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Perforin-Mediated CTL Cytolysis Counteracts Direct Cell-Cell Spread of *Listeria monocytogenes*

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The immune system has evolved various effector cells and functions to combat diverse infectious agents equipped with different virulence strategies. CD8 T cells play a critical role in protective immunity to *Listeria monocytogenes* (Lm), a bacterium that grows within the host cell cytosol and spreads directly to neighboring cells. The importance of CD8 T cells during Lm infection is currently attributed to the cytosolic niche of this organism, which allows it to evade many aspects of immune surveillance. CTL lysis of infected cells is believed to be an essential protective mechanism, presumably functioning to release intracellular bacteria, although its precise role remains to be fully defined. In this study, we examined the contribution of perforin-mediated CTL cytosis to protective immunity against recombinant Lm capable of or defective in cell-cell spread. We found that CTL cytosis is critical for protective immunity to Lm capable of cell-cell spread while protective immunity against spread-defective Lm is largely independent of CTL cytosis. These results demonstrate that an important function of CTL cytosis is to counter the microbial virulence strategy of direct cell-cell spread. We propose a model that advances the current view of the role of CTL cytosis in immunity to intracellular pathogens. *The Journal of Immunology, 2002, 169: 5202–5208.*

*Listeria monocytogenes* (Lm) is an intracellular bacterial pathogen for which a number of microbial virulence factors are known, including gene products mediating host cell binding and internalization (InLA, B), escape from phagosomes (listeriolysin O and phospholipase C), and intercellular spread (ActA) (1, 2). Early control of primary Lm infections in the mouse model requires the participation of innate immune cells including neutrophils, NK cells, and macrophages (3, 4). The adaptive immune response develops over a period of days, and assists in bacterial clearance later in the infection (5, 6). Ag-specific T cells, particularly CD8 T cells, play a critical role in protective immunity to secondary infection. Upon reinfection, memory CD8 T cells respond promptly, mediating rapid bacterial clearance and providing protection against otherwise lethal challenge doses.

CD8 T cells deploy multiple effector functions including lysis of target cells and production of cytokines such as IFN-γ and TNF (3, 7). CTL induce target cell lysis through Fas/Fas ligand interactions or a perforin-dependent mechanism. Perforin-mediated cytosis is the major pathway involved in lysis of tumor or target cells infected by intracellular pathogens (8, 9). CTL recognition of a target cell causes release of perforin and granzymes from intracellular stores, resulting in pore formation and apoptosis of the target cell.

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Materials and Methods

Mice, bacteria, and viruses

C57BL/6 (B6) and perforin-deficient B6 (PKO) (18) mice from National Cancer Institute (Frederick, MD) and The Jackson Laboratory (Bar Harbor, ME), respectively, were maintained in Institutional Animal Care and Use Committee-approved facilities. rLm strains in wild-type 10403S background (wt rLm)-gp and rLm in cell spread-defective background (ΔactA rLm)-gp secreting CTL epitope gp33–41 from lymphocytic choriomeningitis virus (LCMV) were constructed as described (19) on the wild-type strain 10403S and ΔactA backgrounds, respectively. The ΔactA strain was derived from 10403S by an inframe deletion of the acta sequence encoding aa 20–330. Two pairs of isogenic wt rLm-gp and ΔactA rLm-gp strains were used: one erythromycin (Em)-resistant pair with gp33–41 embedded in a dihydrofolate reductase fusion, and one kanamycin (Km)-resistant pair with gp33–41 in an alkaline phosphatase fusion (20). Similar results were obtained with the two pairs of strains, providing cross-validation of results and demonstrating that the gp33–41 epitope context did not impact in vivo growth in naive mice and clearance rates in immune mice (data not shown). Recombinant murine hepatitis virus (MHV) strain S41-P41 (MHV-gfp) expressing enhanced green fluorescent protein (EGFP) was derived from strain MHV-AS9 by targeted recombination of the EGFP gene into the gene 4 sequence. The derivative MHV-gfp-gp33–41 fusion. MHV and LCMV viruses were propagated as described (20, 21).

Animal experiments

Viral immunizations of mice were done i.p. with 6 log10 PFU MHV or 5.3 log10 PFU LCMV Armstrong. Hepatic MHV titers were determined by plaque assay as described (21). Mice were challenged i.v. >21 days after viral immunization with 6 log10 CFU wt rLm-gp or 7.7 log10 CFU ΔactA rLm-gp. CFU rLm in the spleen was determined for days 2 or 3 postchallenge as described (20). Mixed infections involved the Em-resistant wt rLm-gp strain and the Km-resistant ΔactA rLm-gp strain, each given at the same dose as in individual infections, and spleen homogenates were plated on media with Em or Km.

Flow cytometry

Splenocytes were stained with anti-CD8, anti-CD43 mAb (BD PharMingen, San Diego, CA) and D4gp33 tetramers. Intracellular IFN-γ staining was performed with the Cytofix/Cytoperm Plus kit (BD PharMingen) after 5 h of in vitro stimulations with or without 1 μM synthetic gp33–41 (KAYVYFATM) peptide.

Results

Memory CD8 T cells confer protection against wt and cell-spread-defective rLm

We hypothesized that CTL cytolysis plays a critical role in control of secondary listeriosis by limiting direct cell-cell spread of Lm. As a first step toward testing this hypothesis, we determined whether Ag-specific memory CTL enhance clearance of rLm strains capable of or defective in cell-cell spread. We used a previously described system designed to examine the role of Ag-specific memory CTL in protective immunity to Lm (19, 20). B6 mice were immunized with LCMV to establish LCMV-specific memory CTL. Immunized and naive control mice were then challenged with either a cell spread-capable or a cell spread-defective rLm strain secreting the gp33–41 epitope of LCMV (wt rLm-gp or ΔactA rLm-gp, respectively). In this system, protective immunity against the rLm strains is strictly dependent on a pool of recalled CTL specific to the gp33–41 epitope.

To assess the efficacy of Ag-specific memory cells to control infection with rLm capable of cell-cell spread, LCMV-immune and naive B6 mice were challenged with 6 log10 CFU wt rLm-gp. Splenic bacterial titers were high in challenged naive mice on day 2 postinfection (6.11 ± 1.33 log10 CFU per spleen). In contrast, rLm-gp-challenged LCMV-immune mice had significantly lower bacterial loads (2.00 ± 0.41 log10 CFU per spleen, Fig. 1A). Clearance of rLm-gp in LCMV-immune mice was associated with the generation of gp33–41-specific effector CD8 T cells from a memory pool established by LCMV immunization (Fig. 1, B and C). Effector and memory CD8 T cells were distinguished on the basis of binding to 1B11, a mAb that binds activation-associated O-glycan moieties on CD43 (22). The majority of gp33–41-specific CD8 T cells in LCMV-immunized mice were memory cells, displaying low to intermediate surface densities of the activated form of CD43. Two days after rLm challenge of immune mice, most splenic gp33–41-specific CD8 T cells were exhibiting high levels of activated CD43, indicating that these cells had become effectors (Fig. 1C). Thus, memory CD8 T cells established by LCMV immunization mounted a recall response and conferred significant protection against wt rLm-gp challenge.

To assess the ability of Ag-specific memory CTL to mediate the clearance of a cell-spread defective rLm strain, we infected naive and LCMV-immune mice with ΔactA rLm-gp, a rLm strain that both secretes gp33–41 and has an inframe deletion in the acta gene. The ΔactA Lm enter and multiply normally within host cells but do not nucleate actin for locomotion and are unable to spread directly to neighboring cells (23). Challenge of naive mice with 7.7 log10 CFU ΔactA rLm-gp resulted in bacterial loads of 6.06 ± 0.78 log10 CFU per spleen on day 2 postinfection, similar to day 2 bacterial loads of naive mice infected with wt rLm-gp (Fig. 1A). LCMV immunization conferred significant protection against ΔactA rLm-gp challenge. Bacterial loads in immune mice were 3.08 ± 0.85 log10 CFU per spleen, ~1000-fold less than in naive mice (Fig. 1A). This protection was also associated with the generation of IFN-γ-producing gp33–41-specific effector CD8 T cells (Fig. 1, B and C).

Although gp33–41-specific primary and recall responses of CD8 T cells were similar in LCMV-immunized mice challenged with wt and ΔactA rLm-gp (Fig. 1, B and C), we performed additional experiments wherein naive and immune mice were challenged with a mixture of wt and ΔactA rLm-gp. Splenic bacterial loads for each strain were determined by plating on media with Em or Km (see Materials and Methods). Mixed infections allowed direct internally controlled comparisons of protective immunity. To compare protection against wt rLm-gp and ΔactA rLm-gp conferred by immunization in the context of mixed and individual infections, the extent of protection was calculated for each strain in each experiment by subtracting mean titers in log10 CFU in immune mice from mean titers in log10 CFU in naive mice (10). We observed 4.14 ± 0.87 and 4.11 ± 1.50 log10 CFU protection against wt rLm-gp, and 3.02 ± 1.24 and 3.13 ± 1.25 log10 CFU protection against ΔactA rLm-gp in individual and mixed infections, respectively (Fig. 1D). Thus, LCMV immunization conferred protection against both the wt rLm-gp and ΔactA rLm-gp strains in the context of mixed infection, and levels of protection were similar to those observed in individual infections. In addition, the levels of protection against ΔactA rLm-gp appear lower than those for wt rLm-gp, although this difference in protection was not statistically significant (p = 0.09, Student’s t test). Therefore, our results show that Ag-specific memory CD8 T cells are capable of...
mediating clearance of bacteria that spread cell-cell as well as cell-spread-defective bacteria.

Perforin-deficient Ag-specific CD8 T cells do not confer significant protective immunity against rLm capable of cell-cell spread

Perforin-dependent D CD8 T cell cytotoxicity has been shown to be involved in protection against wild-type Lm (10). We hypothesized that the importance of CTL lysis of infected cells in protective immunity to Lm is related to the ability of this bacterium to directly spread cell-cell (17). We thus predicted that protective immunity against bacteria incapable of cell-cell spread would be less dependent on the cytolytic function of CTL. To determine the contribution of CTL cytotoxicity to protective immunity against wt and cell spread-defective Lm, we established a system for comparing protective immunity conferred by normal and perforin-deficient gp33–41-specific memory CD8 T cells. Perforin-deficient mice have been shown to clear MHV, but not LCMV, infections (24, 25). Thus, gp33–41-specific memory was generated in B6 and PKO mice by immunization with a rMHV strain expressing the LCMV gp33–41 epitope within a GFP fusion protein (MHV-gfp). Splenic bacterial loads and CD8 T cell responses were determined 65 h after infection with 6.0 log_{10} CFU wt rLm-gp. MHV-gfp-gp immunization of B6 mice conferred high levels of protection against challenge with wt rLm-gp (~10,000-fold or 4.27 ± 0.70 log_{10} CFU difference between MHV-gfp-gp- and MHV-gfp-immune mice, Fig. 3A). In contrast, splenic bacterial loads of wt rLm-gp in MHV-gfp-gp-immune PKO mice were similar to those of control mice (Fig. 3A). Thus, MHV-gfp-gp immunization of PKO mice conferred little, if any, advantage over control immunization with respect to protective immunity against PKO mice. The control virus, MHV-gfp, expressing GFP alone, did not induce a gp33–41-specific CD8 T cell response (data not shown). Viral clearance in PKO mice was slightly delayed compared with B6 mice, but virus was undetectable by day 7 postinfection in both B6 and PKO mice, in agreement with previous reports that perforin-mediated cytosis is not required for clearance of MHV infections (24, 25) (Fig. 2C). Thus, MHV-gfp-gp was cleared by PKO mice and immunization generated similar levels of gp33–41-specific memory in B6 and PKO mice.

To determine the contribution of CTL cytosis to protective immunity against rLm-gp capable of direct cell-cell spread, we immunized B6 and PKO mice with MHV-gfp-gp and challenged ~21 days later with wt rLm-gp. The specific contribution of gp33–41-specific memory CD8 T cells to protective immunity was deduced from comparisons with control mice immunized with MHV-gfp. Splenic bacterial loads and CD8 T cell responses were determined 65 h after infection with 6.0 log_{10} CFU wt rLm-gp. MHV-gfp-gp immunization of B6 mice conferred high levels of protection against challenge with wt rLm-gp (~10,000-fold or 4.27 ± 0.70 log_{10} CFU difference between MHV-gfp-gp- and MHV-gfp-immune mice, Fig. 3A). In contrast, splenic bacterial loads of wt rLm-gp in MHV-gfp-gp-immune PKO mice were similar to those of control mice (Fig. 3A). Thus, MHV-gfp-gp immunization of PKO mice conferred little, if any, advantage over control immunization with respect to protective immunity against...
The gp33–41-specific CD8 T cells specifiﬁc for gp33–41 peptide. gp33–41 peptides are from one mouse per group of two mice with similar proﬁles in the spleens 5, 8, or 14 days postinfection with MHV-gfp-gp. Pro-memory following MHV-gfp-gp infection.

FIGURE 3. MHV-gfp-gp-immunized PKO mice exhibit little protective immunity against wt rLm-gp despite gp33–41-speciﬁc CD8 T cell recall responses. A. Splenic bacterial burden 3 days after rLm-gp challenge of B6 and PKO mice that were previously immunized with MHV-gfp-gp (I) or control MHV-gfp (C). Data shown are mean ± SD from three independent experiments with two to three mice per group. B. gp33–41-speciﬁc effecter CD8 T cells in the spleen 3 days after rLm-gp challenge of B6 and PKO mice that were previously immunized with MHV-gfp-gp (I) or with control MHV-gfp (C). gp33–41 effecter cells were detected by staining for surface activation marker CD43 and for intracellular IFN-γ, with numbers indicating the percentage of CD8 T cells that produced IFN-γ when stimulated with gp33–41 peptide. Profiles shown are gated on CD8+ cells and are from one representative mouse per group of two to three mice. Similar results were obtained in two more independent experiments.

Perforin-mediated cytosis is not critical for protective immunity against cell-spread-deﬁcient $\Delta$actA rLm

We concluded from the experiments described above that perforin-dependent cytotoxicity contributes most of the CD8 T cell-mediated protective immunity against wt rLm infection in our experimental system. We then sought to examine the role of perforin-mediated target cell killing by CD8 T cells in the control of the cell spread-defective $\Delta$actA rLm-gp strain. B6 and PKO mice immunized with MHV-gfp-gp or the control virus, MHV-gfp, were challenged with 7.7 log$_{10}$ CFU $\Delta$actA rLm-gp. Bacterial numbers and immune responses were assayed 65 h postinfection. MHV-gfp-gp immunization of B6 mice conferred protection against challenge with $\Delta$actA rLm-gp (Fig. 4A), in agreement with our results using LCMV-immunized mice (Fig. 1A). The bacterial numbers in control-immunized B6 mice were 5.45 ± 0.74 log$_{10}$ CFU per spleen, 2.76 ± 0.77 log$_{10}$ CFU more than that in MHV-gfp-gp-immunized B6 mice (2.68 ± 0.27 log$_{10}$ CFU per spleen). Surprisingly, immune PKO mice had signiﬁcantly lower bacterial burdens than control PKO mice (3.65 ± 0.36 log$_{10}$CFU and 5.39 ± 0.49 log$_{10}$CFU per spleen in immune and control mice, respectively, $p = 0.0002$, Student’s $t$ test). Thus, PKO mice exhibited considerable protection against $\Delta$actA rLm-gp (1.78 ± 0.83 log$_{10}$ CFU). Immunization-conferred protection in B6 (2.76 ± 0.77) and PKO mice (1.78 ± 0.83) was not signiﬁcantly different (p = 0.24, Student’s $t$ test), demonstrating a minimal contribution of perforin-dependent killing to protective immunity against $\Delta$actA rLm-gp.

Perforin-independent protection against $\Delta$actA rLm-gp is in striking contrast to the requirement for perforin against wt rLm-gp. gp33–41-speciﬁc CD8 T cell recall responses were similar among immune B6 and PKO mice challenged with the wt and $\Delta$actA.
FIGURE 4. MHV-gfp-gp-immunized PKO mice exhibit significant immunity against ΔactA rLm-gp challenge. A, Splenic bacterial loads in MHV-gfp-gp-immunized (I) or control MHV-gfp-immunized (C) B6 and PKO mice 3 days postchallenge with ΔactA rLm-gp. Data shown are mean ± SD and are from three independent experiments with two to three mice per group. B, Numbers of gp33–41-specific CD8 T cells in MHV-gfp-gp-immunized B6 and PKO mice 3 days after challenge with wt rLm-gp or ΔactA rLm-gp. gp33–41 specific cells were measured by D9gp33 tetramer staining and data shown represent mean ± SD from one representative experiment of three independent experiments.

rLm-gp strains (Fig. 4B), indicating that differences in protection were not due to disparities in levels of CD8 T cell responses but rather due to differing dependence on perforin for protection. In sum, we found that perforin-dependent CD8 T cell cytotoxicity is essential for protective immunity against rLm that directly spreads cell-cell while control of cell spread-defective Lm by CD8 T cells depends on perforin-independent effector mechanisms. We thus conclude that perforin-mediated CTL lysis of infected cells counters the effects of direct cell-cell spread of Lm.

Discussion

Perforin-dependent cytolysis has previously been shown to participate in the clearance of both primary and secondary Lm infections (9, 18). Perforin-deficient mice exhibit delayed clearance of Lm from the spleen, and these mice are severely impaired in protective immunity against Lm. Protection conferred by previous Lm immunization in PKO mice is almost 10,000-fold less than in normal mice and adoptively transferred splenocytes from immune PKO mice are ~100-fold less protective than immune splenocytes from normal mice (18). Protective immunity measured in this original study was likely conferred, at least in large part, by Lm-specific CD8 T cells, thus suggesting a critical role for perforin in CD8 T cell-mediated immunity. Later experiments using Ag-specific CD8 T cell lines derived from PKO mice confirmed the importance of perforin-mediated immunity while revealing effective perforin-independent mechanisms of protection (11, 12). Recent studies have also uncovered a role for perforin in the regulation of CD8 T cell responses, distinct from its role as an effector molecule (26, 27).

In the present study, we examined the relative roles of perforin-dependent CTL cytotoxicity in protective immunity against rLm strains capable of or deficient in direct cell-cell spread. B6 and PKO mice were immunized with either recombinant MHV-gfp-gp expressing the LCMV gp33–41 epitope, or the control virus, MHV-gfp. Immunized mice were challenged with rLm expressing the gp33–41 epitope and protective immunity was measured as the difference in bacterial growth between MHV-gfp-gp and MHV-gfp-immunized mice. Because MHV-gfp-gp and MHV-gfp differ only by the nonamer epitope, protective immunity in our experimental system can be attributed exclusively to epitope-specific CD8 T cells. This is further demonstrated by our finding that MHV-gfp-gp-immunized mice are not protected against rLm that do not express the gp33–41 epitope (data not shown). We found that memory CD8 T cells in normal mice were protective against rLm capable of direct cell-cell spread, while memory CD8 T cells in PKO mice provided little protection against these bacteria. The impaired clearance of rLm in PKO mice could only be attributed to the lack of perforin-mediated cytotoxic effector function in its CD8 T cells, because normal and PKO mice exhibited similar Ag-specific CD8 T cell memory and recall responses, as expected from previous reports (12, 28). Although perforin-deficient memory CD8 T cells conferred little protective immunity against rLm capable of direct cell-cell spread, they provided resistance against the cell spread-defective ΔactA rLm strain, to an extent not significantly different from that conferred by memory CD8 T cells from normal mice. Thus, the life cycle of intracellular bacterial pathogens influences the extent to which perforin contributes to protective immunity. Similarly, it has been shown that the contribution of CD8 T cell cytolyis to viral clearance differs depending on the life cycle of the virus in the host. Although perforin is required for clearance of LCMV infection, it is completely dispensable for protective immunity to vaccinia virus, VSV, and Semliki Forest virus (15). These observations have led to the hypothesis that cytotoxicity is required for the clearance of noncytopathic viruses (such as LCMV) but not of cytopathic or lytic viruses (such as vaccinia, VSV, and Semliki Forest) (15). It is hypothesized that CTL cytotoxicity would have no or minimal impact on cytopathic viruses because they lyse host cells in the process of releasing progeny virus, unless CTL cytotoxicity occurs before virus-induced cell lysis. Such rapid CTL cytolyis of infected cells may explain the role of perforin in certain lytic viral infections (e.g. influenza and ectromelia viruses).

Our data point to a new role for perforin-dependent cytolyis in countering the bacterial virulence strategy of direct intercellular spread. Based on the results of this study, we propose a model that stipulates that the importance of perforin-mediated control is determined by a race between CTL-mediated cytolyis and bacterial spread (Fig. 5). When the rate of bacterial spread exceeds the rate of CTL cytolyis, the role of perforin in control is minimal. This may be the case in primary listeriosis during which Lm efficiently spread during the development of the CD8 T cell response. When the rate of bacterial spread is exceeded by the rate of CTL cytolyis of infected cells, the contribution of perforin to bacterial clearance becomes apparent. Perforin-dependent clearance of Lm during secondary infections supports this notion because memory CTL are known to mount a rapid recall response. Perforin-mediated cytolyis is likely a trivial component of protective immunity to intracellular bacteria that do not directly spread cell-cell, as modeled by the ActA-deficient rLm strain. Because ΔactA rLm disseminates through an extracellular route after bacterial growth causes lysis of infected cells, CD8 T cell-mediated immunity against ΔactA Lm is likely dependent on cytokines such as TNF and IFN-γ that may enhance killing of extracellular bacteria, thereby preventing infection of other cells (Fig. 5). TNF has been shown to mediate antilisterial immunity conferred by perforin-deficient CD8 T cells (11, 14). Although IFN-γ is not required for CD8 T cell-mediated immunity against wild-type Lm (13), a role for IFN-γ may become apparent in the control of ΔactA Lm and this possibility awaits
An alternative interpretation suggested by our model is that perforin-independent control of *Chlamydia* and further investigation. Studies in animal models of intracellular bacterial infections support our hypothesis that the importance of perforin-mediated cytotoxicity in protective immunity is related to the ability of bacteria to directly spread from cell to cell. *Rickettsia* sp. are intracytoplasmic bacteria capable of direct cell-cell spread. In agreement with a prediction of our model, protective immunity against *Rickettsia* depends on perforin-mediated CD8 T cell cytotoxicity (29). Although CD8 T cells have been established to participate in defense against the intracellular pathogens *Chlamydia pneumoniae* and *Mycobacterium tuberculosis*, perforin has been shown to play a minimal role in controlling infections with these bacteria (30, 31). Unlike Lm and *Rickettsia*, these are intracytoplasmic pathogens, some bacteria reside within vacuolar compartments, which may influence the contribution of perforin. However, perforin-mediated cytotoxicity should release and expose both cytosolic and vacuolar bacteria to macrophage and AB-mediated immune mechanisms. An alternative interpretation suggested by our model is that perforin-independent control of *Chlamydia* and

*Mycobacterium* relates to their inability to spread directly from cell to cell without host cell lysis.

The model described above represents a significant step forward in our understanding of the function of CTL cytosis in immunity to intracellular pathogens. The value of perforin-dependent cytotoxicity in protective immunity is currently thought to relate to the intracellular growth of pathogens. By simultaneously manipulating the pathogen and the host, we have demonstrated that perforin-independent killing of infected cells is important not just because Lm grows in the host cell cytosol but more importantly, because Lm spreads directly from cell to cell. Thus, one important function of perforin-dependent cytosis by CD8 T cells is to counter the microbe’s virulence strategy of direct intercellular spread. The relationship between perforin cytotoxicity and bacterial spread provides an example of the many specific interactions between host immune effectors and microbial virulence factors, the balance of which ultimately determines the outcome of the infection. Dissecting these specific interactions and defining immune correlates of protection will aid in the design of effective vaccines against infectious diseases.

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References


