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Primary and secondary immune responses to *Listeria monocytogenes*

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Abstract

Recent studies have revealed the complexity of cytokine and cellular interactions required for resistance to primary *Listeria monocytogenes* infection and have illustrated that resistance to secondary infection may occur through multiple pathways. Analyses of *Listeria* epitope generation and the specificity of protective CD8+ T cells have suggested that future research should focus on secreted protein antigens in specific resistance to infection and have increased our understanding of *Listeria* antigens presented by MHC class I-b molecules.

Introduction

Listeria monocytogenes (LM) is an intracellular bacterial pathogen capable of infecting humans and numerous animal species. Extensive *in vitro* studies of LM pathogenesis have characterized the cell biology of infection, whereby the organism enters the eukaryotic cell in a membrane-bound vesicle but subsequently escapes from the vesicle into the cytoplasm, where it replicates and initiates cell to cell spread through the actions of coordinately regulated virulence factors such as the pore-forming listeriolysin (LLO) and activities which polymerize actin [1]. *In vivo*, a well characterized mouse model of LM infection has yielded significant insight into the nature of innate and specific cell-mediated immunity to bacterial infection of any type. Experiments with gene knockout mice, neutralizing cytokine or cell type-specific depleting antibodies have generated a complex picture of cytokine and cellular interactions required for innate resistance to primary LM infection. Similar reagents have been employed to dissect the specific T cell mediated response to primary and secondary LM infections. The identification of LM antigens that elicit specific CD8+ T-cell responses capable of mediating immunity against infection has facilitated studies aimed at understanding CD8+ T cell effector mechanisms and revealed MHC class I-b-restricted LM antigen presentation to CD8+ T cells. In this review, we summarize recent experiments that have had an impact on our understanding of the cytokine requirements in the innate immune response to LM. In addition, we address experiments that characterize the specificity of CD8+ T cells for LM and their *in vivo* effector mechanisms.

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Innate resistance to primary LM infection

Early studies with severe combined immunodeficiency (SCID) mice that lack mature T and B cells demonstrated a T-cell-independent mechanism for resistance to primary LM infection [2]. Subsequent *in vitro* and *in vivo* studies identified a pathway where cytokines such as interleukin (IL)-12 and tumor necrosis factor (TNF)α are produced by LM-infected macrophages and function to stimulate interferon (1FN)γ production by natural killer (NK) cells [3]. These cytokines activate the microbicidal activities of macrophages and can affect the development of specific immunity to infection [4]. Other studies have demonstrated that neutrophils play a critical role in resistance to primary LM infection [5–7] and have revealed that γδ T cells control the severity of immune-mediated pathology in the livers of LM-infected mice [8,9].

Recent experiments using gene knockout mice and cytokine antibodies have revealed the contribution of multiple cytokines in the innate response to primary LM infection. Studies with IFNγ receptor (IFNγR−/−) [10] and IFNγ gene knockout mice have demonstrated that IFNγ is a critical mediator of resistance to primary LM infection; homozygous IFNγ gene knockout mice exhibit three orders of magnitude less resistance to primary LM infection (lethal dose $[LD]_{50}$~10 LM) than heterozygous controls (LD_{50}~10^4 LM) [11••]. Interestingly, IFNγ gene knockout mice exhibit wild-type levels of resistance to an attenuated LM strain that fails to spread between cells, suggesting that IFNγ may ultimately function to inhibit bacterial cell–cell spread [11••].

Resistance to primary infection also depends on TNFα and lymphotoxin, as shown by the increased susceptibility of mice with disruption of the genes encoding TNF receptor 1 (TNFR1) [12], and lymphotoxin-α and TNFα [13]. Mice that express soluble TNFR1 [14] or lymphotoxin-β inhibitor [15•] were also at greater risk of infection. TNFα may play multiple roles in the innate immune response, including activating macrophages and increasing expression of adhesion molecules required for neutrophil extravasation [16]. Neutralization of IL-12 has also been shown to exacerbate primary LM infection [17•], probably through interference with the production of IFNγ by NK cells [18•]. Together, these studies support the model of macrophage activation described by Unanue and colleagues [3]. An interesting question that has not been addressed relates to the dependence of TNFα and IL-12 on the actions of IFNγ. IFNγ and IFNγR mice should be invaluable in addressing this issue.

In vivo neutralization of IL-1β has been shown to exacerbate primary LM infection by decreasing the production of IFNγ by NK cells [18•] and neutrophil recruitment [19]. Disruption of the gene for IL-6 [20•] or NF-IL6 (nuclear factor-IL6) [21•] resulted in mice with increased susceptibility to LM infection and has shown that IL-6 has an impact on both neutrophil [20•] and macrophage [21•] functions. The demonstration that TNFα, IL-6 and IL-1β are important for both macrophage activation and neutrophil function has provided a link between the effector cells involved in the innate immune response to infection.

These recent experiments have shown that interference with the activities of many different cytokines impairs resistance to primary LM infection. These data have illustrated the complexity of the innate immune response to bacterial infection and have suggested a high degree of interdependence in the various effector pathways of innate resistance. Clearly, LM infection of mice continues to provide an excellent probe to dissect the complex interactions that result in innate resistance to bacterial infection.

Specific resistance to primary LM infection

Gene knockout mouse studies have provided a clearer picture of the role T cells play in response to primary LM infection. MHC class I and class II deficient mice that lack substantial CD8+

and CD4+ T cell subsets exhibited slightly increased susceptibility to LM compared to wild-type mice [22] and sometimes developed chronic infections [23]. However, such mice were less susceptible to primary LM infection than the IFNγ knockout mice described previously. These data suggest that T cells are not the major determinants of resistance to primary LM infection. Consistent with this view, perforin knockout mice that were defective in one pathway of CD8+ T cell mediated lysis appeared to be as resistant to primary LM infection as their heterozygous littermates, although they exhibited defects in secondary resistance [24].

Together, these data suggest that the innate immune response is the major defense against primary LM infection of the mouse and that specific T cell mediated responses play a role in the eventual clearance of the infection. This hypothesis is consistent with the rapid and potent induction of the innate response to infection which limits the rate of LM replication and influences the nature of the gradually developing specific response.

Resistance to secondary LM infection

Resistance to secondary LM infection requires T cells, with MHC class I restricted CD8+ T cells exhibiting the greatest capacity to mediate antilisterial immunity. The original assumption, that CD8+ T cell mediated immunity to LM functioned through IFNγ driven macrophage activation, has recently been explored. Studies with IFNγ antibodies have yielded different results: one study indicated a role for IFNγ in secondary resistance to LM infection [17•]; whereas another found IFNγ independence in the secondary response to LM [25••]. Interestingly, the first study showed no dependence on IL-12 for secondary resistance to LM [17•]. We have recently presented studies combining LM with IFNγ knockout mice to demonstrate that CD8+ T cell mediated resistance to secondary LM infection can develop and be expressed in the complete absence of IFNγ [11••]. Thus, in contrast to the absolute requirement for IFNγ in the primary response to LM, resistance to secondary LM infection can occur in its absence.

Studies indicating that CD8+ T cells from perforin knockout mice are deficient in their ability to mediate antilisterial immunity [24] are consistent with a pathway for IFNγ-independent resistance to secondary LM infection. Studies with LM antigen specific CD8+ T cell lines, however, suggest that perforin gene function is not absolutely required for transfer of antilisterial immunity to naive mice (DW White, JT Harty, unpublished data). These data may indicate that CD8+ T cells can provide antilisterial immunity by either perforin-mediated killing or IFNγ-mediated macrophage activation. The possibility that novel effector mechanisms are employed by LM specific CD8+ T cells, however, requires further exploration.

In contrast to IFNγ and perforin, TNFα appears to be required for resistance to secondary LM infection [25••]. The function of TNFα in resistance to secondary infection is unknown but may relate to the finding that neutrophils are also required for resistance to secondary LM infections [26•,27,28] and that TNFα plays a role in neutrophil recruitment [16]. Thus, at least some effector pathways of the specific immune response depend on elements of the innate immune response. Given the interdependence of the innate and specific immune responses to LM, the challenge lies in developing experimental systems to identify which effector molecules and cells are essential for each process.

Specificity of CD8+ T cells against LM

The findings that secreted LM proteins LLO [29] and p60 [30] are target antigens for CD8+ T cells that transfer significant immunity to naive mice [31•,32], suggested the hypothesis that secreted LM proteins may be the most important target antigens for protective CD8+ T cells. Consistent with this hypothesis, lymphocytic choriomeningitis virus (LCMV)-specific CD8+ T cells lysed target cells that were infected with recombinant LM that expressed an LCMV T

cell epitope in secreted form, but failed to recognize cells infected with LM that expressed the epitope in cytosolic form (JT Harty, H Shen, JF Miller, unpublished data). The basis for this result may be explained by access of the secreted proteins to the cytosolic MHC class I antigen-presentation pathway. In this regard, recent experiments by EG Pamer and co-workers have characterized the efficiency and mechanism of epitope generation from secreted LM antigens. They determined that LM infected cells expressed significant numbers of complexes of LLO and p60 epitope with MHC as early as 2–3 hours postinfection, at levels that were sufficient to allow recognition by antigen-specific CD8+ T cells [33]. These studies have been extended to show that epitope generation from LLO is extremely efficient [34••], probably due, at least in part, to the secreted nature of the antigen. In addition, studies with proteosome inhibitors have demonstrated a major role for the cytosolic pathway of antigen presentation in generating p60-derived peptide epitopes [35•]. These studies support the hypothesis that secreted proteins may be the most relevant targets for protective CD8+ T cells, based on the efficiency of epitope generation from these antigens, which should be freely accessible to the cytosolic antigen-presentation pathway. The significance of these findings for secondary resistance to LM remains to be determined by *in vivo* studies. Recent experiments using attenuated *Salmonella* to deliver the LM antigens p60 and LLO also demonstrate that antigen compartmentalization can affect the efficiency of priming the CD8+ T cell response under vaccine conditions [36••]. Consistent with these data, recombinant LM strains that secrete heterologous CD8+ T cell antigens effectively vaccinate mice against viral infection [37•] and tumor development [38•]. In total, these data support the hypothesis that secreted LM proteins may be the most relevant targets for CD8+ T cell-mediated immunity to infection. Additional experimental support for this hypothesis may have an impact on vaccine design strategies against complex intracellular pathogens, by limiting the candidate antigens to the fraction secreted from these organisms.

H-2M3 presentation of LM antigens

A number of older studies have reported that some LM-specific CD8+ T cells could transfer protective immunity to MHC-mismatched mice, apparently breaking the rules for MHC class I-restricted antigen recognition [39,40]. This intriguing observation may be explained (at least in part) by LM-specific CD8+ T cells restricted by *H-2M3*. The non-polymorphic *H-2M3* gene is linked to the mouse H-2 complex and encodes a non-classical, or class I-b, antigen-presenting molecule, M3 [41]. The M3 molecule was originally shown to bind and bring to the cell surface a mitochondrially encoded peptide initiating with N-formyl-methionine [42]. Subsequent evidence has pointed to *H-2M3* as the restriction element for LM specific 'MHC-unrestricted' CD8+ T cell responses [43,44]. The implication was clear that, during infection, LM peptides initiating with N-formyl-methionine may be presented to CD8+ T cells.

Recent work has focused on identifying LM epitopes presented to *H-2M3*-restricted CD8+ T cells. Surprisingly, such work has led to two divergent outcomes: in the first case, Kurlander's group [45•] concluded that the LM epitope presented to *H-2M3*-restricted CD8+ T cells was in fact a glycolipid. This conclusion was based on biochemical studies showing that this extremely hydrophobic epitope is tightly associated with the bacterial membrane and resists degradation with a number of proteases, yet is sensitive to periodate treatment. On the other hand, we used a genetic approach to identify an *H-2M3*-restricted epitope, also presented to CD8+ T cells, as the formylated amino terminus of a novel N_{out}–C_{in} LM membrane protein (LL Lenz, B Dere, MJ Bevan, unpublished data). The most active peptide is an extremely hydrophobic N-formylated hexapeptide: f-Met-Ile-Gly-Trp-Ile-Ile. A synthetic hexapeptide lacking the formyl group is 100-fold less bioactive, demonstrating the importance of the N-formyl-methionine in binding and presentation of this peptide. The presumed membrane orientation of the protein — an extracytoplasmic amino terminus followed by a ~20 residue transmembrane region and a cluster of positively charged residues which mark the beginning

of the cytoplasmic domain — explains how the amino-terminal formyl-methionine is protected from cytosolic deformylases. Interestingly, Pamer and colleagues have recently identified a hydrophobic, N-formyl-methionine initiated pentapeptide epitope recognized by another *H-2M3*-restricted CD8+ T cell clone (EG Pamer, personal communication).

As expected from previous work on M3, recent studies have shown that this unique molecule presents N-formylated bacterial peptides to CD8+ T cells. However, we are now confronted with the intriguing possibility that M3 may also present bacterial lipids or glycolipids to cells of the immune system. It has become clear recently that a human MHC-like molecule, CD1b, presents specialized lipids derived from the cell walls of *Mycobacteria* to T cells. Two antigens which are presented by this β_2-microglobulin-associated molecule have recently been identified: mycolic acid from *M. tuberculosis* and lipoarabinomannan from *M. leprae* [46, 47]. It is clear that certain non-classical MHC molecules have evolved to bind unique structures present in bacterial membranes and cell walls, and to present these antigenic epitopes to specific TCRαβ T cells.

Conclusions

The LM infection of mice provides a broad spectrum probe to dissect the mechanisms and specificity of resistance to bacterial infection. Recent studies have increased our appreciation of the complex nature of the innate immune response to LM, an activity that requires multiple cell types and cytokines acting in concert to stave off the infection until the specific T cell mediated response develops. Interestingly, many of the effector cells and cytokines identified as important for innate immunity are required for the development and expression of specific T cell mediated immunity to secondary LM infection. In the case of IFNγ, however, studies with gene knockout mice reveal that secondary resistance to LM can overcome the absence of a cytokine that is critical for resistance to primary infection.

Studies with LM-specific CD8+ T cells and their antigens suggest that antigen compartmentalization may affect the development and expression of protective immunity to secondary infection. The demonstration that secreted proteins are important as protective antigens may be applicable to infection by other bacterial and protozoan pathogens. Recent studies have characterized LM antigens presented by the non-polymorphic H-2M3 molecule and revealed both peptide and non-peptide epitopes, thus expanding the nature of candidate antigens capable of stimulating LM-specific CD8+ T cell responses.

Abbreviations

IFN, interferon; IL, interleukin; LCMV, lymphocytic choriomeningitis virus; LLO, listeriolysin; LM, *Listeria monocytogenes*; NK, natural killer; SCID, severe combined immunodeficiency; TCR, T-cell receptor; TNF, tumor necrosis factor.

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