T. gondii* strain ME49 and monitored over a 6-week period. Weight loss as a percentage of initial weight was recorded. Control mice were killed at week 7 as their weight had decreased to 80% of the starting weight. A second independent experiment produced concordant results, including early euthanasia for the control mice but not the infected *Arg1*$^{flox/flox}$*; LysMcre* mice.