Macrophage Inflammatory State Influences Susceptibility to Lysosomal Damage

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Summary sentence: TLR and cytokine stimulation increase macrophage resistance to lysosomal damage through multiple distinct signaling pathways.

Running Title: Inducers of Renitence in Macrophages

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Abbreviations

A2bR, adenosine 2b receptor; Ado, adenosine; ANOVA, analysis of variance; ATP, adenosine triphosphate; AW, acid-washed; BFA, brefeldin A BMM, bone marrow-derived macrophages;, dsRNA, double-stranded RNA; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; Fdx, fluorescein dextran; FLA-ST, flagellin from *Salmonella typhimurium*; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; IFITM3, interferon-induced transmembrane protein 3; IFNβ, interferon-β; IFNγ, interferon-γ; Ifnar1, interferon-α/β receptor 1; IL-4, interleukin-4; IL-10, interleukin-10; IL-13, interleukin-13; *L. monocytogenes*, *Listeria monocytogenes*; ISG, interferon-stimulated gene; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; MDA5, melanoma differentiation-associated protein 5; MNV-1, murine norovirus-1; MOI, multiplicity of infection; MyD88, myeloid differentiation primary response 88; NCOA, nuclear receptor coactivator 7; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Pam3CSK4, Pam3CysSerLys4; PGE2, prostaglandin E2; Poly(I:C), polyinosinic:polycytidylic acid; qPCR, quantitative polymerase chain reaction; R848, Resiquimod; RelA, nuclear factor NF-kappa-B p65 subunit; Relm-α, resistin-like molecule-α; RIG-I, retinoic acid-inducible gene I; TLR, Toll-like receptor; TNFα, tumor necrosis factor-α; TRIF, TIR (Toll-interleukin-1 receptor) domain-containing adapter-inducing interferon-β; WT, wild-type.

Abstract

Macrophages possess mechanisms for reinforcing the integrity of their endolysosomes against damage. This property, termed inducible renitence, was previously observed in murine macrophages stimulated with LPS, peptidoglycan, interferon-γ (IFNγ) or tumor necrosis factor-α (TNFα), which suggested roles for renitence in macrophage resistance to infection by membrane-damaging pathogens. This study analyzed additional inducers of macrophage differentiation for their ability to increase resistance to lysosomal damage by membrane-damaging particles. Renitence was evident in macrophages activated with LPS plus IFNγ, prostaglandin E2, or adenosine, and in macrophages stimulated with interferon-β, but not in macrophages activated with interleukin (IL)-4 or IL-10. These responses indicated roles for macrophage subtypes specialized in host defense and suppression of immune responses, but not those involved in wound healing. Consistent with this pattern, renitence could be induced by stimulation with agonists for Toll-like receptors (TLR), which required the signaling adaptors MyD88 and/or TRIF, and by infection with murine norovirus-1 (MNV-1). Renitence induced by LPS was dependent on cytokine secretion by macrophages. However, no single secreted factor could explain all the induced responses. Renitence induced by the TLR3 agonist Poly(I:C) was mediated in part by the type I interferon (IFN) response, but renitence induced by Pam3CSK4 (TLR2/1), LPS (TLR4), IFNγ or TNFα was independent of type 1 IFN signaling. Thus, multiple pathways for inducing macrophage resistance to membrane damage exist and depend on the particular microbial stimulus sensed.

Introduction

To achieve their diverse functions, macrophages exhibit enormous functional heterogeneity and plasticity (1, 2). The functional state a macrophage assumes is influenced by the tissue in which it resides and the signals it receives within that environment. Extensive efforts have characterized several functional classes of macrophages with distinct roles *in vivo* (1). These macrophages can be generated *in vitro* through exposure to the same polarizing cytokines that induce their generation *in vivo*. Interferon-γ (IFNγ) priming of macrophages followed by overnight stimulation with lipopolysaccharide (LPS) and IFNγ leads to the generation of M(LPS+IFNγ), historically referred to as classically activated macrophages, which are primed to fight infection. Macrophages with similar properties can be generated by stimulation of macrophages with IFNγ and tumor necrosis factor-α (TNFα), or with IFNγ and Toll-like receptor (TLR) agonists that induce macrophages to secrete TNFα. Stimulation of macrophages with interleukin (IL)-4 induces the generation of macrophages specialized in wound healing, commonly referred to as alternatively activated macrophages. TLR stimulation coupled with a second signal, such as that provided by IgG immune complexes, prostaglandin E2 (PGE2), or adenosine (Ado), can reprogram macrophages to adopt an immunosuppressive phenotype (1, 3), referred to as regulatory macrophages. In keeping with consensus guidelines for describing various macrophage activation states, macrophages subtypes herein will be defined by the stimulation conditions that induce their generation rather than by names previously given in the literature, which may have imprecise or misleading meanings (2, 4).

The goal of this study was to examine systematically how macrophages of various inflammatory states differ in their susceptibility to lysosomal damage. Previous work in this lab uncovered a macrophage activity called inducible renitence, which describes the enhanced ability of macrophages stimulated with LPS (M(LPS)), peptidoglycan (M(PGN)), IFNγ (M(IFNγ)), or TNFα

(M(TNFα)) to resist damage to their phagolysosomes following the phagocytosis of membrane-damaging silica microspheres (5). As phagolysosomal damage represents a common threat posed by pathogens, and as the factors found to induce renitence correspond to microbial ligands or host pro-inflammatory cytokines, we reasoned that renitence is a consequence of macrophage activation in response to infections. However, other types of activated macrophages not yet examined might also have mechanisms for limiting damage to their lysosomes.

Of the macrophage activation states examined in previous work, overnight incubation of macrophages in LPS elicited the most pronounced and consistent protection against lysosomal damage (5). Like M(LPS+IFNγ), M(LPS) have been noted for their anti-microbial properties (6, 7). TLR stimulation of macrophages in the absence of IFNγ priming induces the differentiation of macrophages that initially resemble M(LPS+IFNγ) in terms of their pattern of cytokine secretion, but that over several hours transition to an immunosuppressive state (8). The regulatory cascade driving this transition has been proposed to serve as an autoregulatory mechanism used by macrophages to prevent the development of hyperinflammatory responses following TLR activation (9). According to this model, the release of pro-inflammatory cytokines following TLR stimulation is accompanied by the release of low levels of adenosine triphosphate (ATP), which eventually is converted into adenosine (Ado), a signal that promotes the generation of immunosuppressive macrophages. *In vitro*, stimulation of macrophages with LPS and Ado (M(LPS+Ado)) or LPS and prostaglandin E2 (M(LPS+PGE2)) generates such immunosuppressive macrophages (9, 10).

Based on the above model, we predicted that M(LPS) harbor characteristics of these previously defined immunosuppressive macrophages, and that M(LPS+Ado) and M(LPS+PGE2) also exhibit renitence. We sought to test these predictions within the context of two broader aims: (1) to

expand our understanding of the physiological contexts in which renitence acts, and (2) to define other inflammatory stimuli that induce renitence.

We report that only a subset of macrophage subtypes exhibited renitence. These included M(IFNγ+LPS), M(LPS+Ado), M(LPS+PGE2), and macrophages treated with interferon-β (M(IFNβ)) or with ligands of TLRs 2/1, 3, 4 and 7/8. Macrophages that induced little or no renitence included macrophages treated with IL-4 (M(IL-4)), IL-10 (M(IL-10)) or an agonist of TLR 9. Renitence induced by LPS depended on the release of secreted factors. Building upon these observations, we examined the potential for viral infection to induce renitence and the contribution of type I IFN signaling to renitence. Together, this work supports the concept that macrophages increase their resistance to lysosomal damage in the setting of multiple inflammatory states and that the mechanism of renitence depends on cytokine secretion.

Materials and Methods

Mice and macrophage isolation

C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Myd88/Trif*$^{-/-}$ mice were provided by Gabriel Nuñez (University of Michigan). All mice were maintained under specific pathogen-free conditions at the University of Michigan. Bone marrow cells isolated from the femurs and tibia of mice were differentiated into bone marrow-derived macrophages (BMM) through culture for 6-8 days in DMEM containing 10% fetal bovine serum (FBS) and 50 ng/ml recombinant M-CSF (R&D Systems, Minneapolis, MN), as previously described (5). Femurs and tibia from *Ifnar1*$^{-/-}$ mice on a C57BL/6J background were provided by Megan Baldridge (Washington University in St. Louis, MO, USA). *Ifnar1*$^{-/-}$ and wild-type (WT) BMM were differentiated through culture for 6 days in L929-conditioned DMEM containing 20% FBS, 30% L9 supernatant, 1% L-Glutamine, 1% Sodium Pyruvate, 0.1% β-mercaptoethanol and 1% Penicillin/Streptomycin, as described in (11).

Cell culture and stimulation

M(LPS+IFNγ) were generated by priming BMM with 150 U/ml IFNγ (R&D Systems) for 6 h, and then stimulating cells overnight with 150 U/ml IFNγ and 100 ng/ml LPS (from *Salmonella typhimurium*; no. 225; List Biological Laboratories, Campbell, CA). M(IL-4) and M(IL-10) were generated by stimulating macrophages overnight with 20 ng/ml IL-4 or 10 ng/ml IL-10, respectively (R&D Systems, Minneapolis, MN). M(LPS+PGE2) and M(LPS+Ado) were generated by stimulating macrophages overnight with 100 ng/ml LPS and either 200 nM PGE2 (Cayman Chemical, Ann Arbor, MI) or 200 μM adenosine (Sigma-Aldrich, St. Louis, MO), respectively. Studies of macrophages stimulated with TLR agonists were performed with the following reagents: Pam3CSK4 (100 ng/ml); Poly(I:C) (10 μg/ml); ultrapure flagellin from *Salmonella typhimurium* (FLA-ST; 100 ng/ml); R848 (100 ng/ml), ODN 1826 (1

μM). All TLR agonists were purchased from Invivogen (San Diego, CA) except poly(I:C), which was purchased from Tocris (Bristol, United Kingdom).

For experiments in which both RNA isolation and cytokine analyses were performed, 6 x 10^6 cells were plated onto 60-mm dishes (ThermoFisher, Waltham, MA). For experiments in which cytokine analyses were performed and RNA was not isolated, 1 x 10^5 cells were plated onto 24-well plates (ThermoFisher). For assays of lysosomal damage, 8 x 10^4 cells were plated onto 35-mm dishes with attached 14-mm coverglass (MatTek Corporation, Ashland, MA).

Gene expression analysis

RNA was isolated using a Qiagen RNeasy Mini Kit (74104; Venlo, Netherlands) and converted into cDNA using MMLV-Reverse Transcriptase from ThermoFisher (28025013). Quantitative PCR (qPCR) analysis was performed using an Applied Biosystems 7500 Fast Real-Time PCR system (ThermoFisher) and Brilliant II SYBR Green Master Mix (600830; Agilent, Santa Clara, CA). Primer pairs used for amplification of specific gene products are as follows: *Il-12p40* F, AAGACGTTTATGTTGTAGAGGTGGAC; *Il-12p40* R, ACTGGCCAGGATCTAGAAACTCTTT; *Il-10* F, GACTTTAAGGGTTACTTGGGTTGC; *Il-10* R, TCTTATTTTCACAGGGGAGAAATCG; *Relm-α* F, AATCCAGCTAACTATCCCTCCA; *Relm-α* R, CAGTAGCAGTCATCCCAGCA

; *Gapdh* F, AAGGTCGGTGTGAACGGATTT; *Gapdh* R, AATTTGCCGTGAGTGGAGTCATAC. Primers were previously published in (3). Relative expression levels were calculated using the $\Delta\Delta C_T$ method, using *Gapdh* as the reference gene for normalization (12).

Cytokine measurements

Murine IL-12p40, TNFα and IL-10 cytokine concentrations were determined using ELISA DuoSet kits (R&D Systems, Minneapolis, MN).

Particle preparation

3 µm diameter silica dioxide microspheres were purchased from Microspheres-Nanospheres, a subsidiary of Corpuscular Inc (Cold Spring, NY). To clean them of debris, microspheres were acid-washed overnight in 1N HCl, then rinsed extensively with Milli-Q-filtered water.

Measurement of lysosomal damage by ratiometric imaging

BMM were plated onto glass-bottom MatTek dishes in RPMI 1640 containing 10% FBS, 1% GlutaMAX supplement, and 10 U/ml penicillin-streptomycin. Damage to macrophage lysosomes was measured using an assay for ratiometric measurement of pH (13). To label macrophage lysosomes, BMM were incubated overnight with 150 µg/ml fluorescein dextran, average molecular weight 3 kDa (Fdx; ThermoFisher). During this overnight pulse, cells also were treated with inducers of macrophage differentiation. The next day, cells were rinsed in Ringer's buffer (155 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM NaH_2PO_4, 10 mM HEPES, and 10 mM glucose) and returned to unlabeled media for 3-5 hours before the start of imaging. Lysosomal damage was induced by feeding BMM acid-washed microspheres (AW beads) in RPMI lacking serum for 60 min. AW beads were added at a concentration empirically determined to result in uptake of 3 to 4 beads per cell. Measurements of damage were restricted to cells that had internalized 3 to 7 beads. To measure inhibition of cytokine secretion by brefeldin A (BFA), BMM on MatTek dishes were labeled by overnight incubation with Fdx, with or without LPS, then rinsed with RB and chased 4 hours in R10 with or without LPS or 5 mM BFA, before administering AW beads and assaying lysosomal damage.

To monitor dye release, BMM containing Fdx-labelled lysosomes were imaged by fluorescence microscopy after 60 min incubation in the presence or absence of AW beads. For each field of cells imaged, three images were acquired: a phase-contrast image, which allowed enumeration of bead number per cell, and two fluorescent images, captured using a single emission filter centered at 535 nm and two excitation (exc.) filters, centered at 440 nm or 490 nm, the pH-insensitive and pH-sensitive wavelengths, respectively, for fluorescein. Taking the ratio of 535 nm fluorescence intensities captured at exc. 490 and exc. 440 yielded pH information for each pixel in the image. A calibration curve was generated by measuring 490 nm/440 nm excitation ratios of Fdx in BMM exposed to 10 μM nigericin and valinomycin in fixed pH clamping buffers (5). Release of dye from lysosomes was quantified as the percent of pixels in cellular regions which indicated pH greater than 5.5. This measurement of percent Fdx release was made on a per-cell basis and reported as the average percent Fdx release for each condition. Image acquisition and analysis were performed using Metamorph software (Molecular Devices, Sunnyvale, CA) as described in (14). To eliminate bias in sampling, cells in the microscope were identified for imaging using the phase contrast images, which offered no information about membrane damage or Fdx release into cytoplasm. 15 to 20 random fields were collected from each coverslip. Subsequent processing was performed on every cell image in each field; cells were excluded from the analysis only if they were partially outside of the image frame or if they contained fewer than 3 or more than 7 AW beads.

Viral infection and measurement of viral titers

The plaque-purified MNV-1 clone (GV/MNV1/2002/USA) MNV-1.CW3 (15) (referred to here as MNV-1) was used at passage 6 in all experiments. BMM were seeded overnight on MatTek dishes for lysosomal damage assays and in parallel in 24-well plates for measurement of viral titers. Cells were infected with MNV-1 stock at multiplicities of infection (MOI) of 0.05, 0.5 and 5, then kept on ice for

1 hour with gentle shaking. Inoculum was removed and cells were washed twice with cold DPBS with calcium and magnesium and replaced with RPMI 1640 containing 10% FBS, 1% GlutaMAX supplement, and 10 U/ml penicillin-streptomycin. After 18 h of infection, cells were subjected to assays of lysosomal damage, as described above. BMM infected in parallel were replaced in DMEM containing 10% FBS, 10% L9 supernatant, 1% L-Glutamine, 1% non-essential amino acids and 1% HEPES buffer solution and freeze-thawed twice. MNV-1 titer was determined using plaque assays on RAW 264.7 cells as described in (16).

Statistical methods

Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc; La Jolla, CA). For gene expression and cytokine secretion analyses, statistical significance relative to unstimulated cells was determined using a two-tailed, nonparametric Student's t-test (Mann-Whitney). For analyses of lysosomal damage, average percent Fdx release values between groups across multiple experiments (typically three to five independent experiments per condition) were compared using Two-way ANOVA.

Results

Generation and characterization of variously activated macrophages

To assess the ability of variously activated macrophages to undergo renitence, murine BMM were incubated with the appropriate polarizing cytokines in culture. Gene expression and cytokine secretion analysis confirmed that these treatments successfully generated macrophages of the expected subtypes (17). IFNγ and LPS stimulation of BMM first primed for 6h with IFNγ generated M(LPS+IFNγ), producing high levels of IL-12p40 and TNFα, and low levels of IL-10 (Fig. 1A and B). Macrophages treated with 100 ng/ml LPS in combination with either prostaglandin E2 (M(LPS+PGE2)) or adenosine (M(LPS+Ado)) produced low levels of IL-12p40 and TNFα, and high levels of IL-10. Finally, macrophages differentiated in IL-4 (M(IL-4)) produced low levels of IL-12p40 and IL-10, but expressed high levels of *Relm-α* (Fig. 1A). As expected, *Relm-α* was not abundantly expressed in M(LPS+IFNγ), M(LPS+PGE2) or M(LPS+Ado).

M(LPS) exhibited features of M(LPS+IFNγ), M(LPS+PGE2) and M(LPS+Ado). In addition to producing high levels of IL-12p40 and TNFα, M(LPS) also produced high levels of IL-10, to a similar extent to that produced by M(LPS+PGE2) and M(LPS+Ado) (Fig. 1B). Placed on the spectrum of macrophage activation, M(LPS) therefore assumed an intermediate phenotype between that of M(IFNγ+LPS) and M(LPS+PGE2) or M(LPS+Ado).

M(LPS+ IFNγ), M(LPS+PGE2) and M(LPS+Ado) exhibit renitence

We next assessed the ability of macrophages of each activation state to undergo renitence. We previously defined renitence as an inducible activity within macrophages that confers protection against lysosomal damage (5). The methods for inducing and measuring lysosomal damage have been previously described (13). Briefly, BMM lysosomes pulse-chase labeled with Fdx were

subjected to lysosomal damage through challenge with acid-washed, 3 μm diameter silica microspheres (AW beads), which upon phagocytosis have the potential to damage endolysosomal membranes. Ratiometric fluorescence imaging determined the proportion of the dye that was released from lysosomes into the cytoplasm within individual cells. Damage to lysosomes in cells containing three to seven beads was quantified on a per-cell basis and reported as the average percent release of Fdx.

As previously demonstrated (5, 14), M(LPS) challenged with AW beads experienced reduced damage compared to resting macrophages (Fig 1C). M(LPS+IFNγ), as well as M(LPS+PGE2) and M(LPS+Ado), also showed protection from AW bead-mediated damage, similar to that seen in M(LPS). The protective effects seen in M(LPS+PGE2) and M(LPS+Ado) were dependent on the presence of LPS, as single treatment with either PGE2 or Ado did not confer protection. M(IL-4) did not exhibit protection from AW bead-mediated damage, experiencing similar levels of damage as that seen in resting macrophages. The pattern of protected versus unprotected subsets suggests that renitence is an activity characteristic of macrophages specialized in host defense (M(LPS+IFNγ), M(LPS)) and immune suppression (M(LPS+Ado), M(LPS+PGE2)).

Stimulation by a subset of TLR agonists induces renitence

Of the subset of macrophage activation states examined here thus far, renitent macrophages share a common requirement for their generation: exposure to the TLR4 agonist LPS. We previously observed that stimulating cells with peptidoglycan, a TLR2 agonist, induced renitence to a similar degree as that induced by LPS (5). To examine the range of TLRs capable of inducing renitence, we evaluated a panel of TLR agonists. A subset of the tested agonists induced renitence, including the synthetic triacylated lipopeptide Pam3CSK4, a bacterial ligand that activates TLR2/1;

Poly(I:C), an analog of double-stranded RNA (dsRNA) which activates TLR3; and LPS, a component of the cell wall of gram-negative bacteria and canonical TLR4 ligand (Fig. 2A). R848 (Resiquimod), an anti-viral compound that activates TLR7/8, and ODN 1826, a synthetic oligonucleotide containing unmethylated CpG motifs, which activates TLR9, induced much more modest protection against lysosomal damage. Flagellin purified from *S. typhimurium* (FLA-ST), an agonist of TLR5, induced no protection.

We considered whether the differential ability of TLR agonists to induce renitence might reflect differences in the inflammatory state produced by stimulation with the agonists. By examining cytokine secretion levels in macrophages stimulated with each of the TLR agonists, we determined that all TLR agonists tested except FLA-ST were capable of inducing TNFα secretion (Fig. 2B). The level of IL-10 secretion induced by the panel of agonists was more variable, but likewise did not correlate with the ability to induce renitence. However, levels of IL-12p40 secretion differed between the two groups. The agonists with less renitence-inducing potential (R848 and ODN 1826) induced markedly higher production of IL-12p40 than the set of agonists capable of inducing renitence (Pam3CSK4, Poly(I:C), and LPS) (Fig. 2B), suggesting that IL-12p40 secretion correlates inversely with renitence. FLA-ST stimulation did not induce secretion of TNFα, an expected secreted product following TLR stimulation (Fig. 2B). FLA-ST thus seems to have failed to activate its cognate receptor, TLR5. The inability of FLA-ST to activate BMM has been noted previously and is attributed to low levels of TLR5 expression in mouse BMM (18). Taken together, TLR 2/1, 3, 4, 7/8 and 9 signaling induced renitence in murine macrophages to varying degrees.

Renitence induced by TLR ligands requires intact TLR signaling

MyD88 (Myeloid differentiation primary response 88) and TRIF (TIR-domain-containing adapter-inducing interferon-β) are the major signaling adaptors responsible for propagating signaling downstream of TLR, and macrophages deficient in these two adaptors lack functional TLR signaling (19). To determine whether the induction of renitence by TLR ligands depends on the canonical pathways of TLR signaling, we measured lysosomal damage following 60 min AW bead incubation in C57BL/6J (WT), and *Myd88* and *Trif*-deficient (*Myd88/Trif*$^{-/-}$) BMM stimulated with TLR agonists. Stimulation of WT BMM with the panel of TLR agonists (excluding FLA-ST) induced the same pattern of protection as seen in Figure 2A, except that ODN 1826 stimulation of WT BMM (TLR 9) did not confer a significant reduction in lysosomal damage over that seen in resting BMM (Fig. 2C). Impairment of TLR signaling in *Myd88/Trif*$^{-/-}$ BMM eliminated renitence by all agonists tested except ODN 1826. The finding of no exacerbation of damage in ODN 1826-stimulated *Myd88/Trif*$^{-/-}$ BMM suggests that neither signaling adaptor contributes to lysosomal damage responses in ODN 1826-stimulated macrophages. The slightly decreased damage in macrophages stimulated with ODN 1826 may reflect a vulnerability of the damage assay, as exposure to ODN 1826 induced a morphological change in macrophages that may have affected the assay for phagolysosome damage. Cells in ODN 1826 were less spread out than LPS-stimulated or unstimulated macrophages, which may have limited the maximal levels of detectable damage (ie., there were fewer "cytoplasmic" pixels for the Fdx to occupy), and thereby appeared to have less damage. However, we cannot yet exclude the possibility that ODN 1826 induced a non-canonical signal that reduced damage. Together, these results suggest that renitence stimulated by TLR agonists other than ODN 1826 requires functional TLR signaling.

LPS-induced renitence depends on the release of secreted factors

TLR stimulation leads to the activation of signaling pathways that result in the secretion of numerous inflammatory cytokines (20, 21). To determine whether renitence depends on conventional cytokine secretion, lysosomal damage was measured in LPS-treated BMM in the presence and absence of brefeldin A (BFA), which inhibits cytokine secretion by blocking membrane trafficking from the endoplasmic reticulum to the Golgi apparatus (22, 23). BMM were incubated overnight in Fdx, with or without LPS, then chased in medium with or without LPS or BFA for 4 hours prior to challenge with AW beads. The presence of BFA abrogated renitence in LPS-treated BMM, indicating that LPS-induced renitence depends on the release of secreted factors from BMM (Fig. 3).

MNV-1 infection induces renitence

Previously we discovered that infection of macrophages with hemolysin-deficient *L. monocytogenes*, which cannot perforate phagolysosomes, conferred protection from lysosomal damage upon subsequent challenge with AW beads (5). To ask whether an analogous protective effect may be conferred by viral infection, lysosomal damage responses were measured in BMM infected with murine norovirus-1 (MNV-1). BMM were infected with MNV-1 for 1 hour at MOI 0.05, 0.5, and 5, and then were washed and subjected to overnight pulse-chase labeling of lysosomes with Fdx. Compared to resting cells, BMM first subjected to MNV-1 infection showed enhanced protection against lysosomal damage at all MOI tested, although to a lesser degree than that conferred by LPS (Fig. 4A). Renitence capacity increased with increasing viral load, which was confirmed by measurement of viral titers from macrophages infected in parallel with those assayed for lysosomal damage (Fig. 4B). These data indicated that prior virus infection can be a trigger for renitence in macrophages.

The type I IFN response contributes to renitence induced by TLR3

The identity of the secreted factors that promote renitence is unknown. As infection with many viruses, including MNV-1, induces the secretion of type I IFNs, which limit viral infection, we sought to determine whether type I IFN secretion might contribute to renitence (24). The type I IFNs interferon-α (IFNα) and interferon-β (IFNβ) were discovered for their role in anti-viral immunity but they have since been shown to contribute to immunity against bacteria, parasites, and fungi (24). Stimulation of TLR3 or TLR4 induces type I IFN production in many cell types, including macrophages (25-27). Stimulation of the other TLRs induces type I IFN production only in select cell types, including plasmacytoid dendritic cells and conventional dendritic cells [27].

To assess the effect of the type I IFN response on renitence induced by various stimuli, we compared the extent of lysosomal damage in wild-type BMM and BMM lacking IFNα receptor (*Ifnar1*$^{-/-}$), which cannot respond to type I IFNs. Stimulation of macrophages with IFNβ induced renitence to a similar degree as stimulation by agonists of TLRs 2/1, 3, and 4 (Fig 5). Renitence induced by IFNβ and the TLR3 agonist Poly I:C was reduced in *Ifnar1*$^{-/-}$ BMM, indicating that renitence induced by these factors depends on the type I IFN response. As expected, the absence of type I IFN signaling did not affect the degree of renitence induced by TLR2/1 ligand Pam3CSK4, which does not induce type I IFN secretion. Interestingly, the loss of the type I IFN response did not affect renitence induced by LPS, even though LPS induces type I IFN secretion in macrophages (26, 27). TNFα and IFNγ each induced renitence in WT BMM, and this protective effect was not affected by the loss of type I IFN signaling (Fig. 5). IL-10, a secreted cytokine released by M(LPS), M(LPS+Ado), and M(LPS+PGE) (Fig 2A), did not induce renitence in wild-type or *Ifnar-/-* BMM, consistent with earlier findings (5). Therefore, the type I IFN response contributes to renitence induced by IFNβ and by stimulation of TLR3, but not to renitence induced by TNFα, IFNγ, or stimulation of TLR2/1 or TLR4.

These results indicate that the stimuli that induce renitence work through stimulating distinct downstream signaling pathways, and suggest the presence of multiple types of renitence.

Discussion

By systematically evaluating the inflammatory state and renitence capacity of a range of activated macrophages, this study refined our understanding of the immunological stimuli that induce renitence and their signaling requirements. We showed that distinct pathways for inducing renitence exist and vary depending on the stimulus sensed. In general, stimuli associated with microbial infection or generation of a pro-inflammatory state induced renitence. Renitent macrophages include the well-defined M(LPS) and M(LPS+ IFNγ), as well as macrophages stimulated with IFNβ and agonists of TLRs 2/1, 3, and 4. Interestingly, M(LPS+PGE2) and M(LPS+Ado), macrophages implicated in the suppression of immune responses, were similarly equipped to resist damage. However, not all pro-inflammatory macrophages displayed renitence. Modest protection was observed in macrophages stimulated with agonists of TLRs 7/8 and 9. Macrophages associated with wound healing and immune suppression, M(IL-4) and M(IL-10) respectively, were the least renitent, exhibiting a similar susceptibility to lysosomal damage as that seen in resting macrophages.

TLR stimulation of renitence required canonical signaling through MyD88 and TRIF. Renitence in LPS-treated BMM required the secretion of cytokines or other molecules which may function in an autocrine and/or paracrine fashion. Although these molecules were not identified, renitence induced by stimulation of a TLR3 agonist was shown to depend on the type I IFN response. Future work will test whether conditioned media from LPS-treated BMM confers protection to non-renitent macrophages, and if so, which signals are necessary for mediating renitence.

Interestingly, the specific set of cytokine responses contributing to renitence differed depending on the initial signal provided to the macrophage. For example, even though poly(I:C), a TLR3 agonist, and LPS, a TLR4 agonist, both trigger the secretion of type I IFNs (24), renitence induced by TLR3 stimulation required intact type I IFN signaling, but renitence induced by TLR4

stimulation did not (Fig. 5). The differential requirement for type I IFN signaling in these two conditions likely reflects the different signaling pathways activated following stimulation of TLRs 3 and 4. TLR3 activation recruits the signaling adaptor TRIF, whereas stimulation of TLR4 induces signaling through either MyD88 at the cell surface or TRIF from within endosomes (28, 29). The requirement for type I IFN signaling for TLR3 but not TLR4-induced renitence suggests that TLR4 activation induces the secretion of renitence-inducing cytokines through a MyD88-dependent, TRIF-independent pathway. Candidate factors include the pro-inflammatory cytokines TNFα, IL-12, and IL-1β, which are secreted downstream of a MyD88-dependent signaling pathway that leads to activation of NF-κB (20).

The mechanism by which renitence protects lysosomes from damage remains to be determined. When LPS-treated macrophages ingest AW beads, they form large vacuoles near the damaging particles, which retain lysosomal dyes and maintain acidic pH (14). They indicate an osmotic regulatory mechanism of repair or damage-resistance that prevents leakage of lysosome contents into cytoplasm. The molecular mechanism of damage resistance may involve different effectors in response to different stimuli. TLR3 stimulation induces expression of interferon-stimulated genes (ISGs). Of note, several ISGs have been identified that encode proteins involved in inhibition of endosomal entry of viruses. These include interferon-induced transmembrane protein-3 (IFITM3), cholesterol 25-hydroxylase, and nuclear receptor coactivator-7 (NCOA7) (30). Such proteins might also contribute to protection against phagolysosomal damage in settings other than viral infection and may underlie the mechanism of renitence. Renitence induced by TLR 2/1, TLR 4 or cytokines would work through other mechanisms, which may include upregulation of the endosomal sorting complex required for transport (ESCRT) pathway of membrane damage repair (31).

The observation that MNV-1 infection protects against subsequent phagolysosomal damage in macrophages supports the concept that sublethal viral infection can prime cells to defend against future membrane-damaging threats. Whether the induction of renitence promotes the restriction of viral escape from endosomes is not known, and can be investigated in the future using established models for measuring the extent of MNV-1 endosomal escape (32).

The pattern recognition receptor that recognizes MNV-1 is a question of active investigation. MNV-1 is a single-stranded, positive-sense RNA virus whose recognition is mediated by the intracellular sensor MDA5, which traditionally recognizes dsRNA (33). As such, MDA5 presumably recognizes a replication intermediate of MNV-1. Whether stimulation of intracellular sensors induces renitence remains unknown. It is conceivable that stimulation of cytosolic sensors promotes renitence through a different mechanistic pathway than stimulation of TLRs.

Taken together, our current understanding of the stimuli and signaling involved in renitence suggests a model in which TLR stimulation of macrophages triggers downstream signaling (through MyD88, TRIF, or both) that leads to the release of cytokines, which function in an autocrine or paracrine manner to induce a set of renitence-related genes within the originally activated cell or its neighbors. Numerous pro-inflammatory stimuli are capable of inducing renitence and do so through distinct and complex signaling pathways. Although macrophages associated with immunosuppressive properties (M(LPS+Ado), M(LPS+PGE2)) also exhibit renitence, they share with the pro-inflammatory renitent macrophages a common requirement for exposure to a microbial ligand (i.e. LPS). Therefore, renitence is likely a property conferred in the setting of infection. The increased resistance to membrane-damaging pathogens could lead to their containment by activated macrophages. Mechanistically, renitence depends on secreted cytokines, with the specific set of renitence-inducing cytokines differing depending on the initial microbial stimulus sensed. This

suggests that cytokine secretion responses and the induction of renitence are both fine-tuned to the particular infectious or inflammatory setting encountered.

Authorship

A.O.W. participated in the conceptualization, investigation, data analysis, and original writing of the manuscript as well as subsequent revisions. M.M., A.H. and I.A.O. participated in the investigation, data analysis, and revisions of the manuscript. C.E.W. and J.A.S. participated in the conceptualization and data analysis, the investigation, and the review, editing and revision of the manuscript.

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Conflict of Interest Disclosure

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Renitence is a property of M(LPS+IFNγ), M(LPS+Ado), and M(LPS+PGE2)

(A) BMM were treated overnight with LPS and IFNγ (after initial 6 h IFNγ priming), IL-4, or LPS alone, or left unstimulated (Resting). For each condition, mRNA expression of *Il-12p40*, *Relm-α*, and *Il-10* relative to levels expressed in resting BMM was determined by qPCR. Bars represent mean ± SEM calculated from two (*Il-10*) or three (*IL-12p40*, *Relm-α*) independent experiments. Statistical significance relative to expression levels in resting BMM is indicated. *p ≤ 0.05. (B) BMM were subjected to the following treatments for generating M(LPS+IFNγ), (M(IL-4), M(LPS+PGE2) or M(LPS+Ado). As controls, macrophages were left unstimulated (Resting) or treated overnight with LPS (M(LPS)), PGE2 (M(PGE2)), or Ado alone (M(Ado)). Levels of IL-12p40, TNFα, and IL-10 in cell supernatants were measured by ELISA. Each bar represents mean ± SEM of at least three independent experiments. Statistical significance relative to levels of cytokine secretion in resting BMM is shown. *p ≤ 0.05. (C) BMM were subjected to the indicated treatments for generating M(LPS+IFNγ), M(IL-4), M(LPS+Ado) or M(LPS+PGE2), or control macrophages, which included resting macrophages and BMM singly treated with LPS, PGE2, or Ado. BMM in each condition were pulsed overnight with fluorescein dextran (Fdx), followed by a 3 hr chase in unlabeled medium. To initiate membrane damage, cells were incubated with AW beads or received no bead challenge as controls. Ratiometric imaging was performed to measure the extent of Fdx release from lysosomes. Bars represent the average percent Fdx release ± SEM per condition (n= 2 to 4 independent experiments per condition). In the groups of cells receiving AW beads, analysis

was restricted to cells containing 3-7 beads. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

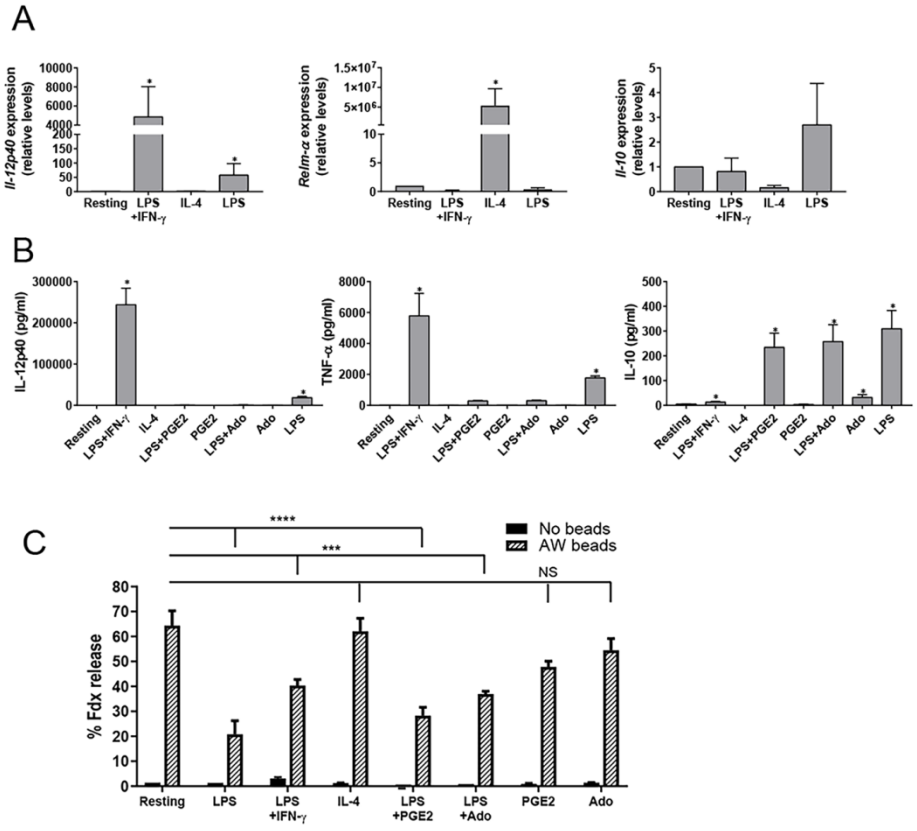

Figure 2: A subset of TLR agonists induces renitence

(A) BMM were pulsed overnight with Fdx while undergoing stimulation with the indicated TLR agonists or left untreated. The next day, cells were chased for 3 h, incubated with AW beads or no beads for 60 min, and imaged to quantify the extent of Fdx release. Bars represent the average percent Fdx release ± SEM per condition (n= 2 to 3 independent experiments per condition). ****p ≤ 0.0001. (B) BMM were stimulated overnight with the indicated TLR agonists or left untreated. Levels of IL-12p40, TNFα, and IL-10 in cell supernatants were measured by ELISA. n = 5 experiments. (C)

BMM were isolated from C57BL/6J (WT) mice and mice deficient in *Myd88* and *Trif* (*Myd88/Trif*$^{-/-}$). Both groups were treated overnight with the indicated TLR agonists concurrent with pulse-chase labeling of lysosomes with Fdx. BMM were then incubated with AW beads for 60 min and assayed for lysosomal damage. Bars represent the average percent Fdx release ± SEM (Beads, n = 3-4; no beads, n=2). NS: no significant difference, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

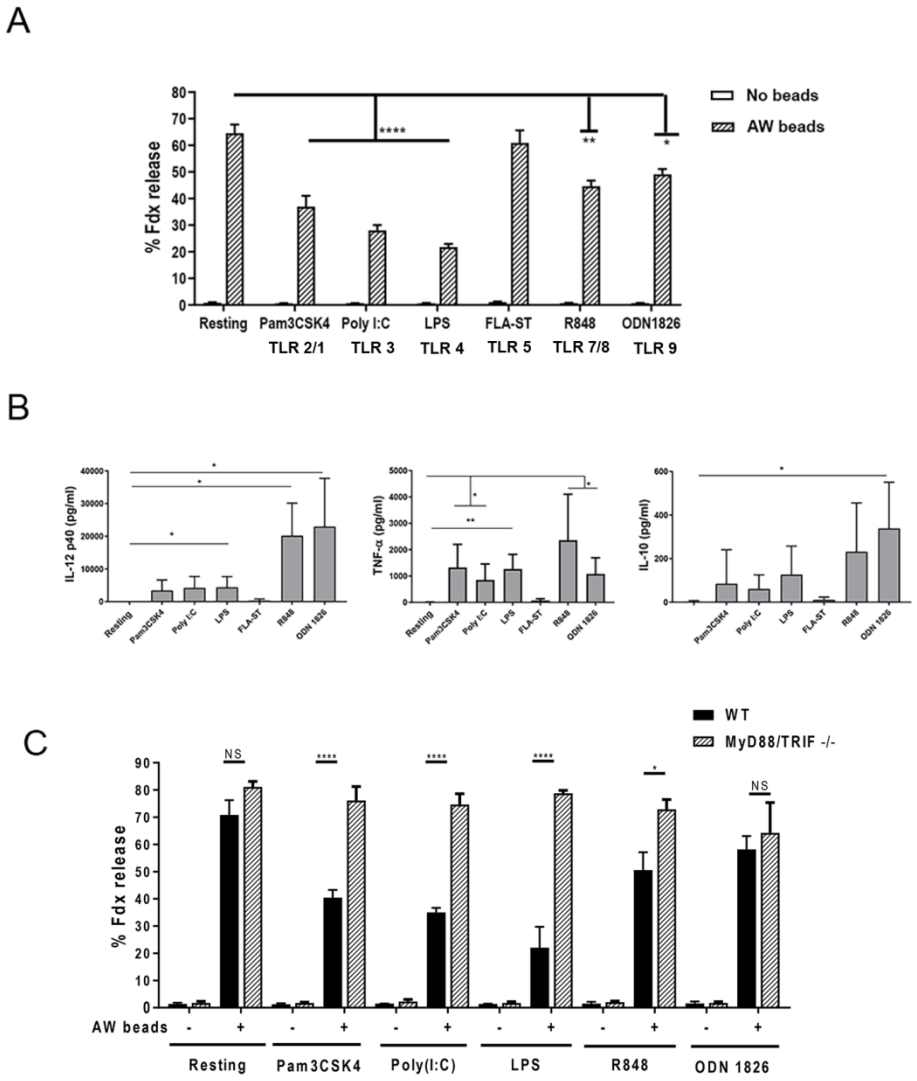

Figure 3. LPS-induced renitence depends on secretion

BMM were incubated overnight with Fdx in the presence or absence of LPS. The following day, cells were rinsed free of Fdx and incubated 4 h in media, with or without LPS and/or BFA (5 μM). Cells were then fed AW beads for 60 min and assayed for lysosomal damage. Bars represent the average percent Fdx release ± SEM (n = 2-3 independent experiments). NS: no significant difference, *$p \leq 0.05$.

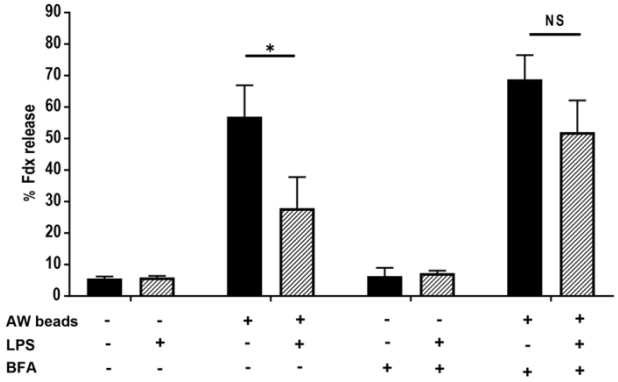

Figure 4: MNV-1 infection induces renitence

(A) BMM were infected with MNV-1 at MOI 0.05, 0.5, and 5 for one hour, washed, and then subjected to pulse-chase labeling of lysosomes with Fdx. BMM were then incubated with AW beads or no beads for 60 min and assayed for lysosomal damage. As controls, lysosome damage was also measured in resting BMM and M(LPS). Bars represent the average percent Fdx release ± SEM per condition (n = 5 independent experiments, except for no-bead conditions, where n = 1-2) $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$. **(B) MNV-1 infects BMM in an MOI-dependent manner at 18 h**rs **post-infection.** BMM were infected with MNV-1 at three different MOI (0.05, 0.5 and 5). Viral titers in cell culture lysates were measured by virus titration using a plaque assay and reported as plaque-forming units/ml (PFU/ml). Bars show MNV-1 infection titers of three different MOIs from 5 independent experiments performed in duplicate or triplicate. These assays were performed in parallel with the viral infections for the lysosomal damage experiments. $^{*}p \leq 0.05$, $^{****}p \leq 0.0001$.

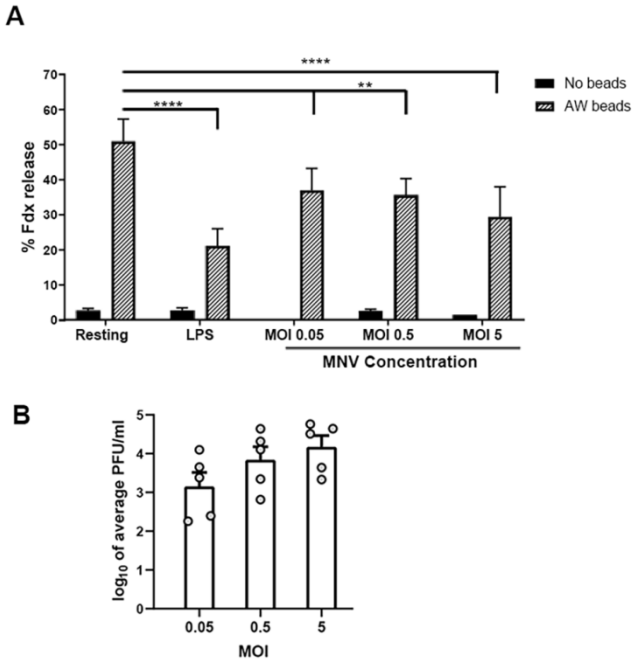

Figure 5. Reinitence induced by stimulation of TLR3 depends on the type I IFN response
C57BL/6J (WT) BMM and *Ifnar1*$^{-/-}$ BMM were treated overnight with Pam3CSK4 (TLR2/1), poly(I:C) (TLR3), LPS (TLR4), TNFα, IFNβ, IFNγ or IL-10, or left untreated while subjected to pulse-chase labeling of lysosomes with Fdx. BMM were then incubated with AW beads for 60 min and assayed for lysosomal damage. Bars represent the average percent Fdx release ± SEM per condition (n = 2-3 independent experiments). NS: no significant difference, ***p* ≤ 0.01, ****p* ≤ 0.001, *****p* ≤ 0.0001.