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# CD8 $\alpha^+$ Dendritic Cells Selectively Present MHC Class I-Restricted Noncytolytic Viral and Intracellular Bacterial Antigens In Vivo<sup>1</sup>

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CD8 $\alpha^+$  dendritic cells (DCs) have been shown to be the principal DC subset involved in priming MHC class I-restricted CTL immunity to a variety of cytolytic viruses, including HSV type 1, influenza, and vaccinia virus. Whether priming of CTLs by CD8 $\alpha^+$  DCs is limited to cytolytic viruses, which may provide dead cellular material for this DC subset, or whether these DCs selectively present intracellular Ags, is unknown. To address this question, we examined Ag presentation to a noncytolytic virus, lymphocytic choriomeningitis virus, and to an intracellular bacterium, *Listeria monocytogenes*. We show that regardless of the type of intracellular infection, CD8 $\alpha^+$  DCs are the principal DC subset that initiate CD8 $^+$  T cell immunity. *The Journal of Immunology*, 2005, 175: 196–200.

The CD8 $^+$  T cell recognizes pathogen-derived peptides presented by MHC-encoded class I (MHC class I) molecules. Although peptide/MHC class I complexes are expressed on the surface of most cells, their expression by dendritic cells (DCs)<sup>3</sup> is essential for priming of pathogen-specific CD8 $^+$  T cell responses (1). DCs are a heterogeneous population of cells. In mice, they are composed of at least six phenotypically distinguishable subsets, some of which have been shown to perform discrete functions during the immune response to pathogens (2–11). Induction of naive CD8 $^+$  T cell responses to cytolytic viruses appears to depend largely on Ag presentation by a single subset of DCs, the CD8 $\alpha^+$ CD11c $^+$  conventional DCs (CD8 DCs), although other DC subsets may be used in the priming process (6–8, 12). In addition, Ag carried in apoptotic cells is also selectively presented to naive CD8 $^+$  T cells by this same subset of DCs (13, 14). The death of cells as a result of viral infection might therefore provide a source of viral Ags particularly suitable for uptake by CD8 DCs. It was not clear whether immunity to noncytolytic pathogens, such as lymphocytic choriomeningitis virus (LCMV), or intracellular bacteria such as *Listeria monocytogenes* (LM) would also rely heavily on the CD8 $^+$  subset of DCs for T cell priming.

LCMV is a negative-stranded RNA virus that can infect cells of the immune system and establish either acute or persistent infec-

tion in its natural host, the mouse. The outcome of infection depends on the viral variant, the route of infection, and the infectious dose. Viral infection involves receptor-mediated uptake of virions into the cell in endoplasmic vesicles. Endosomal acidification allows release of viral nucleocapsids into the cell cytoplasm, where replication of virus is initiated. After transcription, translation, and assembly of viral particles, noncytolytic shedding leads to dissemination of virus by infected cells. The LCMV Armstrong (ARM) 53b strain of virus (and most viral variants isolated from the CNS) causes an acute fulminant systemic infection that is efficiently controlled within 7–10 days by LCMV-specific CD8 $^+$  T cells. In contrast, clone 13 LCMV differs from ARM by only 2 aa, but shows preferential replication in CD11c $^+$  cells. It is associated with immunosuppression and establishes persistent infection (15–19).

LM is a Gram-positive facultative intracellular bacterium with mechanisms that enable it to invade and colonize most cell types. LM infects by inducing phagocytosis by cells through the interaction of internalin A expressed by LM and E-cadherin at the cell's surface (20). Listeriolysin O, a virulence factor that disrupts the phagosomal membrane, allows release of LM from the phagocytic vacuole into the cytoplasm (21). The bacteria then replicate in the cytoplasm and move directly into adjacent cells by propulsion along polymerized host cell actin filaments that extend into pseudopodia (22, 23). Once the bacteria have invaded the neighboring cell, escape from the secondary vacuole into the cytoplasm now requires LM-derived phosphatidylcholine-specific lipase and zinc metalloproteinase, in addition to listeriolysin O. Infection can extend in this manner through many cells without cellular destruction.

DCs are crucial in mounting an effective cytotoxic T cell response to both LCMV and LM (1, 24, 25). This most likely involves a complex interplay of different DC populations encompassing not only conventional DCs, but also plasmacytoid DCs and novel subsets such as Tip DCs (9, 11, 24). In the case of LCMV, the influx of IFN- $\alpha$ -producing plasmacytoid DCs limits viral replication (26). In LM infection, Tip DCs (CD11c $^+$ CD11b $^+$  DCs), so named for their production of TNF- $\alpha$  and inducible NO synthase (iNOS), provide cytokine-directed innate control of infection (9). Although both of these DC types can present pathogen-derived Ags, presentation is very inefficient when compared with conventional DC and was not required for generation of Ag-specific immunity (9).

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; ARM, lymphocytic choriomeningitis virus Armstrong 53b strain; DN, double negative; iNOS, inducible NO synthase; LCMV, lymphocytic choriomeningitis virus; LM, *Listeria monocytogenes*; NP, nucleoprotein; pDC, plasmacytoid DC.

Which DCs are important for priming the CD8<sup>+</sup> T cell immune response to these noncytolytic pathogens and whether they are the same DC subset used by cytolytic pathogens is unclear.

The objective of this study was to determine the DC subsets responsible for the Ag presentation that initiates CD8<sup>+</sup> T cell responses to the noncytolytic virus LCMV, and to the intracellular bacterium LM. We show that, as for lytic viruses, CD8 DCs are the dominant presenting population, implicating these DCs as central to immunity to intracellular pathogens.

## Materials and Methods

### *Mice, virus, and infections*

C57BL/6 (B6) mice were purchased from Taconic Farms, and P14 (transgenic mice that express a CD8<sup>+</sup> TCR specific for the immunodominant MHC class I-restricted epitope of LCMV glycoprotein) TCR transgenic mice were maintained under standard conditions at the animal facility at the University of Washington. Experiments with all mice began when they were between 6 and 10 wk of age. P14-specific TCR transgenic mice express a TCR ( $V\alpha 2$ ) that recognizes the immunodominant MHC class I-restricted epitope of LCMV gp<sub>33-41</sub> (27). Mice were infected either i.v. or i.p. with  $2 \times 10^5$  PFU of LCMV ARM 53b diluted in 200  $\mu$ l of sterile PBS, or i.v. infected with  $1 \times 10^3$  PFU of recombinant isogenic strains of LMs (rLM). rLM encoded either a secreted or nonsecreted fusion protein comprising the LCMV glycoprotein peptide linked to the LCMV nucleoprotein (NP<sub>118-126</sub>), which will be referred to as rLM expressing secreted glycoprotein and rLM expressing nonsecreted glycoprotein, respectively (28–30). Frozen stocks of rLM were grown in brain-heart infusion broth supplemented with 5 mg/ml erythromycin. At mid-log growth phase, culture samples were measured by OD and diluted in PBS for tail vein injection into animals. Bacterial counts were further verified by plating culture dilutions on brain-heart infusion agar plates and incubating overnight. All experiments were undertaken in accordance with ethical guidelines of the University of Washington.

### *DC isolation from spleen*

DCs were isolated essentially as previously described (7, 8, 31). Briefly, spleen fragments were digested for 20 min at room temperature with collagenase/DNase, and then treated for 5 min with EDTA to disrupt T cell-DC complexes. Cells not of the DC lineage were depleted by incubating in predetermined optimal concentrations of purified Abs: anti-CD3 (KT3), anti-Thy-1 (T24/31.7), anti-CD19 (ID3), anti-GR-1 (RB6-8C5), and anti-erythrocyte (TER-119), and then removing the Ab-binding cells with anti-rat Ig-coupled magnetic beads (Dynabeads; Dynal Biotech). Note that, in our hands, plasmacytoid DCs (pDCs) are not depleted using anti-GR-1 mAb (4, 32). The DCs in the enriched populations were gated as CD11c<sup>+</sup> cells before sorting into specific subsets.

### *CFSE labeling of transgenic T cells*

Lymph nodes (inguinal, axillary, sacral, cervical, and mesenteric) were obtained from P14 TCR transgenic mice and CD8<sup>+</sup> T cells purified using a mixture of optimally titered Abs to deplete cells expressing Mac-1 (M1/70), Mac-3 (F4/80), TER-119, GR1 (RB6-8C5), MHC class II (M5/114), and CD4 (GK1.5), followed by sheep anti-mouse and anti-rat Dynabeads (Dynal Biotech). Enriched cells contained 84–96% specific TCR transgenic CD8<sup>+</sup> T cells. These were labeled with CFSE (Molecular Probes) by incubating  $10^7$  purified cells/ml with 5  $\mu$ M CFSE for 10 min at 37°C. Cells were then washed three times in HEPES modified Eagle's medium containing 2.5% FCS.

### *Analysis of in vitro activation of naive T cells by DCs*

A total of  $5 \times 10^4$  enriched CFSE-labeled P14 CD8<sup>+</sup> TCR transgenic cells was added to graded numbers of flow cytometrically sorted DCs in 200  $\mu$ l of RPMI 1640 containing 10% FCS, 50  $\mu$ M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin in 96-well U-bottom plates (Falcon; BD Biosciences). Each culture was performed in duplicate. Cultures were analyzed for proliferation after 60 h. Cells were stained with anti-CD8 $\alpha$  (53-6.7; BD Pharmingen) and anti-CD11c (HL3; BD Pharmingen). Cells were gated on CD11c<sup>+</sup> propidium iodide<sup>-</sup> cells.

## Results

### *Presentation of Ag from a noncytolytic virus*

For i.v. and s.c. routes of infection with cytolytic viruses such as HSV, vaccinia, and influenza virus, Ag presentation to CD8<sup>+</sup> T

cells is largely restricted to the CD8 DC subset (6, 7, 12). By limiting our studies to these cytolytic viruses, it remained possible that the dominant role of CD8 DC was due to their specialized ability to capture Ag from apoptotic or dying cells (14, 33). To determine whether additional or alternative DC subsets were involved in presenting viral Ags from nonlytic viruses, Ag presentation was examined following i.v. infection with LCMV. The use of LCMV also had the advantage of using an authentic mouse pathogen, as earlier studies used virus from other species. One day after i.v. infection with LCMV, DCs were purified from the spleen and separated into subsets based on expression of CD8 $\alpha$  and CD45RA. This divided the DCs into conventional (CD45RA<sup>-</sup>) DCs of CD8 $\alpha$ <sup>-</sup> (double-negative (DN DCs)) or CD8 $\alpha$ <sup>+</sup> (CD8 DCs) phenotypes and the pDCs (CD45RA<sup>+</sup>) (Fig. 1A). In this case, the DN DCs contained more than one DC subtype. Each subset from infected mice was examined for the ability to induce proliferation of naive CFSE-labeled P14 CD8<sup>+</sup> T cells specific for the immunodominant MHC class I-restricted epitope of glycoprotein encoded by aa 33–41 (Fig. 1B, top panel). Similar to our previous finding with cytolytic viruses (7), CD8 DCs were the only subset capable of stimulating naive P14 CD8<sup>+</sup> T cells following i.v. infection with noncytolytic LCMV. Failure of other DC subsets to present LCMV Ags was not due to any form of nonspecific inhibition or toxicity to the DCs or the cultures, as P14 T cells were efficiently stimulated by each DC population when the DCs were directly coated with the glycoprotein peptide (Fig. 1B, lower panel).

Previous studies of Ag presentation during i.v. infection with cytolytic viruses such as HSV focused on the 24-h time point because presentation peaked at this time point (7), possibly due to limited virus replication. In the case of LCMV, however, extensive virus replication occurs in the spleen, raising the possibility that optimal Ag presentation, by either CD8 DC or other subsets, may occur at earlier or later time points. To address this possibility, mice were infected i.v. with LCMV at 6, 12, and 48 h before sampling (Fig. 1C, and data not shown). At any time point, the CD8 DCs were the only subset presenting LCMV glycoprotein Ag to P14 CD8<sup>+</sup> T cells, although responses were very poor at 12 h and undetectable at 6 h after infection.

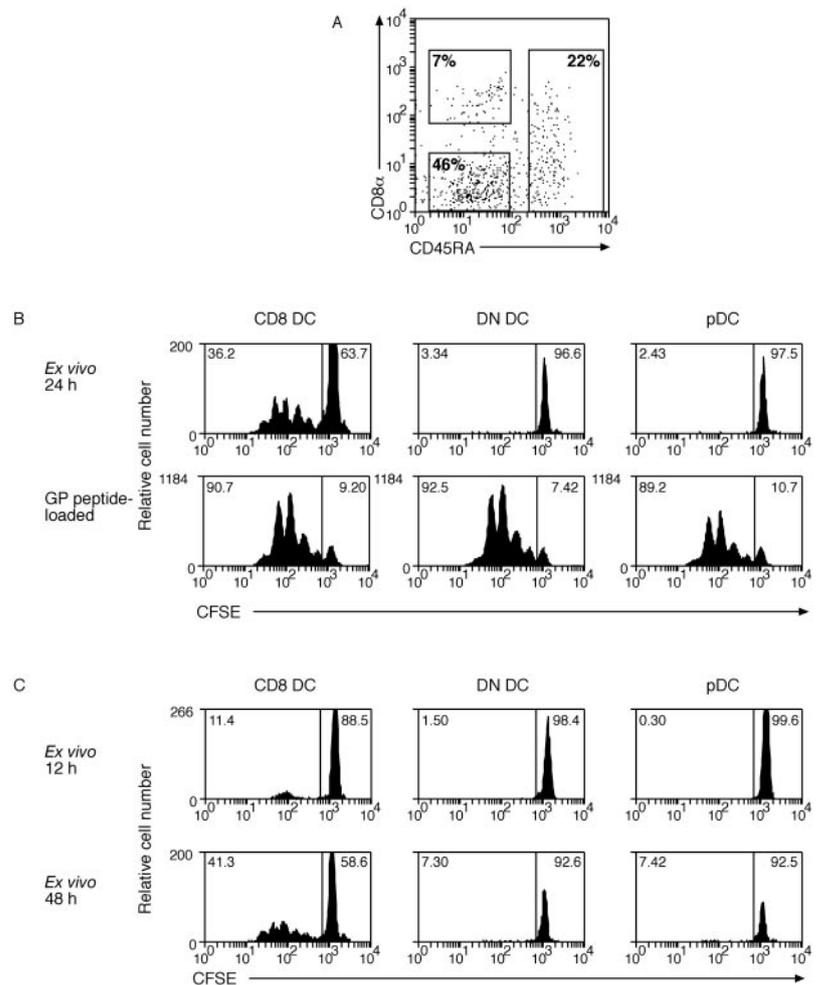
### *Presentation of LCMV Ag does not change with the i.p. route of infection*

In previous analyses, we showed that MHC class I viral Ag presentation was restricted to the CD8 DC subset following i.v. infections, but presentation of inhaled Ags involved the conventional CD8 DC and, in addition, a CD8-negative DC subset (7, 8). Therefore, we examined a second route of infection, namely i.p. infection, to determine whether other DC subsets might contribute to Ag presentation when the virus gained access to DCs from a different inoculation site. LCMV was given via i.p. inoculation, and DCs were purified from the spleen 24 and 48 h after infection. DC subsets were cocultured with CFSE-labeled P14 CD8<sup>+</sup> T cells, and the number of proliferated cells enumerated after 60 h of culture (Fig. 2). Although administration of Ags via the i.p. route provides ready access to peritoneal macrophages that could also potentially contribute to the presentation of viral Ags, the CD8 DC remained the predominant subset of DC priming the immune response in the spleen. No response was detected in a single analysis performed 6 and 12 h after i.p. LCMV infection (data not shown).

### *Intracellular bacterial pathogens also selectively rely on CD8 DC for Ag presentation*

Bacterial pathogens use a number of strategies to allow them to colonize a broad range of cells in the body. Such wide infection implies that infected APCs could be involved in activating naive T

**FIGURE 1.** CD8 DCs are the only subset that activates naive CD8<sup>+</sup> T cells after i.v. infection with LCMV virus. *A*, Sorting gates for DCs enriched from spleen at various time points after infection. Cells were enriched for DCs by magnetic depletion of cells staining for CD3, Thy-1, CD19, anti-Gr-1, and erythrocytes. Enriched cells were then stained with anti-CD11c, anti-CD8 $\alpha$ , and anti-CD45RA. Rectangles show sorting gates for CD8 $\alpha$ <sup>+</sup>CD45RA<sup>-</sup> (CD8), CD45RA<sup>+</sup> (pDC), and CD8 $\alpha$ <sup>-</sup>CD45RA<sup>-</sup> DC (DN DCs). *B*, Mice were infected with LCMV by i.v. inoculation 24 h after infection; DCs were enriched from spleen and sorted by flow cytometry into CD8 DC, DN DC, or pDC before culturing  $2.5 \times 10^4$  cells with  $5 \times 10^4$  CFSE-labeled glycoprotein-specific CD8<sup>+</sup> T cells. Some DCs were coated with 0.1  $\mu$ M glycoprotein peptide for 1 h at 37°C and washed three times before coculture with CFSE-labeled glycoprotein-specific CD8<sup>+</sup> T cells (*lower panel*). Proliferation was analyzed at 60 h of culture. The percentage of proliferating cells for each culture is indicated in the *top left corner* of each histogram (*C*). At 12 and 48 h after infection, DCs were enriched from spleen of infected mice and responses were analyzed as in *B*. Each time point was performed twice with similar results. A 6-h time point was also examined in a single experiment.

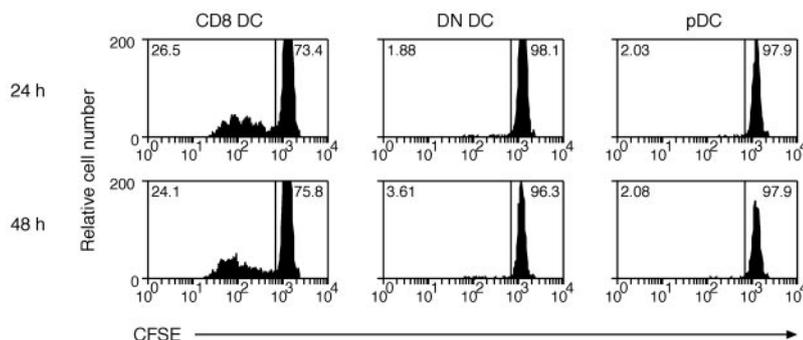


cells during the immune response. In addition to conventional DCs, Tip DCs, a novel DC subset that produces TNF- $\alpha$ /iNOS, are recruited into the immune response against *Listeria*. Tip DCs have limited Ag presentation capacity and are not required for priming of T cells in the immune response, suggesting that other subsets of APCs perform this function. To determine whether intracellular pathogens, such as LM, use a much broader repertoire of DC subsets to prime virus-specific CD8<sup>+</sup> T cells, DCs were isolated from mice infected i.v. with a recombinant form of LM that expressed the LCMV viral peptide determinant, glycoprotein, as a surrogate Ag (28, 30). This allowed us to use the same CD8<sup>+</sup> TCR transgenic mouse line as in previous studies. The recombinant bacterium expresses the glycoprotein Ag as either a secreted or nonsecreted protein, permitting investigation of whether such differences affect the DC subset(s) driving T cell responses. DCs were isolated

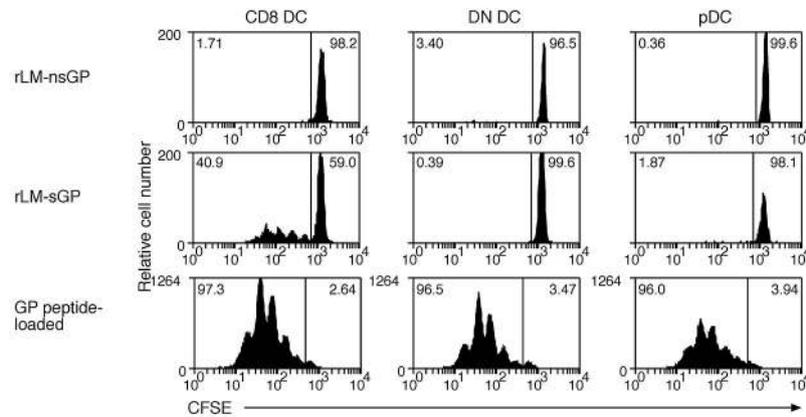
from the spleen of mice 24 or 48 h after infection with rLM and were then cocultured with CFSE-labeled P14 CD8<sup>+</sup> T cells. The number of proliferated cells was enumerated after 60 h of culture (Fig. 3, *upper panels*). As with viral infections, secreted Ag from LM was only presented by the CD8 DC. Presentation of nonsecreted Ag was not detected. This was not due to inhibitory effects of the bacteria, as all DC populations presented exogenously loaded peptide to naive CD8<sup>+</sup> T cells (Fig. 3, *lower panel*).

## Discussion

DCs express specialized Ag-presenting machinery, costimulatory molecules, and cytokines that allow them to initiate immunity to Ags. Although it has been clear for some years that bone marrow-derived cells are necessary to elicit pathogen-specific responses, it has been more difficult to definitively identify which APC was



**FIGURE 2.** CD8 DCs, but not other DCs, activate naive LCMV-specific CD8<sup>+</sup> T cells after i.p. infection with virus. Mice were infected with LCMV by i.p. injection. One or 2 days after infection, DCs were enriched from spleen and then sorted into CD8 DC, DN DC, and pDC before culturing  $2.5 \times 10^4$  cells with  $5 \times 10^4$  CFSE-labeled glycoprotein-specific CD8<sup>+</sup> T cells. Proliferation was analyzed at 60 h of culture. The percentage of proliferating cells for each culture is indicated in the *top left corner* of each histogram. This experiment was performed twice with similar results.



**FIGURE 3.** Only CD8 DCs present rLM expressing the glycoprotein Ag. Mice were infected with nonsecreted (*upper panel*) or secreted (*middle panel*) forms of the rLM glycoprotein (29) by i.v. injection. DCs were enriched from the spleen 24 h after infection and sorted by flow cytometry into CD8 DC, DN DC, or pDC. Purified DC subsets ( $2.5 \times 10^4$  DC) were then cocultured with  $5 \times 10^4$  CFSE-labeled glycoprotein-specific CD8<sup>+</sup> T cells for 60 h. Twenty-four hour infected DCs from each subset were coated with 0.1  $\mu$ M glycoprotein peptide for 1 h at 37°C and washed three times before coculture with CFSE-labeled glycoprotein-specific CD8<sup>+</sup> T cells (*lower panel*). The percentage of proliferating cells for each culture is indicated in the *top left corner* of each histogram. Each experiment was performed twice with similar results. Ag presentation was also examined 12 and 18 h after infection with secreted and nonsecreted forms of rLM glycoprotein (data not shown; each experiment was performed once). No proliferation of naive glycoprotein-specific CD8<sup>+</sup> T cells was detected at either of these time points.

responsible for this function (34, 35). More recently, this was resolved by Jung et al. (1), who used an elegant transgenic mouse model to eliminate CD11c<sup>+</sup> cells by inducible short-term ablation in vivo. Mice that lacked DCs failed to develop T cell responses following either malaria or *Listeria* infection, indicating the crucial importance of DCs to the immune response to these pathogens.

In the mouse, at least six different DC subsets have been described. Although most DC express a common set of pattern recognition receptors, such as TLR2 and 4, some populations have a distinct pattern of TLR expression, raising the idea that different DC populations may be able to discriminate different pathogens and therefore be specialized in initiating the adaptive immune response to those pathogens. Previously, we have determined which DC subsets were responsible for initiation of the immune response to cytolytic viruses. Only a limited number of DCs, and principally (but not exclusively) the CD8 DC, was shown as a key player for priming CD8<sup>+</sup> T cells (6–8, 12). Intriguingly, this subset also expresses the TLR3 that recognizes viral products (36). Whether the CD8 DC subset, or another subset, plays a broader role in handling different types of pathogen-derived Ags remained to be elucidated. Therefore, we extended our earlier studies to examine infections with either a noncytolytic virus, LCMV, or an intracellular bacteria, LM. This revealed that the CD8 DC was also the principal APC recruited to prime naive CD8<sup>+</sup> T cells in these very different types of infections.

The critical importance of disruption of DC function following LCMV infection was first alluded to by Zinkernagel and colleagues (25), who observed that mice infected with LCMV variants (WE or DOCILE strains) developed immune suppression. Similarly, infection with the LCMV clone 13 strain, but not the parental strain LCMV ARM, resulted in generalized suppression of the host immune response (19, 24). Further analysis of the differences between these latter two strains revealed mutations in LCMV clone 13, enabling it to directly target DCs by binding to the laminin receptor,  $\alpha$ -dystroglycan. This receptor is predominantly found on DCs that express the C-type lectin, CD205. As CD8 DCs isolated from spleen are the sole subset that expresses CD205, this implicates the CD8 DCs as direct targets for the action of LCMV that leads to crippling of their ability to activate naive CD8<sup>+</sup> T cells. In contrast, LCMV ARM only poorly infects DCs,

and mice rapidly resolve the infection by mounting an effective virus-specific cytotoxic T cell response (19). These data suggest that activation of naive T cells in LCMV infection may involve transfer of Ag from infected cells to the CD8 DC, and that direct infection of these DCs, as occurs for LCMV clone 13, destroys their capacity to initiate the cytotoxic T cell response.

Clearance of LM infection is dependent on generation of a T cell response, but early containment and bacterial clearance rely on the induction of iNOS (37). Of the different splenic DC populations that could participate in the immune response to LM, a novel subset, Tip DCs have recently been identified as a dominant source of TNF- $\alpha$  and iNOS (9). This subset is the major producer of TNF- $\alpha$  and iNOS during the first 48 h of infection. They are critical to the innate immune defense, and mice lacking these cells ultimately die. Despite this, the apparent absence of this subset from CCR2-deficient mice does not interfere with the normal development of T cell responses implicating another DC subset as responsible for presenting bacterial Ag and priming naive T cells (9). In this report, we investigated which DC subset was responsible for presenting LM Ag. Similar to viral infections, only the CD8 DC subset was able to activate naive CD8<sup>+</sup> T cells to LM.

Intravenous infection of mice with rLM expressing either a secreted or a nonsecreted form of LCMV-derived Ag (NP alone, or an NP-glycoprotein fusion protein) (29) elicits CD8<sup>+</sup> T cell expansion and contraction that are similar for both pathogens, although the magnitude of the response is diminished for the nonsecreted Ag (28, 29). Despite this, mice previously infected with LCMV were protected against rLM challenge only when the Ag was secreted into the cytoplasm of the cell. As an indirect measure of Ag presentation, we analyzed presentation by DC subsets isolated from either rLM expressing secreted glycoprotein- or rLM expressing nonsecreted glycoprotein-infected mice. The amount of Ag presented by the nonsecreted form of the rLM was insufficient to induce proliferation of our transgenic T cells, while Ag presentation from the secreted form was detected on the CD8 DC subset. Our failure to detect presentation by the nonsecreted variant may reflect some loss of function of the DCs in the preparation procedure, or alternately, the potentially lower levels of Ag obtained by cross-presentation of a nonsecreted Ag are more difficult for us to analyze by this approach. Despite this, the differences in the level

of Ag presentation detected via the ex vivo assay suggest that the blunted Ag presentation observed in the nonsecreted form of LM could shorten the developmental phase of effector and memory cells, thereby contributing to the diminished capacity of mice infected with this pathogen to develop potent long-term CD8<sup>+</sup> memory T cells (28, 29, 38). These results reinforce our previous observations of the importance of the CD8 DC subset in priming naive CD8<sup>+</sup> T cells in response to intracellular pathogens. The importance of the interplay between different functionally specialized DC subsets is underlined by LM infection, in which it is the concert of different DC populations that is essential for control and clearance of the pathogen. Investigating the rules governing the cellular and molecular signals regulating these interactions is essential to understanding the generation of long-lasting protective memory.

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## Disclosures

The authors have no financial conflict of interest.

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