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H2-M3-Restricted T Cells Participate in the Priming of Antigen-Specific CD4+ T Cells

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H2-M3-restricted CD8+ T cells provide early protection against bacterial infections. In this study, we demonstrate that activated H2-M3-restricted T cells provide early signals for efficient CD4+ T cell priming. C57BL/6 mice immunized with dendritic cells coated with the MHC class II-restricted listeriolysin O peptide LLO_{220-228} (LLO) generated CD4+ T cells capable of responding to Listeria monocytogenes (LM) infection. Inclusion of a H2-M3-restricted formylated peptide fMIGWII (fMIG), but not MHC class Ia-restricted peptides, during immunization with LLO significantly increased IFN-γ-producing CD4+ T cell numbers, which was associated with increased protection against LM infection. Studies with a CD4+ T cell-depleting mAb indicate that the reduction in bacterial load in fMIG plus LLO immunized mice is likely due to augmented numbers of LLO-specific CD4+ T cells, generated with the help of H2-M3-restricted CD8+ T cells. We also found that augmentation of LLO-specific CD4+ T lymphocytes with H2-M3-restricted T cells requires presentation of LLO and fMIG by the same dendritic cells. Interestingly, the augmented CD4+ T cell response generated with fMIG also increased primary LLO-specific responses by MHC class Ia-restricted CD8 T cells. Coinmunization with LLO and fMIG also increases the number of memory Ag-specific CD4+ T cells. We also demonstrate that CD8 T cells restricted to another MHC class Ib molecule, Qa-1, whose human equivalent is HLA-E, are also able to enhance Ag-specific CD4+ T cell responses. These results reveal a novel function for H2-M3- and Qa-1-restricted T cells; provision of help to CD4+ Th cells during the primary response. The Journal of Immunology, 2006, 177: 5098–5104.

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3 Abbreviations used in this paper: DC, dendritic cell; LLO, listeriolysin O; LM, Listeria monocytogenes; LCMV, lymphocytic choriomeningitis virus.

Materials and Methods

Mice and bacteria

C57BL/6 (B6, H-2b) mice were obtained from The Jackson Laboratory. Mice 6–12 wk of age were used for the experiments described. All animal procedures were conducted in accordance with Canadian Council on Animal Care guidelines. The strains of Listeria used in this study have a LD_{50} of \(-1 \times 10^{5}\) organisms for B6 mice. The wild-type strain of Listeria (LM::3) and a recombinant strain of Listeria XFL203 (referred to as rLM::GP33) were used; the latter strain expresses the gp33–41 (GP33) epitope from lymphocytic choriomeningitis virus (LCMV) (16). A second recombinant strain of Listeria that expresses the 2C agonist peptide SIYRYUYGL (rLM-SIY) was also used (J. J. Priatel, L. Zenewicz, H. Shen, and H.-S. T.).
Abs and peptides

The following mAbs were used: anti-CD4 (GK1.5), anti-CD8α (53-6.7), anti-CD3ε (2C11), anti-IFN-γ (XMG1.2), and anti-TCRβ (H57-597). All Abs were obtained from eBioscience. The listeriolysin O peptide LLO197-209 (LLO) and GP33 peptide were synthesized at the University of British Columbia, Nucleic Acid-Protein Service Unit. The IMIG peptide was synthesized at Sigma-Genosys.

In vivo mAb depletion

CD4⁺ T cells were depleted by i.p. injection of 85 μg of anti-CD4 mAb GK1.5 twice at intervals of 3 days beginning 3 days following immunization. Efficacy of depletion in mice treated with GK1.5 mAb in PBS was always >95%, as measured by staining with H57-597, GK1.5, and 53-6.7 mAbs.

Bone marrow-derived DCs

Bone marrow-derived CD11c⁺ DCs were generated by 8 days of culture as described by Lutz et al. (17). The nonadherent cell population consisted of >95% CD11c⁺ cells following positive selection using the MiniMACS system (Miltenyi Biotec) according to the manufacturer’s specifications. For activation and peptide loading of DCs, bone marrow-derived DCs were incubated for 3 h at 37°C with 1 μg/ml LPS and the indicated peptides in the figures. The DCs were collected following incubation and extensively washed with 1× PBS. Approximately 1× 10⁶ peptide-coated DCs were injected i.v. via the tail vein.

Detection of Ag-specific T cells

The frequency and number of CD4⁺ and CD8⁺ T cells specific for LLO in the context of I-A朇, GP33 in the context of H-2Db, or IMIG in the context of H-2-M3 was determined by intracellular cytokine staining for IFN-γ as described by Hamilton et al. (18). Synthetic peptides were used at concentration of 2.5 μM (IMIG) or 5 μM (LLO or GP33). The CellQuest software program (BD Biosciences) was used for data acquisition and analysis.

Results

H2-M3-restricted CD8⁺ T cells participate in the priming of Ag-specific CD4⁺ T cells

To assess the potential influence of MHC class Iβ-restricted T cells on the priming of Ag-specific CD4⁺ T cells, mice were immunized with DCs coated with LLO with or without the dominant H2-M3-binding formylated peptide found in LM, IMIG (19). The frequency of responding LLO-specific CD4⁺ and IMIG-specific CD8⁺ T cells were analyzed 6 days following immunization by restimulating splenocytes with the appropriate peptides and measuring IFN-γ production. Immune response to LLO in mice with LLO-coated DCs resulted in the generation of a small but significant population of LLO-specific CD4⁺ T cells 6 days postimmunization. Surprisingly, priming of LLO-specific CD4⁺ T cells concurrently with H2-M3-restricted CD8 T cells, achieved through pulsing the DCs with the combination LLO and IMIG peptides, resulted in a 2.2-fold increase in the frequency (Fig. 1A) of LLO-specific CD4⁺ T cells, which translated to a 2.1-fold increase in the total number of LLO-specific CD4⁺ T cells (Fig. 1B). Immunization with unpulsed DCs or DCs pulsed with IMIG alone did not result in the generation of LLO-specific CD4⁺ T cells. Therefore, the observed increase in the total number of LLO-specific CD4⁺ T cells correlated with the activation of IMIG-specific CD8⁺ T cells because immunization with LLO alone generated fewer LLO-specific CD4⁺ T cells. As expected, recognition of the formylated peptide in the context of H2-M3 molecules on DCs induced robust proliferation and expansion of IMIG-specific CD8⁺ T cells, as determined by measuring both their frequency and total numbers following restimulation of splenocytes from mice immunized with either IMIG or the combination LLO and IMIG (Fig. 1, C and D).

CD4⁺ T cells generated with H2-M3-restricted T cell help improves protective immunity

To determine whether Ag-specific CD4⁺ T cells generated with H2-M3-restricted T cell help were associated with enhanced protective immunity, we infected previously immunized mice with wild-type LM 6 days following immunization to boost preprimed Ag-specific CD4⁺ T cell populations. We found that control and IMIG immunized mice, which lack preprimed LLO-specific CD4⁺ T cells, did not generate a measurable LLO-specific response 3 days following infection (Fig. 2A). Interestingly, mice previously immunized with a combination LLO and IMIG contained ~9.5-fold higher number of Ag-specific CD4⁺ T cells, relative to Ag-specific CD4⁺ T cells generated in mice without H2-M3-restricted T cell help (Fig. 2A). As shown in Fig. 2B, priming with IMIG in the absence or presence of LLO led to significant numbers of IMIG-specific CD8 T cells. To establish whether an increase in the total number of LM-specific CD4⁺ T cells is associated with improved protection, the bacterial load in the spleens from these mice were quantified. We found that mice immunized with LLO demonstrated a 0.65 log reduction in bacterial load relative to control and IMIG immunized mice. Remarkably, mice immunized with both LLO and IMIG demonstrated a 1.3 log reduction in bacterial loads, relative to control and IMIG immunized mice (Fig. 2C). The greater efficacy of combined IMIG and LLO immunized mice to eliminate LM may be due to either activated IMIG-specific CD8⁺
T cells and/or LLO-specific CD4 T cells. To distinguish between the relative contributions of these two cell types in bacterial clearance, mice immunized with DCs coated with fMIG or LLO and fMIG combined were depleted of CD4^+ T cells 3 days before infection and on the day of infection with wild-type LM. In this experiment, the presence of fMIG-specific CD8^+ T cells lead to a reduction of bacterial colonies in the spleen when compared with control mice, although this reduction is not statistically significant (p < 0.1). Consistent with our previous results, the combination LLO and fMIG immunized mice treated with PBS alone resulted in a significant 2.4 and 1.3 log reduction in the levels of bacteria in their spleens 3 days following infection, relative to control and fMIG-immunized mice, respectively (Fig. 2D). Importantly, the additional protection observed in combined LLO and fMIG immunized mice was abrogated by treatment with the GK1.5 mAb, which eliminates CD4^+ T cells in vivo (Fig. 2D). This result indicates that the reduction in bacterial load in fMIG plus LLO immunized mice is likely due to augmented number of LLO-specific CD4^+ T cells, generated with the help of H2-M3-restricted CD8^+ T cells. We also found generating LLO-specific CD4^+ T lymphocytes with H2-M3-restricted T cells activated on separated DCs, as opposed to the same DC, did not generate as many Ag-specific CD4^+ T cells (Fig. 2E). This result is observed even when twice the number of LLO-coated DCs were used for priming CD4^+ T cells (Fig. 2E). Thus, the observed increase in Ag-specific CD8^+ T cells, generated with help from activated H2-M3-restricted T cells functioning in close proximity rendered these mice more resistant to LM infection. These results are in agreement with a recent study that demonstrates impaired immune response to LM infection in H2-M3-deficient mice (20).

**Effective priming of Ag-specific CD4^+ T cells generated with help from nonclassical H2-M3-restricted T cells augment conventional CD8^+ T cell responses**

To rule out the possibility that conventional MHC class Ia-restricted CD8^+ T cells may function analogously to nonconventional H2-M3-restricted T cells in augmenting the number of Ag-specific CD4^+ T cells, we immunized mice with LLO-coated DCs with and without fMIG or GP33 peptide from LCMV. We found that mice immunized with DCs coated with either fMIG or GP33 peptide presented by MHC class I b or MHC class I a, respectively,
lead to similar numbers of Ag-specific effector T cells 6 days following DC immunization (data not shown). This finding is consistent with previous studies that demonstrated quicker expansion of Ag-specific CD8 T cells occurred when using peptide-coated DC immunizations (21). These mice were subsequently infected 6 days later with rLM-GP33 to boost the preprimed effector cells. The number of Ag-specific CD4+ and CD8+ T cells was analyzed 7 days later to study the ability of conventional CD8+ T cells to influence CD4+ T cell responses. As anticipated, the presence of fMIG-specific T cells was observed again only in mice immunized with the combination LLO and fMIG (Fig. 3A). In agreement with our previous observations, the priming of LLO-specific CD4+ T cells with H2-M3-restricted T cell help resulted in a significant 3.6-fold increase in the total number of LLO-specific CD4+ T cells, relative to the response generated in the absence of help from fMIG-specific CD8+ T cells (Fig. 3B). More importantly, conventional MHC class Ia-restricted T cells are intrinsically different from MHC class Ia-restricted T cells. Similar results were obtained when GP33 was substituted with a second MHC class Ia-restricted peptide SIYRYYGL, followed by infection with rLM-SIY (Fig. 4), indicating the weaker generation of CD4+ T cell responses is not a unique property of GP33 and is likely representative of all MHC class Ia-restricted peptides.

These studies also yielded a surprising finding. We found mice immunized with either the combination LLO and fMIG or LLO and GP33 possessed a significant 3.3- and 3.4-fold higher number of GP33-specific CD8+ T cells, respectively, compared with mice immunized with LLO alone (Fig. 3C). It is likely LLO-specific CD4+ T cells, whose number is in turn augmented as a result of copriming with fMIG, mediate augmentation of the GP33-specific CD8+ T cell response. We also found that mice immunized with GP33-coated DCs in the absence or presence of fMIG had similar numbers of GP33-specific CD8+ T cells 7 days following infection with rLM-GP33 (Fig. 3D). This finding is in agreement with a previous study that found fMIG-specific H2-M3-restricted CD8+ T cells are unable to directly augment classical CD8+ T cell responses (23).

Enhancement of memory CD4 and CD8 T cell responses by H2-M3-restricted T cells

Following the effector phase of an immune response, the majority of Ag-specific CD4+ T cells undergo apoptosis with only a small population surviving, which constitutes long-lived memory T cells (24). To determine whether H2-M3-restricted T cells are able to produce quantitatively and qualitatively enhanced Ag-specific CD4+ memory T cells, mice were immunized with LLO-coated DCs in the presence or absence of fMIG peptide. Following infection of mice with rLM-GP33, Ag-specific CD4+ T cells were enumerated 30 days later to determine whether H2-M3-restricted T cells help generate a larger pool of memory CD4+ T cells. As shown in Fig. 5A, there was a 4-fold increase in the absolute number of LLO-specific CD4+ T cells generated with H2-M3-restricted T cell help, relative to mice immunized without help from

**FIGURE 3.** Ag-specific CD4+ T cells generated with help from nonclassical H2-M3-restricted T cells augment MHC class Ia-restricted CD8+ T cell responses. A–C, Mice were immunized with 1 × 10⁶ DCs coated with either LLO, LLO plus fMIG, or LLO plus GP33. Error bars, Mean ± SD of three mice per group. D, Mice were immunized with GP33 or GP33 plus fMIG. At day 6 postimmunization, all mice were infected with 5 × 10⁵ CFUs of rLM-GP33. The number of Ag-specific CD4+ and CD8+ T cells in the spleen of infected mice was determined 7 days later, as described in Fig. 1. Error bars, Mean ± SD of four mice per group. A, Total number of fMIG-specific CD8+ T cells in the spleen of mice immunized with the indicated peptide(s). ***, p < 0.004, compared with LLO plus GP33 immunization. **, p < 0.006, compared with LLO immunization. B, Total number of LLO-specific CD4+ T cells in the spleen of mice immunized with the indicated peptide(s). *, p < 0.01 and ***, p < 0.001, compared with LLO plus GP33 immunization. C, Total number of GP33-specific CD8+ T cells in the spleen of mice immunized with the indicated peptide(s). *, p < 0.01 and ***, p < 0.006, compared with LLO immunization. D, Total number of GP33-specific CD8+ T cells in the spleen of mice immunized with the indicated peptide(s).
activated fMIG-specific CD8\(^+\) T cells. Furthermore, Ag-specific CD4\(^+\) T cells generated with H2-M3-restricted T cell help produced ∼20% more IFN-γ on a per-cell basis, as determined by evaluating the geometric mean fluorescence intensity of Ab against IFN-γ (Fig. 5B). Our findings thus indicate that H2-M3-restricted T cells support the enhancement of Ag-specific CD4\(^+\) T cells both quantitatively and qualitatively with respect to their increased persistence during the memory phase following infection, as well as their ability to produce higher amounts of IFN-γ, respectively. In agreement with our earlier observation that copriming with LLO plus fMIG can lead to enhanced primary GP33-specific CD8 T cell responses, we found that mice previously immunized with a combination LLO also fMIG also possess a larger population of memory GP33-specific CD8\(^+\) T cells (Fig. 5C). Impressively, there was not a significant difference in the number of Ag-specific CD8\(^+\) T cells between LLO and fMIG and GP33 and fMIG immunized mice, which represented a primary and secondary GP33-specific CTL response, respectively. Similar expansion of GP33-specific CD8 T cells was observed by immunizing mice with DC-GP33, DC with combined GP33 and LLO, or DC with combined GP33 and fMIG (data not shown). Our data thus indicate that the qualitatively and quantitatively enhanced Ag-specific CD4\(^+\) T cells associated with fMIG copriming can lead to a vigorous primary response by GP33-specific CD8\(^+\) T cells.

Qa-1-restricted CD8\(^+\) T cells enhance Ag-specific CD4\(^+\) T cell responses

Because humans do not possess an ortholog of H2-M3 and human CD8\(^+\) T cells do not recognize fMIG, we wanted to establish whether another murine MHC class Ib molecule with a human equivalent could perform similarly in augmenting CD4\(^+\) T cell responses. In this regard, the mouse Qa-1 and human HLA-E molecules are functional counterparts, based on their ability to bind class I leader sequence-derived peptides and serve as a ligand for the CD94/NKG2A receptor complex (25, 26). Both Qa-1 and HLA-E are able to bind and present the peptide GMQFDRGYL (GroEL), which is an immunodominant epitope of Salmonella typhimurium (7, 27). Recent work suggests that Qa-1-restricted CD8\(^+\) T cells can suppress the response of successfully activated CD4\(^+\) T cells through an interaction that depends on expression of Qa-1 molecules on the Th cell (28–31). Because it has been established, Qa-1-restricted CD8\(^+\) T cells can regulate ongoing CD4\(^+\) T cell responses, we wanted to determine whether Qa-1-restricted CD8\(^+\) T cells can also influence the priming of Ag-specific CD4\(^+\) T cells (28). To address this, mice were immunized with DCs coated with LLO with or without GroEL, and analyzed 6 days later. The priming of LLO-specific CD4\(^+\) T cells with help from Qa-1-restricted T cells led to a significant 3-fold increase, relative to mice that received just LLO-coated DCs alone (Fig. 6A). In agreement with this observation, day 6 immune mice receiving LLO-coated DC immunizations in the presence of GroEL also contained a ∼2-fold higher number of Ag-specific CD4\(^+\) T cells, relative to immunizations with DC-LLO alone 7 days following infection with wild-type LM (Fig. 6B). Because LM does not express GroEL, one could account for the modest increase in the number of LLO-specific CD4\(^+\) T cells after LM infection in LLO plus GroEL immunized mice. Because Qa-1 performs similar functions as H2-M3, these observations also explain why mice can mount effective immune responses to a mutant LM strain that is unable to add formyl groups to nascent polypeptides (32). Furthermore, these results suggest that Qa-1-restricted CD8\(^+\) T cells may be involved in immune regulation on many levels, depending on when they are used during an immune response.
B, Total number of LLO-specific CD4+ T cells in the spleen of infected immune mice. *, p < 0.04 and **, p < 0.005, compared with control. ***, p < 0.04, compared with LLO. 

Discussion

In this study, we have demonstrated a novel function for H2-M3-restricted T cells in enhancing Ag-specific CD4+ T cell responses following immunization, as well as during the acute, effector and memory phase following LM infection. During bacterial infection, H2-M3-restricted T cells are able to rapidly exert their effector functions upon receptor engagement by reaching peak frequencies earlier than their classical CD8+ T cell counterparts. This early response enables them to provide early protection to the infected host. In this study, we have determined some of the basic parameters by which H2-M3-restricted CD8+ T cells participate in the priming of Ag-specific CD4+ T cells. We have demonstrated the necessity of activating H2-M3-restricted T cells on the same, as opposed to separate DC, for enhancing the priming of Ag-specific CD4+ T cells, suggesting cytokines functioning in a short and paracrine manner may be mediating this effect. Consistent with this notion, recent work by other groups have also demonstrated early production of cytokines, such as IFN-γ, by up-regulating cytokines to the priming of Ag-specific CD4+ T cells in enhancing Ag-specific CD8+ T cell responses following immunization (A), or 7 days post LM-GP3 infection of day-6 immune mice (B). A, Total number of LLO-specific CD4+ T cells in the spleen of mice immunized with the indicated peptide(s), *, p < 0.04 and **, p < 0.005, compared with control. ***, p < 0.04, compared with LLO. B, Total number of LLO-specific CD4+ T cells in the spleen of infected immune mice. *, p < 0.02, compared with LLO. Error bars, Mean ± SD of four mice per group. Data are representative of two independent experiments.

Helper CD4+ T lymphocytes also exert other pleiotropic effects including the ability to cross-link CD40 receptors on DCs. The engagement of CD40 on DCs leads to enhanced survival, increased expression of costimulatory molecules and increased secretion of inflammatory cytokines, all of which provide favorable conditions for stimulating CD8 T cells (37). Interestingly, our work has also demonstrated higher numbers of Ag-specific CD4+ T cells resulted in a subsequent increase in conventional Ag-specific CD8+ T cell numbers, which was approximately equivalent to the quantity found in mice immunized with DCs coated with the respective MHC class Ia-specific peptide, during both the effector and memory phase following an immune response to LM infection. One explanation could be increased numbers of Ag-specific CD4+ T cells expressing CD40L could more efficiently license DCs to become more efficient APCs by either inducing the up-regulation of B7 molecules or their ability to produce IL-12, both of which has been reported to dramatically improve CTL induction (37). Alternatively, increased frequency of CD40L expressed by Ag-specific CD4+ T cells could also induce the up-regulation of 4-1BBL on DCs, which can induce both CD8+ T cell activation and survival (42). Consistent with this, 4-1BBL-deficient mice have reduced effector and memory CD8+ T cells following infection with LCMV (43, 44). These results suggest H2-M3-restricted T cells, although unable to directly augment numbers of classical Ag-specific CD8+ T cells, are able to do so indirectly.

The functions of H2-M3-restricted T cells have long been enigmatic with respect to their specific contributions during immune responses. In this study, we have demonstrated a novel function for these cells in augmenting the number of Ag-specific CD4+ and CD8+ T cells, which could provide a novel method for enhancing weak immune responses to syngeneic tumors because they are more dependent on CD4+ T cell help. More importantly, having identified a similar function for Qa-1, whose human equivalent is HLA-E, this study emphasizes the significance of targeting this

clustering those restricted by Qa-1, function as immunoregulatory cells that have a significant influence on adaptive immunity.

We have also demonstrated increased number of Ag-specific CD4+ T cells were able to confer a greater ability to eliminate LM from the spleen of infected mice. Because LM replicates primarily within macrophages (36), the augmented number of IFN-γ-producing Ag-specific CD4+ T cells may be amplifying the host response by activating resident and newly recruited macrophages, or other innate cells including neutrophils and NK cells, to become more bactericidal. CD4+ Th cells perform these functions by secreting cytokines, such as IFN-γ, and by up-regulating CD40L, both of which positively influence macrophages to become more efficient at removing harmful pathogenic organisms (37, 38). The importance of IFN-γ to host defense against LM infection has also been demonstrated in a number of previous studies showing increased susceptibility in mice with disrupted genes for IFN-γ (39) or lack the IFN-γ receptor (40), resulting in both impaired innate cell activity, failure to recruit other innate effector cells and the inability of macrophage cells to produce antimicrobial products despite the presence of normal cytokotoxic CD8+ and CD4+ Th cell responses. Furthermore, in agreement with work done by other labs (23), mice immunized with DCs coated with fMIG were also able to modestly reduce bacterial levels in the spleens of infected immune mice, although this decrease was found not to be statistically significant 3 days following infection. The degree of variability observed in both the splenic bacterial burden and magnitude of the fMIG-specific T cell response generated between genetically identical mice may be due to differential environmental factors that shaped their H2-M3-restricted T cell repertoire (21, 41).

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and other nonpolymorphic MHC class Iib molecules for future vaccine design strategies, which will help induce optimal CD4+ and CD8+ T cell responses and thus, protective immunity.

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Disclosures
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