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Original Article

Potent suppression of arginase 1 expression in murine macrophages by low dose endotoxin

Michael J Surace, Liwu Li

Department of Biological Sciences, Virginia Tech, Blacksburg, VA 24061-0910

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Abstract: Macrophages can respond to diverse signals and adopt multiple phenotypes. Although interleukin-4 (IL-4) is shown to potently induce the expression of arginase 1 and contribute to differentiation of macrophages to the anti-inflammatory M2 phenotype, other modulators may potentiate or reduce the effect of IL-4. In this report, we focus on the combinatorial effects of IL-4 with all-trans retinoic acid (ATRA) and lipopolysaccharide (LPS). ATRA has been shown to contribute to the resolution of inflammation, however it has not been linked to arginase 1 expression in macrophages. We demonstrate that although ATRA alone has no effect on the expression or activities of arginase 1, ATRA can dramatically potentiate the induction of arginase 1 by IL-4. On the other hand, high doses of LPS, such as those seen in septic shock, can induce the expression of both M1 and M2 mediators in macrophages. The effects of subclinical doses of LPS, which are prevalent in humans with adverse health conditions, on macrophage differentiation are not well studied. We demonstrate that low dose LPS can effectively suppress the expression of arginase 1 induced by IL-4 and ATRA. Mechanistically, we report that the interleukin-1 receptor-associated kinase 1 (IRAK-1) and Toll-interacting-protein (Tollip) are involved in the suppressive effect of low dose LPS. Our study reveals dynamic modulation of arginase 1 expression in macrophages by competing agonists, and bears relevance for potential intervention of chronic diseases.

Keywords: Low grade inflammation, molecular mechanisms, arginase expression, macrophages

Introduction

Macrophages, derived from terminally differentiated monocytes, are dedicated first responders and mediators of the innate immune inflammatory response [1, 2]. Quiescent macrophages are capable of being activated to an inflammatory (M1) or anti-inflammatory (M2) state by different cytokines [3] and by pathogen-associated molecular patterns from bacteria, virus, and fungus [4, 5]. The cell biology of M1 and M2, as well as various sub-categorizations of macrophages has been studied extensively. Altered macrophage activation has been associated with diverse inflammatory diseases such as atherosclerosis [6], arthritis [7], Crohn's disease [8], multiple sclerosis [9], Parkinson's [10], and Alzheimer's diseases [11]. However, the aspects of signaling that lead to macrophage polarization are not clearly understood [3].

Interleukin 4 (IL-4) is a Th-2 cytokine that signals macrophages to polarize to the M2 phenotype [12], which is associated with resolution of inflammation [5]. Arginase 1 expression is one of the most relevant markers of murine M2 activation [13]. The arginase 1 enzyme competes for substrate with the NOS (nitric oxide synthase) enzymes, particularly iNOS, which is associated with inflammation and necessary for generation of nitric oxide [14], which is used to produce peroxynitrite, a defensive reactive oxygen species [15]. The Th2 cytokine IL-4 induces arginase 1 expression primarily by JAK1/3 activation/dimerization of STAT6 and CEBPβ [16].

ATRA is known to be beneficial in skin healing [17] and to be protective of macrophage activation in atherosclerosis [18] by reducing inflammation, but the mechanism is not well understood. Through intracellular nuclear receptors,

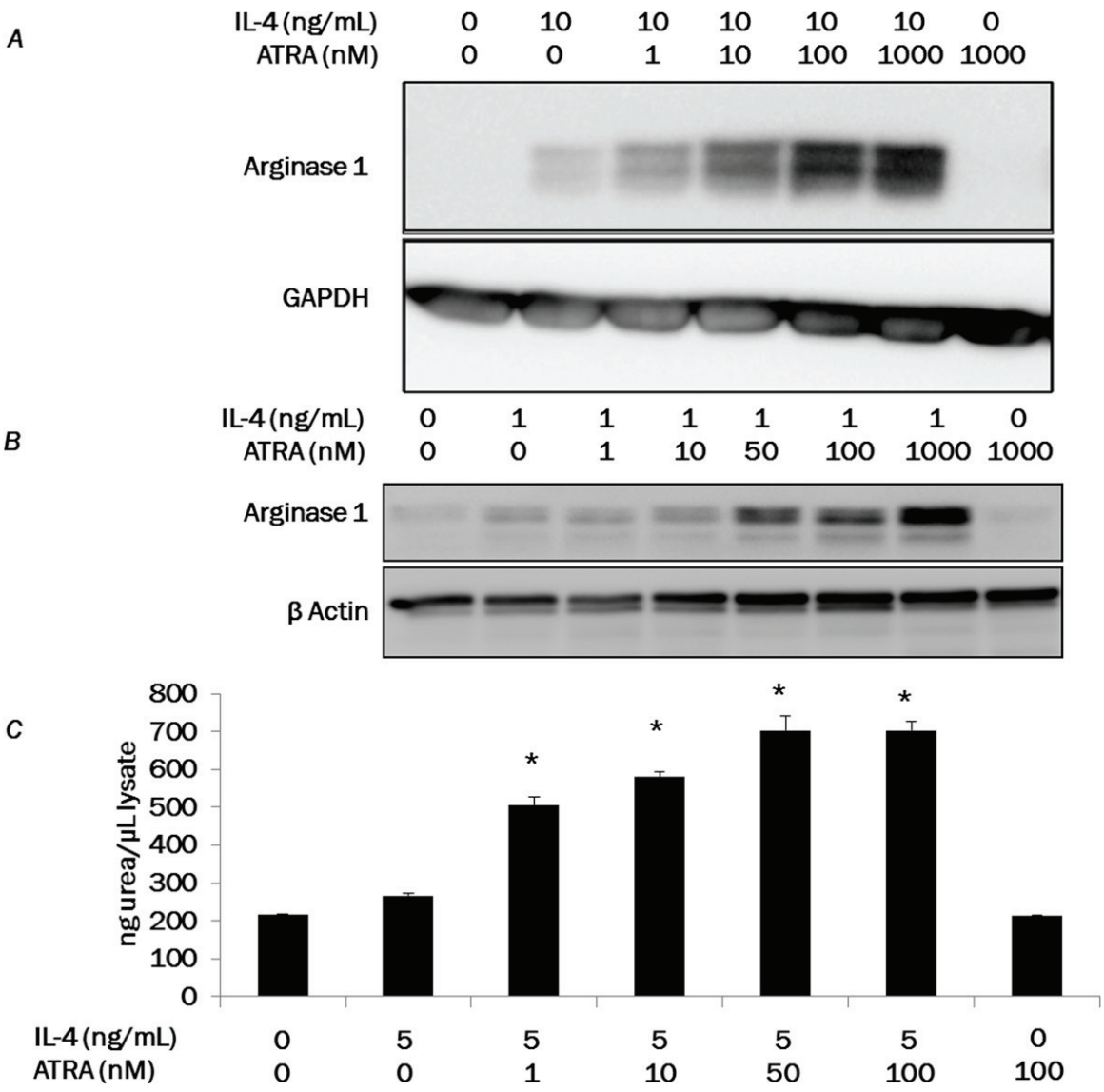

Figure 1. ATRA potentiates the effects of IL-4 in the induction of arginase 1. WT BMDM were treated with 10 (A) or 1 (B) ng/mL IL-4 or IL-4 with increasing concentrations of ATRA for 8 h. Arginase 1 protein was analyzed by western blot. GAPDH or β actin levels were determined and as served as equal loading controls. C. Arginase 1 activities were measured and quantitated with cells treated as indicated on the figure legend. Error bars are SEM. Results are representative of the trend observed in 3 or more experiments. *P < 0.05.

ATRA affects the expression of hundreds of genes, CEBPβ among them [19]. The effects of ATRA on macrophage phenotype are not well-studied.

LPS is a Gram-negative bacterial inflammogen that is detected by TLR4, and is capable of inducing both pro- and anti-inflammatory mediators [14, 20]. Mechanistically, high dose LPS activates a plethora of downstream pathways that lead to the activation of NF-kB, AP1, CREB, and STAT [1], eventually resulting in the induction of both pro- and anti-inflammatory mediators [14]. In contrast, low dose LPS preferentially skews the mild expression of pro-inflammatory mediators through limited downstream signaling networks [20]. Specifically, IRAK-1 and Tollip are shown to be selectively involved in the effect of low dose LPS in the induction of pro-inflammatory mediators [20, 21].

Although it is clear that low dose LPS can skew macrophages toward a mild pro-inflammatory

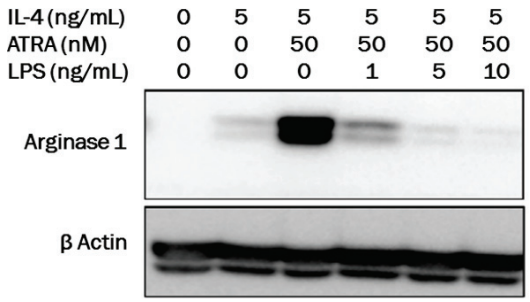

Figure 2. Low dose LPS suppresses arginase 1 expression. WT BMDM were treated with 5 ng/mL IL-4 plus 50 nM ATRA in the absence or presence of low doses of LPS for 8 h. Arginase 1 protein levels were analyzed by western blot. The levels of β actin were determined as equal loading controls. Results are representative of the trend observed in 3 or more experiments.

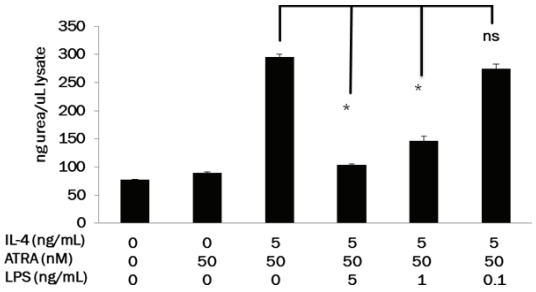

Figure 3. Low dose LPS suppresses arginase activity. WT BMDM were treated with 5 ng/mL IL-4 plus 50 nM ATRA in the absence or presence of low doses of LPS for 8 h. Arginase activities were measured and compared. Error bars are SEM. Results are representative of the trend observed in 3 or more experiments. *P < 0.05.

state, it is not known whether low dose LPS is capable of suppressing the induction of anti-inflammatory mediators by M2-promoting agents such as IL-4.

The current study aims to determine whether ATRA may augment the expression of arginase 1 in macrophages induced by IL-4. Furthermore, we plan to test whether low dose LPS may be capable of suppressing the expression of arginase 1 in macrophages.

Methods

Mice and Cell Culture

Wild-type (WT) C57BL/6 mice were obtained from the Charles River Laboratory. *IRAK-1*$^{-/-}$ mice with a C57BL/6 background were kindly provided by Dr. James Thomas, University of Texas Southwestern Medical School. Tollip$^{-/-}$ mice in C57BL/6 background were obtained from Dr. Kimberly Burns while at the Institute of Biochemistry, University of Lausanne, Lausanne, Switzerland. All mice were housed and bred at Derring Hall Animal Facility in compliance with approved Animal Care and Use Committee protocols at Virginia Polytechnic Institute and State University. Bone marrow-derived macrophages (BMDMs) from 6–10 week-old C57BL/6, *IRAK-1*$^{-/-}$, and Tollip$^{-/-}$ mice were isolated and cultured, as described previously [22]. BMDM were cultured in 150 mm non-tissue culture polystyrene vessels. Medium was DMEM with 10% FBS, 100 U/mL penicillin, and 100 µg/ml streptomycin preconditioned by culture with L-929 fibroblast, 0.2 µm-filtered, then mixed 1:3 with non-conditioned medium. BMDM were cultured in 1:3 medium at 37°C, 5% CO_2. Three days post-dissection, 20 mL additional 1:3 medium was added. Three days later, BMDM were harvested by washing with PBS to remove from 150 mm plates, resuspended in DMEM supplemented with 1% FBS to reduce basal activation, plated in new vessels, and treated 24 hours later.

Ligands

LPS (0111:B4, Sigma) and recombinant IL-4 (404-ML, RnD Systems) stocks were reconstituted in phosphate buffered saline (PBS) with 0.02% bovine serum albumin (BSA). ATRA (R2625, Sigma) was reconstituted in anhydrous dimethyl sulfoxide (DMSO). PBS/BSA and/or DMSO were included as vehicle in control samples.

Western Blot

BMDM were treated in 1% FBS DMEM, washed 1x with PBS, then harvested in lysis buffer (50 mM Tri-HCl, 2% SDS, 10% glycerol). 10 µg total protein was used for SDS-PAGE and transferred to a PVDF membrane, blocked 1 hour at room temp with 5% skim milk in tris-buffered saline with 0.05% Tween 20. Membranes were incubated in primary antibodies for arginase 1 (sc-18354, Santa Cruz), β Actin (sc-47778, Santa Cruz), or GAPDH (sc-25778, Santa Cruz) overnight at 4°C. Blots were stripped with ReBlot Plus Mild (Chemicon). HRP-conjugated secondary antibodies were incubated 1 hour at room temperature. Detection was performed with Amersham ECL Plus chemiluminescent detection system (GE Healthcare) and the LAS-3000 geldoc and Multi Gauge software (Fujifilm).

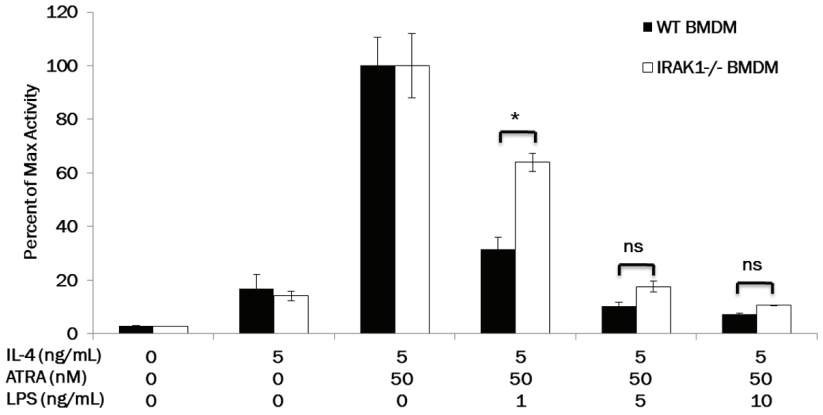

Figure 4. The suppression of arginase 1 activities by LPS is dependent on IRAK-1. WT and IRAK-1 deficient BMDM were treated with 5 ng/mL IL-4 plus 50 nM ATRA in the absence or presence of low doses LPS for 8 h. Arginase 1 activities were measured. Error bars are SEM. Results are representative of the trend observed in 3 or more experiments. *P < 0.05.

Double bands are observed for arginase 1 in rodent samples with some antibodies, with the lower band corresponding to the 41 KDa human liver arginase.

Arginase Activity Assay

This assay is based on Corraliza's modification [23] of Schimke's method [24]. Cells were plated in 1% FBS DMEM at a concentration 5·10^5 cells/well in a 24 well plate. The next day cells were treated, washed once with RT PBS, and lysed in the wells in 50 µL 0.1% triton x-100 with protease inhibitors by shaking at 37°C, 200 RPM for 30 min. 50 µL of 10 mM $MnCl_2$ in 50 mM Tris-HCl (pH 7.8) was added to each well followed by 10 minute incubation at 55°C. 25 µL of each sample was mixed with 25 µL 500 mM L-arginine (pH 9.7) in a 1.5 mL tube, incubated 1 hour at 37°C, then 400 µL acid (1:3:9, Sulfuric Acid: Phosphoric Acid: ddH_2O) and 25 µL 9% ISPF in EtOH were added to each tube. Tubes were boiled for 45 minutes, then 200 µL was moved to a polystyrene 96-well plate to determine abs540, which was calibrated against a standard curve of urea using linear regression.

Statistical Analysis

One-way ANOVA was performed on data from three experiments using SPSS statistical software. Independent comparisons were made using Bonferroni post hoc test with a significance level of 0.05.

Results

ATRA potentiates the induction of arginase 1 by IL-4

Although ATRA is associated with resolution of inflammatory phenotype in a variety of contexts, the underlying mechanism is not well understood. We thus tested whether it may potentiate the expression of arginase 1 induced by IL-4. We observed that BMDM treated with IL-4 alone moderately expressed the M2 marker arginase 1 (**Figure 1A** and **1B**). Arginase enzyme activity was likewise moderately induced by IL-4 (**Figure 1C**). ATRA alone did not induce the protein level or activity of arginase 1. Instead, treatment with IL-4 and ATRA together dramatically enhanced arginase 1 expression and activity in a dose-dependent manner (**Figure 1A-C**). Lower doses of IL-4 required more ATRA to observe the effect, but ATRA similarly potentiated the expression of arginase 1 in the presence of 1, 5, or 10 ng/mL IL-4. As expected, arginase 2 protein and RNA were not induced by IL-4 or ATRA (data not shown). Our data reveal that ATRA potentiates the induction of arginase 1 by IL-4 in macrophages.

Low dose LPS suppresses arginase 1 induction by IL-4 and ATRA

Although low dose endotoxemia is clinically associated with chronic inflammation, the underlying molecular mechanisms are not well studied. We thus tested whether low dose LPS may suppress the expression of arginase 1 in macrophages. As shown in **Figure 2**, treatment with LPS as low as 1 ng/mL potently suppressed the expression of arginase 1 induced by IL-4 and ATRA.

To confirm that the suppression of arginase 1 by low dose LPS was correlated with the suppression of arginase 1 enzyme activity, we further measured the activity of arginase 1 in BMDM treated with various combinations of LPS, IL-4 and ATRA. As shown in **Figure 3**, treatment with LPS as low as 1 ng/mL potently sup-

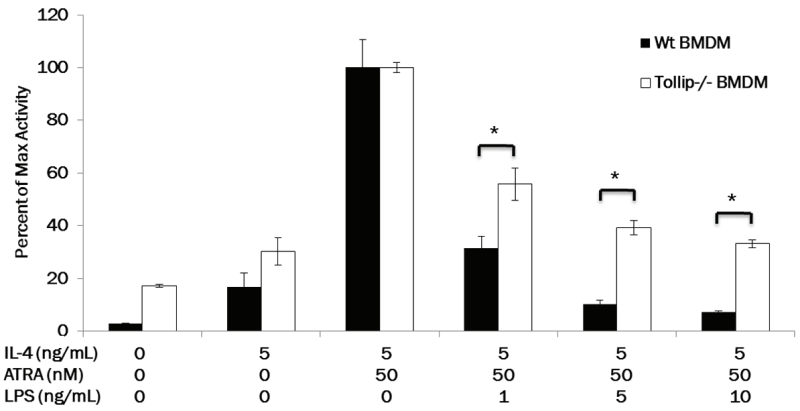

Figure 5. The suppression of arginase 1 activities by LPS is dependent on Tollip. WT and Tollip deficient BMDM were treated with 5 ng/mL IL-4 plus 50 nM in the absence or presence of low doses LPS for 8 h. Arginase 1 activities were measured. Error bars are SEM. Results are representative of the trend observed in 3 or more experiments. *P < 0.05.

pressed the arginase activity induced by IL-4 and ATRA.

Suppression of arginase 1 by low dose LPS is IRAK-1-dependent

IRAK-1 is utilized by TLR4 as an intracellular signaling molecule. We thus tested the role of IRAK-1 in the suppression of arginase 1 by LPS. WT and IRAK-1$^{-/-}$ BMDM were treated with 5 ng/mL IL-4, 50 nM ATRA and 1, 5, or 10 ng/mL LPS for 8 hours. As shown in **Figure 4**, the effect of low dose LPS (1 ng/mL) in suppressing arginase activity was significantly reduced in IRAK-1$^{-/-}$ macrophages. Higher doses of LPS had similar suppressive effect in WT and IRAK-1$^{-/-}$ cells.

Suppression of arginase 1 by low dose LPS is Tollip-dependent

Tollip, a novel intracellular adaptor molecule, was recently shown to be specifically involved in mediating the pro-inflammatory effect of low dose LPS [21]. We hereby tested the role of Tollip in the suppression of arginase 1 by low dose LPS. WT and Tollip$^{-/-}$ BMDM were treated with 5 ng/mL IL-4, 50 nM ATRA and 1, 5, or 10 ng/mL LPS for 8 hours. In WT BMDM, arginase activity induced by ATRA and IL-4 was significantly reduced by 1 ng/mL LPS (**Figure 5**). In contrast, the suppression by low dose LPS was significantly reduced in Tollip$^{-/-}$ cells.

Discussion

Our findings show novel synergistic activation of arginase 1, a key anti-inflammatory enzyme, in macrophages by IL-4 and ATRA, explaining in part the observed anti-inflammatory properties of ATRA [17, 18]. Additionally, we show that low dose LPS is effective in suppressing arginase 1 induced by IL-4 and ATRA in macrophages via IRAK-1 and Tollip.

The therapeutic effect of ATRA has been controversial, with high doses of ATRA eliciting toxic effects [25] and low doses being effective in the treatment of chronic inflammatory diseases [26]. Herein we demonstrate that ATRA potentiates IL-4-induced arginase 1 expression in macrophages at a concentration 1000-fold less than the non-toxic concentration of ATRA used to treat patients acute promyelotic leukemia [25]. Our study provides a potential cue for future combinatorial usage of ATRA with IL-4 in skewing innate macrophages into an anti-inflammatory state, and likely regimen for treating inflammatory diseases.

Low grade endotoxemia is caused by a variety of factors, including high-fat diet [27, 28], smoking [29], and alcohol [30], which are associated with increased risk of diseases of inflammation including atherosclerosis, obesity, and diabetes [31]. However, limited studies are available with regard to its pathological consequences and underlying mechanisms. Current studies regarding LPS almost exclusively address the septic shock effects of high dose LPS (>100 ng/mL) [32]. Our data demonstrate that low doses of LPS can effectively suppress expression and activity of arginase 1 induced by M2 skewing mediators such as IL-4 and ATRA. This bears significant implications in chronic diseases associated with low dose endotoxemia, and suggests that the population of circulating monocytes in individuals with low dose endotoxemia may skew the activation of macrophages.

Mechanistically, our observation indicates that IRAK-1 and Tollip are critically involved in the

effects of low dose LPS. IRAK-1 and Tollip are key molecules involved in the TLR4 pathway responsible for the sensing of LPS by macrophages. We previously demonstrated that IRAK-1 and Tollip are required for the suppression of RARα in macrophages [20, 33]. Our current studies further reveal that low dose LPS functionally suppresses ATRA-mediated augmentation of arginase 1 expression.

Collectively, this study provides evidence that demonstrate the suppressive effect of low dose LPS on arginase 1 induced by IL-4 and ATRA, as well as the underlying mechanisms involving IRAK-1 and Tollip. Our current study alludes to dynamic integration of signals involved in the skewing of macrophages, as well as potential molecular targets helpful for future development of combinatory therapies in the treatment of chronic inflammatory diseases.

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Address correspondence to: Dr. Liwu Li, Life Science 1 Building, Washington Street, Department of Biology, Virginia Tech, Blacksburg, VA 24061. Tel: 540-231-1433; Fax: 540-231-4043; E-mail: lwli@vt.edu

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