Localized Reactive Oxygen and Nitrogen Intermediates Inhibit Escape of *Listeria monocytogenes* from Vacuoles in Activated Macrophages

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Localized Reactive Oxygen and Nitrogen Intermediates Inhibit Escape of *Listeria monocytogenes* from Vacuoles in Activated Macrophages

Jesse T. Myers,*† Albert W. Tsang,‡ and Joel A. Swanson*‡†

*Listeria monocytogenes* (*Lm*) evade being killed after phagocytosis by macrophages by escaping from vacuoles into cytoplasm. Activated macrophages are listericidal, in part because they can retain *Lm* in vacuoles. This study examined the contribution of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) to the inhibition of *Lm* escape from vacuoles. *Lm* escaped from vacuoles of nonactivated macrophages within 30 min of infection. Macrophages activated with IFN-γ, LPS, IL-6, and a neutralizing Ab against IL-10 retained *Lm* within the vacuoles, and inhibitors of ROI and RNI blocked inhibition of vacuolar escape to varying degrees. Measurements of *Lm* escape in macrophages from gp91phox−/− and NO synthase 2−/− mice showed that vacuolar retention required ROI and was augmented by RNI. Live cell imaging with the fluorescent probe dihydro-2',4,5,6,7',7'-hexafluorofluorescein coupled to BSA (DHFF-BSA) indicated that oxidative chemistries were generated rapidly and were localized to *Lm* vacuoles. Chemistries that oxidized DHFF-BSA were similar to those that retained *Lm* in phagosomes. Fluorescent conversion of DHFF-BSA occurred more efficiently in smaller vacuoles, indicating that higher concentrations of ROI or RNI were generated in more confining volumes. Thus, activated macrophages retained *Lm* within phagosomes by the localization of ROI and RNI to vacuoles, and by their combined actions in a small space of the oxidase complex (10, 11). Both ROI and RNI contribute to murine resistance to *Lm* infection and to the listericidal activities of activated macrophages (2, 12–16). However, it is not known whether ROI and RNI affect escape from the vacuole or subsequent microbial functions.

The present studies examined the contributions of ROI and RNI to *Lm* retention in vacuoles. The timing of *Lm* escape from vacuoles was measured, then gp91phox−/− and NOS2−/− knockout mice, which are unable to generate superoxide and NO, respectively (17, 18), were used to define the relative contributions of ROI and RNI to inhibition of escape in activated macrophages. Finally, fluorescent methods were used to measure the timing of ROI and RNI generation in vacuoles. These studies demonstrate that escape occurs within the first 30 min after entry, and that the combined actions of ROI and RNI inhibit *Lm* escape from vacuoles in activated macrophages.

**Materials and Methods**

**Bacterial preparation**

*Lm* strains 10403S and DP-L2161 (gifts from D. Portnoy, University of California, Berkeley, CA) were maintained on brain-heart infusion agar plates. For experiments, one or two bacterial colonies were added to 5 ml of brain-heart infusion broth, shaken overnight at room temperature, and diluted 1/6 the following morning, and shaken at 37°C for 1.5 h to obtain an OD600 of 0.500. Bacteria were washed by pelleting and resuspending in Ringer’s buffer (155 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 2 mM NaHPO4, 10 mM HEPES, and 10 mM glucose, pH 7.2) three times before addition to macrophages.

**Macrophages**

Female NOS2−/−, gp91phox−/−, and homozygous wild-type (C57BL/6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). NOS2−/− and gp91phox−/− mice had been backcrossed for at least 10 generations onto a C57BL/6 background. Bone marrow-derived macrophages were cultured as previously described (19). After 5–9 days of growth, cells were replated into 6-, 24-, or 96-well tissue culture dishes. The Journal of Immunology, 2003, 171: 5447–5453.
To compare sizes of Lm-containing vacuoles from activated and non-activated macrophages, cells were plated on 25-mm circular coverslips (3.0 × 10^4) overnight, then mounted on the temperature-controlled stage of an inverted microscope and maintained at 37°C. Cells were pulsed for 5 min with 100 μl of Ringer’s buffer containing hemolysin-deficient Lm mutant hly (strain DP-2.16) plus 1.0 mg/ml Lucifer Yellow and then washed with 15 ml of Ringer’s buffer. Cells were excited with 436 nm of light, and the coverslip was scanned for vacuoles containing Lm, which were identified as fluorescent compartments with darker regions created by dye displaced by bacteria. Phase contrast and fluorescence images (excitation, 436 nm; emission, 480 nm) were acquired of each Lm-containing vacuole. In MetaMorph, regions were drawn around the bacteria and the vacuoles in which they were contained, and the pixel area of each vacuole was logged into Excel.

**Listerial assay**

Macrophages were plated onto 13-mm coverslips and incubated overnight in medium alone (control) or medium plus one of the following combinations of ingredients: 1) IFN-γ and LPS, 2) IFN-γ, LPS, plus IL-6 added during the experiment, or 3) IFN-γ, LPS, and α-IL-10, plus IL-6 added during the experiment. Macrophages were then incubated with Lm for 30 min, washed, and incubated for the indicated times in medium with gentamicin (50 μg/ml) for 0.5–7.5 h, after which macrophages were fixed and stained with DAPI to determine the number of bacteria per infected macrophage.

**Results**

**Lm escape from vacuoles within 30 min**

To visualize the chemistries involved in the listerial response of activated macrophages, it was important to know the time frame in which those chemistries would be active. A drop in pH is required for Lm to escape from the vacuole into the cytosol (21). The proton ATPase inhibitor BFA1 prevents endocytic vacuole acidification and can be used to prevent phagosomal escape of Lm (22). Non-activated macrophages were treated with BFA1 at various times after infection with Lm. Bacteria that had escaped into the cytosol were identified by their association with filamentous actin, as indicated by staining with TR-phalloidin. When cells were treated with BFA1 immediately following infection, only ~20% of bacteria engulled by macrophages managed to escape into the cytoplasm (Fig. 1). Later additions of BFA1 showed an increased amount of escape that leveled off after 30 min, at which point addition of BFA1 had no measurable effect on the escape of Lm. Therefore, nearly all vacuolar escape occurred within 30 min of infection. The mechanisms used by activated macrophages to retain Lm within the vacuole must be active during this brief period following entry.

**Imaging with dihydro-2′,4′,5,6,7,7′-hexafluoro-2′-amidotetracarboxylic acid (DHF-BSA)**

Macrophages were plated on 25-mm circular coverslips (2.5 × 10^4) and then mounted in a temperature-controlled stage at 37°C, mounted on a cooled microscope (TE-300; Nikon, Tokyo, Japan) equipped with a cooled CCD camera (Quantix; Photometrics, Tuscon, AZ), filter wheel (A 10–2; Sutter Instruments, Novato, CA), and a phase contrast objective (N.A.1.4). Cells were pulsed for 5 min with Lm (MOI ~1) along with DHF-BSA (1 mg/ml Molecular Probes). TR-dextran (m.w., 10,000; 0.1 mg/ml) was included in the pulse to verify uptake of the nonfluorescent DHF-BSA into phagosomes. Coverslips were then washed three times with 5 ml PBS and mounted on glass slides with Prolong Antifade (Molecular Probes). For each coverslip, 50 macrophages with DAPI-labeled bacteria were scored for colocalization of bacteria with filamentous actin.

**Fluospectra**

Imaging with dihydro-2′,4′,5,6,7,7′-hexafluoro-2′-amidotetracarboxylic acid (DHF-BSA) was used to quantify fluorescent conversion of DHF-BSA. Regions were traced around phase contrast images of Lm-containing phagosomes. The corresponding region was copied to the F485 and F580 images, and the average F485 and F580 pixel intensities of the region were logged into an Excel spreadsheet (Microsoft, Redmond, WA) along with the time the image was acquired. To calculate the average amount of conversion of DHF-BSA (see Fig. 6C), the individual phagosomal fluorescence intensities were pooled and averaged for each condition, and a background value of 127 (average F485 in a phagosome without probe; calculated in a separate experiment) was subtracted. Values were then displayed as a percentage of the response measured in wild-type activated macrophages. For experiments comparing oxidation of DHF-BSA with phagosomal area (see Fig. 7), F485, F580, time, and pixel area were recorded for Lm phagosomes of activated macrophages. Phagosomes were grouped into three arbitrary size groupings representing small, medium, and large phagosomes. The F485 intensity was divided by the F580 intensity to normalize for the amount of probe within the phagosome. Student’s t test (2-tailed distribution) was used to calculate the statistical significance of the differences between samples.
Activation of bone marrow-derived macrophages

Overnight treatment of peritoneal macrophages with IFN-γ is sufficient to control the growth of Lm (4). The same treatment of bone marrow-derived macrophages, however, does not lead to as high a degree of listericidal activity (23). Therefore, we sought to enhance activation of bone marrow-derived macrophages with additional factors to augment the standard IFN-γ and LPS method of activation. α-IL-10, a cytokine secreted by macrophages that down-regulates activation (24), was included in an overnight activation medium along with IFN-γ and LPS. IL-6 was also included during the infection because it has been shown to increase the listericidal activity of macrophages when added at the start of infection (25). Activation of macrophages with IFN-γ and LPS alone initially reduced the number of bacteria per macrophage, as measured by staining infected cells with DAPI and counting the number of bacteria per cell, but bacterial numbers increased over 8 h (Fig. 2). In contrast, macrophages activated with IFN-γ, LPS, IL-6, and α-IL-10 controlled the growth of bacteria for the duration of the experiment. Because activation with this cocktail of ingredients resulted in more efficient containment of Lm, this activation protocol was used for all additional experiments.

ROI and RNI both inhibit vacuolar escape by Lm

The ability of activated macrophages to inhibit Lm escape was used to develop assays for the microbicidal activities of ROI and RNI. Macrophages were infected at a low MOI (~0.1), such that each infected macrophage initially contained only one bacterium. Two hours after infection, cells were fixed and stained with TR-phalloidin, and the percentage of infected macrophages with actin-positive bacteria was determined. The effects of various RNI and ROI inhibitors on the ability of Lm to escape from the vacuole into the cytoplasm were tested (Fig. 3). Activation of macrophages with IFN-γ, LPS, IL-6, and α-IL-10 reduced Lm access to cytoplasm. Pretreatment of activated macrophages with the NO synthase inhibitor L-NMMA led to a dramatic increase in the escape of Lm from vacuoles, indicating a role for NO in the prevention of Lm escape. Treatment of activated macrophages with the superoxide scavenger SOD produced a small, but significant (p > 0.05), increase in Lm escape. The hydrogen peroxide scavenger catalase, alone or in combination with SOD, did not significantly increase vacuolar escape, suggesting that hydrogen peroxide did not contribute to the retention of Lm in vacuoles of activated macrophages. Treatment of activated macrophages with L-NMMA, SOD, and catalase together resulted in the greatest amount of escape from the vacuole, similar to that observed in nonactivated macrophages.

As a separate approach for analyzing the contributions of ROI and RNI to inhibition of Lm escape, vacuolar escape was measured in macrophages from NOS2−/− and gp91phox−/− mice, in which elimination of RNI or ROI, respectively, was specific and complete (Fig. 4A). Lm escape in nonactivated macrophages from NOS2−/− and gp91phox−/− mice was not significantly different from that in wild-type macrophages. However, although activation of wild-type macrophages greatly reduced vacuolar escape of Lm, escape from activated gp91phox−/− macrophages was only slightly

FIGURE 3. The effects of ROI and RNI inhibitors on escape of Lm from phagosomes. Cytoplasmic localization of Lm was determined by colocalization with TR-phalloidin. The percentage of infected macrophages with TR-positive bacteria was recorded in nonactivated macrophages and macrophages treated with IFN-γ, LPS, IL-6, and α-IL-10 (1, L, 6, and α). Macrophages were treated with various combinations of 1 mM L-NMMA, 150 U/ml SOD, and 1500 U/ml catalase as indicated. Activated macrophages inhibited escape, and treatment of activated macrophages with L-NMMA, SOD, and/or catalase prevented that inhibitory activity to varying degrees. Values represent the mean ± SEM. (n = 3).

FIGURE 4. Enhanced phagosomal escape of Lm in gp91phox−/− and NOS2−/− macrophages. The percentage of infected macrophages with TR-positive bacteria was determined in activated and nonactivated macrophages from wild-type, gp91phox−/−, and NOS2−/− mice, and in activated wild-type, gp91phox−/−, and NOS2−/− macrophages treated with 1 mM L-NMMA (A). Activated macrophages from gp91phox−/− mice did not inhibit escape any more than nonactivated macrophages. Activated macrophages from NOS2−/− mice showed intermediate levels of escape inhibition. B, Effect of phagosomal escape in macrophages treated with 100 μM FeTPPS. FeTPPS reversed some of the inhibition, indicating a possible role for peroxynitrite in blocking escape. Data represent the mean ± SEM (n = 3).
less than that from nonactivated gp91phox−/− macrophages. These results differed significantly from data obtained with the ROI scavengers, SOD and catalase (Fig. 3), indicating that they may not have inhibited ROI completely. Activation of NOS2−/− macrophages led to a partial reduction in Lm escape, similar to the effects of L-NMMA on wild-type macrophages (Fig. 4A). L-NMMA did not have additional effect on escape in NOS2−/− macrophages. Therefore, L-NMMA was a specific and effective inhibitor of iNOS. To examine the role of peroxynitrite, a microbical molecular entity formed by the reaction of superoxide and NO, on Lm escape, macrophages were treated with the peroxynitrite-specific scavenger FeTPPS (26) (Fig. 4B). FeTPPS had no effect on phagosomal escape in nonactivated macrophages, but escape in activated macrophages treated with the scavenger was approximately double that observed in control cells (p < 0.01).

In summary, the Lm escape studies indicated significant contribution of both ROI and RNI to the inhibition of escape from activated macrophages. The inability of macrophages from gp91phox−/− mice to inhibit escape suggests that ROI are essential. The substantial, albeit incomplete, inhibition in NOS2−/− mice indicated its secondary importance, and the partial inhibition by FeTPPS indicated a role for peroxynitrite.

Localization of oxidative activity using DHFF-BSA

To visualize the timing and localization of the oxidative chemistries generated within the Lm vacuole, live cell imaging was performed with the probe DHFF-BSA. This is a relatively nonfluorescent molecule conjugated to BSA that, when oxidized, becomes highly fluorescent (27). Macrophages were incubated for 5 min with Lm, DHFF-BSA, and TR-dextran, a fluorescent indicator of the volume of fluid taken into vacuoles and phagosomes. Time-lapse sequences of Lm vacuoles containing DHFF-BSA showed a rapid conversion of the relatively nonfluorescent probe to a fluorescent-like molecule (Fig. 5, A and B). Dye conversion was restricted to vacuoles containing bacteria. Probe-loaded macropinosomes, identifiable by their labeling with TR-dextran, generally exhibited little DHFF-BSA fluorescence, even in cells containing vacuoles with very bright DHFF-BSA signals (Fig. 5, C–E). On rare occasions, fluorescent vacuoles with no apparent bacteria were observed; the fluorescence in these vacuoles was less than that in those that contained bacteria. It was unclear whether these vacuoles contained unseen factors, such as bacterial proteins, which generated a response by the macrophage.

One concern was that repeated exposure to 485 nm light in time-lapse sequences contributed to the oxidation of DHFF-BSA. To circumvent this problem, data were collected by scanning the coverslip using phase contrast optics to identify Lm vacuoles, then taking only one set of images for each vacuole (phase contrast, F485, F580). Quantitative data were plotted as a function of time after infection, then analyzed as a population of vesicles.

Fluorescent conversion of the probe was detectable almost as soon as images could be acquired (Fig. 6A). DPI, an inhibitor of flavoproteins that prevents the generation of both ROI and RNI (28, 29) completely abrogated fluorescent conversion of DHFF-BSA in activated macrophages (Fig. 6B). Thus, chemistries restricted to the phagosomes were converting DHFF-BSA very soon after vacuole formation.

These chemistries were quantified by measuring the fluorescence in digital images. The degree of DHFF-BSA conversion was determined by calculating the average fluorescence intensity under various conditions (Fig. 6C). Nonactivated macrophages were capable of generating fluorescent vacuoles, although their average fluorescence intensity was less than that of Lm vacuoles in activated macrophages.

The specificity of DHFF-BSA oxidation was examined using gp91phox−/− and NOS2−/− macrophages. Phagosomes from gp91phox−/− macrophages exhibited minimal dye conversion; their average fluorescence was similar to that observed in macropinosomes and in Lm vacuoles of DPI-treated macrophages (Fig. 6C). Thus, ROI were required for the oxidation of DHFF-BSA, and RNI alone were not sufficient to oxidize DHFF-BSA. Phagosomes from NOS2−/− macrophages as well as macrophages treated with L-NMMA demonstrated a reduced average phagosomal fluorescence compared with wild-type activated macrophages (Fig. 6C). Hence, RNI were not essential, but enhanced the fluorescent conversion of the probe, indicating either that some dye conversion resulted from oxidation by peroxynitrite or that NO somehow enhanced ROI generation.

Although most Lm vacuoles of activated macrophages inhibited escape, a smaller percentage showed dye conversion. We hypothesized that although the oxidative chemistries affecting Lm escape and dye conversion were qualitatively similar, their effects on Lm

**FIGURE 5.** Localization of phagosomal oxidative activity with DHFF-BSA. Macrophages were infected for 5 min in the presence of DHFF-BSA and TR-dextran. A time-lapse series showing, at 30-s intervals, phase contrast (A) and fluorescence images (excitation, 485 nm; B) of a phagosome containing Lm (arrowhead) and DHFF-BSA. Arrowheads indicate the bacterium in corresponding positions in the images. Phase contrast (C) and fluorescence images exciting at 485 nm (D) and 580 nm (E) were taken of a macrophage infected with Lm. The arrow shows an Lm-containing phagosome, and arrowheads show macropinosomes loaded with the probe. DHFF-BSA conversion was restricted to the Lm vacuole. Bar = 5 μm.
FIGURE 6. Rapid conversion of DHFF-BSA in Lm phagosomes. Images of phagosomes containing Lm, DHFF-BSA, and TR-dextran were taken over a period of 25 min following a 5-min infection. The average fluorescence intensity (excitation, 485 nm) was recorded in activated macrophages (A) and activated macrophages treated with 10 μM DPI (B). Each dot represents the fluorescence intensity of an individual phagosome. Time zero was defined as the beginning of the 5-min infection. The average fluorescence intensity (excitation, 485 nm) of Lm phagosomes, relative to wild-type activated macrophages, was determined in activated and nonactivated wild-type, gp91phox−/−, and NOS2−/− macrophages as well as in wild-type macrophages treated with either DPI (activated only) or L-NMMA (C; ±SEM). Measurements were also taken of probe-loaded macropinosomes in macrophages not exposed to Lm. Macropinosomes, Lm vacuoles of DPI-treated wild-type macrophages, and macrophages from gp91phox−/− mice showed background levels of fluorescence. Limited, submaximal conversion was detected in Lm vacuoles of nonactivated macrophages and in activated NOS2−/− macrophages.

FIGURE 7. DHFF-BSA converted more efficiently in smaller phagosomes. Representative images of phagosomes, with areas of 132 (A), 264 (B), and 531 (C) pixels, respectively. Bar = 2 μm. Fluorescence was normalized for the amount of ingested dye by dividing of DHFF-BSA (F485) by the intensity of TR-dextran fluorescence (F580). Normalized fluorescence was recorded for phagosomes of three different size groupings (±SEM).

Discussion

Activated macrophages, which are critical components of the cell-mediated immune response against Lm (30–33), control the growth and replication of Lm by retaining the bacteria within the phagocytic vacuole (4). ROI and RNI are important microbicidal mediators and contribute to the listericidal activity of macrophages. We show here that ROI and RNI directly affect the retention of Lm in vacuoles of activated macrophages.

The role of ROI in the inhibition of escape was characterized using gp91phox−/− macrophages as well as the ROI-scavenging enzymes, SOD and catalase. Activation did not improve the ability of gp91phox−/− macrophages to retain Lm within vacuoles (Fig. 4A), thereby indicating a requirement for ROI in enhanced vacuolar retention by activated macrophages. ROI alone (L-NMMA-treated or NOS2−/− macrophages) could also reduce Lm escape, although this reduction was not as great as when RNI were also present. Results obtained with SOD and catalase did not produce a phenotype as pronounced as when gp91phox−/− macrophages were used. Acidification of the vacuolar compartment may have decreased the activity of SOD and catalase. Alternately, the rapid, NO-dependent conversion of superoxide to peroxynitrite, which should occur more rapidly than the scavenging of superoxide by SOD (34), may have consumed superoxide before it could be scavenged by SOD.

RNI also aided in the retention of Lm in vacuoles of activated macrophages. Activated NOS2−/− or L-NMMA-treated macrophages showed decreased Lm phagosomal escape, but these effects were not as complete as in wild-type macrophages (Fig. 4A). RNI alone were not sufficient to retain Lm within vacuoles, because gp91phox−/− macrophages, producing RNI, but not ROI, could not inhibit escape after activation. The effects of the NO inhibitor L-NMMA on the escape and oxidation of DHFF-BSA were similar to the effects observed in NOS2−/− macrophages (Figs. 4A and...
6C). However, preincubation of cells with L-NMMA for 15 min was necessary for complete suppression of NO generation (our unpublished observations).

Our data are consistent with a model in which two types of chemistries contribute to retention of \textit{Lm} in vacuoles: one mediated by ROI alone, and another ROI-mediated chemistry that is also dependent on the presence of RNI. This is similar to the RNI-dependent and RNI-independent listericidal activities observed by Muller et al. (12). One mechanism by which RNI could have enhanced the activity of ROI was via the generation of peroxynitrite, which is formed by the reaction of superoxide and NO. Peroxynitrite is highly reactive and microbialicidal (34–37). Evidence for peroxynitrite-mediated vacuolar retention was demonstrated by the increased percentage of cytoplasmic \textit{Lm} in activated macrophages treated with FeTPPS, a peroxynitrite scavenger with little SOD-mimetic activity (26). Attempts to localize nitrotyrosine, a reaction product of peroxynitrite chemistry, have not succeeded. It is also possible, however, that RNI enhance ROI-mediated phagosomal retention by peroxynitrite-independent means. Along with being a microbialicidal molecule, NO is also commonly employed as a signaling molecule. NO-mediated chemistries may have primed the macrophage to deliver a more potent oxidative burst.

Although RNI appear to be a significant contributor to antilisterial activity in our system, the importance of NO and RNI in human macrophages is unclear. Cytokines that lead to \textit{iNOS} expression appear to be more tightly regulated in human macrophages, but this does not preclude the possibility that human macrophages use RNI more efficiently to control the growth of \textit{Lm}.

If peroxynitrite has a role in the vacuolar retention of \textit{Lm}, then superoxide and NO must be concomitantly present in the vacuole within 30 min of infection, before the bacteria had escaped from the vacuole. Therefore, it was necessary to determine whether the localization and timing of the generation of oxidative chemistries within the \textit{Lm} vacuole were consistent with the generation of peroxynitrite. An imaging method was developed, using the fluorogenic probe DHFF-BSA, to study the timing and localization of the oxidative chemistries generated by macrophages into the \textit{Lm} vacuole. While it is relatively common to measure the oxidative burst with fluorogenic probes either extracellularly or throughout the cytoplasm, to our knowledge, ROI/RNI have not previously been measured within a bacterial phagosome, the location where oxidative chemistries are most likely to have an effect. Fluorescent conversion of DHFF-BSA was reported to be a result of oxidation by hydrogen peroxide (27). DHFF-BSA oxidation was ROI dependent, as judged by the lack of fluorescence generated by \textit{g91\textsuperscript{phox}−/−} macrophages (Fig. 6C). RNI alone were insufficient for probe oxidation, but fluorescent conversion of DHFF-BSA was reduced in both \textit{NOS2−/−} and L-NMMA-treated macrophages, indicating that RNI contribute to the ROI-mediated oxidation of DHFF-BSA. Because NO can diffuse across membranes, it was unclear whether RNI-mediated conversion of DHFF-BSA was due to a localized generation of RNI into phagocytic vacuoles or if constitutive generation of NO throughout the cell produced levels of RNI within vacuoles that were sufficient for probe oxidation. Although the reactivity of DHFF-BSA with peroxynitrite has not been previously examined, 2',7'-dichlorofluorescein, a molecule similar to DHFF, is readily oxidized by peroxynitrite (39, 40), suggesting the RNI-mediated enhancement of probe oxidation could be due to reaction with peroxynitrite. Conditions that led to fluorescent conversion of DHFF-BSA were similar to those that retained \textit{Lm} within the vacuole, with the exception of NO expression and L-NMMA-treated macrophages. In that case, escape was at an intermediate level between activated and nonactivated control macrophages (Fig. 4A), whereas fluorescent conversion of DHFF-BSA in those cells was at the same level as in nonactivated macrophages (Fig. 6C). This raises the possibility that there might be a small ROI/RNI-independent contribution to vacuolar retention of \textit{Lm} that does not lead to fluorescent conversion of DHFF-BSA. Overall, however, DHFF-BSA oxidation was a good indicator of the chemistries that led to vacuolar retention of \textit{Lm} and demonstrated a rapid generation of oxidative chemistries localized to \textit{Lm} phagosomes.

Many \textit{Lm} vacuoles were not fluorescent or were only slightly more fluorescent than background. This may have been a result of loss of probe, via fusion with lysosomes, or leakage through pores formed by the pore-forming toxin listeriolysin O (LLO). Also, vacuoles that were only partially formed after the 5-min pulse would have lost probe when the noninternalized bacteria were washed off. Nevertheless, many nonconverting vacuoles must have contained probe, since they were TR-dextran positive. These vacuoles may have been imaged before ROI/RNI were generated within the vacuole. Another possibility is that the concentrations of the oxidative chemistries required to retain \textit{Lm} in the vacuole are less than those concentrations required to convert DHFF-BSA. Accordingly, some \textit{Lm} vacuoles will have had ROI/RNI levels sufficient to block \textit{Lm} escape, but insufficient to convert DHFF-BSA.

Because measurement of oxidative activity with DHFF-BSA was not strictly quantitative, it is difficult to compare the responses we observed with measurements that have been made using other methods. Presumably, cells with a more prodigious oxidative burst, such as peritoneal macrophages, would show an increased conversion of DHFF-BSA, although experiments comparing the conversion of DHFF-BSA by different cell types and different activation states have not been performed.

The rapid and localized response of the oxidative burst within \textit{Lm} vacuoles suggests recognition of the bacteria by the macrophage and invites speculation as to how the signals are generated for this response. The bacteria were not serum-opsonized, although we cannot rule out that something secreted by the macrophages opsonized them rapidly. In the absence of opsonization by Abs or complement, pattern recognition receptors are likely to contribute to the generation of an immune response. The most likely candidates for the start of the signaling cascade are Toll-like receptors (41, 42). It remains to be seen whether oxidation of DHFF-BSA is a result of Toll-like receptor signaling.

Interestingly, DHFF-BSA was oxidized more efficiently in small, tight phagosomes than in large spacious ones. Antimicrobial chemistries generated into a tight-fitting phagosome would presumably be more concentrated than if they were generated within a larger, spacious phagosome. The average size of \textit{Lm}-containing vacuoles was similar in activated and nonactivated macrophages (our unpublished observations), indicating that activated macrophages do not restrict phagosomal size to enhance the effectiveness of their oxidative chemistries. Spacious phagosome formation by \textit{Lm} may be a defense mechanism that reduces the effectiveness of ROI and RNI within the phagosome.

Although it is now clear that the combined actions of ROI and RNI are necessary for retention of \textit{Lm} in vacuoles, their mechanism of action remains unknown. Rapid ROI/RNI-mediated killing could have led to the retention of \textit{Lm} within vacuoles. Preliminary experiments have shown no effect of activation on the survival of hemolysin-deficient mutant hly \textit{Lm} in macrophages (our unpublished observations), making it unlikely that ROI/RNI directly...
killed Lm in our system. Alternately, ROI/RNI may have inactivated the pore-forming toxin LLO, thereby preventing the escape of Lm into the cytosol. ROI and RNI could act cooperatively (e.g., generating peroxynitrite) or independently to inactivate LLO or to stimulate other antilisterial mechanisms. It is also possible that NO enhances the function of the NADPH-oxidase complex, either by direct chemical modification of component proteins or by acting as a second messenger to stimulate other mechanisms that potentiate the activity of ROI.

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References