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Epitope-specific vaccination limits clonal expansion of heterologous naïve T cells during viral challenge

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Abstract

Despite robust secondary T cell expansion primed by vaccination, the impact on primary immune responses to heterotypic antigens remains undefined. Here we show that secondary expansion of epitope-specific memory $CD8^+$ T cells primed by prior infection with recombinant pathogens limits the primary expansion of naïve $CD8^+$ T cells with specificity to new heterologous antigens, dampening protective immunity against subsequent pathogen challenge. The degree of naïve T cell repression directly paralleled the magnitude of the recall response. Suppressed primary T cell priming reflects competition for antigen accessibility, since clonal expansion was not inhibited if the primary and secondary epitopes were expressed on different dendritic cells. Interestingly, robust recall responses did not impact antigen-specific NK cells, suggesting adaptive and innate lymphocyte responses possess different activation requirements or occur in distinct anatomical locations. These findings have important implications in pathogen vaccination strategies that depend on the targeting of multiple T cell epitopes.

Graphical abstract

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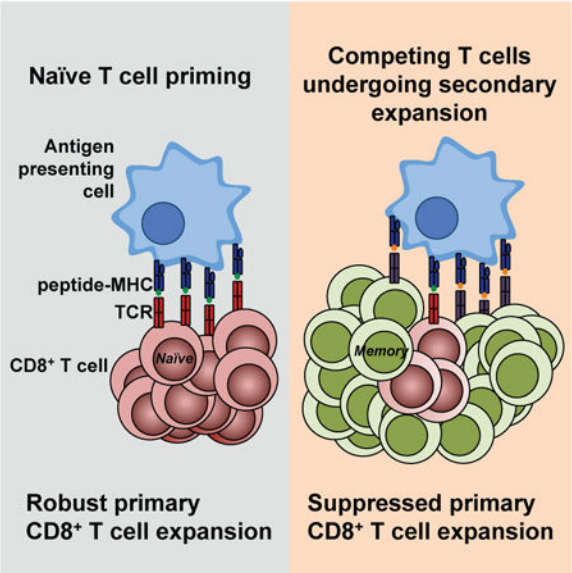

Introduction

The safety and protective efficacy of vaccination using intracellular microbial vectors such as recombinant *Listeria monocytogenes*, adenovirus, or vaccinia virus engineered to contain $CD8^+$ T cell epitopes is well-documented (Weiskirch and Paterson, 1997, Koup and Douek, 2011, Orr et al., 2007). Many of these vaccination strategies aim to generate large numbers of memory $CD8^+$ T cells that are capable of mounting a robust and protective recall response during future infection. Although the generation of more accelerated and robust secondary responses is typically considered to be beneficial, cross-reactivity of vaccine-elicited $CD8^+$ T cells can negatively impact naïve immune responses to variants of the targeted epitopes encountered during an actual pathogen exposure, a phenomenon previously termed “original antigenic sin”. Although this phrase was first described in the setting of humoral responses against repeated exposure to influenza (Davenport et al., 1953, Fazekas de St and Webster, 1966, Kim et al., 2009), this phenomenon has also been observed in recall $CD8^+$ T cell responses against variants of lymphocytic choriomeningitis virus (LCMV) in mice and dengue virus in humans (Klenerman and Zinkernagel, 1998, Mathew and Rothman, 2008, Mongkolsapaya et al., 2003). The prevailing explanation for original antigenic sin is that large numbers of preexisting memory cells can suppress the small numbers of naïve immune cells responding to variants of the priming antigen. However, a recent study showing that low-avidity memory $CD8^+$ T cells, even at extremely high precursor frequency, did not suppress high-avidity naïve $CD8^+$ T cells suggests that this phenomenon may be limited to instances where the memory $CD8^+$ T cells have a high avidity for the priming epitopes (Zehn et al., 2010).

Nonetheless, all of these prior studies describe how naïve T cells may be competing with memory T cells against the same epitope or epitope variant. To the best of our knowledge, how a large epitope-specific recall response impacts new heterotypic T cell responses remains undefined. Here we utilize a prime-boost strategy to investigate the influence that

immunodominant memory CD8$^+$ T cell populations may have on the priming of both adaptive and innate lymphocyte responses.

Results

Vaccination with recombinant Listeria monocytogenes suppresses naïve heterologous responses after MCMV challenge

To investigate how pre-existing memory CD8$^+$ T cells may affect the generation of heterologous naive T cell responses, we first engineered recombinant *Listeria monocytogenes* (Lm) strains that express the CD8$^+$ T cell epitopes found in MCMV-encoded M45 (amino acids 985-993, Lm-M45) and M38 (amino acids 316-323, Lm-M38). These two epitopes have been previously shown to drive robust CD8$^+$ T cell responses during MCMV infection (Munks et al., 2006), and were thus attractive candidates for vaccination. To confirm that these Lm strains were effective tools for generating memory CD8$^+$ T cell populations, we immunized C57BL/6 mice with Lm-M45 or Lm-M38 and challenged 4-6 weeks later with MCMV (Fig 1A). Antigen-specific CD8$^+$ T cell responses were measured using peptide/MHC class I tetramers (Munks et al., 2006), in conjunction with intracellular IFN-γ staining after peptide stimulation (data not shown). Infection with recombinant Lm produced epitope-specific CD8$^+$ T cell responses that peaked at day 7 post-infection (PI), followed by contraction to form a stable memory pool (data not shown). Following MCMV challenge in mice previously infected with Lm-M38 (Fig 1B) or Lm-M45 (Fig 1C), we observed enhanced epitope-specific memory CD8$^+$ T cell responses compared to mice given PBS during initial priming. To investigate the impact of these M45- or M38-specific recall CD8$^+$ T cell responses on heterologous naïve CD8$^+$ T cell responses generated during MCMV infection, we measured the M38-specific CD8$^+$ T cell response in the cohort previously vaccinated with Lm-M45 or, reciprocally, the M45-specific CD8$^+$ T cell response in animals previously vaccinated with Lm-M38. Interestingly, we found that the naïve heterologous responses (i.e. M45-specific CD8$^+$ T cell response in Lm-M38 primed mice, and M38-specific CD8$^+$ T cell response in Lm-M45 primed mice) elicited by MCMV in these vaccinated animals were diminished compared to animals that had been mock-immunized with PBS (Fig 1, B and C). This suppression of new CD8$^+$ T cell responses in the spleen was also observed in other tissues such as blood and liver (Fig S1A). Thus, immunization of mice with recombinant Lm successfully generated epitope-specific memory CD8$^+$ T cells, which when recalled, correlated with diminished heterologous primary CD8$^+$ T cell responses.

During MCMV infection, a subset of NK cells can respond specifically to the viral glycoprotein m157 through the activating receptor Ly49H (Arase et al., 2002). These MCMV-specific NK cells have recently been shown to exhibit many adaptive immune features, including antigen-specificity, clonal expansion, long-lived memory, and recall responses during MCMV infection (Sun and Lanier, 2011). Therefore, we also investigated whether a large secondary CD8$^+$ T cell response might influence the generation of an antigen-specific primary NK cell response during viral infection. Using a previously-described experimental system (Sun et al., 2009), we adoptively transferred Ly49H$^+$ NK cells into Ly49H-deficient hosts previously infected with Lm-M45 (or PBS), and then

challenged with MCMV (Fig S1B). Following MCMV challenge, M45-specific $CD8^+$ T cell responses were significantly larger in Lm-M45 primed mice compared with PBS primed mice (Fig 1D, Fig S1C); however, unlike heterologous naïve $CD8^+$ T cell responses, which were also suppressed in these studies (data not shown), the $Ly49H^+$ NK cell population proliferated and expanded in a manner that was independent of the presence or magnitude of the M45-specific $CD8^+$ T cell recall response (Fig 1D, Fig S1C). Thus, robust recall $CD8^+$ T cell responses have no impact on NK cell activation or proliferation, and suggest either differential priming requirements or compartmentalized priming of innate versus adaptive lymphocytes following viral challenge.

To confirm that the suppressed clonal expansion of heterologous naïve $CD8^+$ T cells by prolific recall responses was not limited to epitopes found in M45 and M38, mice were primed with Lm-Ova and challenged with MCMV-Ova 30 days later. In these studies, a large secondary $CD8^+$ T cell response specific to Ova (SIINFEKL) suppressed a primary $CD8^+$ T cell response to M45 (Fig 1E), and this diminished response measured at day 7 PI was not due to delayed kinetics, as the M45-specific $CD8^+$ T cell numbers contracted on a similar time scale in control and suppressed mice (data not shown). In separate experiments, diminished expression of Nur77, CD69, and CD25 was observed in the suppressed $CD8^+$ T cells early after infection (Fig S1, D and E), suggesting that the suppressed T cells do not receive the same quality of TCR signal or degree of activation as the control T cells (Moran et al., 2011) even though both groups are encountering antigen and undergoing clonal proliferation.

Furthermore, to demonstrate that suppression of naïve heterologous $CD8^+$ T cells by a recall response was not restricted to bacteria priming followed by virus boost, we primed mice with MCMV (or PBS) first, followed by challenge with Lm-M45 6 weeks later. Because Lm-M45 also carries the Ova epitope, and Lm encodes the endogenous fMIGWII epitope which is presented on the non-classical MHC class I molecule H2-M3 (Lenz et al., 1996), these two epitopes represent the primary $CD8^+$ T cell responses, whereas the M45 epitope represents the recall. In this prime-boost scheme, we observed that the large M45-specific recall $CD8^+$ T cell response in the animals pre-treated with MCMV correlated with significantly diminished primary Ova- and fMIGWII-specific $CD8^+$ T cell responses in all organs compared to PBS controls (Fig 1F, Fig S1F). Together, these findings suggest that the inhibition of primary heterologous $CD8^+$ T cell responses by a robust recall response is neither dependent on the type of pathogen, nor the type of MHC class I.

The overall size of recall determines the degree of heterologous $CD8^+$ T cell suppression

Having observed that large secondary $CD8^+$ T cell responses were able to suppress heterologous primary responses during MCMV challenge, we asked whether the size of that recall response might dictate the degree of primary $CD8^+$ T cell suppression. To address this question, we primed one group of mice with Lm-Ova plus Lm-M38, another group with Lm-Ova alone, and a third group with PBS. This strategy generated three groups with titrated numbers of antigen-specific memory $CD8^+$ T cells (Fig S2A), which were reflected in a scaled recall response in all organs following challenge with MCMV-Ova (Fig 2A, Fig S2B). In agreement with our previous data, the groups with antigen-specific recall $CD8^+$ T cell

responses potently suppressed the primary M45-specific CD8$^+$ T cell response compared to the PBS treated group (Fig 2B, Fig S2B). Notably, the group primed with both Lm-Ova and Lm-M38 showed a greater degree of suppression than the group primed with Lm-Ova alone (Fig 2B, Fig S2B), suggesting that the overall size of the recall correlates with amount of suppression observed in the primary heterologous CD8$^+$ T cell response.

As an alternative approach, we transferred different numbers of memory OT-1 CD8$^+$ T cells (containing a TCR specific to the Ova epitope) into mice and challenged with MCMV-Ova. (Memory OT-I cells were generated by adoptive transfer of naïve CD45.1$^+$ OT-1 into CD45.2$^+$ WT mice followed by Lm-Ova infection; 30 days later, spleens were harvested and CD8$^+$ OT-1 cells were sorted and transferred). On day 7 following MCMV-Ova challenge, titrated recall OT-1 responses were observed in spleen and multiple peripheral organs (Fig 2C, Fig S2C). Consequently, the degree of suppression of the primary M45-specific CD8$^+$ T cell response correlated with the magnitude of the recall OT-1 response (Fig 2D, Fig S2C), consistent with a previous study that investigated the impact of naïve P14 cells on endogenous CD8$^+$ T cells responding to different LCMV epitopes (Butz and Bevan, 1998). Although these findings are restricted by the transgenic mice and recombinant pathogens currently available, they broadly suggest that the size of recall responses play an important role in the degree of suppression observed in primary heterologous CD8$^+$ T cell responses against pathogens.

Suppression of CD8$^+$ T cell responses impacts pathogen load during challenge

To investigate the impact of suppressed CD8$^+$ T cell responses during repeated pathogen exposure, susceptibility to infection was determined in animals where naïve CD8$^+$ T cell responses were suppressed by heterologous recall responses. WT mice were primed with MCMV (or PBS as a control), and 30 days later challenged with Lm-M45 + Lm-M38 (which both contain Ova). Similar to the findings in Fig 1F, robust M45- and M38-specific recall responses suppressed the primary Ova-specific CD8$^+$ T cell response in MCMV-primed but not PBS-primed mice (data not shown). Next, these primed mice were given a third injection 30 days later with Lm-Ova, and bacterial titers (CFU) determined after challenge. In mice where the Ova-specific CD8$^+$ T cell response had previously been suppressed (MCMV infection followed by Lm-M45 + Lm-M38), sharply increased bacterial titers were recovered from the spleen and liver compared with control mice (PBS followed by Lm-M45 + Lm-M38) following bacteria challenge (Fig 2E), demonstrating that the heterologous suppression of naïve CD8$^+$ T cell clonal expansion during epitope specific vaccination can have detrimental consequences to host immunity.

Naïve CD8$^+$ T cell suppression is not due to lack of antigen availability

We next sought to determine the underlying mechanisms behind the heterologous CD8$^+$ T cell suppression. In order to rule out the possibility that the antigen-specific recall response was simply clearing infected cells more effectively and thus eliminating antigen available for optimal priming of new naïve CD8$^+$ T cells, we evaluated viral titers throughout the course of MCMV challenge in the blood of Lm-primed and PBS-primed mice. We observed no differences in the amount of virus in blood and organs regardless of prior immunization with Lm expressing either CD8$^+$ T cell epitope from MCMV (Fig 3, A and B), suggesting that

loss of antigen availability is not the cause of primary CD8$^+$ T cell suppression during a recall response. The similar viral titers observed between the two groups of mice may be due to the multiple immune evasion strategies employed by MCMV (Sun and Lanier, 2009).

Although previous studies have demonstrated that a short exposure to antigen is sufficient to drive a productive CD8$^+$ T cell response (Kaech and Ahmed, 2001, van Stipdonk et al., 2001), we nevertheless wanted to confirm that antigen availability over time was not an important factor in primary CD8$^+$ T cell suppression in our prime-boost studies. Using a replication-deficient *Listeria* strain, Lm-M45(ΔActA), the amount and duration of antigen should be comparable between groups, as there will be limited antigen presentation on APCs. Thus, we first primed mice with MCMV, and then challenged with ActA-deficient Lm-M45 (which also contains the Ova epitope). Although the M45-specific recall response was weak (likely due to the replication-deficient Lm), the primary CD8$^+$ T cell response to Ova was still suppressed in MCMV-primed mice compared to the control group (Fig 3C, Fig S3A). Similarly, transfer of memory OT-1 followed by challenge with Lm-M45 and treatment with ampicillin at days 1, 3, and 5 PI (which efficiently eradicates Lm in target tissues) (Williams and Bevan, 2004), resulted in a robust Ova-specific recall response, but suppression of the M45-specific primary response (Fig 3D, Fig S3B). Taken together, these results suggest that overall antigen availability is not the determining factor in the suppression of primary CD8$^+$ T cell responses by large recall responses during pathogen challenge.

Naïve CD8$^+$ T cell suppression is due to competition for antigen access on APCs

We next investigated whether competition with memory cells for antigen access on APCs was restricting the primary CD8$^+$ T cell response. First, we attempted to block the ability of OT-1 cells to see antigen on APCs by using an antibody against MHC class I/SIINFEKL complexes (Porgador et al., 1997, Blair et al., 2011). Following OT-1 transfer and challenge with MCMV-Ova, one group of mice received SIINFEKL blocking antibody at days 0, 2, and 4 PI, whereas another group received control IgG antibody. The SIINFEKL blocking antibody partially inhibited the OT-1 response during infection, resulting in a partial rescue of the primary CD8$^+$ T cell response to M45 (Fig 4A, Fig S4A), suggesting that limiting the expansion of memory CD8$^+$ T cells can reduce suppression of heterologous primary CD8$^+$ T cell responses.

To further address the question of antigen accessibility, we primed mice with Lm-M45, and then challenged 30 days later with bone-marrow derived dendritic cells (BMDCs) co-pulsed or separately pulsed with M38 and M45 peptide. (The M38 peptide is presented by H2-K^b whereas the M45 peptide is presented by H2-D^b, thereby eliminating competition of peptide binding for the same MHC class I.) In the separately-pulsed DC group, the priming of the secondary response (M45) is distinct from the primary response (M38) at the level of the APC. When previously immunized mice were challenged with BMDCs that had been co-pulsed with both peptides, such that both epitopes are presented by the same DC, the primary M38-specific CD8$^+$ T cell response was suppressed by the recall M45 response (Fig 4B). However, immunized mice challenged with separately-pulsed BMDCs showed a more robust M38-specific CD8$^+$ T cell expansion, rescued fully in the blood and partially in the

spleen (Fig 4B), providing further evidence that competition for antigen presented by APCs is responsible for the suppression of primary heterologous responses by recall responses.

Finally, we generated mixed bone marrow chimeric mice using bone marrow from $K^{b-/-}D^{b-/-}$ mice and H2-$M3^{-/-}$ mice, where half of the hematopoietically-derived DCs are able to present M3/fMIGWII following *Listeria* infection, and the other half are able to present MCMV-specific peptides of interest concurrently with Ova (SIINFEKL) on K^b or D^b, but neither population of DCs is able to present all epitopes (Fig S4B). These mixed chimeric mice were primed with MCMV or PBS, and then challenged with Lm-M45 5 weeks later. In MCMV-primed mice, the large M45-specific recall response suppressed the primary Ova-specific response in all organs compared to PBS primed controls (Fig 4C, Fig S4C). However, no suppression of the primary M3/fMIGWII-specific response was observed in MCMV-primed chimeric mice (Fig 4C, Fig S4C), demonstrating naïve heterologous $CD8^+$ T cells can respond optimally if they are not competing with memory $CD8^+$ T cells for access to antigen on the same APC.

Discussion

In this study, we provide evidence for immune suppression mediated by recall $CD8^+$ T cell responses where heterologous naïve $CD8^+$ T cell populations are unable to proliferate to their full potential. This observed suppression is in part due to decreased antigen access on APCs, likely because small numbers of naïve $CD8^+$ T cells are “crowded out” by the large population of memory $CD8^+$ T cells. The competition of naïve and memory T cells for access to the APCs may also result in differential interactions with co-stimulatory molecules on the APC surface, in addition to MHC and antigen. Furthermore, a larger overall recall response led to greater suppression of new heterologous responses. Our findings build upon our current understanding of cross-competition during primary infection, and provide a new model of clonal competition between heterologous memory and naïve T cells, whereas previous studies on $CD8^+$ T cell competition relied on antigen affinity for a given epitope (Kedl et al., 2000, Smith et al., 2000, Grufman et al., 1999, Kedl et al., 2002). Although our findings resemble the results from original antigenic sin papers, in that new responses are inhibited by anamnestic responses, the previous studies demonstrate suppression of the same (or similar) epitopes, whereas we now demonstrate suppression of heterologous immune responses.

Interestingly, large secondary $CD8^+$ T cell responses had no impact on the expansion of MCMV-specific NK cells. Previous studies have suggested that APC engagement by NK cells during MCMV is needed for optimal activation (Degli-Esposti and Smyth, 2005, Krug et al., 2004); however, the current study provides evidence that antigen presentation (of viral m157) may not be restricted to professional APCs, and that NK cells may not need to see antigen on professional APCs for their priming, as MCMV is also known to infect monocytes, hepatocytes, or endothelial cells (Shellam et al., 2006). An alternative hypothesis could be that differential kinetics of m157 expression compared to processing/presentation of viral peptides on MHC class I may explain why MCMV-specific NK cells are not inhibited by antigen-specific memory $CD8^+$ T cells. Furthermore, naïve T and NK cells may be primed at anatomically distinct sites, with naïve $CD8^+$ T cells competing with central

memory $CD8^+$ T cells in draining lymph nodes, whereas NK cells may compete with effector memory $CD8^+$ T cells in the spleen and liver. None of these explanations are mutually exclusive, and further investigation is required to determine whether location or timing of antigen presentation can account for the differences in suppression between naïve T cells versus NK cells mediated by the recall response.

Our overall findings using mice primed with specific recombinant pathogens suggest that vaccination strategies where individuals are primed against a specific $CD8^+$ T cell epitope (or set of epitopes) can in certain circumstances impede new T cell responses to a given pathogen of interest, and be detrimental during subsequent pathogen encounter. Although the epitopes investigated in our study all represent immunodominant T cell epitopes, our findings are consistent with a companion study in which the degree of suppression of naïve heterotypic $CD8^+$ T cell responses by robust recall responses correlates with affinity of the ligand (Oberle et al manuscript), and suggests that even greater suppression of heterotypic clonal expansion in our system (than what we have measured) may be occurring within subdominant T cell populations. Additionally, a previous study suggested that pre-existing anti-viral immune memory to epitopes naturally expressed by adenovirus resulted in the decreased effectiveness in priming new heterologous $CD4^+$ and $CD8^+$ T cell responses in individuals when using adenoviral vectors expressing HIV proteins (Frahm et al., 2012). We believe our current findings may provide an explanation for this observation, and inform current and future vaccine strategies that involve epitope-specific priming of T cell responses against pathogens such as HIV, malaria, and tuberculosis.

Materials and Methods

Mice and chimeras

All mice used in this study were bred and maintained at MSKCC in accordance with the Institutional Animal Care and Use Committee. Mice were immunized at 6-10 weeks of age. In certain experiments, WT CD45.1 mice were lethally irradiated (~900 gy), and injected with a 1:1 mixture bone marrow from M3-deficient (kindly provided by Dr. Chyung-Ru Wang, Northwestern University) and $K^{b-/-}D^{b-/-}$ mice (kindly provided by Dr. Ming Li, MSKCC); 8 weeks following bone marrow reconstitution, chimeric mice were immunized.

Bacterial and Viral Infections

Recombinant *Listeria monocytogenes* expressing MCMV- and Ova-derived epitopes were generated, as previously described (Orr et al., 2007). Lm-M45 and Lm-M38 were grown in BHI plus 10 μg/mL chloramphenicol until mid-log phase, and Lm-Ova was grown in BHI plus 1 μg/mL erythromycin, before dilution in PBS to appropriate concentration for injection. Unless indicated, mice were infected with 10^4 colony-forming units (CFU) of Lm intravenously. In certain co-infection experiments, 5×10^3 CFU of each Lm strain was used. In certain challenge experiments, 2×10^5 CFU of recombinant Lm strains were used. A dose of 10^6 CFU was used for Lm-M45(ΔActA). Bacterial titers were determined in organs by plating on BHI plates containing 1 μg/mL erythromycin, as previously described (Sun and Bevan, 2003).

MCMV (Smith strain) was passaged serially through BALB/c mice two to three times, and viral stocks were prepared from salivary glands. MCMV-Ova was prepared in culture using NIH3T3 cells. Mice were infected with 7.5×10^3 plaque-forming units (PFU) by intraperitoneal injection. In NK cell transfer experiments, recipient Ly49H-deficient mice were infected with 7.5×10^2 PFU of MCMV one day after receiving approximately 5×10^5 purified splenic Ly49H$^+$ NK cells.

Flow Cytometry Analysis

Single-cell suspensions were generated from the various organs and stained with the indicated fluorophore-conjugated antibodies (BD Biosciences, eBioscience, BioLegend, and Tonbo). MHC class I tetramers were generated by conjugating K^b/SIINFEKL (Ova), K^b/SSPPMFRV (M38), or D^b/HGIRNASFI (M45) monomers (NIH Tetramer Facility) to streptavidin-phycoerythrin (BD Biosciences/Life Technologies). The M3/fMIGWII tetramer was kindly provided by Dr. Eric Pamer (MSKCC). Flow cytometry was performed on a LSR II cytometer (BD), cells were sorted on an Aria (BD), and data were analyzed with the FlowJo software (Tree Star).

Adoptive Transfers

NK cells were enriched by removing T, B, and red blood cells from total splenocyte suspensions using rat IgG against mouse CD4, CD8, CD19, and Ter119 (University of California at San Francisco Core Facility, 10 μg of each antibody per spleen), followed by anti-rat IgG-coupled magnetic beads (QIAGEN) and magnetic depletion. Approximately 5×10^5 enriched NK cells were injected intravenously into recipient mice 1 day before MCMV infection.

Memory OT-I CD8$^+$ T cells were isolated by tetramer staining and cell sorting (greater than 99% purity), and indicated amount of cells were injected intravenously into recipients prior to infection. In certain experiments, 500 μg/mouse of an anti-K^b/SIINFEKL antibody (clone 25-D1-16, Bio-X-Cell) or control IgG1 (clone MOPC-21, Bio-X-Cell) was administered intraperitoneally on days 0, 2, and 4 following infection with MCMV-Ova.

DCs were prepared by isolating primary bone marrow from WT mice and culturing in complete RPMI+10% FBS and 20 ng/mL GM-CSF for 6 days. Following 6 days of culture, a cocktail of TLR ligands (2 μg/ml CpG + 10 μg/ml Poly I:C) were added overnight to induce maturation, and relevant peptide was added to culture at a concentration of 500 ng/mL for 2 hours before injection. Mice were injected subcutaneously with 10^6 peptide-pulsed BMDCs.

Viral qRT-PCR

DNA was isolated from indicated organs using a genomic purification kit (QIAGEN). Following isolation, concentration of DNA was measured using Nanodrop for each sample and 3 μL was added into mastermix containing iQ Sybr Green (Biorad) and primers specific to MCMV DNA (For:TCGCCCATCGTTTCGAGA and Rev:TCTCGTAGGTCCACTGACCGA). Copy number was determined by comparing Cq values to a standard curve of known dilutions of an MCMV plasmid.

Statistical Analysis

Results are presented as mean, with error bars representing ± standard deviation. Data were analyzed on GraphPad Prism 6 software using a two-tailed paired Student's *t* test without standard scatter assumptions, and differences were considered significant at p values of less than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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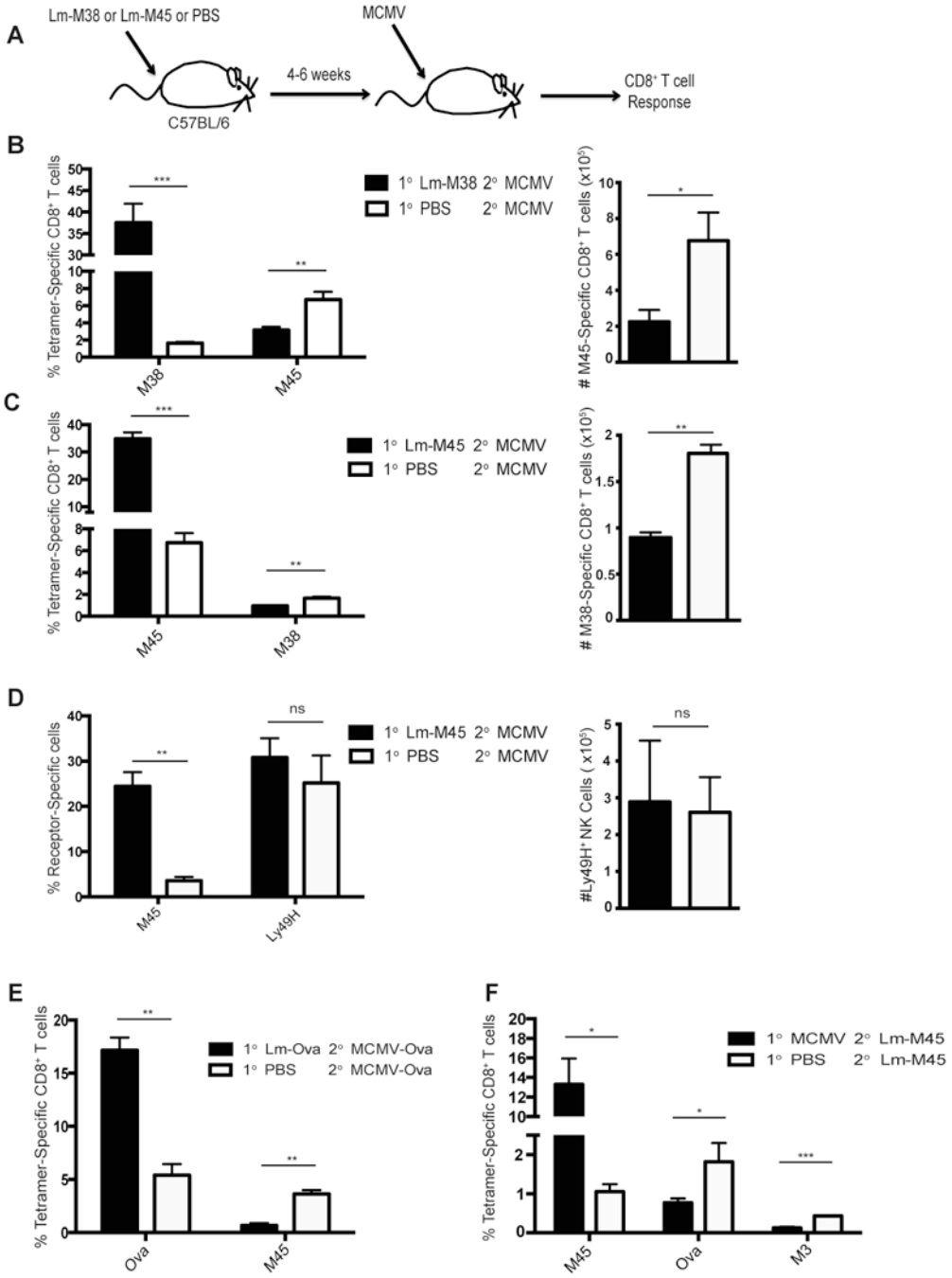

Figure 1. Previous priming with recombinant Lm generates large recall CD8$^+$ T cell responses following MCMV challenge that limit heterologous CD8$^+$ T cell responses

(**A**) Schematic of CD8$^+$ T cell prime-boost strategy against MCMV-specific epitopes. (**B-C**) Percentage and absolute number of epitope-specific CD8$^+$ T cells in the spleen were determined by tetramer staining at day 7 following MCMV challenge in mice vaccinated with Lm-M38 (B) or Lm-M45 (C) 4-6 weeks earlier. (**D**) Ly49H-deficient mice that had been primed with Lm-M45 4-6 weeks prior received adoptively transferred Ly49H$^+$ NK cells and were challenged with MCMV. Percentages of M45-specific CD8$^+$ T cells and

Ly49H$^+$ NK cells in spleen were measured at day 7 following MCMV challenge. Mice primed with PBS and challenged with MCMV served as a control for all experiments (AD). (**E**) Mice were primed with Lm-Ova (or PBS as a control) and subsequently challenged with MCMV-Ova 4-6 weeks later. Ova- and M45-specific CD8$^+$ T cell responses were measured in the spleen at day 7 after challenge. (**F**) Mice were primed with MCMV (or PBS as a control) and challenged 4-6 weeks later with Lm-M45. Epitope-specific CD8$^+$ T cell responses were measured in the spleen at day 7 following challenge. Bars represent mean ± s.e.m; n ≥ 3 per replicate; data are representative of 3-4 experiments; *p<0.05, **p<0.01, ***p<0.001.

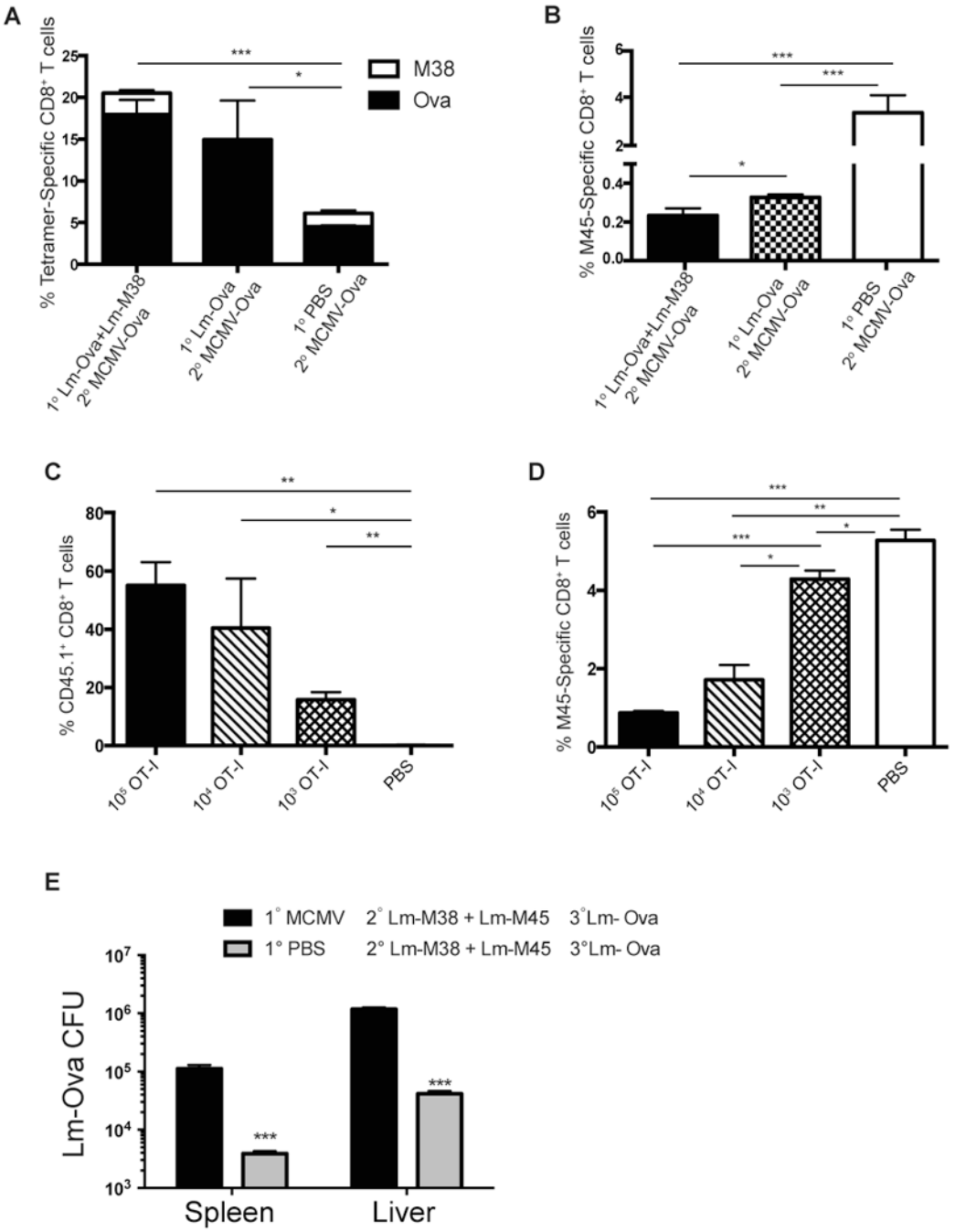

Figure 2. The size of recall response correlates with degree of heterologous CD8$^+$ T cell suppression

(**A-B**) Mice were primed with 2, 1, or 0 Lm strains and then challenged with MCMV-Ova 4-6 weeks later. Percentages of recalled M38- and Ova-specific CD8$^+$ T cells (A) or primary M45-specific CD8$^+$ T cells (B) in the spleen are shown for day 7 after challenge. (**C-D**) 10-fold dilutions of memory OT-I CD8$^+$ T cells (CD45.1) were transferred into WT mice (CD45.2), and mice were challenged with MCMV-Ova. Percentages of recalled OT-I cells (C) or primary M45-specific CD8$^+$ T cells (D) are shown for day 7 following challenge. (**E**)

WT mice were primed with MCMV (or PBS as a control), infected 4-6 weeks later with Lm-M45 + Lm-M38 (both containing Ova), and challenged 4-6 weeks later with Lm-Ova. Lm-Ova colony forming units (CFU) in spleen and liver of both groups of mice were determined at day 2 following challenge. Bars represent mean ± s.e.m; n ≥ 3 per replicate; data are representative of 2-4 experiments; *p<0.05, **p<0.01, ***p<0.001.

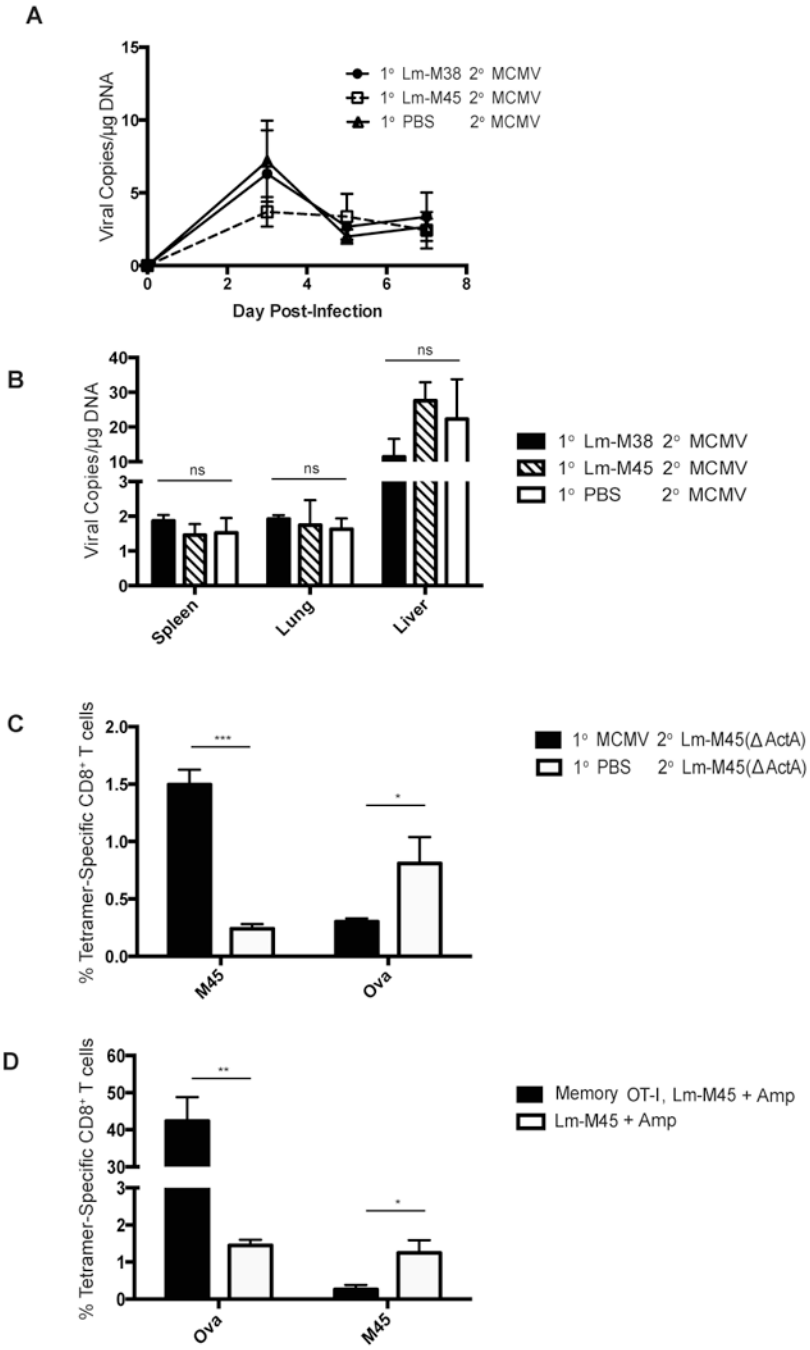

Figure 3. Suppressed CD8$^+$ T cell response in primed mice following pathogen challenge is independent of pathogen titer

(**A**-**B**) MCMV titers are shown in the blood at various time points (A), and in relevant organs at day 3 PI (B) from Lm-M38 or Lm-M45 primed mice followed by MCMV challenge 4-6 weeks later. (**C**) Mice were primed with MCMV and then challenged with Lm-M45(ΔActA) 4-6 weeks later. Percentages of secondary M45-specific CD8$^+$ T cell responses and primary Ova-specific CD8$^+$ T cell responses are shown for the spleen at day 7 after challenge. (**D**) 10^5 memory OT-I cells (CD45.1) were transferred into WT mice (CD45.2), mice were

infected with Lm-M45 (also containing Ova epitope), and treated with ampicillin at days 1, 3, and 5 PI. Percentages of secondary Ova-specific $CD8^+$ T cell responses and primary M45-specific $CD8^+$ T cell responses are shown for the spleen at day 7 PI. Bars represent mean ± s.e.m; n ≥ 3 per replicate; data are representative of 2-4 experiments; *p<0.05, **p<0.01, ***p<0.001.

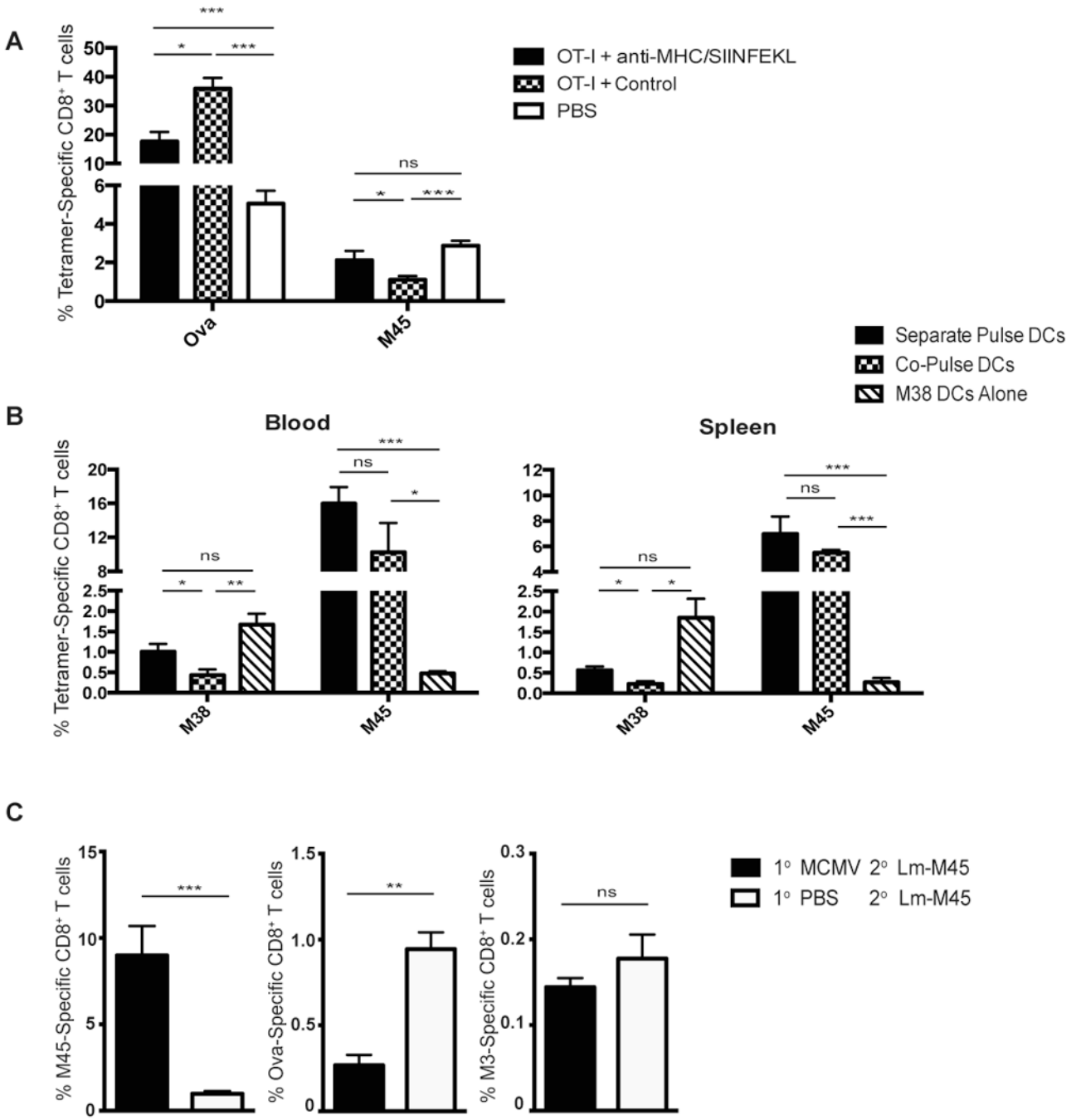

Figure 4. Competition for MHC class I access on APCs dictates suppression of naïve heterologous CD8$^+$ T cells by recall response

(**A**) Mice received 10^5 OT-I cells (CD45.1) and were infected with MCMV-Ova while also receiving an anti-K^b/SIINFEKL antibody or control IgG at days 0, 2, and 4 PI. Percentages of secondary Ova- and primary M45-specific CD8$^+$ T cell responses in the spleen are shown at day 7 PI. (**B**) Mice were primed with Lm-M45 and then challenged 30 days later with indicated peptide-pulsed BMDCs. Percentages of secondary M45- and primary M38-specific CD8$^+$ T cell responses in the spleen and blood are shown at day 7 following DC injection.

(**C**) Mixed $M3^{-/-}$:$K^{b-/-}D^{b-/-}$ bone marrow chimeric mice were primed with MCMV or PBS, and challenged 5 weeks later with Lm-M45. Percentages of secondary M45- and primary Ova- or M3-specific $CD8^+$ T cell responses in the spleen are shown at day 7 following Lm-M45 challenge. Bars represent mean ± s.e.m; n ≥ 3 per replicate; data are representative of 2-4 experiments; *p<0.05, **p<0.01, ***p<0.001.