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Division of labor between subsets of lymph node Dendritic Cells determines the specificity of the CD8 recall response to influenza infection

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Abstract

Cytotoxic T lymphocytes are important targets for vaccines against a wide variety of infections that enter the body via mucosal tissues. To induce effective immunity these vaccines must include the most protective epitopes and elicit rapid recall responses at the site of infection. Although live attenuated viruses are sometimes used to induce cellular immunity against recurrent influenza infections, the mechanisms that determine the magnitude of the response to individual viral components are very poorly defined. Heterosubtypic infections in B6 mice illustrate an additional level of complexity, when the antigen-specificity of the response shifts dramatically between primary and secondary challenge. This model provides a unique opportunity to identify the mechanisms that regulate memory CD8 T cell reactivation *in vivo* and control the specificity of the recall response by pathogen-specific CTL. We show that multiple factors contribute to the changing pattern of immunodominance during secondary infection, including the location of the memory CD8 T cells at the time of reinfection and their ability to directly recognize migratory CD103⁺ DC as they arrive in the lung draining lymph node.

Keywords

Antigen presentation; immunodominance; memory CD8 T cells

Introduction

Cytotoxic T lymphocytes (CTL) play an important role in immunity against many intracellular pathogens, however the level of protection can be influenced by many factors including the breadth of the response and the ability of the pathogen-specific T cells to find newly infected host cells before the infection spreads. Viruses that undergo constant mutation rapidly evade protective antibody responses and can repeatedly infect the same host. In some cases virus-specific CTL which recognize highly conserved internal viral proteins can provide protection against the new infection by reducing or preventing early viral replication. This protection relies on rapid reactivation of pathogen-specific memory CD8 T cells which must reach the site of viral replication before the infection spreads.

Dendritic cells (DC) are a specialized family of antigen-presenting cells (APCs) that link innate recognition of invading pathogens to the generation of an appropriate adaptive immune response. Upon activation, the DCs undergo a well-defined maturation process

when they become less endocytic and more capable of presenting antigen to naïve lymphocytes. The subsets of DC that participate in priming pathogen-specific CTL are well described [1;2] however the APCs that participate in memory CD8 T cell reactivation *in vivo* are far less clearly defined. DC are known to participate in the recall response to influenza infection [3-5] but no specific roles for individual subsets have been defined. *In vitro* studies originally suggested that memory T cells have minimal requirements for costimulatory signals and can respond to a wide range of APCs including non-professional cells such as macrophages [6]. Recent studies indicate that the picture is considerably more complicated *in vivo*, since data from our lab and others have shown that central memory CD8 T cells (T_{cm}) fail to recognize some antigen-bearing DCs in the draining lymph nodes (DLN) of the respiratory tract during the recovery from influenza infection [7;8]. Here we show that the ability of influenza-specific memory CD8 T cells to recognize specific APCs in the mediastinal lymph node (MLN) plays an instrumental role in determining the specificity of the CD8 recall response during heterosubtypic challenge.

The influenza genome encodes multiple peptide sequences which are recognized by antigen-specific CD8 T cells. The individual epitopes have been classified as dominant or subdominant based on the size of the T cell response that is elicited during infection. Serologically distinct strains of influenza virus were previously used to follow primary and secondary responses by virus-specific CD8 T cells with minimal interference from neutralizing antibodies [9]. In B6 mice primary infection with the reassortant HKx31 virus produced a co-dominant response by nucleoprotein (NP) and acid polymerase (PA)-specific CD8 T cells but the dominance hierarchy changed substantially during heterosubtypic challenge with the serologically distinct strain A/PR8/8/68 (PR8) when NP-specific CD8 T cells became dominant by a ratio of more than 5:1 in most tissues [10]. The shift toward NP dominance was preserved when the order of the infections was reversed [6]. Although some progress has been made toward elucidating the mechanisms that promote this change in epitope dominance [6;11;12] our understanding of the *in vivo* process remains incomplete. The revised dominance hierarchy is likely to be important for protective immunity since there is evidence that NP-specific CD8 T cells are more effective at clearing infectious virus from lungs, than PA-specific cells [13].

Here 5-Bromo-2-deoxyuridine (BrdU) incorporation has been used to follow the early proliferative response by endogenous influenza-specific memory CD8 T cells during heterosubtypic challenge. We show that NP-specific CD8 T cells undergo an early proliferative response in the DLN ~4 days after recall, while PA-specific CD8 T cells remain relatively dormant. The early proliferative response of the NP-specific memory CD8 T cells was dependent on their ability to directly recognize CD103⁺ DC, which arrived in the MLN soon after reinfection. In contrast, the PA-specific memory CD8 T cells that were recovered from the MLN preferentially recognized CD8⁺ DC and entered the recall response with slightly delayed kinetics.

Results

NP-specific CD8 T cells undergo an early proliferative response in the MLN

To determine where virus-specific memory CD8 T cells begin cell division after heterosubtypic challenge, HKx31 primed mice were reinfected with PR8 and groups of three mice were given BrdU on consecutive days after reinfection. The virus-specific CD8 T cells in different tissues were analyzed for BrdU incorporation 4hrs later, using MHC I tetramer analysis (Figure 1). The ratios of NP31 and PA-specific CD8 T cells remained stable until ~4d after PR8 infection (Figures 1A and S1A) when the numbers of BrdU⁺ CD8 T cells in the lungs and MLN began to increase dramatically (Figure 1B and S1B). A shift toward NP dominance became visible ~5dpi when higher percentages of NP31 than PA-specific CD8 T

cells incorporated BrdU indicating that they were recruited into the recall response more efficiently than the PA-specific cells. Shorter pulses of BrdU were used for some experiments to determine where the virus-specific T cells were located as they initiated first-strand DNA synthesis (Figure 1C). The largest numbers of BrdU+ CD8 T cells were consistently found in the lungs and MLN showing that some virus-specific memory CD8 T cells were being reactivated at both sites. However the greatest disparity in the rates of BrdU incorporation between the NP31 and PA-specific cells was found in the MLN 4-5dpi indicating that the shift toward NP dominance occurred primarily at this site (Figure 1C). During the later stages of the response (5-7dpi) NP31 and PA-specific cells incorporated BrdU at very similar rates (Figure 1D). Similar results were obtained with mice that were recalled >6 months after primary infection (Figure 1C and S2) when processed viral antigens had been cleared from the tissues [7;14].

Competition for rare APCs does not promote changing epitope dominance

Some antigen-experienced CD8 T cells maintain constitutive lytic activity and can kill their APCs during recall [15-17]. Competition for rare APCs can also influence the epitope dominance hierarchy during infection [18]. To determine whether competition for antigen-bearing APCs was responsible for the change in epitope dominance during heterosubtypic influenza infection, we used two closely related strains of influenza virus to adjust the relative precursor frequencies of recall-sensitive NP and PA-specific memory CD8 T cells before secondary challenge [19].

HKx31 and E61-13-H17 (H17) are reassortant viruses which differ by only two amino acids in the NP epitope [20;21]. Previous studies have shown that these viruses stimulate overlapping repertoires of NP-specific CD8 T cells in B6 mice [19]. To assess the extent of the overlap, groups of B6 mice were infected with H17 or HKx31 and lymphocytes in the lungs were analyzed for ratios of NP and PA-specific CD8 T cells 10dpi, using tetramers that contained the NP peptides from the HKx31 (NP31) and H17 (NP68) viruses (Figure 2A). The HKx31, H17 and PR8 viruses all encode identical PA peptides.

The HKx31 and H17 viruses both induced large effector CD8T cell responses in the lungs, which included approximately equal ratios of NP and PA-specific cells, and were cleared from the lungs with very similar kinetics [7]. The tetramer analyses showed that less than 10% of the NP68-specific CD8 T cells that had been primed during H17 infection were recognized by the NP31 tetramer, indicating that only small numbers of NP-specific memory CD8 T cells were likely to participate in the recall response to PR8 infection. Some NP31-specific memory CD8 T cells which expressed CD11a at high levels were detected in the H17 primed animals 60dpi (Figure S3A). Since HKx31 and PR8 encode identical internal proteins all of the NP31-specific cells had the potential to participate in the recall response in the HKx31 primed animals.

To determine whether low precursor frequencies of NP31-specific memory CD8 T cells in the H17 primed mice were sufficient to enhance the response by the PA-specific CD8 T cells during recall, separate groups of mice were primed with H17 and challenged with PR8 30dpi. The PA and NP31 tetramers were used to follow the changing ratios of virus-specific CD8 T cells (Figure 2B). Some mice also received BrdU on 5 or 7dpi and were analyzed for dividing virus-specific CD8 T cells 4h later (Figure 2C). Although there were much smaller numbers of NP31-specific CD8 T cells in the circulation of the H17 primed mice at the time of infection, than in the HKx31 primed animals, they were still recruited into the secondary response more efficiently than the PA-specific cells causing the ratios in the lungs to equalize by 7dpi (Figure 2B). Although small numbers of NP31-specific CD8 T cells were detected in the unprimed mice by 7d after PR8 infection, there were much higher numbers in the H17 primed animals which showed that the recall response was predominantly derived

from reactivated memory CD8 T cells (Figure S3B). Overall these data indicate that competition for APC was not a principal cause of the shift toward NP dominance after reinfection [19].

Entry into the MLN is a rate-limiting step for early proliferation by NP-specific cells after transfer

Previous studies have shown that NP antigen is produced at higher concentrations than PA antigen during influenza infection [11]. A mathematical model has also suggested that high concentrations of NP antigen contribute to the change in epitope dominance after recall [22]. In the absence of any other variables we would expect to find similar patterns of epitope dominance in intact mice and transfer recipients that receive equal ratios of NP and PA-specific memory CD8 T cells before infection. Although the responses of transferred memory CD8 T cells have been analyzed previously conflicting results were obtained. One study found more a balanced response after transfer than the ratios found in intact animals, while others reported similar shifts toward NP-dominance in both groups of animals [6;19]. We broadened the approach to determine whether the timing of the transfers, tissue-of-origin or numbers of donor CD8 T cells substantially influenced the specificity of the secondary response.

To generate virus-specific CD8 T cells for transfer, congenic CD45.1+ mice were infected with HKx31. Lymphocytes were isolated from different tissues either 1 or >6 months after infection for transfer to CD45.2+ recipients. Total lymphocytes were used for these studies to avoid interference from residual staining antibodies or a loss of activated CD8 T cells during purification. The donor CD8 T cells were phenotyped (Figure S4A and B) and analyzed for ratios of NP31 and PA-specific cells at the time of transfer. One day after transfer, the recipient mice were infected with PR8 and the ratios of NP31 and PA-specific CD8 T cells were measured 7dpi (Figure 3A & 3B).

The donor lymphocytes that were harvested 30dpi contained approximately equal ratios of NP31 and PA-specific cells in all of the tissues, except the MLN where there were slightly more NP than PA-specific cells (~3:1) and some of the NP-specific cells showed signs of a response to recent antigen stimulation (CD69+, CD127-) as in our previous studies [7]. The NP-specific CD8 T cells in the other lymphoid tissues were not activated, but universally lacked CD62L expression (Figure S4A). Relatively few PA-specific CD8 T cells were activated and they included some CD62L+ cells in the lymphoid tissues [23]. All of the donor lymphocytes that were harvested >6 months after HKx31 infection contained approximately equal numbers of NP and PA-specific CD8 T cells which universally expressed markers that are characteristic of Tcm cells, including CD62L (Figure S4B).

Secondary infection with PR8 induced substantial increases in the numbers of transferred virus-specific CD8 T cells in all of the recipient animals, but did not result in dramatic skewing toward NP dominance 7dpi in any of the recipients (Figure 3A & 3B). Very similar results were obtained when donor T cells were harvested either one or six months after HKx31 infection. Increasing the numbers of transferred CD8 T cells did not substantially change the results except in the MLN where there were slightly higher numbers of NP than PA-specific CD8 T cells after infection (Figure S4C).

Our BrdU studies showed that the MLN is an important site of memory CD8 T cell reactivation (Figure 1) however the MLNs of the naïve recipient mice were very small and could not accommodate many virus-specific CD8 T cells after transfer. To increase the size of the MLN before transfer we used mice that were recovering from a prior unrelated respiratory virus infection as recipient animals (Figure 3C). The mice were challenged with PR8 one day after transfer. After 7dpi there were approximately equal ratios of donor NP

and PA-specific cells in most tissues except the MLN where NP was consistently dominant. Together these data indicated that access to the MLN before reinfection was a critical step in the early proliferative response by the NP-specific memory CD8 T cells.

NP-specific memory CD8 T cells are more sensitive to reactivation by CD103+ migratory DC than PA-specific cells

Memory T cells often maintain constitutive effector functions and mount vigorous responses to infections, but also have the potential to be highly pathogenic and can cause severe immune damage in the lungs. Consequently the recall response by pathogen-specific CTL must be very tightly regulated. To determine which APCs were responsible for the early proliferative response by the NP-specific memory CD8 T cells during PR8 infection, we isolated individual subsets of DCs from the MLN (pooled from 16-20 animals) after recall and compared their ability to reactivate NP and PA-specific memory CD8 T cells *in vitro* (Figure 4).

At least five different types of DC have been identified in the lymph nodes of infected animals [24;25], including two populations of migratory DC which carry antigens from the site of infection and can be distinguished using CD103 and CD11b expression [26;27]. To isolate these subsets, CD11c+ cells were first enriched using magnetic beads and then subdivided by sterile sorting using an exclusion gate to remove unwanted cells. The CD11c+ cells were subdivided using the sorting gates shown in Figure 4C. Memory CD8 T cells were purified from the MLN of other mice that had been infected with HKx31 32d previously (n = 15-20 mice) by staining with anti-CD8 antibodies before sorting. All purified cells were gated to exclude doublets. After purification, the CD8 T cells were labeled with CFSE and cultured with individual populations of APCs for 4 days (Figure 4A and B). Similar numbers of DC were added to each well.

The MLN increased substantially in size between 1-4 days after recall (Figure 4C) when large numbers of migratory CD103+ and CD11b+ DC began to arrive from the lungs [1;26]. Although a relatively small percentage of the PA-specific memory CD8 T cells proliferated in the cultures that received CD103+ DC (20%), a much larger percentage of NP-specific CD8 T cells underwent extensive cell division in the same wells (74%). Some CD8+ DC were also recovered from the MLN 2dpi and induced proliferative responses from the virus-specific memory CD8 T cells after enrichment, but did not favor preferential expansion of the NP-specific cells. In contrast, there was very little response from either population of virus-specific CD8 T cells during restimulation with CD11b+ DC. Cumulative data from three independent experiments are shown in Figure S5. The only DC subset that induced significantly different responses from the NP and PA-specific memory CD8 T cells was the CD103+ subset. The presorted cells from these experiments were used to compare the ratios of CD103+ and CD8+ DC in the MLN before purification (Figure 4D). Cumulative data from three experiments show that there were substantially higher percentages of CD103+ than CD8+ DC in the MLN 2dpi infection (i.e. 29% + 4 and 8% + 1 of the CD11c+ cells respectively). This difference was less pronounced by 4dpi when the ratios of CD103+ DC had declined substantially (18% + 5), while the CD8+ DC were slightly increased (11% + 1). The change in the ratios of CD103+ and CD8+ DC was consistent with the *in vivo* BrdU studies which showed that the NP and PA-specific CD8 T cells proliferated at similar rates later in the recall response (Figure 1D). Although the CD11b+ DC reached very high numbers in the MLN after recall, they induced very little response from the virus-specific memory CD8 T cells during *in vitro* culture.

Together the data from these experiments are consistent with the idea that CD103+ DC act as early sentinels of infection in the lungs and trigger an early wave of memory CD8 T cell reactivation in the MLN. As the response progressed, the numbers of CD8+ DC increased

(Figure 4E) and amplified the size and breadth of the response by triggering a more diverse range of pathogen-specific CTL, including the PA-specific memory CD8 T cells.

Discussion

The epitope dominance hierarchies that are established during viral infections are highly reproducible and sometimes reveal a close relationship between the frequencies of naïve CTL which are specific for each viral epitope and the magnitude of the response to antigenic challenge [28], however the CTL response to primary influenza infection does not adhere to this pattern. Although limiting dilution studies, and quantifications of tetramer-positive T cells, have shown that the repertoire of naïve CD8 T cells in C57BL6 mice contains more PA than NP-specific CD8 T cells [29;30], the two cell populations were equally represented during the response to viral infection [10]. A recent study found that the pattern of epitope dominance in this model was closely related to the rate at which antigen-specific CTL were recruited into the response and the extent of their subsequent proliferation [30]. As a result, the numbers of NP-specific CD8 T cells were able to catch up with the PA-specific cells in most tissues before the contraction of the effector response. The responsible APCs were not identified in this study, but others found that migratory CD103+ and CD11b+ DC were both able to prime naïve NP-specific CD8 T cells *in vitro* [8;31]. The APC that prime naïve PA-specific CD8 T cells have not been examined. We have used similar approaches to analyze the characteristics of the recall response by NP and PA-specific memory CD8 T cells during heterosubtypic challenge. Our data show that antigen-presentation by CD103+ DC in the MLN induced early proliferation by the NP-specific memory CD8 T cells and caused a substantial change in the epitope dominance hierarchy over the course of the secondary effector response.

Most mature DC are short lived cells which survive *in vivo* for a few days and are quickly replaced by other APC during normal homeostasis [2;32]. Some DC are also deleted by virus-specific T cells during antigen presentation [15;16] which further accelerates the turnover of antigen-bearing APCs and promotes tight immune control during infection. We show that this rapid turnover of different DC subsets plays an important role in the kinetics of memory CD8 T cell reactivation during the recall response. It was previously shown that lymphoid-resident CD8+ DC can acquire antigens from other cells for highly efficient cross-presentation to T cells [33] and can efficiently reactivate virus-specific memory CD8 T cells after influenza infection [8]. This second study reported efficient reactivation of NP-specific memory CD8 T cells by lymphoid-resident CD8+ DC, but minimal reactivation during restimulation with a mixed population of migratory DC that were isolated from the MLN soon after influenza infection [8]. We have further dissected the reactivation properties of the influenza-specific memory CD8 T cells by examining their response to separate populations of CD103+ and CD11b+ migratory DC (Figure 4A and 4B). Our data show that NP-specific memory CD8 T cells were able to recognize CD103+ DC after isolation from the MLN 2dpi, as well as the lymphoid-resident CD8+ DC. In contrast, the PA-specific memory T cells preferentially recognized the CD8+ DC, but not the migratory CD103+ DC. Neither population of virus-specific memory CD8 T cells was efficiently reactivated by the CD11b+ DC, which were present in the MLN in very high numbers during the recall response (Figure 4C). It is possible that the large numbers of CD11b+ DC in the mixed cultures masked the response by the CD103+ DC in the earlier study [8] however it is clear that CD103+ DC can provide sufficient costimulatory signals to reactivate some influenza-specific memory CD8 T cells.

Theoretical studies have previously suggested that different concentrations of antigen contribute to the changing pattern of epitope dominance [22]. While experimental studies confirmed that NP antigen was produced at higher concentrations than PA antigen during

influenza infection [11] they did not examine the downstream impact on antigen-presentation by different DC subsets in the MLN. Our transfer studies show that a difference in the concentrations of NP and PA antigen is not sufficient to drive NP dominance during the recall response by itself, since equal populations of NP and PA-specific CD8 T cells were recovered from the recipient animals after PR8 infection (12). These data show that the difference in the antigen concentrations did not impact all APC equally and may have been relevant only during antigen-presentation by CD103+ DC in the MLN.

Previous studies have shown that CD8+ DC have a unique capacity for highly efficient antigen cross-presentation [33] and can acquire antigens by engulfing other cells during viral infections [34]. Others have shown that the NP and PA antigens differ in their ability to access the antigen cross-presentation pathway [19]. These observations combined with our studies (Figure 4) suggest that highly efficient antigen cross-presentation by CD8+ DC may be necessary to overcome low concentrations of PA antigen during memory CD8 T cell reactivation in the MLN. In contrast, we show that the NP antigen was presented to virus-specific memory CD8 T cells by a more diverse range of APCs [6] which included migratory CD103+ DC in the MLN (Figure 4A).

Peripheral CD103+ DC are developmentally related to CD8α+ DC [35] and are capable of some antigen cross-presentation *in vivo* [1;31] but the relative efficiency of the processing pathway has not been directly compared with CD8+ DC. We have found that secondary PR8 infections in μMT and wild type B6 mice produce very similar shifts toward NP dominance during heterosubtypic challenge (data not shown), which indicates that virus-specific antibodies are not required for the early reactivation of the NP-specific memory CD8 T cells. It is possible that the CD103+ DC acquired NP antigen for cross-presentation to memory CD8 T cells using an antibody-independent mechanism of phagocytosis. Alternatively, some of the CD103+ DC may have been directly infected with influenza virus before leaving the lungs and were thus able to reactivate the NP-specific memory CD8 T cells using a relatively inefficient cytosolic mechanism for processing endogenous antigens. In support of this suggestion, earlier studies showed that PR8 infection produced a more balanced pattern of immunodominance when administered together with neutralizing antibodies to block active viral infection [19]. We were also able to detect some influenza genome in migratory DC that were purified from the MLN 2d after secondary PR8 infection, using nested PCR analysis (Data not shown).

Our transfer studies previously showed that CD11b+ DC were able to stimulate naïve CD8 T cells in the MLN 30dpi, but were not recognized by similar central memory CD8 T cells [7]. Similarly, endogenous virus-specific memory CD8 T cells failed to recognize the CD11b+ DC that were isolated from the MLN during the recall (Figures 4A and 4B). Together these data suggest that CD11b+ DC are generally poor APCs for memory CD8 T cells and that their principal function during the recall response may be to provide antigens for cross-presentation by CD8+ DC, and/or enhance the response of partially-activated memory T cells which have been recently stimulated by other APCs. Others have shown that CD11b+ DC also play a role in stimulating naïve CD4 T cells after influenza infection [26].

In summary our data show that after secondary influenza infection antigen-presentation to memory CD8 T cells in the MLN is achieved primarily by CD103+ and CD8+ DC, while the more persistent CD11b+ DC play very little role. The response to the CD103+ DC was sufficiently large to reshape the epitope dominance hierarchy. We suggest that transient antigen-presentation in the MLN exclusively by short-lived CD103+ and CD8+ DCs helps protect the lungs from excessive immune damage by limiting the duration of the response by pathogenic memory CTL. It remains to be determined whether early-antigen presentation by

migratory CD103⁺ DC also helps shape the dominance hierarchy during recall responses to other intracellular pathogens.

Materials and methods

Mice and reagents

C57BL/6 and congenic CD45.1⁺ mice were purchased from Charles River through the NCI animal program. At 8-20 weeks of age, mice were anesthetized by i.p. injection with avertin (2,2,2-tribromoethanol) before i.n. infection with 600 50% egg infectious doses (EID₅₀) of HKx31 or E61-13-H17 influenza virus for primary infections. Mice were given 300 EID₅₀ of PR8 for recall responses. Stocks of influenza virus were grown in chicken eggs, titered, and stored as described previously [36]. Anesthetized mice were given 10⁴ pfu vesicular stomatitis virus (VSV) by i.n. inoculation.

Sample preparation for flow cytometry

Blood was removed from anesthetized mice by exsanguination or perfusion with PBS containing 75units/ml heparin. The lymph nodes were dissociated to produce single cell suspensions using glass slides. The spleens were pushed through cell strainers and red blood cells were lysed with ammonium chloride. Lymphocytes were released from the chopped lung tissues by digestion with 150 U/ml collagenase (Life Technologies, Rockville, MD) in RPMI, 1 mM MgCl₂, 1 mM CaCl₂ and 5% FCS, at 37°C for one hour. Filtered cells were spun on 44/67% Percoll gradients at 400g for 20 mins. Washed lymphocytes were stained with tetramers (supplied by the NIH Tetramer Facility) that were specific for the influenza virus NP₃₆₆₋₃₇₄/D^b and PA₃₂₄₋₃₃₃/D^b epitopes as described previously [9;10]. Enriched lymphocytes were stained with either PE or APC-conjugated tetramers and anti-CD8 (clone 53.6.72) for 1 h. at room temperature. All other markers were stained at 4°C using mAb specific for CD45.1, CD69, CD127, CD62L, PD-1, CD43 and CD27 (EBioscience or BD Pharmingen, San Diego CA). Fixed samples were analyzed on a Becton-Dickinson FACSCalibur or LSR-II flow cytometer and analyzed using Flowjo software (Tree Star Inc.).

DC purification, analysis and culture

MLN from pools of 16-20 animals were digested with collagenase IV (Worthington Biochemical Corp) 1mg/ml in RPMI containing 10mM HEPES, 50mM Pen/Strep, 1.2mM CaCl₂, 2%FCS, 100units/ml DNase at 37°C 20 mins and an additional 40mins after the addition of collagenase D 1mg/ml (Roche). EDTA was used to disrupt T cell/DC conjugates and CD11c⁺ cells were enriched by magnetic bead separation (Miltenyi Biotec) using antibodies to Fc receptors to prevent non-specific binding. The enriched CD11c⁺ cells were further fractionated by sterile FACS sorting using antibodies to CD11b, CD103 and CD8. Unwanted cells were excluded using antibodies against CD3, B220, NK1.1 and SIGLECF. The recovered DC were 97-99% pure. Purified CD8⁺ T cells were labeled with CFSE and cultured in RPMI containing 10% FBS and 10ul/ml rIL-2 for 4 days. Mixed cultures contained purified DCs (approx 5:1 ratio) or synthetic peptides at 1ug/ml. After 4 days the cultures were analyzed for virus-specific CD8 T cells by MHC I tetramer analysis and CFSE-dilution.

BrdU analysis

Mice received 0.8mg BrdU (Sigma, St Louis MO) in 200μl in PBS via i.p. or i.v. injection. Lymphocytes were harvested from the tissues and processed as described above. Antigen specific CD8 T cells were stained with antibodies to CD8 and MHC I tetramers at RT for 1hr as described previously [37]. The washed cells were fixed overnight with 2% paraformaldehyde and washed twice with PBS. Cellular DNA was denatured with 50 Kunitz

units of Bovine pancreas Dnase-1 (Sigma, St Louis MO) for 60 min at 37°C. The cells were washed with PBS containing 5% FCS and 0.5% IGEPAL and stained with FITC-conjugated anti-BrdU antibodies (Becton-Dickenson CA) for 45 min at 4°C.

Statistics

Unless otherwise stated the experiments were repeated twice using 3 individual animals per group. Significance was determined using an unpaired Student's T test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

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Abbreviations

CFSE	5-(and 6-) carboxyfluorescein diacetate succinimidyl ester
DLN	draining lymph nodes
MLN	mediastinal lymph node
NP	nucleoprotein
PA	Acid polymerase

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Figure 1.

NP-specific memory CD8 T cells undergo an early proliferative response in the MLN after heterosubtypic challenge.

C57BL/6 mice were infected with HKx31 and challenged with PR8 30dpi. BrdU was given by i.v. injection on the days shown and virus-specific CD8 T cells were analyzed 4h later.

A) MHC class I tetramer analysis was used to compare the ratios of NP and PA-specific CD8 T cells in different tissues. The mean percentages of tetramer+ cells within the CD8 gates are shown + SD from 4 animals. Three independent experiments gave similar results. **B)** Gated populations of CD8+ T cells were analyzed by tetramer analysis. Parentheses indicate the percentages of tetramer+ cells that incorporated BrdU. Representative animals from groups of 3 are shown.

HKx31 primed mice were recalled with PR8 either **C)** 42d and 7 months or **D)** 30d after primary infection. BrdU was given 4 days after recall and tetramer+ CD8 T cells were analyzed 2h later.

Percentages of BrdU+ cells within the tetramer gates are shown as the mean +SD from 3 individual animals. Two independent experiments gave similar results.

Figure 2.

Competition for rare APCs does not cause changing epitope dominance.

C57BL/6 mice were infected with HKx31 or E61-13-H17 influenza virus.

A) MHC tetramers were used to compare the percentages of NP31, NP68 and PA-specific CD8 T cells in the lungs 10dpi. A pool of 3 mice is shown.

C57BL/6 mice were primed with E61-13-H17 and recalled with PR8 30dpi. **B)** Percentages of NP31 and PA-specific cells within the CD8 gate are shown. **C)** BrdU was injected days 5 or 7dpi and lymphocytes were analyzed 4hrs later. Mean percentages of BrdU+ cells within the tetramer gates are shown +SD from 3 individual animals. Two independent experiments gave similar results.

Figure 3.

Entry into the MLN is a rate-limiting step in the CD8 recall response.

Lymphocytes were recovered from CD45.1+ mice (n=10) either **A)** one or **B)** 8 months after HKx31 infection and transferred to uninfected CD45.2+ recipients. The mice were recalled with PR8 24hrs after transfer and analyzed for ratios of NP and PA-specific cells 7dpi. The percentages of tetramer+ cells within the CD8 gates are shown. The ratios of NP and PA-specific cells before transfer are shown in parentheses. Three independent experiments gave similar results.

C) Lymphocytes were harvested from CD45.1+ mice (n=10) 6 months after HKx31 infection. Recipient CD45.2+ animals were preinfected with VSV 15 days before transfer. The recipient mice were infected with PR8 24h after transfer and gated populations of CD45.1+ CD8 T cells were analyzed for percentages of tetramer+ cells 7dpi. The mean +SD from 3 individual animals are shown. Two independent experiments gave similar results.

Figure 4.

NP and PA-specific memory CD8 T cells respond to different DC in the MLN.

DC were isolated from the MLN **A**) 2d or **B**) 4d after secondary PR8 infection. CD8 T cells were purified from the MLN between 30-60 days after HKx31 infection, labeled with CFSE and cultured with the purified DC. Histograms show gated NP and PA-specific CD8 T cells. Four independent experiments gave similar results.

C) Example of the sorting strategy that was used for DC isolation. The MLN were harvested from pools of 16-20 animals after secondary infection with PR8. Unwanted cells were excluded from the sorting gates using antibodies to CD3, B220, NK1.1 and SIGLECF. The plots show representative staining for cells within the CD11c gate.

D) The ratios between different DC subsets in the MLN changed as the response to influenza infection progressed. The ratios of the DC subsets were calculated as percentages within the CD11c gate. The cumulative data are the means + SD from 3 independent experiments (* P<0.05).

E) The numbers of CD8⁺ DC in the MLN increase between 2-4dpi. The numbers of DC were calculated from two independent experiments. Each data point shows DC isolated from a pool of 16-20 animals.