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Cutting Edge: Priming of CD8 T Cell Immunity to Herpes Simplex Virus Type 1 Requires Cognate TLR3 Expression In Vivo

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Despite its potential for involvement in viral immunity, little evidence links TLR3 to adaptive antiviral responses. Here we show that TLR3 is required for the generation of CD8 T cell immunity to HSV-1. The magnitude of the gB-specific CD8 T cell response after flank infection by HSV-1 was significantly reduced in mice lacking TLR3. Impaired CTL induction was evident in chimeric mice lacking TLR3 in bone marrow (BM)-derived cells. Among the dendritic cell subsets, TLR3 was expressed by CD8α+ dendritic cells, known to be involved in priming HSV-1–specific CD8 T cells. Use of mixed BM chimeras revealed that TLR3 and the MHC class I-restriction element must be expressed by the same BM-derived cell for effective priming. These data imply that a cognate linkage between TLR3 and MHC class I is required for efficient CTL priming to HSV-1. The Journal of Immunology, 2010, 184: 2243–2246.

Recognition of conserved viral moieties by TLRs is an important step in the shaping of the subsequent immune responses to virus infections. Many TLRs have been identified and all, with the exception of TLR3, recruit the intracellular adaptor molecule MyD88 for downstream signaling. In contrast, TLR3 only uses TIR domain-containing adaptor-induced INF-β (TRIF), which in combination with MyD88, is also used by TLR4 (1). Although it is well established that these receptors play a profound role in innate antiviral immune responses (2), their precise contribution to antiviral T cell responses is much less clear. Based on its ability to bind dsRNA (3), and elegant work linking TLR3 signaling directly to cross-presentation of virus infected cells by dendritic cells (DCs) (4), it was widely expected that TLR3 would play a dominant role in antiviral T cell immunity (5). However, TLR3 appears to be entirely dispensable for the generation of T cell responses against reovirus, lymphocytic choriomeningitis virus, murine cytomegalovirus, or vesicular stomatitis virus (6). Whether this is a consequence of the specific nature of these infections, or rather an indication that antiviral T cell responses are regulated independently of TLR3 remains to be elucidated.

HSV is a DNA virus with tropism for epithelial cells. After infection, complex immune mechanisms are activated, including type I IFN secretion by plasmacytoid DCs (7–9) and stimulation of HSV-1–specific T cells by CD8α+ DCs and CD103+ DCs (10, 11). Whereas HSV-1–induced secretion of type I IFN requires signaling through TLR2 or TLR9 and MyD88 (7–9), it is currently unclear whether HSV-1–specific CD8 T cell responses are regulated by TLR-mediated signals. This study was therefore designed to analyze whether TLR signals were required for optimal priming of HSV-1–specific CD8 T cell responses in vivo.

Materials and Methods

Mice and virus

TLR3−/−, MyD88−/−, and TRIF−/− mice (12, 13) were generously provided by S. Akira (Osaka University, Osaka, Japan). H-2Kb−/− mice (14) were obtained through the National Institutes of Allergy and Infectious Diseases Exchange Program. These mice, C57BL/6 (B6), CD45.1, and gB-specific TCR transgenic CD8 T cell (gBT-I) mice were bred and maintained under specific pathogen-free conditions in the Department of Microbiology and Immunology at The University of Melbourne. HSV-1 KOS strain was propagated and titrated using Vero cells (CCL, Parkville, Australia). Mice were infected with 104 PFU of HSV-KOS on the skin by flank scarification (10, 11, 15, 16). Infectious virus from the site of infection (1 × 2-cm piece of full-thickness skin) was measured by the standard assay of PFU on confluent Vero cells as described (16).

Flow cytometry analysis of antiviral CD8 T cells

Antiviral CD8 T cells specific for gB498–505 from HSV-1 were detected in the spleen using H-2Kb–restricted gB498–505–specific tetramers. After labeling with anti-CD8 mAb (53.6–7), anti-CD44 mAb (IM7), and tetramer staining, viable CD8+ CD44+ tetramer+ cells were measured by flow cytometry. Intracellular gB498–505–induced IFN-γ production was determined as previously described (17).
Ag presentation assay

DCs were enriched from the brachial lymph nodes of mice infected 2 d earlier with HSV-1 on the flank and isolated as previously described (11). Briefly, DC subsets were sorted as follows: CD8α− DCs (CD11c+, CD8α−), CD103+ DCs (CD11c+, CD8α+, CD103−), dermal DCs (dDC) (CD11c+, CD8α+, CD103−, CD205+, CD256−), and Langerhans cells (LCs) (CD11c+, CD8α+, CD103+, CD205+, CD326−). Serial dilutions were incubated with CFSE-labeled TCR transgenic gBT-I cells for 60 h in vitro. Proliferation was analyzed by CFSE dilution using flow cytometry.

Bone marrow chimeras

CD45.1 or TLR3−/− mice were lethally irradiated with 2 × 550 cGy, and reconstituted with 5 × 10^6 T-depleted B6 or TLR3−/− bone marrow (BM) cells as described elsewhere (10, 15). In some cases, mice were reconstituted with 1:1 mixes of TLR3−/− and H-2Kb−/− BM. The mice were left for 8–10 wk before use. Only those mice that showed <15% remaining host cells were included in the study.

Quantitative reverse transcription PCR

RNA was prepared from purified DC populations using the RNeasy Mini Kit (Qiagen, Victoria, Australia). Quantitative expression of Gapdh and TLR3 was measured using the QuantiTect SYBR Green PCR Kit (Qiagen) and a light cycler (Roche, Victoria, Australia). The specific primers were as follows: Gapdh: 5′-CATTTCGATGGCAGG-3′; 5′-GCCTCGGTCTCGTAAAGAATCC-3′; TLR3: 5′-AAAATCATCGGGCGGAACTG-3′; 5′-AGGTCGAGGGCAGCGAA-3′; TLR3 expression was determined as a ratio to Gapdh.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software.

Results and Discussion

To determine whether TLR signals participate in the generation of HSV-1–specific CD8 T cells in vivo, we infected MyD88−/−, TRIF−/−, and C57BL/6 mice epidermally with HSV-1 by flank skin scarification (10, 11, 15, 16), and assessed the primary, endogenous HSV-1–specific CD8 T cell response 7 d later in the spleen by tetramer staining for the immunodominant H-2Kb–restricted gB epitope, gB498–505 (17). Analyzing the relative proportion of gB-specific CD8 T cells (Fig. 1A) and their absolute numbers in the spleen (Fig. 1B), we found that priming did not require MyD88. However, in the absence of TRIF, the percentage (Fig. 1A) and magnitude (Fig. 1B) of the gB-specific CD8 T cell response was significantly reduced, demonstrating that priming of HSV-1–specific CD8 T cells is MyD88-independent, but requires TRIF. Given that both TLR3 and TRIF signal through TRIF (1), we next examined whether TLR3 was required for priming of HSV-1–specific CD8 T cells.

Using TLR3−/− mice (3), we found that the proportion of gB-specific CD8 T cells (Fig. 1C), and the absolute number, as measured by tetramer staining (Fig. 1D) or the ability to secrete IFN-γ (Fig. 1E), was significantly reduced. As the reduction of the CD8 T cell response in the absence of TRIF was reiterating in TLR3−/− mice, we conclude that in vivo priming of HSV-1–specific CD8 T cells requires a signal that is mediated through TLR3 and TRIF. Thus, to the best of our knowledge, the current study provides the first direct demonstration that a TLR3-mediated signal is required for optimal priming and acquisition of effector function by virus-specific CD8 T cells in vivo.

A major function of CD8 T cells is the elimination of virus-infected cells. Because the magnitude of the HSV-1–specific CD8 T cell response was reduced in the absence of TLR3, we reasoned that this might affect viral replication. Two days after infection, viral replication in skin samples at the site of infection was not different between TLR3−/− mice and wild-type mice (Fig. 2). However, by day 7 postinfection viral titers were significantly increased in TLR3−/− mice (Fig. 2). Given that T cell priming begins 4–5 d postinfection and peaks between days 7 and 10, the observation that only the later phase of viral replication (day 7) was affected by the absence of TLR3 suggests a causal link between the reduced T cell response and impaired viral clearance. Colonna and colleagues (8) had previously reported that although HSV-1–induced type I IFN secretion required MyD88 and TLR9, viral replication after s.c. or corneal infection was regulated in a TLR9-independent manner. Our findings now resolve this
puzzling observation and identify TLR3 as an important element in the efficient control of viral replication of HSV-1.

Casanova and colleagues have recently reported that a dominant mutation in the thr3 gene increases susceptibility of individuals to HSV-1–induced encephalitis (18), posing the interesting question as to whether a similar association also occurs in mice. When monitoring TLR3−/− and B6 mice after HSV-1 flank infection over a 20-d period; however, no signs of herpes simplex encephalitis were evident in either of these mouse strains. The reduced HSV-1–specific CD8+ T cell response and increased viral replication in TLR3−/− mice could be explained by lack of TLR3 signaling on nonhematopoietic, radiosensitive tissue cells, such as keratinocytes (19) or fibroblasts, which have been implicated in TLR3-dependent antiviral immune responses to HSV-1 (18). Alternatively, the TLR3 signal might be required on radiosensitive hematopoietic cells, consistent with the demonstration that TLR expression is required for dendritic cell activation during systemic LPS-driven inflammation (20).

To discriminate between these two possibilities, we generated chimeric mice, in which TLR3 was either absent from the BM (TLR3−/− > CD45.1) or the parenchyma (CD45.1 > TLR3−/−). After allowing at least 8 wk for reconstitution, these chimeras were infected with HSV-1 on the flank and the gB-specific CD8+ T cell response was assessed 7 d later in the spleen by tetramer (Fig. 3). This showed that CD8+ T cell responses were reduced in mice lacking TLR3 in the BM compartment, but not those lacking parenchymal expression. It should be noted that the data in Fig. 3 is expressed as relative change to control chimeras, because the magnitude of the T cell responses in chimeras varied between experiments. These findings demonstrate that TLR3 expression on parenchymal cells or radiosensitive hematopoietic cells was dispensable for the priming of HSV-1–specific CD8+ T cells. Thus, our data indicates that a radiosensitive, hematopoietic cell type is the target for the essential TLR3 signal.

Our previous work has established that CD8α− DCs and CD103+ DCs present HSV-1 Ag to CD8+ T cells after skin infection (10, 11). To assess whether these DCs represent the critical TLR3-expressing BM-derived cell type, we examined TLR3 mRNA expression by DC subsets isolated from cutaneous lymph nodes. This showed that CD8α+ DCs, but not LCs, dDCs, or CD103+ DCs expressed TLR3 (Fig. 4A), extending previous evidence for TLR3 expression by splenic CD8α− DCs (4, 21). A precursor-product relationship has recently been proposed for CD8α− DCs and CD103+ DCs (22). Although both subsets share some functions (10, 11, 23), the absence of TLR3 on CD103+ DCs outlines an important difference between these subsets, which could be useful for further dissection of their immediate relationship.

As an initial test for whether TLR3 expression by CD8α− DCs is required for efficient presentation of HSV-1–derived Ag to CD8+ T cells, we measured ex vivo Ag presentation by CD8α− DC, LCs, dDCs, and CD103+ DCs from brachial lymph nodes 2 d after HSV-1 infection (11). Consistent with our previous observations (10, 11), only CD8α− DCs (Fig. 4B), but not LCs, dDCs, or CD103+ DCs presented Ag at this stage of the infection, irrespective of whether TLR3 was present or not (data not shown). Although TLR3−/− CD8α− DCs appeared less effective in inducing proliferation of gB-specific T cells when compared with TLR3–competent CD8α+ DCs (Fig. 4B), this difference did not reach statistical significance. Given that ex vivo assays require isolation of DCs from tissues, which causes their activation (24) and may negate an in vivo dependence on TLR3 for T cell priming, we resorted to mixed BM chimeras. Reconstitution of lethally irradiated mice with a 1:1 mixture of TLR3−/− BM and BM from H-2Kb−/− mice created a situation in which DCs either expressed H-2Kb or TLR3, but not both. Presentation of gB498–505 strictly depends

![FIGURE 3](http://www.jimmunol.org/) A BM-derived cell is required to express TLR3 for efficient priming of HSV-1–specific CD8+ T cells in vivo. Lethally irradiated mice were reconstituted with either CD45.1+ or TLR3−/− BM. Eight weeks post-reconstitution, mice were infected on the flank with HSV-1 and 7 d later the number of splenic gB-tetramer+ CD8+ T cells was determined by flow cytometry. Data are expressed as percent change (mean ± SEM) of control chimeras varied between experiments. These findings demonstrate that TLR expression is required for dendritic cell activation during systemic LPS-driven inflammation (20).

![FIGURE 4](http://www.jimmunol.org/) TLR3 expression by CD8α− DCs and requirement for the presenting DC to express TLR3 in vivo. A, CD8α− DCs selectively express TLR3. DC subsets (LCs, dDC, CD103+ DCs, and CD8α− DCs) were sorted from skin-draining lymph nodes of naive mice and TLR3 expression was assessed by real-time PCR. Values are expressed relative to GAPDH levels and the data are pooled from at least two independent experiments (mean ± SEM). B, Ex vivo Ag presentation of HSV-1–derived Ag to TCR transgenic HSV-1 gBT-I. CD8α− DCs were sorted from the brachial lymph nodes of mice infected epidemically with HSV-1 2 d earlier and proliferation was assessed by incubating serial dilutions of DC subsets with CFSE-labeled gBT-I for 60 h. Data are pooled from two independent experiments (each assay performed in duplicate wells) and are expressed as mean ± SEM. C, TLR3 is required on the same DCs presenting the Ag to HSV-1–specific CD8+ T cells in vivo. Lethally irradiated mice were reconstituted with a 1:1 mixture of H-2Kb−/− and TLR3−/− BM. After 8 wk, mice were infected on the flank with HSV-1 and 7 d later the number of gB-tetramer+ CD8+ T cells was determined in the spleen by flow cytometry by tetramer. Data are expressed as percent change (mean ± SEM) of control chimeras (CD45.2 > CD45.1) and are pooled results from four independent experiments (n = 5–7 per experiment). Asterisks indicate statistically significant differences versus control chimeras as assessed by Student t-test. ***p < 0.0001.
on the presence of functional H-2Kb (10). Thus, the only cell type capable of priming HSV-1–specific CD8 T cells in these mice will lack TLR3. The gB-specific CD8 T cell responses in these mixed chimeras was significantly reduced compared with control chimeras (Fig. 4C). Bearing in mind that only the CD8α+ DCs expressed TLR3, these findings are consistent with the notion that CD8α+ DCs require a TLR3-mediated signal for efficient priming of HSV-1–specific CD8 T cells in vivo.

Even though no obvious role for TLR3 in adaptive immunity to a number of viral infections has been uncovered so far (5, 6), signaling through this receptor was shown to enhance cross-priming in mice injected with virus-infected cells (4). Our study now shows that in vivo priming of HSV-1–specific CD8 T cells depends on a TLR3-mediated signal that is received by the same DCs presenting HSV-1 Ag to CD8 T cells, most likely the CD8α+ DCs (10, 11, 15). Potentially, differences in DC maturation cause the impaired priming of HSV-1–specific CD8 T cells in vivo. When analyzing maturation markers on freshly isolated CD8α+ DCs from the brachial lymph nodes of HSV-1–infected mice we found that TLR3−/− CD8α+ DCs upregulated CD86 and MHC class II as effectively as control mice (data not shown), indicating that maturation, as measured by these markers, was not affected. It has recently been documented that upregulation of costimulatory molecules does not necessarily correlate with a DC effector phenotype capable of inducing efficient CD4 T cell differentiation in vivo (25). It is therefore tempting to speculate that CD8α+ DCs isolated from HSV-1–infected TLR3−/− mice correspond to incompletely activated DCs (25) with impaired abilities to prime CD8 T cells. Identifying the exact molecular events responsible for the effects observed in this study will provide novel insights into the priming requirements for virus-specific immunity.

Disclosures
The authors have no financial conflicts of interest.

References