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Osteopontin Modulates the Generation of Memory CD8+ T Cells during Influenza Virus Infection

Junko Morimoto,* Kayoko Sato,† Yosuke Nakayama,† Chiemi Kimura,* Kiichi Kajino,‡ Yutaka Matsui,§ Tadaaki Miyazaki,* and Toshimitsu Uede*§

The adaptive immune system generates memory cells, which induce a rapid and robust immune response following secondary Ag encounter. Memory CD8+ T cells are a critical component of protective immunity against infections and cancers. Therefore, understanding the mechanism whereby memory CD8+ T cells are generated and maintained is important for inducing effective memory CD8+ T cell response. Recent studies have demonstrated that the inflammatory cytokine IL-12 favors the generation of terminal effector CD8+ T cells rather than memory precursor effector CD8+ T cells by regulating the expression of the transcription factor T-bet. In this study, we report that the inflammatory cytokine osteopontin (Opn) modulates memory CD8+ T cell generation during influenza virus infection. Although Opn wild-type and Opn knockout (KO) mice had similar numbers of virus-specific effector CD8+ T cells, virus-specific effector CD8+ T cells generated in Opn KO mice showed low levels of T-bet expression and an increased memory precursor cell population compared with cells generated in Opn wild-type mice. This resulted in the persistently increased number of memory CD8+ T cells in Opn KO mice. Studies with bone marrow-derived dendritic cells demonstrated that Opn deficiency in bone marrow-derived dendritic cells results in low levels of IL-12 production in response to the stimulation with influenza virus. Thus, we hypothesize that Opn modulates the generation of memory precursor effector CD8+ T cells by regulating cytokine milieu during the acute phase of virus infection. This finding may provide new insight into the role of Opn in adaptive immune response. The Journal of Immunology, 2011, 187: 000–000.

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Abbreviations used in this article: BAL, bronchoalveolar lavage; BM, bone marrow; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; i.n., intranasally; KO, knockout; LCMV, lymphocytic choriomeningitis virus; mLN, mediastinal lymph node; NP, nucleoprotein; Opn, osteopontin; WT, wild-type.

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D uring a viral infection, naive CD8+ T cells recognize Ags presented by professional APCs and expand to generate a large number of Ag-specific effector CD8+ T cells that participate in the elimination of the virus. These expanded CD8+ T cells then undergo contraction, leaving behind a small population of memory T cells that has a potential to expand drastically in response to the secondary Ag stimulation. Although enhanced secondary response is dependent on IL-2 and helper T cells (1, 2), it is also influenced by the frequency of memory precursors. A recent study, using a lymphocytic choriomeningitis virus (LCMV) infection system, has demonstrated that effector CD8+ T cells are divided into two dominant populations, killer cell lectin-like receptor G1 (KLRG1)highCD127 (IL-7 receptor α-chain)low cells and KLRG1lowCD127 (IL-7 receptor α-chain)high cells. KLRG1lowCD127high cells have a limited life span (terminal effector T cells), whereas KLRG1highCD127low cells have a potential to give rise to long-lived memory CD8+ T cells (3). The factors and mechanisms that underlie the development of terminal effector CD8+ T cell and long-lived memory CD8+ T cell fates are not well defined. It has been proposed that several signals, including TCR stimulation (signal 1), costimulation (signal 2), and inflammatory cytokines (signal 3), are involved in the process (4–9). Thus, a better understanding of how the generation of memory precursor effector CD8+ T cells is regulated may facilitate induction of effective memory responses and the development of more effective vaccines against various pathogens. The T-box transcription factors T-bet and eomesoderm have been shown to cooperate in regulating the differentiation and function of cytotoxic CD8+ T cells (10). T-bet expression is rapidly induced in effector CD8+ T cells, whereas eomesoderm expression is induced in memory CD8+ T cells (10). Recently, several studies have demonstrated that the inflammatory cytokine IL-12 is a key factor that determines the expression of T-bet in effector CD8+ T cells and controls memory precursor generation (3, 11). Large amounts of IL-12 promote the T-bet expression and the generation of terminal effector CD8+ T cells. In contrast, under conditions of low IL-12 levels, Ag-stimulated CD8+ T cells express low levels of T-bet and have a tendency to become long-lived memory CD8+ T cells (3). The role of IL-12 in controlling the generation of memory precursor effector CD8+ T cells is further supported by the finding that IL-12-deficient mice generate more memory CD8+ T cells following Listeria monocytogenes infection, although these mice generate weaker primary CD8+ T cell responses than do wild-type (WT) mice (12). However, the involvement of other inflammatory cytokines in the regulation of effector and memory CD8+ T cell fates is still unclear.

Osteopontin (Opn), a glycosylated phosphoprotein, plays a critical role in many physiological and pathophysiological processes, including cancer, bone remodeling, and inflammatory
responses (13–15). The importance of Opn in the regulation of these processes has been shown in various cell types (16). A previous report showed that Opn promotes a Th1-type immune response by promoting IL-12 production of macrophages through binding to αβ integrin and suppressing IL-10 production by macrophages by interacting with a spliced variant of CD44, CD44v (17). Studies with Opn knockout (KO) mice have indicated that Opn contributes to host resistance against several microbes, including HSV-1, rotavirus, and Mycobacterium bovis (17–19). A recent study demonstrated that Opn enhances the survival of self-reactive activated CD4+ T cells through inhibition of the transcription factor Foxo3a and regulation of the expression of proapoptotic proteins such as Bim, Bak, and Bax, resulting in the relapse and progression of experimental autoimmune encephalomyelitis (EAE) (20). This finding suggests that Opn might affect memory T cell generation and maintenance.

In this study, we investigated the role of Opn in the generation of memory CD8+ T cells during influenza virus infection. In contrast to what we had expected, Opn KO mice had an increased number of virus-specific memory CD8+ T cells, although Opn WT and Opn KO mice generated comparable numbers of virus-specific effector CD8+ T cells. Virus-specific effector CD8+ T cells generated in Opn KO mice showed low levels of T-bet expression and contained more KLRG1lowCD122high memory precursor effector CD8+ T cells compared with cells generated in WT Opn mice. We found that virus Ag-loaded dendritic cells (DCs), but not virus-specific effector CD8+ T cells, produced Opn, which promotes IL-12 production in a paracrine and/or autocrine fashion. Thus, we hypothesize that Opn deficiency attenuates the expression of T-bet in virus-specific effector CD8+ T cells and the generation of terminal effector CD8+ T cells by regulating IL-12 production in DCs during the acute phase of influenza virus infection, although there might be other mechanisms behind this phenomenon.

Materials and Methods

**Viruses, mice, and infection**

Influenza virus A/HKx31 (HKx31; H3N2) was provided by Dr. Y. Kawaoka (University of Wisconsin–Madison, Madison, WI). Heterologous A/PRL/8 (PR8; H1N1) influenza virus and HKx31 were grown in the allantoic cavity of 10–12-day-old embryonated hen’s eggs and stored at −80°C. WT C57BL/6 (Opn WT) mice were purchased from Japan SLC. Opn KO mice were backcrossed 10 times to C57BL/6 mice. For primary infection, ~10-d-old embryonated hen’s eggs and stored at ∼10-fold to C57BL/6 mice. For primary infection, C57BL/6 (Opn WT) mice were purchased from Japan SLC. Opn KO mice were backcrossed 10 times to C57BL/6 mice. For primary infection, mice were anesthetized and infected intranasally (i.n.) with 10^4 PFU of influenza virus A/HKx31 (HKx31; H3N2) at 37°C for 24 h in 96-well plates with or without 10^8 PFU heat-inactivated influenza HKx31 virus. BMDC stimulation was also performed under the presence of 10 μg/ml rOpn (R&D Systems), 1 μg/ml purified anti-CD61 (β integrin) (BioLegend), and 100 μg/ml rGRGDs peptide (Sigma-Aldrich).

**Flow cytometric analysis and sorting**

Anti-mouse CD8 (53–67.7), F4/80 (C1:3-A-1), Gr-1 (RB6-8C5), CD11b (M1/70), IFN-γ, granzyme B (16G6), CD69 (29.G2), CD62L (MEL-14), CD127 (SB), CD13 (145-2C11), CD25 (PC6), CD27 (LG3.1A10), CD122 (TM-β1), KLRG1(2F1), CD11c (N418), CD212 (114), and I-A/E (M15/114.15.2) were all commercially obtained (BD Pharmingen and BioLegend). Cells were evaluated with a FACS Calibur (BD Biosciences), and the obtained data were analyzed with FlowJo software (Tree Star). To isolate influenza virus-specific CD8+ T cells, the splenocytes were pre-incubated with H-2Db tetramers bound to influenza virus-derived peptide ASNENMETM, then incubated with anti-mouse CD8 mAb and sorted for tetramer+CD8+ T cells with a FACS Aria (BD Biosciences). To isolate splenic DCs, spleens were first incubated with 2 mg/ml collagenase D (Roche) at 37°C for 0.5 h, and then cells were stained with anti-mouse CD11c and I-A and sorted for CD11c+I-A+ cells with a FACS Aria.

**Real-time PCR analysis**

Total RNA was extracted from the lungs using TRIzol reagent (Invitrogen). RNA from sorted tetramer+CD8+ cells was prepared using an RNAeasy Plus Micro kit (Qiagen). First-strand cDNA was synthesized from total RNA by using reverse transcriptase and random primers. Real-time PCR analysis was carried out using the LightCyther Fast Start DNA Master SYBR Green I system (Roche Diagnostics). The primer sequences were as follows: CXCL9, 5′-TTGGGAGTTCGAGGAACCC-3′ and 5′-CCCGAGTACTAGCAGGTTGTG-3′; CCL3, 5′-CAAGCCGACGTTGACAAGT-3′ and 5′-GGGTTTCCAGTTTGATGCTG-3′; CCL4, 5′-ACACAGTCTATCGCTAC-3′ and 5′-TCCACACCCCCCTTGGTCT-3′; and T-bet, 5′-CTGGTTGACCTAGGTTGCCC-3′ and 5′-TTCATCTCТГТГТССССССС-3′. Cytokine detection

Lungs, mLN, and spleens were homogenized with tissue lysis/extraction reagent (Sigma-Aldrich) containing protease inhibitor mixture (Roche Diagnostics). Cytokine levels in the plasma, tissue homogenates, and culture medium were determined using ELISA kits (Opn and IL-12p70 from R&D Systems; IL-6, IFN-γ, and IL-12p40 from BD Biosciences).

**Immunohistochemistry**

Sections of paraffin-embedded lung tissue were prepared for immunohistochemistry. After inhibition of endogenous peroxidase activity, the sections were incubated with anti-mouse Opn Ab (O-17; BIBL Japan). The sections were then treated with HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and visualized with 3,3′-diaminobenzidine (Nichirei, Japan). The sections were counterstained with hematoxylin. Pulmonary epithelial cells were defined by the morphological characteristics, having flattened shape and facing or close to alveolar lumen. Foamy cells containing granular materials were defined as pulmonary macrophages.

**Analysis of virus-specific CD8+ T cell responses**

Virus-specific CD8+ T cells were identified using MHC class I tetrameric complexes specific for the H-2Dβ-restricted immunodominant epitope of the influenza virus nucleoprotein (DNP366-374 ASNENMETM) (MBL Japan). To measure virus-specific cytokine expression, splenocytes prepared from infected mice were stimulated with 0.5 μg/ml DNP366-374 peptide (Sigma-Aldrich) in the presence of 1 μg/ml Golgiplag (BD Pharmingen) for 6 h. Cells were then stained for relevant surface molecules, fixed, and permeabilized with Cytofix/Cytoperm reagent (BD Pharmingen) and stained for intracellular cytokine. Intracellular granulocyte B staining was also done using Cytofix/Cytoperm reagent. For in vitro proliferation assays, 2 × 10^6 splenocytes from infected mice were labeled with CFSE and incubated at 37°C for 3 to 9 days at 96-well plates with or without 0.5 μg/ml NP366-374 peptide. All cell cultures were maintained in RPMI 1640 supplemented with antibiotics, 2-ME, and 10% FBS.

**Cell preparation and stimulation**

Bronchoalveolar lavage (BAL) fluid cells were collected with three 500-μl aliquots of PBS containing 10 mM EDTA at the indicated times post-infection with influenza virus. BAL cell suspensions were centrifuged, and the pellets were then treated with RBC lysis solution. Single-cell suspensions were also prepared from spleens and mediastinal lymph nodes (mLN). To generate bone marrow-derived DCs (BMDCs), 2 × 10^6 BM cells/ml were cultured in RPMI 1640 supplemented with antibiotics, 10% FBS, and 20 ng/ml rGM-CSF (Wako Japan). The culture medium was replaced on day 3, and cells were collected on day 6. For BMDC stimulation, 1 × 10^7 BMDCs were incubated at 37°C for 24 h in 96-well plates with or without 10^8 PFU heat-inactivated influenza HKx31 virus. BMDC stimulation was also performed under the presence of 10 μg/ml rOpn (R&D Systems), 1 μg/ml purified anti-CD61 (β integrin) (BioLegend), and 100 μg/ml rGRGDs peptide (Sigma-Aldrich).

**In vivo cytolytic assay**

C57BL/6 mouse splenocytes were pulsed with 5 μg/ml NP peptide at 37°C for 1 h and used as target cells. Target cells were then labeled with 1 and 0.3 μg/ml CFSE. Recipient mice were injected i.v. with 5 × 10^6 target cells, and 24 h later, splenocytes were analyzed for CFSE+ target cells.

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Statistical analysis

Statistical comparisons between groups were performed with a two-tailed unpaired Student t test. Data were considered statistically significant when the p value was <0.05.

Results

Influenza virus infection induces Opn expression in the lungs

A previous report showed that Opn, expressed by inflammatory cells, is responsible for the resistance to microbial infection by acting as a Th1-type cytokine (17). Therefore, we first investigated whether Opn expression in the lungs, which are the primary site of influenza virus infection, is augmented during influenza virus infection. For primary infection, mice were infected i.n. with HKx31 influenza virus. Following infection, Opn levels in the lung tissues and plasma increased, with a peak between day 3 and day 10 (Fig. 1A). We next performed immunohistochemistry to identify cells expressing Opn in the infected lungs. In the normal lungs, Opn expression, if any, was weak in pulmonary epithelial cells. On the other hand, in the infected lungs, strong Opn expression was detected mainly in macrophages in both the alveolar space and interstitial tissues, and moderate Opn expression was also detected in pulmonary epithelial cells (Fig. 1B).

Opn deficiency does not affect the magnitude of lung inflammation

Influenza virus infection causes severe illness and pulmonary damage, associated with an infiltration of inflammatory cells such as neutrophils and macrophages. Following infection with 10⁴ PFU HKx31 influenza virus, which is a nonlethal dose but causes severe illness, Opn WT and Opn KO mice showed similar progressive body weight loss, with a peak at day 7, followed by similar recovery of body weight (Fig. 2A). Because Opn is a chemotactic factor for inflammatory cells (21, 22), we collected BAL fluid at day 9 and determined the numbers of macrophages, neutrophils, and NP-specific CD8⁺ T cells that recognize the immunodominant influenza viral epitope by flow cytometric analysis. We observed that the numbers of macrophages, neutrophils, and NP-specific CD8⁺ T cells infiltrated into the infected lungs were comparable between Opn WT and Opn KO mice (Fig. 2B). We also found that two groups showed similar levels of protein expression of IL-6, IFN-γ, and gene expression of CXCL9, CCL3, and CCL4 chemokines in the lungs at various time points postinfection (Fig. 2C, 2D). Furthermore, histological analysis showed that two groups had similar levels of inflammatory foci in the lungs (Fig. 2E). Consistent with these data, Opn WT and Opn KO mice showed similar viral loads in the lungs (Fig. 2F). Thus, Opn does not influence the severity of lung inflammation, despite the evidence that Opn is strongly induced in the lungs following influenza virus infection.

Opn deficiency does not affect the generation of virus-specific CD8⁺ T cells

Studies in a mouse model have demonstrated that influenza virus infection induces robust proliferation of virus Ag-specific CD8⁺ T cells. Influenza virus-specific CD8⁺ T cells promote efficient elimination of the virus by producing effector cytokines and directly killing virus-infected cells, resulting in host recovery. In fact, mice that lack MHC class I-restricted T cells show delayed viral clearance (23, 24). Influenza virus-specific CD8⁺ T cells are primed and undergo clonal expansion in the draining mLN before migrating into the lungs (25). We detected sharp increases in Opn levels in the mLN following infection, and such increases in Opn levels were also evident in the spleen (Supplemental Fig. 1A). Since upregulation of Opn levels was detectable in the mLN and spleen shortly postinfection, we next examined whether Opn plays any role in the generation of virus-specific CD8⁺ T cells. Opn WT and Opn KO mice showed similar numbers of NP-specific CD8⁺ T cells in the mLN, with a peak at day 7 (Fig. 3A). NP-specific

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Opn is induced in the lungs after influenza virus infection. Opn WT mice were infected i.n. with HKx31. A, Lungs were removed at indicated time points and lung homogenates were assessed for Opn concentrations. Blood samples were collected under EDTA, centrifuged to remove cells, and plasma levels of Opn were measured (n = 5/group at each time point). Data are presented as the means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.005 (versus day 0). B, The lung sections (before infection and day 7) were stained with anti-Opn Ab (brown) and counterstained with hematoxylin (blue). Epithelial cells and lung macrophages were marked by red asterisks and arrows, respectively. The region framed with square on upper panels were magnified and depicted in lower panels. Scale bars, 30 μm.
CD8^+ T cells were also detectable in the spleen, with a peak at day 10 (Fig. 3B). However, the numbers of NP-specific CD8^+ T cells in the spleen were much higher than those in the mLN. This finding does not simply indicate the migration of cells that had been activated in the mLN, but rather that the spleen can be a site of activation for naive NP-specific CD8^+ T cells. Again, there was no difference in the numbers of NP-specific CD8^+ T cells in the spleen between two groups (Fig. 3B).

Taking into account the finding that Opn KO mice could generate quantitatively similar numbers of NP-specific CD8^+ T cells in both the mLN and the spleen compared with Opn WT mice, we next analyzed whether Opn deficiency affects the effector functions of NP-specific CD8^+ T cells. We measured the expression of granzyme B, a major mediator of cytotoxicity, and IFN-γ production by NP-specific CD8^+ T cells around the time of peak response of virus-specific CD8^+ T cells (day 9). The NP-specific CD8^+ T cells generated in the mLN and spleen of Opn KO mice showed similar granzyme B levels compared with cells in Opn WT mice (Fig. 3C). We also found that there was no significant change in the number of IFN-γ-producing CD8^+ T cells in the spleen between Opn WT and Opn KO mice after NP peptide stimulation in vitro (Fig. 3D). These data suggest that Opn is dispensable for the generation of functional virus-specific effector CD8^+ T cells.

Opn deficiency induces an increase in the number of memory CD8^+ T cells

A previous study showed that Opn promotes the survival of activated self-reactive T cells by regulating the transcription factors Foxo3a and NF-κB and altering the expression of proapoptotic proteins such as Bim, Bak, and Bax, which results in the persistence of EAE (20). Therefore, we examined whether Opn affects the generation of memory CD8^+ T cells. We found that Opn KO mice maintained
higher numbers and frequencies of memory CD8+ T cells in the mLNs and spleen compared with Opn WT mice at 60 d postinfection (Fig. 4A, 4B), suggesting that Opn deficiency promotes the generation and/or survival of virus-specific memory CD8+ T cells. We then analyzed the phenotype of memory CD8+ T cells. The expression levels of CD127 and CD62L, by NP-specific memory CD8+ T cells generated in Opn KO mice were similar to those observed in cells in Opn WT mice. Most NP-specific memory CD8+ T cells in both groups showed CD62Llow phenotype, indicative of effector memory T cells (Fig. 4C).

Because Opn WT and Opn KO mice generated similar numbers of NP-specific effector CD8+ T cells at the peak of the response (day 10) (Fig. 3A, 3B), we considered the possibility of reduced contraction of effector CD8+ T cells in Opn KO mice, resulting in the persistence of more memory CD8+ T cells. To investigate this hypothesis, we analyzed the number and frequency of NP-specific CD8+ T cells in the mLNs and spleen at the end of the contraction phase (day 17). Two groups showed similar numbers and frequencies of NP-specific CD8+ T cells at day 17 (Fig. 4D, 4E). The contraction in the spleen of Opn WT mice was ∼4.6-fold, whereas it was ∼5.4-fold in Opn KO mice, which was determined by comparing the numbers of NP-specific CD8+ T cells at day 10 and day 17. These data suggest that the increase in the numbers of NP-specific memory CD8+ T cells in Opn KO mice is not simply due to reduced contraction.

BM is an important site for supporting the homeostatic proliferation of memory CD8+ T cells (26, 27). Indeed, in this study, NP-specific memory CD8+ T cells were detectable in the BM at 60 d postinfection (Fig. 5A). Although, the the level of CD127 expression by NP-specific memory CD8+ T cells in the BM was comparable between Opn WT and Opn KO mice (data not shown), Opn KO mice contained a much higher proportion of NP-specific memory CD8+ T cells compared with Opn WT mice (Fig. 5A), consistent with the data obtained in the mLN and spleen (Fig. 4A, 4B). Recent reports have shown that Opn negatively regulates the size of hematopoietic stem cell pool in the BM niche (27, 28). To determine whether the increased number of memory CD8+ T cells in Opn KO mice is a consequence of a higher proliferation rate of memory cells in the BM, we analyzed the frequencies of endogenous memory CD8+ T cells in the BM and spleen of naive Opn WT and Opn KO mice. We found that Opn WT and Opn KO mice had similar frequencies of CD62LhighCD44high and CD62LlowCD44high CD8+ T cells in the BM and spleen (Fig. 5B), suggesting that Opn does not affect the size (homeostatic proliferation) of the memory CD8+ T cell pool.

Opn deficiency favors the generation of memory precursor effector CD8+ T cells

Following a viral or bacterial infection, naive CD8+ T cells expand and differentiate into effector CD8+ T cells, which can be categorized as memory precursor effector CD8+ T cells (KLRG1lowCD127high) or terminal effector CD8+ T cells (KLRG1highCD127low). The former population has a potential to become long-lived memory CD8+ T cells, whereas the latter population does not (3). Moreover, a recent study has demonstrated that in influenza virus-infected mice, the KLRG1lowCD127high population within NP-specific effector CD8+ T cells is also identified as memory precursors (29). Because increased Opn levels were detectable in the mLN and spleen during the acute phase of influenza virus infection (Supplemental Fig. 1A), we next examined whether Opn affects the generation of memory precursor effector CD8+ T cells. We observed that NP-specific effector CD8+ T cells generated in Opn KO mice at day 9, which is the peak of response, contained a higher proportion of memory precursor effector T cells, which were defined as a KLRG1lowCD127high population, compared with the cells in Opn WT mice (Fig. 6A). Additionally, the proportion of terminal effector CD8+ T cells, which were defined as a KLRG1highCD127low population, was lower in Opn KO mice (Fig. 6A). The expression levels of the other activation markers, including CD62L, CD44, CD27, CD122 (IL-15R β-chain), and CD25, by NP-specific effector CD8+ T cells, as well as by naive CD8+ T cells, were comparable between the two groups (Fig. 6B).

The transcription factor T-bet is implicated in the formation of terminal effector CD8+ T cells (3). Quantitative RT-PCR analysis of NP-specific effector CD8+ T cells isolated from spleens at 9 d postinfection showed that T-bet expression was induced in NP-
specific effector CD8+ T cells in Opn KO mice, but at a significantly lower level than that observed in cells in Opn WT mice (Fig. 6C). These data suggest that Opn is involved in the expression of T-bet in virus-specific effector CD8+ T cells, whereas its absence favors the generation of memory precursor effector CD8+ T cells.

**FIGURE 4.** Opn-deficient mice maintain increased numbers of virus-specific memory CD8+ T cells. Mice were infected i.n. with HKx31. At 60 d following infection, mLNs and spleens were obtained, and absolute numbers of NP-specific CD8+ T cells (A) and frequencies of NP-specific CD8+ T cells within the total CD8+ T cells (B) were determined by flow cytometry. Data are representative of three independent experiments with more than six mice per group. C, At 60 d following infection, the expressions of CD127 and CD62L by NP-specific CD8+ T cells in the mLN and spleen were determined by flow cytometry. Histograms and plots are gated on NP-specific CD8+ T cells. Numbers represent the percentages of CD62Llow cells within the NP-specific CD8+ T cells. Data are representative of three independent experiments. At 17 d following infection, mLNs and spleens were obtained, and absolute numbers of NP-specific CD8+ T cells (D) and frequencies of NP-specific CD8+ T cells within the total CD8+ T cells (E) were determined by flow cytometry. Data are presented as the means ± SEM. Data are representative of three independent experiments with four mice per group. *p < 0.05, **p < 0.01.
Virus Ag-loaded DCs, but not virus-specific CD8+ T cells, produce Opn

We next asked whether Opn is acting intrinsically or extrinsically in virus-specific CD8+ T cells to regulate T-bet expression and memory precursor effector CD8+ T cell generation, since Opn is expressed by several immune cells, including T cells and APCs (30). To address this question, we first examined Opn expression by NP-specific effector CD8+ T cells at 9 d postinfection. Surprisingly, NP-specific effector CD8+ T cells did not produce Opn even after in vitro NP peptide stimulation (Fig. 7A, left panel), although these cells were capable of producing large amounts of effector cytokine IFN-γ (Fig. 7A, right panel). As shown in Supplemental Fig. 1A, increased Opn levels were detectable in the mLN and spleen shortly after virus infection. Therefore, we next investigated whether DCs are capable of producing Opn in response to the stimulation with influenza virus using GM-CSF–induced BMDCs. We found that BMDCs stimulated with heat-inactivated influenza virus produced large amounts of Opn (Fig. 7B). Moreover, we also found that DCs isolated from the spleen at day 7 postinfection produced Opn (Fig. 7B). These data suggest that virus Ag-loaded DCs, but not virus-specific CD8+ T cells, are the Opn-producing cells in the mLN and spleen. Thus, Opn negatively regulates the generation of memory precursor effector CD8+ T cells by a cell-extrinsic rather than a cell-intrinsic mechanism.

Opn regulates the production of IL-12 by DCs through an RGD sequence–independent mechanism

Recently, several studies have revealed that the inflammatory cytokine IL-12 is a key factor in modulating the expression of the transcription factors T-bet and eomesodermin during a CD8+ T cell response (3, 11). High amounts of IL-12 during LCMV infection increase the expression of T-bet and induce the generation of terminal effector CD8+ T cells, whereas low amounts of IL-12 decrease the expression of T-bet and favor the generation of memory precursor effector CD8+ T cells (3). Because Opn is a Th1-type cytokine (17), we investigated whether Opn deficiency affects IL-12 production using BMDCs. Although both Opn WT BMDCs and Opn KO BMDCs showed increased IL-12p40 production in response to the stimulation with heat-inactivated HKx31, the IL-12p40 production level was significantly reduced in Opn KO BMDCs (Fig. 8A). This finding was consistent with in vivo data showing that Opn KO mice contained lower amounts of IL-12p40, as well as IL-12p70, in the mLN and spleen compared with Opn WT mice at day 9 following infection (Fig. 8B). Opn deficiency in BMDCs did not affect the expression of TLR7, which recognizes single-stranded RNA viruses such as influenza virus (31) (data not shown). To further determine the involvement of Opn in the production of IL-12 by DCs, Opn WT BMDCs were stimulated with heat-inactivated HKx31 in the presence of rOpn. We found that exogenous Opn was capable of augmenting IL-12p40 production by BMDCs (Fig. 8C). Signaling through αvβ3 integrin by binding to the RGD sequence within Opn is involved in IL-12 production by macrophages (17). To determine whether the role of αvβ3 integrin in IL-12 production in macrophages can be observed also in DCs, Opn WT BMDCs were stimulated with heat-inactivated HKx31 in the presence of anti-CD61 (β3 integrin) Ab. Although BMDCs expressed β3 integrin (Fig. 8D), treatment with anti-CD61 Ab did not affect IL-12p40 production (Fig. 8E). Additionally, treatment with GRGDS peptide, which inhibits the interaction between RGD-recognizing integrins and Opn, had no effect on IL-12p40 production (Fig. 8E). These findings suggest that Opn regulates IL-12 production by DCs through an RGD sequence-independent mechanism. Furthermore, the levels of CD212 (IL-12R β-chain) expression by NP-specific effector CD8+ T cells were similar between Opn WT and Opn KO mice (Fig. 8F). Thus, we hypothesize that the autocrine and/or paracrine signaling induced by Opn promotes IL-12 production by DCs during the acute phase of influenza virus infection, and this results in the upregulation of T-bet expression in NP-specific effector CD8+ T cells and the promotion of terminal effector CD8+ T cell generation.

Virus-specific memory CD8+ T cells generated in Opn-deficient mice are functional

Memory T cells can proliferate rapidly and robustly in response to the secondary Ag stimulation (32). Because we found that Opn KO mice had increased numbers of NP-specific memory CD8+ T cells following influenza virus infection, we examined whether NP-specific memory CD8+ T cells generated in Opn KO mice are functional. At 60 d postinfection, splenocytes obtained from Opn KO mice had increased numbers of NP-specific memory CD8+ T cells (3). Because Opn is a Th1-type cytokine (17), we investigated whether Opn deficiency affects IL-12 production using BMDCs. Although both Opn WT BMDCs and Opn KO BMDCs showed increased IL-12p40 production in response to the stimulation with heat-inactivated HKx31, the IL-12p40 production level was significantly reduced in Opn KO BMDCs (Fig. 8A). This finding was consistent with in vivo data showing that Opn KO mice contained lower amounts of IL-12p40, as well as IL-12p70, in the mLN and spleen compared with Opn WT mice at day 9 following infection (Fig. 8B). Opn deficiency in BMDCs did not affect the expression of TLR7, which recognizes single-stranded RNA viruses such as influenza virus (31) (data not shown). To further determine the involvement of Opn in the production of IL-12 by DCs, Opn WT BMDCs were stimulated with heat-inactivated HKx31 in the presence of rOpn. We found that exogenous Opn was capable of augmenting IL-12p40 production by BMDCs (Fig. 8C). Signaling through αvβ3 integrin by binding to the RGD sequence within Opn is involved in IL-12 production by macrophages (17). To determine whether the role of αvβ3 integrin in IL-12 production in macrophages can be observed also in DCs, Opn WT BMDCs were stimulated with heat-inactivated HKx31 in the presence of anti-CD61 (β3 integrin) Ab. Although BMDCs expressed β3 integrin (Fig. 8D), treatment with anti-CD61 Ab did not affect IL-12p40 production (Fig. 8E). Additionally, treatment with GRGDS peptide, which inhibits the interaction between RGD-recognizing integrins and Opn, had no effect on IL-12p40 production (Fig. 8E). These findings suggest that Opn regulates IL-12 production by DCs through an RGD sequence-independent mechanism. Furthermore, the levels of CD212 (IL-12R β-chain) expression by NP-specific effector CD8+ T cells were similar between Opn WT and Opn KO mice (Fig. 8F). Thus, we hypothesize that the autocrine and/or paracrine signaling induced by Opn promotes IL-12 production by DCs during the acute phase of influenza virus infection, and this results in the upregulation of T-bet expression in NP-specific effector CD8+ T cells and the promotion of terminal effector CD8+ T cell generation.

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FIGURE 5. Opn does not affect the size of memory CD8+ T cell pool. A, At 60 d following infection with HKx31, the frequencies of NP-specific CD8+ T cells within the total CD8+ T cells in the BM were determined by flow cytometry. Data are representative of three independent experiments with more than four mice per group. B, BM and spleens were obtained from naïve Opn WT and Opn KO mice, and the frequencies of CD62L<sup>hi</sup>CD44<sup>hi</sup> CD8+ T cells (right panel) within the total CD8+ T cells were determined by flow cytometry. Data are presented as the means ± SEM. Data are representative of two independent experiments with five mice per group. *p < 0.05.
WT and Opn KO mice were labeled in vitro with CFSE and stimulated with NP peptide. After 72 h, we assessed the proliferation of NP-specific memory CD8+ T cells by analyzing CFSE dilution. NP-specific memory CD8+ T cells generated in Opn KO mice, as well as cells in Opn WT mice, retained the ability to proliferate in response to the stimulation with NP peptide, as most cells had divided many times (Fig. 9A). To analyze the capacity of NP-specific memory CD8+ T cells generated in Opn KO mice to respond to a secondary challenge in vivo, mice were first infected i.n. with HKx31, then challenged i.p. with serologically distinct PR8 strain 60 d later. At 6 d after secondary infection, Opn KO mice contained significantly higher numbers of NP-specific CD8+ T cells in the spleen than did Opn WT mice (Fig. 9B). This reflects the persistence of more memory CD8+ T cells rather than the extensive proliferation of memory CD8+ T cells in Opn KO mice, since the in vitro data showed that the degree of memory CD8+ T cell proliferation was comparable between Opn WT and Opn KO mice (Fig. 9A). We next assessed the in vivo cytolytic activity of NP-specific memory CD8+ T cells by injecting CFSE-labeled target cells presenting the NP epitope into recipient Opn WT or Opn KO mice and measuring target cell clearance. Naive Opn WT and Opn KO recipients did not eliminate NP-pulsed target cells (Fig. 9C, left panels), whereas primed Opn KO mice, as well as primed Opn WT mice, showed complete elimination of NP-pulsed target cells (Fig. 9C, right panels), suggesting that NP-specific memory CD8+ T cells generated in Opn KO mice have unimpaired cytolytic activity.

We finally attempted to determine whether the increased number of NP-specific memory CD8+ T cells in Opn KO mice directly affects viral control during a secondary response. Unexpectedly, both primed (memory) Opn WT and Opn KO mice were able to protect from a lethal PR8 challenge (Fig. 10A). However, we found that primed Opn KO mice had significantly lower viral loads than did primed Opn WT mice at day 4 after challenge (Fig. 10B), although both mice eventually cleared virus from the lungs by day 7 (data not shown; virus titers were under the limit of detection). Importantly, there was no difference in viral loads at day 4 between naive Opn WT and Opn KO mice, suggesting that lower viral loads observed in primed Opn KO mice was due to the increased number of memory CD8+ T cells. In fact, primed Opn KO mice contained more virus-specific CD8+ T cells in the mLN at day 4 after challenge compared with primed Opn WT mice (Fig. 10C), whereas naive mice did not generate virus-specific CD8+ T cells at this period (data not shown). Therefore, increased number of memory CD8+ T cells in Opn KO mice affects the control of influenza virus during the early stage of the recall response. Note that the enhanced memory CD8+ T cell response in Opn KO mice was associated with the increased levels of inflammatory cytokine such as IFN-γ and IL-10 and slightly higher levels of TNF-α and IL-6 in the lungs (Fig. 10D).

**FIGURE 6.** Augmented generation of KLRG1lowCD127high memory precursor cells in Opn-deficient mice. Mice were infected i.n. with HKx31. A, At 9 d following infection, spleens were obtained and the expressions of CD127 and KLRG1 by NP-specific CD8+ T cells were determined by flow cytometry (right panels). CD127 and KLRG1 expressions by naive CD62L+CD8+ T cells were also determined (left panels). Plots are gated on CD62L+CD8+ T cells (for naive cells) and NP-specific CD8+ T cells (for effector cells). The frequencies of KLRG1highCD127low and KLRG1lowCD127high cells within the NP-specific CD8+ T cell populations are shown in bar graphs. Data are presented as the means ± SEM. Data are representative of three independent experiments with four mice per group. B, The expressions of CD62L, CD44, CD27, CD122, and CD25 by naive CD8+ T cells (upper panels) and NP-specific CD8+ T cells (lower panels) were analyzed by flow cytometry. Shaded histograms represent Opn WT mice. Open histograms represent Opn KO mice. C, At 9 d following infection, NP-specific CD8+ T cells in the spleens were sorted and analyzed by real-time RT-PCR for T-bet expression. The amount of T-bet was normalized against the expression of G3PDH (n = 7). Data are presented as the means ± SEM. *p < 0.01, **p < 0.005.
Discussion

During acute viral and bacterial infections, large numbers of Ag-specific CD8+ T cells are generated. After Ag clearance, most effector CD8+ T cells undergo apoptotic cell death, leaving behind a small population of memory cells. The formation of immunological memory is an important feature of adaptive immunity, because memory cells have special features that differentiate them from naive and effector CD8+ T cells regarding life span, proliferative ability, and effector cytokine production in response to the Ag stimulation. Thus, understanding the intrinsic/extrinsic factors that regulate formation of memory CD8+ T cells is critical not only for the generation of effective memory responses during infection but also for effective vaccination. In the present study, we have examined the role of Opn in the generation of memory CD8+ T cells during influenza virus infection. We show that Opn KO mice are capable of generating unimpaired virus-specific effector CD8+ T cells following primary infection, but they have substantially more memory CD8+ T cells because of increased formation of memory precursor effector CD8+ T cells during the acute phase of infection.

A previous study showed that Opn promotes the survival of activated T cells by regulating the expression of the proapoptotic proteins, resulting in the persistence of inflammation in the CNS (20). However, in the present study, we found that Opn WT and Opn KO mice had similar numbers of virus-specific CD8+ T cells at the end of the contraction phase (day 17), indicating that Opn does not significantly affect the survival of virus-specific CD8+ T cells. The observed difference between the EAE model and the infectious model may reflect the persistence of high levels of Opn expression. In the EAE model, upregulation of Opn expression was detectable in microglia during the acute phase as well as during the remission phase (33). In contrast, in the setting of virus infection, Opn levels in the mLN and spleen reached a peak between day 3 and day 7 and returned to almost basal levels between day 10 and day 13. Thus, it is likely that Opn at the basal level is neither able or sufficient to affect the contraction of virus-specific CD8+ T cells.

Common γ-chain cytokines such as IL-7 and IL-15 support the maintenance of memory CD8+ T cells after Ag clearance (1, 34, 35). The persistence of more virus-specific memory CD8+ T cells in Opn KO mice is not explained by extensive homoeostatic proliferation since the levels of CD127 and CD122 expression by NP-specific memory CD8+ T cells were similar between Opn WT and Opn KO mice (Fig. 4C and data not shown). Furthermore, we found no differences in IL-7 and IL-15 mRNA expression in the mLN and spleen between Opn WT and Opn KO mice at 60 d postinfection (Supplemental Fig. 2). BM is a major reservoir and the site of recruitment of memory CD8+ T cells (27), and this was evident in our study (Fig. 5A). Interestingly, a recent report has demonstrated that within the BM niche, Opn, expressed by endosteal bone surface, acts as a physiological negative regulator of hematopoietic stem cell proliferation (28). However, it seems unlikely that such a mechanism is operative in virus-specific memory CD8+ T cells in BM. Our data demonstrated that naive adult Opn KO mice contained similar numbers of CD8+ T cells that show a memory phenotype in the BM compared with Opn WT mice (Fig. 5B), suggesting that Opn at physiological levels does not affect the memory CD8+ T cell pool in the BM.

CD4+ T cells are required not only for cytotoxic CD8+ T response but also for memory CD8+ T cell generation. The requirement of CD4+ T cells for CTL responses is mostly dependent on the type of pathogens. CD4+ T cells are necessary for the generation of primary CTL responses against HSV infection (36), whereas optimal CTL responses, but not memory CD8+ T cell generation, against L. monocytogenes and LCMV can occur even in the absence of CD4+ T cells (1, 37). The critical role of CD4+ T cells in the generation of memory CD8+ T cells is also evident in influenza virus infection (38). However, it is not likely that the increase in the number of memory CD8+ T cells in Opn KO mice is due to the increased number or functions of CD4+ T cells since Opn WT and Opn KO mice showed comparable numbers of IFN-γ+CD4+ T cells in the spleen (Supplemental Fig. 3A) and similar levels of virus-specific Ab generation at 10 d postinfection (Supplemental Fig. 3B).

Signals from IL-12 enhance T-bet expression in CD8+ T cells and favor the generation of terminal effector CD8+ T cells (3, 11). We found that virus-specific effector CD8+ T cells generated in Opn KO mice expressed lower levels of T-bet, and this might be due to weaker signals from IL-12. A previous study has demonstrated that T-bet−/− P14 cells (LCMV GP33-41-specific CD8+ T cells) express lower levels of CD122 compared with T-bet+/+ P14 cells. However, the lowered expression of T-bet in T-bet−/− P14 cells is sufficient to maintain CD122 expression (3), consistent with our data showing that the expression level of CD122 in virus-specific effector CD8+ T cells generated in Opn KO mice was similar to that in cells in Opn WT mice (Fig. 6B). Opn expression has been observed to be induced in T cells through TCRS; however, the intrinsic role of Opn in the T cell development is not well understood. A previous report has demonstrated that T-bet regulates Opn expression in activated CD4+ T and CD8+ T cells, which results in the skewing of CD4+ T and CD8+ T cells toward the Th1 and Tc1-type CD8+ T cell pathways (39). However, it seems unlikely that such a T-bet/Opn axis is operative in virus-

FIGURE 7. Virus Ag-loaded DCs produce Opn. A. At 9 d following infection with HKx31, splenocytes from Opn WT mice were cultured with or without NP peptide for 24 h and NP-specific CD8+ T cells were sorted. Productions of Opn or IFN-γ by 5 × 105 sorted NP-specific CD8+ T cells during 48 h in vitro culture were measured by ELISA. Data are presented as the means ± SEM. Data are representative of two independent experiments with four mice per group. B. BMDCs (1 × 105) from Opn WT mice were stimulated with heat-inactivated HKx31 virus for 24 h (left panel) and 1.5 × 105 sorted splenic DCs at day 7 postinfection were cultured for 48 h (right panel), and Opn levels in the culture supernatants were analyzed by ELISA (n = 9). Data are presented as the means ± SEM.
specific effector CD8\(^+\) T cells since purified NP-specific effector CD8\(^+\) T cells from Opn WT mice are not capable of producing Opn even after in vitro stimulation despite being fully functional as evidenced by their production of large amounts of effector cytokine IFN-\(\gamma\) (Fig. 7A).

The level of Opn was increased in the mLN and spleen in the acute phase of virus infection. At day 6 postinfection, a large amount of influenza virus RNA was detected in the lungs, whereas virus RNA was hardly detectable in the mLN and spleen (Supplemental Fig. 1B). Following intranasal virus infection, virus Ag-loaded DCs migrate into the mLN from the lungs and participate in the priming of naive T cells (25). We found that the generation of virus-specific CD8\(^+\) T cells in the spleen reached a peak at day 10 postinfection (Fig. 3B) and DCs isolated from the spleen at day 7 produced Opn (Fig. 7B). These data suggest that virus Ag-loaded DCs also migrate into the spleen and/or that splenic DCs are stimulated with virus Ag that reached the spleen through blood flow. Furthermore, we detected the increased number of DCs in the spleen, as well as in the mLN, during the acute phase of virus infection (Supplemental Fig. 1C). Therefore, the upregulation of Opn levels in the mLN and spleen reflects the increased number of Opn-producing DCs, although we do not exclude the possibility that small amounts of influenza virus contribute to the increased levels of Opn in these tissues. Note that DCs may not be the only source of Opn in this experimental setting. It is possible that other cells, including macrophages, also contribute to the production of Opn.

IL-12 is a critical mediator for protective immune response against microbial infection. Concordantly, IL-12p35–deficient mice fail to efficiently eradicate \textit{L. monocytogenes} (12). A previous study with IL-12p40–deficient mice has demonstrated that IL-12 is not required for the efficient clearance of influenza virus from the lungs or for the optimal cytokine production by virus-specific CD8\(^+\) T cells (40), consistent with our data demonstrating that Opn KO mice expressed low amounts of IL-12 and showed unimpaired virus clearance from the lungs. Also, note that Opn KO mice generated unimpaired antiviral immune responses following primary infection, although Opn KO mice had fewer terminal effector CD8\(^+\) T cells than did Opn WT mice. This might be explained by a previous finding that terminal effector CD8\(^+\) T cells and memory precursor effector CD8\(^+\) T cells have comparable levels of cytotoxic activity and IFN-\(\gamma\) production (3).

Opn is a chemotactic factor for inflammatory cells, including neutrophils and macrophages (21). In this study, we found, in contrast to what we had expected, that Opn KO mice showed unimpaired recruitment of inflammatory cells into the lungs.

![FIGURE 8. Opn promotes IL-12 production by DCs. A, BMDCs from Opn WT and Opn KO mice were stimulated with heat-inactivated HKx31 virus for 24 h, and IL-12 p40 levels in the culture medium were analyzed by ELISA. Data are presented as the means ± SEM. Data are representative of two independent experiments with five mice per group. B, At 9 d following infection with HKx31, mLN and spleens were obtained and homogenates were assessed for IL-12p40 and IL-12p70 concentrations. Data are presented as the means ± SEM. Data are representative of two independent experiments with five mice per group. C, BMDCs from Opn WT mice were stimulated with heat-inactivated HKx31 virus for 24 h in the presence of rOpn, and IL-12 p40 levels in the culture medium were analyzed by ELISA. Data are presented as the means ± SEM. Data are representative of two independent experiments with five mice per group. D, The expression of CD61 (\(\beta_3\) integrin) by BMDCs from Opn WT mice was analyzed by flow cytometry. Shaded histogram represents unstained control. E, BMDCs from Opn WT mice were stimulated with heat-inactivated HKx31 virus for 24 h in the presence of anti-CD61 Ab or GRGDSP peptide, and IL-12 p40 levels in the culture medium were analyzed by ELISA. Data are presented as the means ± SEM. Data are representative of two independent experiments with five mice per group. F, At 9 d following infection, the expressions of IL-12R \(\beta\)-chain by NP-specific CD8\(^+\) T cells in the mLN and spleens were determined by flow cytometry. Histograms are gated on NP-specific CD8\(^+\) T cells. Shaded histograms represent Opn WT mice and open histograms represents Opn WT mice. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.005\).](http://www.jimmunol.org/)}
Opn WT and Opn KO mice showed similar levels of inflammatory cytokines and chemokines in the lungs following primary infection. Therefore, we predict that these inflammatory factors are sufficient for the recruitment of inflammatory cells into the lungs in Opn KO mice. It has been reported that Opn regulates the development of several disorders by modulating DC migration to the draining LNs (41–43). In our study, the numbers of class II^+ CD11c^+CD103^- DCs in the mLN, which have been known as tissue DCs that recognize virus Ag in the lungs and prime CD8^+ T cells in the mLN (25), were comparable between Opn WT and Opn KO mice (Supplemental Fig. 4), consistent with the data showing that both groups generated similar numbers of NP-specific effector CD8^+ T cells in the mLN. Therefore, our data suggest that following influenza virus infection, Opn, produced by Ag-loaded DCs in the mLN and spleen, is required for further production of IL-12 by DCs rather than subsequent migration of DCs. In fact, a recent report demonstrated that IFN-β plays a critical role in DC migration into the mLN following influenza virus infection (44).

The increased number of memory CD8^+ T cells in Opn KO mice correlated with enhanced viral control during the early stage of the recall response. However, note that enhanced memory CD8^+ T cells in Opn KO mice correlated with a viral control during the early stage of the recall response. Naive mice were i.n. infected with PR8 as a primary infection. Primed (memory) mice were i.n. challenged with PR8 at 60 d after primary HKx31 infection. A, Mice that had lost >30% of their original body weight were considered moribund and euthanized. Data are representative of two independent experiments with more than six mice per group. B, Lungs were removed at 4 d after challenge and virus titers were determined by Madin–Darby canine kidney cell assay. Data are presented as the means ± SEM. Data are representative of three independent experiments with more than five mice per group. C, At 4 d following challenge, mLNs were removed and absolute numbers of NP-specific CD8^+ T cells were determined by flow cytometry. Data are representative of three independent experiments with more than five mice per group. D, Lungs were removed at day 4 after challenge and lung homogenates were assessed for IFN-γ, IL-10, TNF-α, and IL-6 concentrations. Data are presented as the mean ± SEM. Data are representative of two independent experiments with six mice per group. *p < 0.05, **p < 0.01.
T cell response was also associated with accelerated pulmonary inflammatory responses (Fig. 10D). Therefore, it seems that there is a tradeoff between rapid viral clearance and accelerated lung damage.

In summary, our results demonstrate that Opn deficiency promotes the generation of memory precursor effector CD8+ T cells, without affecting primary immune responses against influenza virus infection, which results in the induction of effective recall response. In other words, Opn, induced by virus Ag-loaded DCs during the acute phase of infection, favors the generation of terminal effector CD8+ T cells rather than memory precursor effector CD8+ T cells. Because influenza virus has varying antigenicity, induction of more terminal effector CD8+ T cells rather than memory precursor effector CD8+ T cells might be reasonable in the context of influenza virus infection. We hypothesize that the regulation of IL-12 production in DCs during the acute phase of infection is one possible mechanism responsible for the increased number of memory precursor effector CD8+ T cells in Opn KO mice, although there might be other mechanisms behind this phenomenon. Thus, our findings provide new insight for the role of Opn in adoptive immune responses and may facilitate induction of effective memory responses and the development of more effective vaccines.

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Disclosures

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References


