Genetic Ablation of Arginase 1 in Macrophages and Neutrophils Enhances Clearance of an Arthritogenic Alphavirus

Kristina A. Stoermer, Adam Burrack, Lauren Oko, Stephanie A. Montgomery, Luke B. Borst, Ronald G. Gill and Thomas E. Morrison

*J Immunol* 2012; 189:4047-4059; Prepublished online 12 September 2012; doi: 10.4049/jimmunol.1201240 http://www.jimmunol.org/content/189/8/4047

Supplementary Material

http://www.jimmunol.org/content/suppl/2012/09/12/jimmunol.1201240.DC1

References

This article cites 88 articles, 29 of which you can access for free at: http://www.jimmunol.org/content/189/8/4047.full#ref-list-1

Why The *JI*? Submit online.

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Genetic Ablation of Arginase 1 in Macrophages and Neutrophils Enhances Clearance of an Arthritogenic Alphavirus

Kristina A. Stoermer,*† Adam Burrack,*† Lauren Oko,* Stephanie A. Montgomery,*‡
Luke B. Borst,§ Ronald G. Gill,* and Thomas E. Morrison*‡

Chikungunya virus (CHIKV) and Ross River virus (RRV) cause a debilitating, and often chronic, musculoskeletal inflammatory disease in humans. Macrophages constitute the major inflammatory infiltrates in musculoskeletal tissues during these infections. However, the precise macrophage effector functions that affect the pathogenesis of arthritogenic alphaviruses have not been defined. We hypothesized that the severe damage to musculoskeletal tissues observed in RRV- or CHIKV-infected mice would promote a wound-healing response characterized by M2-like macrophages. Indeed, we found that RRV- and CHIKV-induced musculoskeletal inflammatory lesions, and macrophages present in these lesions, have a unique gene-expression pattern characterized by high expression of arginase 1 and Ym1/Chi3l3 in the absence of FIZZ1/Relmα that is consistent with an M2-like activation phenotype. Strikingly, mice specifically deleted for arginase 1 in neutrophils and macrophages had dramatically reduced viral loads and improved pathology in musculoskeletal tissues at late times post-RRV infection. These findings indicate that arthritogenic alphavirus infection drives a unique myeloid cell activation program in inflamed musculoskeletal tissues that inhibits virus clearance and impedes disease resolution in an arginase 1-dependent manner.

C

hikungunya virus (CHIKV), Ross River virus (RRV), Mayaro virus, o’nyong-nyong virus, and others are positive-sense, ssRNA viruses in the Alphavirus genus of the family Togaviridae (1). These mosquito-transmitted alphaviruses cause a debilitating musculoskeletal inflammatory disease in humans and are emerging disease threats because of their ability to cause explosive epidemics. Past epidemics include a 1979–1980 epidemic of RRV disease in the South Pacific that involved >60,000 patients (2) and a 1959–1962 epidemic of o’nyong-nyong fever in Africa that involved ≥2 million infections (3). Since 2004, CHIKV has caused major epidemics in multiple countries in the Indian Ocean region, with estimates on the order of 1–6 million cases (4) and, for the first time, it has caused disease outbreaks in Europe and the Pacific Region (5–7). During these outbreaks, ≥106 CHIKV viremic travelers have been identified in the United States (8). The spread of the peri-urban mosquito Aedes albopictus into Europe and the Americas, along with high viremia in infected travelers returning from areas with CHIKV activity, increases the risk that this virus will continue to spread to new regions. In fact, >200 human cases of CHIKV disease in Italy were traced to a single infected traveler, and CHIKV was subsequently detected in local A. albopictus mosquitoes (6, 9). In addition, the Pan American Health Organization and the Centers for Disease Control and Prevention recently released a preparedness guide anticipating the introduction of CHIKV in the Americas (10).

Clinical manifestations following infection with arthritis/myositis-associated alphaviruses are most commonly characterized by fever, intense pain in the peripheral joints, myalgias, and an impaired ability to ambulate (2, 11, 12). The joint pain associated with arthritogenic alphavirus infections is typically symmetrical, with fingers, wrists, elbows, toes, ankles, and knees most commonly affected (13). A number of studies indicate that musculoskeletal pain lasts for months to years in a subset of persons infected with RRV or CHIKV; however, the cause of these long-lasting symptoms is unclear (13–23). More severe disease, including neurologic manifestations, myocarditis, and death, have been reported. These atypical outcomes are associated with age and underlying medical conditions (24). There are no licensed antivirals or vaccines for any of the arthritis/myositis-associated alphaviruses; treatment is limited to supportive care with analgesics and anti-inflammatory drugs (2, 25).

Monocytes/macrophages constitute the major inflammatory infiltrates in musculoskeletal tissues of CHIKV- or RRV-infected humans, nonhuman primates, and mice (26–33), and numerous studies have implicated macrophages in the pathogenesis of these infections. However, how macrophages are activated during alphavirus infection and the precise macrophage effector functions that affect the pathogenesis of arthritogenic alphaviruses have not been defined. Although macrophages at inflammatory sites often have

*Department of Immunology, University of Colorado School of Medicine, Aurora, CO 80045; †Graduate Program in Immunology, University of Colorado School of Medicine, Aurora, CO 80045; ‡Department of Microbiology, University of Colorado School of Medicine, Aurora, CO 80045; and §Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27607

Received for publication April 30, 2012. Accepted for publication August 10, 2012.

This work was supported by National Institutes of Health-National Institute of Allergy and Infectious Diseases Research Grant K22 AI079163 and funding from the Arthritis National Research Foundation (to T.E.M.). K.A.S. was supported by National Institutes of Health-National Institute of Allergy and Infectious Diseases Training Grant T32 AI052066 and National Institutes of Health-National Center for Research Resources Colorado Clinical and Translational Sciences Institute Grant TL1 RR025778.

Address correspondence and reprint requests to Dr. Thomas E. Morrison, Department of Microbiology, University of Colorado School of Medicine, 12800 East 19th Avenue, Mail Stop 8333, Aurora, CO 80045. E-mail address: thomas.morrison@ucdenver.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: Arg1, arginase 1; BMDM, bone marrow-derived macrophage; CHIKV, Chikungunya virus; dpi, days postinoculation; MDSC, myeloid-derived suppressor cell; qPCR, quantitative PCR; RRV, Ross River virus; WT, wild-type.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/$16.00
a spectrum of overlapping phenotypes, subsets with distinct functions have been described (34–36). Classically activated (M1) macrophages have a proinflammatory phenotype and high antigen presentation capacity, and they contribute to host defense against infectious pathogens and tumors. In contrast, alternatively activated (M2) macrophages have high phagocytic capacity and promote tissue repair/remodeling during wound healing. In addition, M2 macrophages dampen inflammation and have immunoregulatory functions. The activation of M2 or M2-like macrophages occurs in response to tissue damage in a variety of tissue types, including musculoskeletal tissues, and this response can be activated in the presence or absence of an infectious pathogen (37–42). These findings, and the findings that tissue repair mediated by Th2 cells is a primary defense against helminth infections (43), have led to the hypothesis that M2 macrophages are a component of a larger Th2-mediated response that evolved as a wound-repair response (44). Consistent with this model, recent studies reported an important role for IL-4Re-dependent M2 macrophages in the resolution of tissue damage during respiratory syncytial virus infection (45).

Interestingly, the expression of arginase 1 (Arg1), a central metabolic enzyme of the liver that catalyzes the hydrolysis of L-arginine to urea and ornithine (46), is associated with M2 macrophages or M2-like cells (47, 48). In addition, the expression of Arg1 by human and murine monocytes/macrophages, neutrophils, and myeloid-derived suppressor cells (MDSCs) (49–51) has emerged as a major regulator of immune responses. Arg1 activity in myeloid cells impairs effective immunity against intracellular pathogens, such as *Mycobacterium tuberculosis* and *Toxoplasma gondii*, exacerbates tumor growth by suppressing T cell function, and limits T cell–driven inflammatory tissue damage by suppressing effector T cell functions and promoting regulatory T cell activation (52–56).

Previous studies in mouse models showed that arthritogenic alphavirus infection results in severe inflammation and tissue damage in skeletal muscle tissue, joint-associated tissues, and tendons (26, 27, 57, 58). We hypothesized that the severe damage to musculoskeletal tissues of RRV- or CHIKV-infected mice promotes a wound-healing response characterized by M2-like macrophages. Given the immunosuppressive activity of these cells, we further reasoned that this wound-healing response would impair the clearance of virus from inflamed tissues. Indeed, we found that Arg1 activity in macrophages and neutrophils had dramatically reduced viral loads, as well as improved tissue pathology, at late, but not early, times post-RRV infection. Our findings suggest that Arg1 activity in myeloid cells has a critical role in regulating the clearance of arthritogenic alphaviruses from inflamed and injured musculoskeletal tissues.

Materials and Methods

**Viruses**

The T48 strain of RRV was isolated from *Aedes vigilax* mosquitoes in Queensland, Australia (59). Prior to cDNA cloning, the virus was passaged 10 times in suckling mice, followed by two passages on Vero cells (60). The SL15649 strain of CHIKV was isolated from a serum sample collected from a febrile patient in Sri Lanka in 2006. This virus was passaged twice in Vero cells prior to cDNA cloning (27). Stocks of infectious RRV or CHIKV were generated from cDNAs, as previously described (27, 28). CHIKV was isolated from a serum sample collected from a febrile patient in Sri Lanka in 2006. This virus was passaged twice in Vero cells prior to cDNA cloning (27). Stocks of infectious RRV or CHIKV were generated from cDNAs, as previously described (27, 28).

**Cells**

BHK-21 cells (ATCC CCL10) were grown as previously described (61). Bone marrow from the femur and tibia of C57BL/6 mice was harvested in RPMI 1640 (HyClone) with 10% FBS. Following RBC lysis, bone marrow cells were plated in 10-cm dishes in DMEM (Sigma) containing 10% FBS, 5% horse serum, and 20% L cell-conditioned media for 6 to 24 days to differentiate into macrophages. Following differentiation, cells were scraped, counted, and plated in 48- well plates in DMEM containing 10% FBS. The following day, cells were treated or not with LPS (100 ng/ml; Sigma) and IFN-γ (15 ng/ml; BD Pharmingen) or recombinant mouse IL-4 (5 ng/ml; R&D Systems) for 24 h. Cells were washed with PBS and then lysed in TRIZol Reagent (Life Technologies). For CHIKV sequence-

**Flow cytometry and cell sorting**

Leukocytes isolated from enzymatically digested tissues were incubated with anti-mouse FcγRIIIA (2.4G2; BD Pharmingen) for 20 min on ice to block specific Ab binding to FcγRIIIA (CD16) before staining in Perm buffer (Invitrogen), 1% paraformaldehyde (PFA), PBS, 2% FBS) with the following Abs from eBioscience: anti–CD11b-FITC and anti–F4/80–allophycocyanin. Cells were fixed overnight in 1% paraformaldehyde and analyzed on an LSR II using FACSData software (Becton Dickinson). For cell sorting, mice were sacrificed at 7 or 10 days postinoculation (dpi), and the quadriceps muscles were processed as described above. Cells were stained in FACS staining buffer with anti–CD11b–FITC and anti–F4/80–allophycocyanin Abs (eBioscience). CD11b+ F4/80+ cells were sorted under BSL2 conditions on a FACSaria using FACSData software (Becton Dickinson). FlowJo software (Tree Star) was used for all analyses. For morphological analysis of FACS-sorted CD11b+ F4/80+ cells, a Thermo Shandon Cytospin 4 was used, and slides were stained with Wright-Giemsa stain (Sigma-Aldrich).

**Real-time RT-quantitative PCR**

RNA was isolated using a PureLink RNA Mini Kit (Life Technologies). For analysis of host gene expression, 0.2–1 μg total RNA was reverse transcribed using Superscript III (Life Technologies), random oligo-deoxithymidylic acid primers, and RNaseOUT. Real-time quantitative PCR (qPCR) experiments were performed using TaqMan gene-expression assays and a LightCycler 480 (Roche). 18S rRNA and hypoxanthine guanine phosphoribosyl transferase were used as endogenous controls. Real-time qPCR was performed with the LightCycler 480. The relative fold induction of amplified mRNA was determined using the Ct method (63). For absolute quantification of viral RNA, a sequence-tagged (small caps) RRV-specific RT primer (4415-5’-ggcagtattcgtgaattcgatgcCGTGTCGGTAGTCTTGCACAT-3’), CHIKV-specific primer (1036-5’-ggcagtattcgtgaattcgatg-3’) during qPCR to enhance specificity. For CHIKV, CHIKV sequence-
specific forward (CHIKV: 874 5'-AAAGGCACATGCCTCACTAC-3') and reverse (CHIKV: 961 5'-GGCTGGGGTCATCGTTATTC-3') primers were used with an internal TaqMan probe (CHIKV: 899 5'-CCGCTGGATAGCAGTGTTGGT-3'). To create standard curves, 10-fold dilutions, from 10^3 to 10^9 copies, of RRV or CHIKV genomic RNAs, synthesized in vitro, were spiked into RNA from BHK-21 cells, and reverse transcription and qPCR were performed in an identical manner.

**MLR**

The capacity of defined macrophage populations to inhibit T cell reactivity in vitro was assessed by macrophage coculture with naive C57BL/6 T cells responding to allogeneic BALB/c splenic stimulator cells. The alloreactive MLR was performed largely as previously described (64). Briefly, 2 × 10^6 responding B6 lymph node cells were stimulated with 3 × 10^5 irradiated (2000 rad) allogeneic BALB/c spleen cells in triplicate cultures consisting of Eagle's MEM supplemented with 10% FCS in 96-well, flat-bottom plates. To assess macrophage suppressive activity, graded numbers of the indicated macrophage populations were added to these MLR cultures. Ratios indicate the number of macrophage cells added relative to the number of responding lymph node cells. Total MLR culture volume was 200 μl after the addition of test macrophage cells or media. On day 4 of the MLR, cultures were assessed for proliferation by measuring the [3H]thymidine incorporation into DNA following a 6-h pulse. The proliferation of each group was calculated as a percent of the proliferation of C57BL/6 responder cells alone to irradiated BALB/c stimulator cells.

**Western blots**

Protein lysates were separated by Tris-HCl-buffered 10% SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes. Membranes were blocked in 5% milk in PBS containing 0.1% Tween and incubated in the appropriate Abs against the indicated proteins. GAPDH expression was used as a loading control. Anti-mouse Arg1 Ab (V-20) was obtained from Santa Cruz; anti-mouse GAPDH Ab (clone 71.1) was obtained from Sigma-Aldrich. Membranes were imaged on a ChemiDoc XRS Plus imager (Bio-Rad), and bands for Arg1 and GAPDH were quantified using Bio-Rad Image Lab software.

**Histological analysis**

At the times indicated, mice were sacrificed and perfused with intracardiac injection of 1× PBS, and the indicated tissues were dissected and fixed in 4% paraformaldehyde (pH 7.3). Tissues were embedded in paraffin, and 5-μm sections were prepared. To assess histopathological changes, such as tissue inflammation and damage, tissue sections were stained with H&E and evaluated by light microscopy. In all mice, the presence, distribution, and severity of histologic lesions were scored blindly by two pathologists. Lesions consisted primarily of myocyte necrosis, myocyte regeneration, inflammation, edema, and fibrosis. For all tissue changes, the following scoring system was used: 0, absent; 1, minimal, <10% of tissue affected; 2, mild, 10–24% of tissue affected; 3, moderate, 25–39% of tissue affected; 4, marked, 40–50% of tissue affected; 5, severe, ≥60% of tissue affected. Scores of histologic lesions for individual mice were plotted for analysis.

**Immunohistochemistry**

For immunohistochemical analysis of Arg1 expression, deparaffinized slides were blocked in normal serum (VECTASTAIN ABC Kit; Vector Labs) for 20 min, incubated in anti-Arg1 Ab (H-52; Santa Cruz) for 1 h, and incubated in biotinylated secondary Ab for 30 min. For resolution of the staining, slides were incubated in VECTASTAIN ABC reagent and developed in DAB Peroxidase Substrate Solution (Vector Labs). Slides were counter-stained with Mayer’s hematoxylin (ThermoScientific).

**Statistical analysis**

All data were analyzed using GraphPad Prism 5 software. Data were evaluated for statistically significant differences using either a two-tailed, unpaired t test with or without the Welch correction or an ANOVA test followed by the Tukey multiple-comparison test. A p value < 0.05 was considered statistically significant. All differences not specifically indicated to be significant were not significant (p > 0.05).

**Results**

**Arg1 is highly expressed in inflammatory lesions of RRV-infected mice**

To study the pathogenesis of arthritogenic alphaviruses, we developed mouse models of RRV- and CHIKV-induced disease in which the major pathological outcomes—arthritis, myositis, and tenosynovitis—are consistent with the disease signs experienced by infected humans (26–28). To determine the extent to which the inflammation and damage to musculoskeletal tissues caused by infection with arthritogenic alphaviruses activate genes associated with wound repair or M2 macrophages, total RNA was isolated from quadriceps muscle tissue and ankle/foot tissues (data not shown) of PBS- or RRV-inoculated mice at various dpi and analyzed by real-time RT-qPCR for the expression of Arg1, a signature gene of M2 macrophage activation in mice (Fig. 1A). At early times postinoculation (3 and 5 dpi), Arg1 expression was undetectable or low in skeletal muscle tissue. At 7 dpi, a time point associated with the development of extensive skeletal muscle tissue inflammation and injury (28), the relative abundance of Arg1 transcripts increased dramatically relative to 18S RNA expression (472-fold increase over mock-inoculated mice, p = 0.0005). Arg1 transcripts remained significantly elevated at 10 and 14 dpi (183-fold increase, p = 0.02 and 63-fold increase, p = 0.03 over mock-inoculated mice, respectively). Similar results were obtained when Arg1 expression data were normalized to hypoxanthine guanine phosphoribosyl transferase expression (data not shown). To correlate the temporal induction of Arg1 with corresponding tissue inflammation and injury, we analyzed the same RNA samples for the expression of a variety of cytokines (Fig. 1B), and tissues derived from quadriceps muscles of uninfected (mock) or RRV-infected mice were stained with H&E and evaluated by light microscopy. The expression of IL-1β, TNF-α, IL-6, and IL-10 mRNAs was similar to that of Arg1, with relatively low expression at 3 and 5 dpi (except for IL-1β mRNA, which was high at 5 dpi), peak expression occurring at 7 dpi, and expression levels remaining elevated at both 10 and 14 dpi (Fig. 1B). In contrast, the relative expression of IL-4 and IL-13 transcripts was very low or undetectable at all time points examined. The expression of IL-12b mRNA in this tissue exhibited a distinct pattern, with very low expression until 10 dpi (Fig. 1B). The expression of Arg1, IL-1β, TNF-α, IL-6, and IL-10 correlated with cellular infiltration and damage of skeletal muscle tissue, which begins at 5 dpi and is most prominent at 7, 10, and 14 dpi (Fig. 1C). Consistent with the RT-qPCR analyses, abundant Arg1 protein was detected by immunoblot analysis in both quadriceps muscle tissue and ankle joint tissue at 10 dpi and was still detectable at 20 dpi (Fig. 1D). These data demonstrate that Arg1 expression in tissues of RRV-infected mice correlates temporally with musculoskeletal tissue inflammation and injury. In addition, the expression of Arg1 correlated with the expression of IL-1β, TNF-α, IL-6, and IL-10 but not IL-4, IL-13, or IL-12b.

In addition to Arg1, Ym1/Chi3l3 and FIZZ1/Relm-α are well-established markers of canonical M2 macrophage activation (65). Similar to Arg1 (Fig. 1A), we detected high levels of Ym1 expression in whole quadriceps tissues of RRV-infected mice at 7 dpi (807-fold increase over mock-inoculated mice, p = 0.0007) (Fig. 1E). In contrast, FIZZ1 expression in the same tissues was actually reduced in RRV-infected mice relative to control mice (66-fold decrease compared with mock-inoculated mice, p = 0.03) (Fig. 1E). These findings suggest that at least a subset of macrophages infiltrating these tissues has a unique M2-like phenotype characterized by abundant expression of Arg1 and Ym1 in the absence of FIZZ1.

Arg1 is induced in CD11b+ F4/80+ musculoskeletal tissue infiltrates

To identify Arg1-expressing cell type(s) within the musculoskeletal inflammatory lesions, we enzymatically digested quadriceps muscles at 7 d post-RRV inoculation, isolated leukocytes, and FACS-sorted the CD11b+F4/80+ macrophage population and...
the CD11b^F4/80^- cell population (Fig. 2A, 2B). RT-qPCR analysis of Arg1 expression in these different cell populations was compared with Arg1 expression in unstimulated and IL-4–stimulated bone marrow-derived macrophages (BMDMs) (Fig. 2C), because IL-4 is a cytokine known to strongly induce Arg1 expression in macrophages (66). As expected, IL-4 stimulation strongly induced Arg1 expression in BMDMs (Fig. 2C). The CD11b^F4/80^- macrophages sorted from inflamed quadriceps tissues of RRV-infected mice had high expression of Arg1 relative to the unstimulated BMDMs and significantly higher expression of Arg1 compared with the CD11b^-F4/80^- cells (92-fold increase over CD11b^-F4/80^- cells, p = 0.0028) (Fig. 2C). These findings suggest that at least a subset of the CD11b^F4/80^- macrophages is a major source of Arg1 expression in the inflamed musculoskeletal tissues of RRV-infected mice.

Interestingly, Arg1-expressing myeloid cells were demonstrated to have immune-suppressive activity (53, 54, 67). To test whether the Arg1-expressing CD11b^F4/80^+ macrophages present in the inflamed musculoskeletal tissues of RRV-infected mice have functional properties consistent with Arg1-expressing myeloid cells, FACS-sorted CD11b^F4/80^+ macrophages isolated from quadriceps muscles of mock- or RRV-infected mice at 10 d post-RRV inoculation were evaluated for the ability to suppress T cell proliferation ex vivo in response to allogeneic (BALB/c) stimulator cells in a standard MLR. As shown in Fig. 3, CD11b^F4/80^+ muscle tissue macrophages impaired T cell proliferation in a dose-dependent manner.

Arg1 is highly expressed in inflammatory lesions of CHIKV-infected mice

We recently developed a mouse model of CHIKV-induced musculoskeletal inflammatory disease based on s.c. inoculation of mice with a low-passage clinical isolate of CHIKV (27). We used this model to determine the extent to which Arg1 induction in mus-
culoskeletal inflammatory lesions is unique to RRV infection or whether it is common to arthritogenic alphavirus infection. Relative to uninfected mice, Arg1 mRNA expression in the left ankle/foot of CHIKV-infected mice was relatively low at 3 dpi (5.9-fold increase over mock-inoculated mice, \( p = 0.024 \)), dramatically induced at 7 dpi (116.5-fold increase over mock-inoculated mice, \( p = 0.02 \)), and remained significantly elevated at 14 dpi (14.6-fold increase over mock-inoculated mice, \( p = 0.012 \)) (Fig. 4A). CHIKV RNA loads in the left ankle/foot at these time points were highest at 3 dpi in comparison with 7 and 14 dpi (Fig. 4B). Thus, similar to RRV-infected mice, the expression of Arg1 correlated with injury and infiltration of CHIKV-infected left ankle/foot tissues with CD11b+F4/80+ macrophages, neutrophils, NK cells, and T cells, which is low at 3 dpi and high at 7 dpi as previously reported (27). Consistent with the RT-qPCR analyses, Arg1 protein was highly expressed at 10 dpi in ankle/foot tissue of CHIKV-infected mice (Fig. 4C). In addition, Arg1 protein was readily detected by immunohistochemistry in tissue sections collected at 10 dpi from the ankle/foot of CHIKV-infected mice but not mock-inoculated control mice (Fig. 4D). These findings indicate that high Arg1 expression in inflamed musculoskeletal tissues is a common characteristic of arthritogenic alphavirus infection.

LysMcre-mediated deletion of Arg1 ablates RRV- and CHIKV-induced Arg1 expression

Because of the established role of myeloid cell Arg1 activity in wound repair and regulation of immune and inflammatory responses (50), we sought to specifically investigate the role of myeloid cell Arg1 during infection with arthritogenic alphaviruses. We bred Lysozyme M Cre-expressing mice (LysMcre) (62) with mice possessing \( \text{lxx} \)P sites on either side of exons 7 and 8 in the Arg1 gene (Arg1\( ^{\text{lxx}} \)) (52) to generate mice in which Arg1 expression is specifically ablated from macrophages and neutrophils (LysMcre; Arg1\( ^{\text{lxx}} \)), as previously reported (52). In con-

**FIGURE 2.** Tissue-infiltrating macrophages express Arg1. Three- to four-week-old C57BL/6J mice were inoculated with \( 10^3 \) PFU of RRV in the left rear footpad. At 7 dpi, quadriceps muscles were enzymatically digested, and CD11b\( ^{-} \)F4/80\( ^{-} \) and CD11b\( ^{+} \)F4/80\( ^{-} \) cellular infiltrates were FACS sorted. (A) Representative dot plots of total cells, double-positive and double-negative populations, along with their postsort purities. (B) Postsort purities of CD11b\( ^{-} \)F4/80\( ^{-} \) (n = 4) and CD11b\( ^{+} \)F4/80\( ^{-} \) (n = 4) populations. Each bar represents the arithmetic mean ± SEM. (C) RT-qPCR analysis of Arg1 mRNA expression in unstimulated or IL-4–stimulated (5 ng/ml) BMDMs (n = 3), FACS-sorted CD11b\( ^{-} \)F4/80\( ^{-} \) cells (n = 4), and FACS-sorted CD11b\( ^{+} \)F4/80\( ^{-} \) cells (n = 4). Data are normalized to 18S rRNA levels and are expressed as the relative expression (n-fold increase) over Arg1 expression in unstimulated BMDMs. Each data point represents the arithmetic mean ± SEM. ***\( p = 0.0028 \), unpaired \( t \) test with Welch correction.

**FIGURE 3.** Macrophages isolated from musculoskeletal tissues suppress T cell proliferation ex vivo by a mechanism that is partially Arg1 dependent. Three- to four-week-old C57BL/6J mice (n = 3) or LysMcre;Arg1\( ^{\text{lxx}} \) mice (n = 3) were inoculated with \( 10^3 \) PFU of RRV in the left rear footpad. At 10 dpi, quadriceps muscles were enzymatically digested, and CD11b\( ^{+} \)F4/80\( ^{-} \) cellular infiltrates were FACS sorted and analyzed for immune-suppressive activity in an MLR. C57BL/6J lymph node cells and irradiated BALB/c splenocytes were incubated in the absence or presence of FACS-sorted CD11b\( ^{-} \)F4/80\( ^{-} \) cells at 1:2, 1:4, 1:8, and 1:16 ratios in the presence of tritiated thymidine. Control wells contained irradiated BALB/c splenocytes only, C57BL/6J lymph node cells only, or C57BL/6J lymph node cells incubated in the presence of C57BL/6J-irradiated splenocytes. Thymidine incorporation was quantified after 4 d. Each data point represents the arithmetic mean ± SEM and is representative of two independent experiments. *\( p < 0.05 \), ANOVA, followed by the Tukey multiple-comparison test.
and foot). Images are representative of three mice/group.

arginase 1 (Arg1) is induced in inflamed musculoskeletal tissues of CHIKV-infected mice. Three- to four-week-old C57BL/6J mice were mock inoculated (0 dpi) or inoculated with 10^3 PFU of CHIKV in the left rear footpad. (A) RT-qPCR analysis of Arg1 mRNA expression in the left ankle/foot tissue of mock-inoculated (n = 5) or CHIKV-inoculated mice at 3 (n = 6), 7 (n = 6), and 14 dpi (n = 8). Data are normalized to 18S rRNA levels and are expressed as the relative expression (n-fold increase) over Arg1 expression in left ankle/foot tissue of mock-inoculated mice. Each data point represents the arithmetic mean ± SEM. *p < 0.05, unpaired t tests with Welch correction. (B) CHIKV genomes in the left foot/ankle of mock- or CHIKV-infected mice at 3, 7, and 14 dpi were quantified by absolute RT-qPCR, as described in Materials and Methods. Horizontal lines indicate the mean, and the dashed line indicates the limit of detection. (C) Immunoblot analysis of Arg1 expression in ankle/foot tissues of mock-inoculated or CHIKV-inoculated mice at 10 dpi. GAPDH was used as a loading control. Data are representative of two independent experiments. (D) Five-micrometer sections generated from the liver (upper panels) or ankle/foot (lower panels) at 10 dpi were stained for Arg1 expression by immunohistochemistry (original magnification ×200—all images, liver and foot). Images are representative of three mice/group.

contrast to BMDMs generated from Arg1<sup>F/F</sup> mice, Arg1 protein expression was dramatically reduced in BMDMs from LysMcre; Arg1<sup>F/F</sup> mice following treatment with IL-4 (Supplemental Fig. 1). Importantly, LysMcre;Arg1<sup>F/F</sup> mice expressed Arg1 in the liver at levels similar to control mice (Supplemental Fig. 1), indicating that this conditional knockout deletes Arg1 specifically from myeloid cells. We next tested the extent to which genetic ablation of Arg1 in macrophages and neutrophils prevented the induction of Arg1 at the sites of inflammation following RRV infection. Compared with RRV-infected Arg1-sufficient mice, RRV-infected LysMcre;Arg1<sup>F/F</sup> mice had significantly reduced Arg1 mRNA (Fig. 5A) and protein (Fig. 5B) levels in quadriceps muscles at 7 and 10 dpi, respectively. Similarly, Arg1 expression at 7 dpi was dramatically reduced in the inflamed ankle/foot tissue of CHIKV-infected LysMcre;Arg1<sup>F/F</sup> mice compared with Arg1-sufficient mice (Fig. 5C). These data suggest that infiltrating macrophages and/or neutrophils are the predominant source of Arg1 expression in the musculoskeletal tissues of RRV- and CHIKV-infected mice.

Previous studies in humans and mice, including differential cell counts on Giemsa-stained cytopsins of collagenase-digested murine quadriceps muscles at various times post-RRV infection [a procedure that has been used successfully to isolate viable neutrophils (68)], demonstrated that musculoskeletal tissue infiltrates associated with RRV infection are primarily mononuclear (26, 28, 33, 58, 69–72). To confirm that LysMcre-mediated ablation of Arg1 prevented Arg1 expression in macrophages at the sites of RRV-induced musculoskeletal tissue inflammation and injury, we isolated inflammatory infiltrates from enzymatically digested quadriceps muscles at 10 d post-RRV inoculation and FACS-sorted CD11b<sup>F4/80</sup> macrophages. As shown in Fig. 6A, the CD11b<sup>F4/80</sup> cells FACS sorted from WT (mean, 82% purity [range, 75–95%]) and LysMcre;Arg1<sup>F/F</sup> mice (mean, 82% purity [range, 77–85%]) were morphologically indistinguishable. RT-qPCR analysis of Arg1 expression in these cells was compared with Arg1 expression in unstimulated and IL-4–stimulated BMDMs (Fig. 6B). Again, CD11b<sup>F4/80</sup> macrophages sorted from inflamed quadriceps tissues of RRV-infected WT mice had high expression of Arg1 relative to the unstimulated BMDMs (Fig. 6B). In contrast, CD11b<sup>F4/80</sup> macrophages FACS sorted from LysMcre;Arg1<sup>F/F</sup> mice had significantly reduced Arg1 expression compared with CD11b<sup>F4/80</sup> macrophages FACS sorted from control mice (Fig. 6B), indicating that Arg1 is efficiently deleted from the tissue-infiltrating inflammatory macrophages in LysMcre;Arg1<sup>F/F</sup> mice. These findings further confirm that tissue-infiltrating macrophages are a major source of Arg1 expression in the musculoskeletal inflammatory lesions of RRV-infected mice. To test whether deletion of Arg1 from CD11b<sup>F4/80</sup> infiltrates had an impact on functionality, we tested the ability of FACS-sorted CD11b<sup>F4/80</sup> macrophages from control mice and LysMcre;Arg1<sup>F/F</sup> mice to suppress T cell proliferation in an MLR and found that suppressive activity was, in part, Arg1 dependent (Fig. 3).

Our analysis of gene expression in whole tissue revealed a gene-expression pattern characterized by high Arg1 and Ym1 in the absence of FIZZ1 (Fig. 1). To explore the extent to which this gene-expression pattern reflected the gene-expression pattern of tissue-infiltrating macrophages, as well as the extent to which deletion of Arg1 impacted the expression of these genes, we quantified the expression of Ym1 and FIZZ1 in FACS-sorted CD11b<sup>F4/80</sup> cells. CD11b<sup>F4/80</sup> macrophages sorted from RRV-infected WT mice and LysMcre;Arg1<sup>F/F</sup> mice had similar expression levels of Ym1 and little to no expression of FIZZ1 relative to the unstimulated BMDMs (Fig. 6B). These results indicate that deletion of Arg1 did not alter the expression of other genes typically associated with the polarization of murine macrophages toward an M2 phenotype and suggest that at least some of the macrophages infiltrating musculoskeletal tissues of RRV-infected mice have a unique M2-like gene expression pattern.

Impact of LysMcre-mediated deletion of Arg1 on acute RRV disease

To assess the effects of LysMcre-specific ablation of Arg1 on acute RRV-induced disease (days 1–10 postinfection), LysMcre;Arg1<sup>F/F</sup> mice and control Arg1-sufficient mice were inoculated with virus diluent only (mock) or RRV and evaluated for multiple parameters of acute disease. Control mice and LysMcre;Arg1<sup>F/F</sup> mice exhibited no significant differences with regard to RRV-induced
effects on weight gain (Fig. 7A) or the development of musculoskeletal disease, as evidenced by defects in hind limb gripping

FIGURE 5. Expression of Arg1 in inflamed tissues of RRV- or CHIKV-infected mice is ablated in LysMcre;Arg1 F/F mice. (A) RT-qPCR analysis of Arg1 mRNA expression in quadriceps muscles of mock-infected (n = 3) or RRV-infected WT (n = 7), Arg1 F/F (n = 6), LysMcre/+ (n = 4), and LysMcre;Arg1 F/F (n = 5) mice at 7 dpi. Data are normalized to 18S rRNA levels and are expressed as the relative expression (n-fold increase) over Arg1 expression in quadriceps tissue of mock-inoculated mice. Each data point represents the arithmetic mean ± SEM. (B) Immunoblot analysis of Arg1 protein expression in quadriceps muscles of mock-infected (mock) or RRV-infected WT and LysMcre;Arg1 F/F mice at 10 dpi (n = 3 mice/group). GAPDH was used as a loading control. Arg1 and GAPDH band intensities were quantified, and Arg1 expression was normalized to GAPDH and expressed as the fold increase over Arg1 expression in mock-infected mice. (C) RT-qPCR analysis of Arg1 mRNA expression in the left ankle/foot tissue of mock-inoculated Arg1-sufficient mice (n = 5), CHIKV-inoculated Arg1 F/F mice (n = 6), or CHIKV-inoculated LysMcre;Arg1 F/F (n = 6) at 7 dpi. Data are normalized to 18S rRNA levels and are expressed as the relative expression (n-fold increase) over Arg1 expression in left ankle/foot tissue of mock-inoculated mice. Each data point represents the arithmetic mean ± SEM and is cumulative from two independent experiments. *p < 0.05, ***p < 0.001, ANOVA followed by the Tukey multiple-comparison test.

FIGURE 6. Gene expression of CD11b+F4/80+ tissue-infiltrating macrophages. (A) Wright–Giemsa staining of FACS-sorted CD11b+F4/80+ cells isolated from quadriceps muscles of RRV-infected WT or LysMcre;Arg1 F/F mice at 10 dpi. (B) RT-qPCR analysis of Arg1, Ym1, and FIZZ1 expression in unstimulated or IL-4–stimulated (5 ng/ml) BMDMs (n = 3) and FACS-sorted CD11b+F4/80+ cells isolated from quadriceps muscles of RRV-infected WT (n = 4) or LysMcre;Arg1 F/F mice (n = 4) at 10 dpi. Data are normalized to 18S rRNA levels and are expressed as the relative expression (n-fold increase) over expression in unstimulated BMDMs. Each data point represents the arithmetic mean ± SEM. ***p = 0.007, unpaired, two-tailed t tests with Welch correction.
infiltrating leukocytes and CD11b^+F4/80^+ macrophages in musculoskeletal tissue at 7 dpi were quantified by flow cytometry. Similar total numbers of inflammatory infiltrates (Fig. 7E), as well as the total number and percentages of CD11b^+F4/80^+ macrophages (Fig. 7F–H), were isolated from inflamed quadriceps muscles of RRV-infected WT mice and LysMcre;Arg1^F/F^ mice. These findings indicate that genetic ablation of Arg1 in macrophages and neutrophils does not alter the initial recruitment of...
mice were inoculated with $10^3$ PFU of RRV by injection in the left rear footpad. At 7, 14, and 21 dpi, mice were sacrificed and perfused by intracardial injection with $1 \times$ PBS, and total RNA was isolated from the right quadriceps muscles. RRV genomes were quantified by absolute RT-qPCR, as described in Materials and Methods. Horizontal lines indicate the mean, and dashed lines indicate the limit of detection. ***$p < 0.001$, ANOVA followed by the Tukey multiple-comparison test.

Arg1 activity in tumor-associated macrophages, MDSCs, and macrophages responding to parasitic infections functions in immune suppression (50). We found that CD11b$^+$F4/80$^+$ cells FACS sorted from inflamed muscle tissue suppressed T cell proliferation ex vivo by a mechanism that was, in part, Arg1 dependent (Fig. 3). Thus, we hypothesized that the high levels of Arg1 expression detected at the sites of musculoskeletal tissue inflammation and damage would inhibit the clearance of RRV from these sites. To investigate this hypothesis, control or LysMcre;Arg1$^{F/F}$ mice were inoculated with RRV, and viral burdens in quadriceps muscles at 7, 14, and 21 dpi were quantified by RT-qPCR (Fig. 8). At 7 dpi, RRV RNA levels were similar in infected control and LysMcre;Arg1$^{F/F}$ mice, further confirming that specific ablation of Arg1 in macrophages and neutrophils dramatically enhances the clearance of RRV from musculoskeletal tissues and diminishes muscle tissue pathology at late times post-RRV infection, establishing a critical role for myeloid cell Arg1 in the pathogenesis of these infections. Previous studies in humans and mice demonstrated that musculoskeletal tissue infiltrates associated with RRV infection are primarily mononuclear (26, 28, 33, 58, 69–72), and studies of RRV and CHIKV infection in mice implicated macrophages in contributing to disease severity. Treatment of mice with macrophage toxic agents prior to virus inoculation (57, 58, 70), or inhibition of macrophage recruitment (74, 75), reduced acute disease signs associated with RRV or CHIKV infection. These studies suggested that macrophages function as pathogenic effectors during the acute stage of these virus-induced inflammatory diseases; however, specific macrophage-activation programs and effector mechanisms that mediate these effects have not been defined.

Macrophages have heterogeneous phenotypes and may exert various, and even opposite, functions, depending on their activation state and anatomical location (34, 35). We hypothesized that RRV/CHIKV infection and the associated inflammation and tissue damage activate a wound-healing response characterized by activation of M2 or M2-like macrophages. Indeed, our gene-expression studies revealed a dramatic increase in Arg1 and Ym1 expression that was associated with peak inflammatory tissue pathology, suggesting that tissue damage may drive the polarization of at least some of the macrophages infiltrating these tissues toward an M2-like phenotype. Consistent with this notion, gene-expression analyses of FACS-sorted muscle tissue infiltrates from RRV-infected WT mice indicated that CD11b$^+$F4/80$^+$ macrophages expressed abundant Arg1 and Ym1. In addition, LysMcre-mediated deletion of Arg1 dramatically reduced Arg1 expression in inflamed musculoskeletal tissues of RRV- and CHIKV-infected mice. Taken together, these data suggest that macrophages and/or neutrophils are the predominant Arg1-expressing cells at the sites of inflammation in mice infected with arthritogenic alphaviruses.
Interestingly, the Arg1-expressing macrophages present in RRV-induced musculoskeletal inflammatory lesions of WT C57BL/6 mice arise in an inflammatory environment with little to no expression of IL-4 or IL-13 mRNA. Furthermore, based on their gene-expression pattern, these cells do not appear to fit into the canonical definition of M2/alternatively activated macrophages, which are defined as macrophages that have been stimulated with IL-4/IL-13 via the IL-4Rα-chain (35). In contrast to our analyses, which detected expression of Arg1 and Ym1 in the absence of FIZZ1 in both whole tissue and FACS-sorted CD11b+F4/80+ macrophages, IL-4/IL-13–stimulated macrophages typically coexpress the prototypic murine M2 genes Arg1, Ym1, and FIZZ1. Similarly, macrophages in mice infected with various parasites, macrophages from RSV-infected mice, endotoxin-tolerant macrophages, and tumor-associated macrophages skewed toward M2 polarization also coexpress Arg1, Ym1, and FIZZ1 (37, 45, 76–78). More recently, studies identified an IL-4/IL-13/STAT6–independent, STAT3-dependent pathway by which macrophages are activated to express Arg1 in the absence of both Ym1 and FIZZ1 (79), demonstrating that unique signaling pathways can uncouple Arg1 expression in macrophages from other canonical M2 genes. We found that expression of Arg1 in inflamed muscle tissue of RRV-infected mice correlated with the expression of mRNAs encoding STAT3-activating cytokines, including IL-6 and IL-10, suggesting that Arg1 may be induced in RRV-infected mice by a STAT3-dependent pathway. An additional STAT6-independent pathway leading to induction of Arg1 was identified following treatment of macrophages with macrophage stimulating protein (80). Future studies in our laboratory are aimed at defining the specific signaling pathways that regulate Arg1 expression in the context of RRV and CHIKV infection.

As mentioned above, we found that macrophages isolated from musculoskeletal inflammatory lesions of RRV-infected mice expressed high levels of Arg1 and Ym1, with little to no expression of FIZZ1. This expression pattern is similar to the gene-expression pattern of MDSCs that infiltrate the heart in response to Trypanosoma cruzi infection (81). Thus, the CD11b+F4/80+Arg1+ cells present in the inflamed musculoskeletal tissues of RRV/CHIKV-infected mice may share overlapping features with mononuclear MDSCs. Arginase activity, NO, and reactive oxygen species production are the main effectors of the immunosuppressive activity of MDSCs (82). We found that Arg1-expressing macrophages sorted from the inflamed quadriceps of RRV-infected mice suppressed T cell proliferation ex vivo by a mechanism that was,
in part, Arg1 dependent. These findings suggest that the macrophages present in RRV-induced musculoskeletal inflammatory lesions have immunosuppressive activity similar to MDSCs.

Conditional deletion of Arg1 from macrophages and neutrophils had minimal impact on the development of RRV-induced acute disease, including effects on body weight, outward disease signs, cellular infiltration of tissues, and viral loads at 7 dpi. These findings are consistent with our gene-expression analyses that indicated Arg1 expression in musculoskeletal tissues was low until 7 dpi. Strikingly, LysMCre-mediated deletion of Arg1 had a dramatic impact on viral loads at late times postinoculation. These findings suggest that Arg1 activity in tissue-infiltrating myeloid cells inhibits viral clearance. In addition, the reduced viral loads in LysMCre;Arg1F/F mice was associated with improved skeletal muscle tissue pathology, suggesting that the enhanced clearance of RRV in these mice reduced the duration of tissue damage. Additionally, deletion of Arg1 did not alter the expression of other genes typically associated with murine macrophages polarized toward an M2 phenotype, suggesting that the enhanced viral clearance in LysMCre;Arg1F/F mice is due to the loss of Arg1.

Although we do not know the exact mechanisms by which Arg1 in macrophages and/or neutrophils inhibits RRV clearance, previous investigations on alphavirus clearance from the CNS demonstrated that both antiviral Abs and T cells are critical for the clearance of infectious virus and viral RNA (83–86). Arg1 activity in myeloid cell populations was shown to directly suppress T cell effector functions in various contexts, primarily by depletion of the amino acid l-arginine, the substrate of Arg1 (53, 54, 67). Depletion of l-arginine from the microenvironment inhibits T cell effector functions and proliferation via several mechanisms, including decreasing the expression of the CD3ζ-chain and preventing the expression of the cell cycle regulators cyclin D3 and cyclin-dependent kinase 4 (87). Ongoing studies in our laboratory are investigating the hypothesis that Arg1-expressing myeloid cells inhibit antiviral effector T cells at the sites of infection.

Human RRV and CHIKV disease is characterized by a prolonged disease course, with most cases requiring weeks to months for complete resolution of musculoskeletal pain and inflammation. Taken together, our findings suggest that Arg1 activity in tissue-infiltrating macrophages and neutrophils may contribute to the duration and/or severity of the disease and could promote the development of chronic disease that is reported by a subset of RRV/CHIKV-infected persons. Thus, therapeutically targeting Arg1 activity or induction could inhibit the duration of disease. Arg1 is a central metabolic enzyme in the liver that catalyzes the hydrolysis of l-arginine to urea and ornithine (46). Thus, systemic inhibition of arginase would likely be associated with toxicity. However, treatment of dystrophin-deficient mdx mice, a model of Duchenne muscular dystrophy, with the Arg1 substrate, L-arginine, improved muscular function (88, 89). Similarly, L-arginine supplementation of dystrophin-deficient mdx mice inhibits viral clearance. In addition, the reduced viral loads in LysMCre;Arg1F/F mice was associated with improved skeletal muscle tissue pathology, suggesting that the enhanced clearance of RRV in these mice reduced the duration of tissue damage. Additionally, deletion of Arg1 did not alter the expression of other genes typically associated with murine macrophages polarized toward an M2 phenotype, suggesting that the enhanced viral clearance in LysMCre;Arg1F/F mice is due to the loss of Arg1.

Acknowledgments

We thank Matthew Maulis for valuable assistance with tissue processing and embedding and Philip Pratt and Sonia Soto for assistance with flow cytometry and flow sorting, respectively.

Disclosures

The authors have no financial conflicts of interest.

References


Fig. S1. Specific deletion of Arg1 in LysMcre;Arg1^{fl/fl} mice. Protein extracts were generated from unstimulated or IL-4 stimulated (5 ng/ml) bone marrow-derived macrophages (BMDMs) or livers of Arg1^{F/F} mice and LysMcre;Arg1^{fl/fl} mice. Immunoblot analysis of Arg1 expression demonstrated a specific loss of Arg1 expression in IL-4-stimulated BMDMs of LysMcre;Arg1^{F/F} mice (left panel). In contrast, LysMcre had no impact on the expression of Arg1 in the liver (right panel). GAPDH expression was used as a loading control.