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OUTSTANDING OBSERVATION

Defect in efferocytosis leads to alternative activation of macrophages in *Francisella* infections

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The macrophage is a versatile cell type that can sense and respond to a particular need based on the conditions of the microenvironment. Some studies have recently suggested that pathogens can directly influence the polarization of macrophages. As *Francisella* infections are characterized by intense necrotic infiltrates in the lung as well as in distal sites of infection, we sought to investigate whether pulmonary *Francisella* infections could cause the polarization of alternatively activated macrophages (M2/aaMs). Our results indicate that *Francisella* infections can cause the polarization of M2/aaM *in vivo* and that macrophages can be polarized toward an M2/aaM phenotype more potently if dead cell debris is used for stimulation in the presence and absence of *Francisella* infections. Finally, we also demonstrate that efferocytosis is inhibited in macrophages infected with *Francisella*, thus providing a potential explanation for the lack of clearance and eventual accumulation of dead cell debris associated with this disease.
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Macrophage activation and differentiation is currently an area of intense research. There are now several types of macrophages and at least two classification systems for macrophages. They include classically activated macrophages and alternatively activated macrophages (aaMs or M2). M2/aaMs can also be further classified into regulatory or wound-healing macrophages according to the markers they express as well as their functionality.[1,2] The unique subsets of M2/aaMs and their corresponding functions arise in response to a distinctive set of stimuli that have recently been shown to be either dependent or independent of interleukin-4. Interestingly, macrophages have also recently been proposed to be highly adaptable to the stimuli present in a given local milieu and can alter their activity to meet the needs present in a specific circumstance.[3]

Tularemia is a disease caused by the Gram-negative coccobacillus species in the genus *Francisella*. The pneumonic form of the disease is characterized by an initial delay in the immune response of approximately 48–72 h. Following this delay, there is a massive influx of inflammatory cells[4,5] as well as the onset of hypercytokinemia and sepsis.[6,7] We have also recently observed that the fatal outcome of the disease correlates with the release of damage-associated molecular patterns (DAMPs) or alarmins that have been described to be potent inflammatory mediators derived from various modes of cell death.[8–10]

Importantly, recent studies have also provided a link between endogenous immune mediators (DAMPs) released in cell death and their potential ability to affect macrophage polarization.[11–13] In light of these studies and in conjunction with the striking pathology associated with pulmonary *Francisella* infections, we hypothesized that the extent of cell death generated in response to infection could have an important role in macrophage polarization. The aim of this study was to assess the generation of M2/aaMs during a pulmonary *Francisella* infection and determine whether dead cell debris could stimulate the differentiation of macrophages into M2/aaMs. Our experiments have led us to propose another potential route by which macrophages with an M2/aaM phenotype could be derived during *Francisella* infections.

RESULTS

Markers of alternative activation of macrophages are upregulated *in vivo* in response to respiratory *Francisella novicida* infections

To determine whether alternatively activated macrophages were generated in pulmonary *F. novicida* infections, we initially screened the lungs of mice infected with 500 CFU of bacteria as well as sham-infected mice (mocks) for the transcripts of common M2/aaM markers. These included *ym-1*, *mrc-1*, *arg-1* and *fizz-1*. We found that *ym-1* and *mrc-1* were constitutively expressed in the lungs of mock-infected mice, and there was no change at the transcript level throughout the 72 h post-infection (HPI). However, both *fizz-1* and *arg-1* were significantly upregulated by about 15-fold over mock levels by 72 HPI (Figures 1c and d; $P<0.005$). Immunofluorescent (IF) microscopy revealed that both Ym-1 and MR-1 were constitutively

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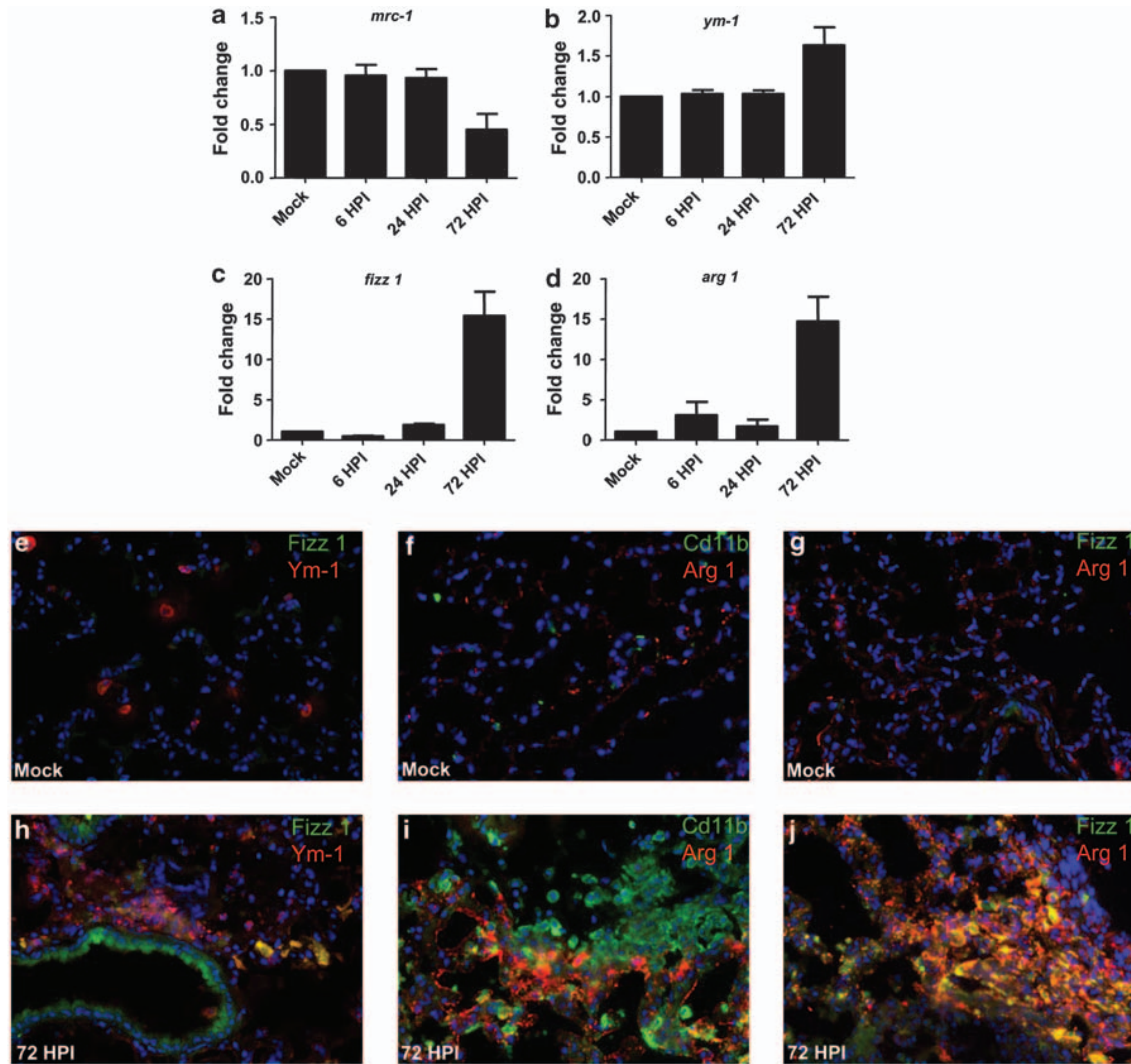

Figure 1 M2/aaMs are upregulated and expressed in the lungs in response to an intranasal infection with *F. novicida*. Expression of mRNA for *mrc-1* (**a**), *ym-1* (**b**), *fizz-1* (**c**) and *arg-1* (**d**) in the lungs of mice (n=3–4) intranasally infected with 500 CFU of *F. novicida*. Immunofluorescent microscopy was also performed in order to verify the presence of M2/aaM markers in the lung. (**e, h**) Fizz-1 (Alexa 488) and Ym-1 (Rhodamine red) staining in a mock lung (**e**, ×400) and in a lung at 72 HPI (**h**, ×400). Fizz-1 expression was observed predominantly at low levels in the bronchial epithelium in mocks and at early times post-infection. (**f, i**) Arginase 1 (Rhodamine red) and CD11b (Alexa 488) in a mock lung (**f**, ×400) and in a lung at 72 HPI (**i**, ×400). (**g, j**) Arginase 1 (Rhodamine red) and Fizz-1 (Alexa 488) staining in a mock lung (**g**, ×400) and in that at 72 HPI (**j**, ×400).

expressed in the lungs of mock mice and did not increase until 72 HPI (Figures 1e and h and data not shown). The expression of Fizz-1 was limited to the bronchial epithelium and remained largely restricted to this structure until 72 HPI (Figures 1e, g, h and j). At 72 HPI, Fizz-1 expression was apparent on infiltrating cells both near and within lesions as well as on other infiltrating cells dispersed throughout the tissue. Arginase 1 expression was present at low levels in mock lungs and increased sharply at 72 HPI. At 72 HPI, Arginase 1 staining colocalized with CD11b and also with Fizz-1, especially on the periphery of lesions (Figures 1i and j).

Dead cell debris is a potent stimulator of Arginase 1 production in macrophages in the presence or absence of infection

We then tested whether M2/aaM differentiation could be driven directly by infection with *F. novicida* and, in addition, whether dead cell debris was also capable of driving M2/aaM differentiation. This was determined by incubating infected and uninfected J774A.1 macrophages with or without dead cell debris and analyzing for the production of the M2/aaM marker Arginase 1. The results indicate that Arginase 1 production could be elicited by necrotic debris and that necrotic debris seemed to be a potent enhancer of Arginase 1 production even in the absence of infection (Figures 2 a2, b2 and c2). Similar results were obtained using bone marrow-derived macrophages (Supplementary Figure 1). Our data also indicate that the percentage of Arginase 1-positive cells was greatest when exposed to dead cell debris regardless of whether *Francisella* was present or not (Figure 2d).

Macrophages infected with *F. novicida* are less efficient at efferocytosis

We next hypothesized that infected cells were less competent at clearing dead cells, thus leading to an accumulation of dead cell debris. In order to test this, we first infected J774A.1 cells at a multiplicity of infection (MOI) of 100 for 24 h with *F. novicida*.

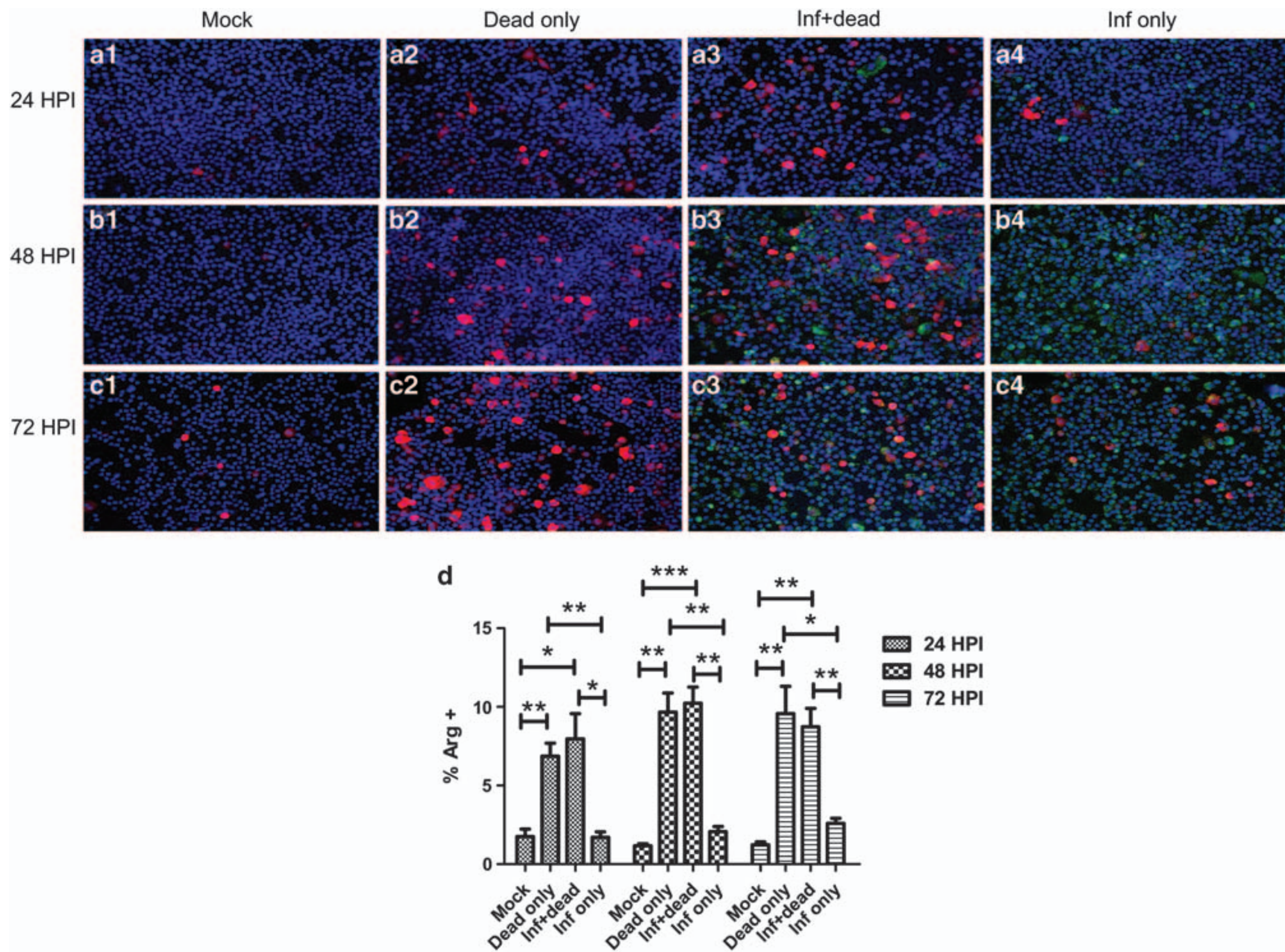

Figure 2 Arginase 1 is highly upregulated in the presence of necrotic cell debris. (**a**–**c**) J774A.1 cells that have received phosphate-buffered saline (**a1**, **b1**, **c1**, **a2**, **b2** and **c2**) or have been infected with *F. novicida* (MOI 100; **a3**, **a4**, **b3**, **b4**, **c3** and **c4**). After infection, necrotic cell debris was added onto the cells (**a2**, **a3**, **b2**, **b3**, **c2** and **c3**) at a ratio of 10:1 (dead cells:live cells) and allowed to incubate until the cells were harvested. Cells were then fixed and IF microscopy was performed to observe the expression of Arginase 1 (Rhodamine red) and to visualize the presence of *F. novicida* (Alexa 488). The graph in (**d**) represents the average percentage of Arginase 1 cells from three different experiments. Statistical analysis was performed using Student's *t*-test (*$P<0.05$; **$P<0.01$; ***$P<0.005$).

We then added non-viable cells labeled with LeukoTracker dye to the uninfected and infected cells and incubated them together for 1 h at 37 °C. We then measured the amount of fluorescent signal retained by these groups or processed the coverslips for visualization via IF microscopy. Our results indicate that uninfected cells had more non-viable cells associated with them when compared with cells that had been infected (Figures 3a and b). This result was corroborated by verifying that the total amount of fluorescent dye retained in the uninfected cells was significantly higher than the amount of dye retained in infected cells (Figure 3c). Another interesting observation was an apparent difference in the number of cells that were moderately stained with dye. In the uninfected samples, many cells appeared to accumulate dye as well as remain associated with non-viable cells (Figures 3a, b and d). These cells were noticeably reduced in frequency and were relatively absent in the infected wells.

As CD36 has been reported to be an important receptor for clearing dead cell debris, the expression of CD36 was compared in response to non-viable cell debris in either the presence or absence of infection as described above. We observed that CD36 expression was abundant in uninfected samples, whereas it was markedly reduced in the presence of infection (Figures 3e–g and Supplementary Figure 2).

DISCUSSION

Francisella infections are typically characterized by an initial delay in the immune response by 48–72 h post-infection. Our recent studies have also indicated that following this initial delay, there is an aberrant immune response characterized by hypercytokinemia and spillage of DAMPs or alarmins into the extracellular milieu. This occurs during both *F. novicida* and SchuS4 infections.[6,7] These studies, in conjunction with the data from Lai *et al.*[14] and our unpublished findings, confirm that late in the infection there is an abnormally high attrition of cells that could be caused by apoptosis, pyroptosis, necrosis and/or secondary necrosis. Our observations indicate that the kinetics of hypercytokinemia generated in response to *Francisella* infections correlate with the onset of massive cell death events. Hypercytokinemia, extensive cell death and release of DAMPs are the hallmarks of severe sepsis, which is the likely cause of death in experimental animals[15] and is known to occur in septic patients.[16] In light of this evidence, we propose that alternatively activated macrophages are generated at the later stages of *Francisella* infection as a result of badly damaged lung tissue associated with severe sepsis in an attempt to promote wound healing and repair.

The plasticity of macrophages has recently taken center stage among studies on the innate immune response to infectious diseases.

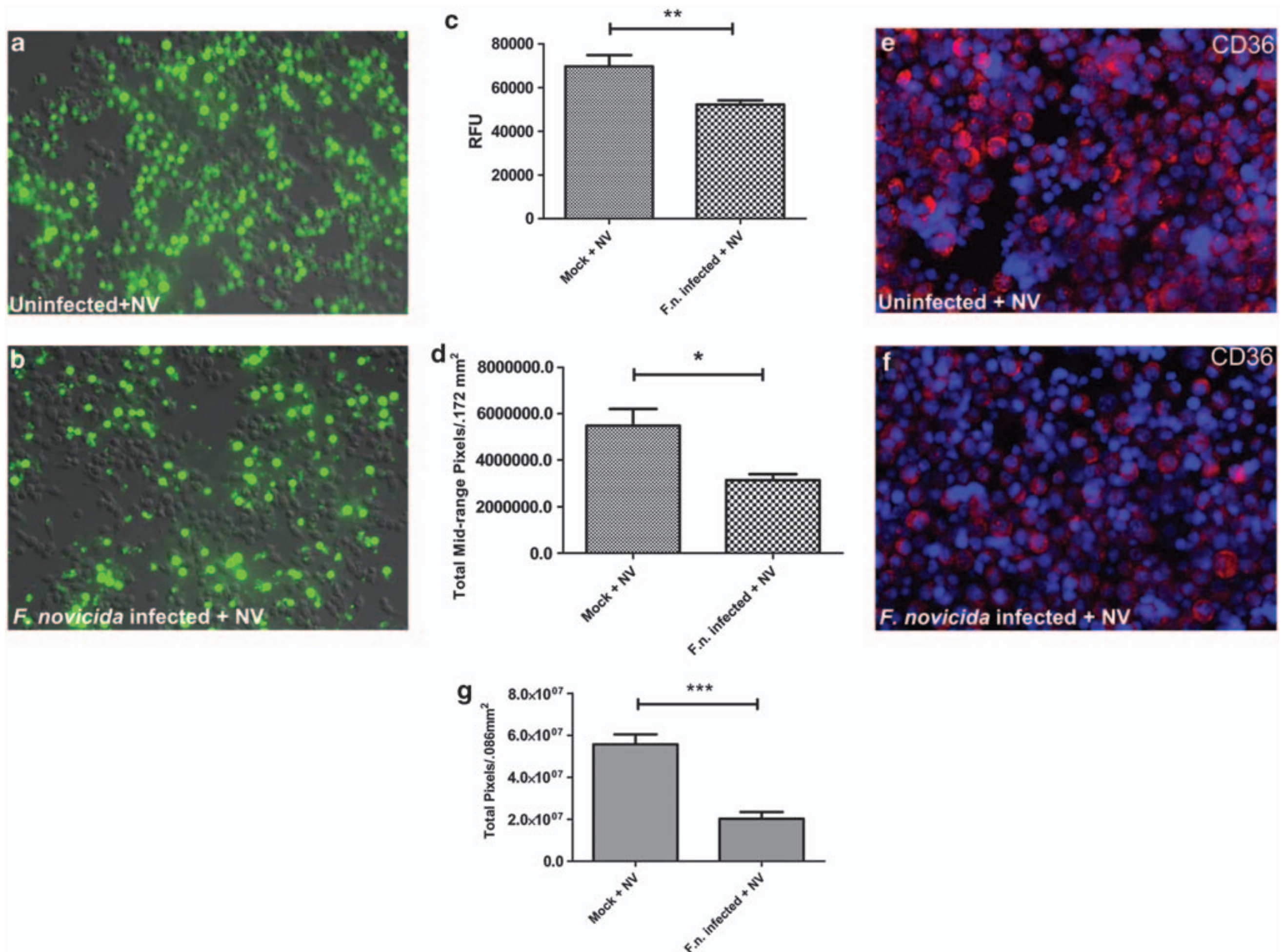

Figure 3 *F. novicida*-infected macrophages are less efficient at efferocytosis. Briefly, mock J774A.1 macrophages and infected J774A.1 cells infected at an MOI of 100 for 24 h. Labeled non-viable (NV) cells were then added to both groups at a ratio of 10:1 and allowed to incubate for 1 h. (**a**, **b**) Representative images showing the adherence of labeled NV cells to uninfected or infected J774A.1 cells (×200). (**c**) The amount of LeukoTracker dye recovered from either mock J774A.1 cells with labeled NV cells or *F. novicida*-infected J774A.1 cells with labeled NV cells (n=6 per group). (**d**) The total mid-range intensity pixels quantified using IP Labs 4.0 in the aforementioned groups (n=3 per group; representative of two independent experiments). (**e**–**g**) The expression of CD36 on J774A.1 cells in the presence or absence of infection in response to NV cells (**e**, **f** are at ×400). (**g**) Quantification of total pixels

Recent reviews have documented the many faces of macrophage differentiation, including those routes that direct macrophages toward the alternatively activated pathway. New evidence has even suggested that certain pathogens, including *Francisella*, may be capable of manipulating macrophage differentiation in order to potentially create a less hostile environment for the pathogen.[17,18] However, the studies herein have indicated that necrotic cell debris is also capable of inducing alternative macrophage differentiation in a manner that is both superior and independent of infection with *F. novicida*. This finding underscores the important role that host-derived endogenous immune mediators or DAMPs may have in potentiating the pathogenesis of inflammatory diseases.

Importantly, we have also described a potential mechanism to account for the accumulation of dead cell debris in response to infection with *F. novicida*. Our results indicate that upon infection of macrophages with *F. novicida*, efferocytosis, or the uptake and clearing of dead cells, is significantly hindered when compared with uninfected cells. Butchar *et al.*[19] have also shown that the mRNA transcripts for multiple signaling pathways and receptors are down-regulated in response to either *F. novicida* or SchuS4 infections. Strikingly, data from the above studies indicate that both CD14 and CD36 are both dramatically downregulated at the transcript level. These two receptors have both been implicated in the recognition and uptake of both apoptotic and necrotic cell debris.[20–22] Our results here indicate that CD36 is sharply downregulated in infected macrophages at the protein level in response to non-viable cells. The results of others have recently shown CD14 is also downregulated as a result of infection.[19] Collectively, these two lines of evidence directly suggest that receptors important for efferocytosis are downregulated and may provide an explanation as to how dead cell debris may accumulate and eventually contribute to the exacerbation of disease. In our model, we propose that the highly infective and cytotoxic nature of this pathogen, coupled with the downregulation of efferocytosis receptors, ultimately leads to the dangerous accumulation of dead cell debris laden with DAMPs commonly observed as necrotic-like lesions *in vivo* (Figure 4). This accumulation of dead cell debris ultimately contributes to the formation of alternatively activated macrophages and the development of severe sepsis.

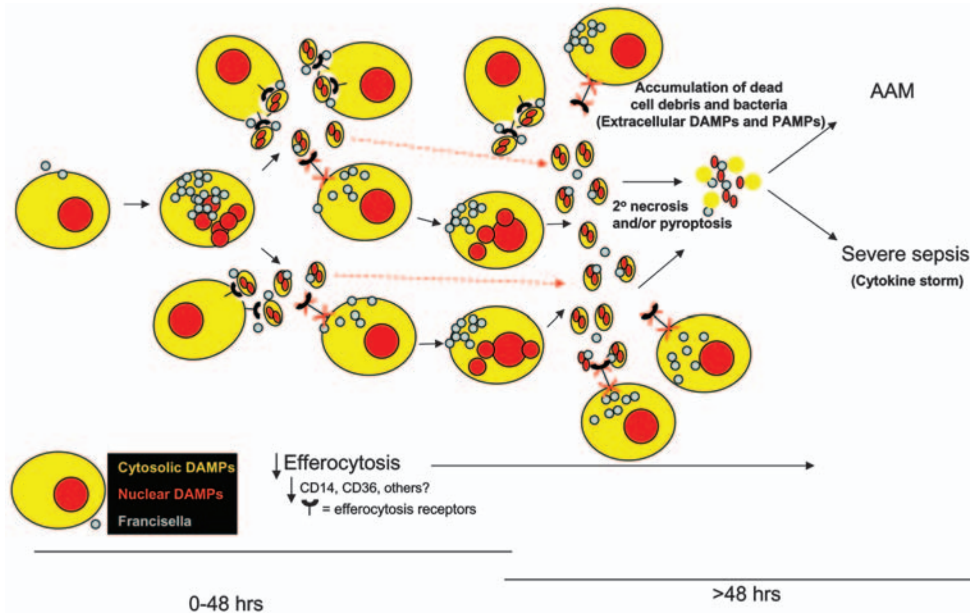

Figure 4 Defect in efferocytosis leads to the accumulation of dead cell debris and accumulation of DAMPs. Initially, *Francisella* infects target cells (macrophages in this case). Once infected, bacteria proliferate intracellularly and egress from the cell via induction of cell death. Infected macrophages are less efficient at efferocytosis owing to the downregulation of key receptors (CD14, CD36 and potentially others). As more and more cells become infected with time post-infection, less efficient efferocytosis, coupled with an increase in infected macrophages that are themselves dying, could lead to secondary necrosis and ultimately accumulation of DAMPs. Finally, the accrual of this dead cell debris in conjunction with persistent bacterial infection leads to the differentiation of alternatively activated macrophages and the onset of severe sepsis, including hypercytokinemia and multiple organ failure.

Our results help support the notion of the plasticity of macrophages and their respective functions. We have shown that M2/aaMs can be generated in the lungs of mice in response to *F. novicida* and have shown that dead cell debris can also be utilized to generate macrophages with an alternative phenotype. Our current findings, coupled with our previous work involving the release of DAMPs, help to underscore the central role that host tissue destruction and cellular attrition have in potentiating the immune response to *Francisella*. These findings further implicate the prominent role of infection-induced collateral host tissue damage in shaping the immune response in this particular model of severe sepsis.

METHODS

Initial screening for markers associated with alternative macrophage differentiation was accomplished using real-time reverse transcriptase-PCR performed on a TaqMan Low Density Array with customized markers selected. Briefly, C57BL/6 (6–8 weeks old) mice were intranasally infected with 500 CFU per 20 μl of *F. novicida*. Mice were killed at 6 HPI, 24 HPI and at 72 HPI. In addition, mice treated with phosphate-buffered saline were also taken for controls. Lungs were harvested in 1 ml of TRIzol and RNA was extracted and used to prepare cDNA for the TaqMan low-density array. In a separate experiment, lungs of infected and control mice were harvested for IF microscopy as described elsewhere.[6] IF microscopy was performed on at least three different sets of mice for Fizz-1, Ym-1, CD11b and Arginase 1 in order to confirm the presence of M2/aaMs in the lungs of mice infected with *F. novicida*. All animal work was conducted in compliance with the IACUC protocols at the University of Texas at San Antonio.

For *in vitro* experiments J774A.1 cells were used. The cells were seeded at a density of 5×10^4 cells per well in 16-well chamber slides or at a density of 2×10^5 cells per well on coverslips in 24-well plates. The cells were then either treated with phosphate-buffered saline (mock) or infected with *F. novicida* (MOI of 10–1000 and 100, respectively, as shown in Results section) for 2 h. Following the 2-h infection period, the cells were pulsed with 50 $\mu g\,ml^{-1}$ of gentamicin for 1 h. Necrotic cells were generated by five freeze–thaw cycles and placed in the appropriate wells after the 50 $\mu g\,ml^{-1}$ pulse of gentamicin. The ratio of dead to live cells was approximately 10:1. The cells were then fixed at various time points and probed for the presence of Arginase 1 and *F. novicida* by IF microscopy. The percentage of Arginase 1-positive cells was determined by counting cells in at least four different fields per experimental group at a magnification of ×200. The results shown are representative of three independent experiments. The same experimental setup was used to assess the efficiency in the uptake of non-viable cells. Approximately 2×10^6 cells per sample of (10:1 ratio) J774A.1 cells were labeled with LeukoTracker solution and washed with phosphate-buffered saline according to the manufacturer's protocol (Cell Biolabs Inc., San Diego, CA, USA). Cells were then subjected to a final concentration of ethanol of 33% in Dulbecco's modified Eagle's medium+10% fetal bovine serum (D10) for 1 h at 37 °C in order to produce non-viable cells. Viability was assessed based on trypan blue assay. Labeled non-viable cells were then added onto either mock J774A.1 cells or J774A.1 cells infected for 24 h with an MOI of 100 and incubated for 1 h at 37 °C. Samples were then washed extensively with five phosphate-buffered saline washes and either fixed with 70% ethanol and processed for IF microscopy or lysed with 1% Triton X-100. The cell lysates were then immediately assessed for the amount of LeukoTracker dye present in each sample as described in the manufacturer's protocol. In the assessment of CD36 using IF microscopy, non-viable cells were unlabeled. Murine immunoglobulin A anti-CD36 was used followed by fluorescein isothiocyanate-conjugated anti-murine immunoglobulin A (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to assess the presence of CD36 on J774A.1 cells. Pixel quantification and pseudocoloring were performed using IP Labs 4.0 (Scanalytics, Fairfax, VA, USA). Statistics were calculated using the Student's *t*-test in GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Immunology and Cell Biology website (http://www.nature.com/icb)