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Phenotype and function of protective, CD4-independent CD8 T cell memory

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Abstract

While the need for CD4 T cells in the generation of CD8 T cell memory has been well documented, the mechanism underlying their requirement remains unknown. Here, we detail an immunization method capable of generating CD8 memory T cells that are indifferent to CD4 T cell help. Using a subunit vaccination that combines polyIC and an agonistic CD40 antibody, we program protective CD4-independent CD8 T cell memory. When cells generated by combined polyIC/CD40 immunization are compared to cells produced following a CD4-dependent vaccination, *Listeria monocytogenes*, they display dramatic differences, both phenotypically and functionally. The memory cells generated in a CD4-deficient host by polyIC/CD40 immunization provide protection against secondary infectious challenge, whereas cells generated by LM immunization in the same environment do not. Interestingly, combined polyIC/CD40 immunization generates long-term memory cells with low Blimp-1 and elevated Eomes expression despite high expression of Blimp-1 during the primary response. The potency of combined polyIC/CD40 to elicit CD8⁺ T cell memory in the absence of CD4 T cells suggests that it could be considered as a vaccine adjuvant in clinical situations where CD4 responses/numbers are compromised.

Keywords

CD8⁺ T cell; CD4⁺ T cell; Vaccine; Memory

Introduction

The goal of vaccination is to produce memory cells that can protect the host against secondary challenge. The induction of neutralizing antibody constitutes the source of protective immunity in the majority of currently available vaccinations against more common infectious agents [1]. However, there is reason to believe that the induction of cellular immunity is imperative for the development of therapeutic vaccines against more recalcitrant chronic infectious diseases and cancer. Unfortunately, the majority of our current vaccines either do not induce relevant levels of cellular immunity, or they do so via the use of an attenuated infectious agent [2]. Besides the fact that not all infectious agents can be safely attenuated, the use of attenuated agents is rife with concerns over manufacturing, transportation, and safety. Thus, a form of vaccination capable of inducing

substantial levels of cellular immunity without requiring the use of a whole pathogen is preferred.

Although pieces of the CD8 T cell memory puzzle are beginning to come together [3], the complete picture of CD8 memory is still elusive. This is particularly true in regard to the role of CD4 T cell help in CD8 T cell memory. While it is known that CD4 T cells are important in CD8 T cell memory, there are numerous conflicting reports in the literature regarding the CD4 dependence of pathogens. Some reports indicate that CD8 primary responses are dependent upon CD4 T cell help [4], while others report that only memory responses are dependent on CD4 help [5–8]. The timing of CD4 T cell help is also a source of debate. Though the majority of reports indicate that CD4 T cell help is necessary during the priming of CD8 T cell responses [5, 8], there are reports that indicate CD4 help is required during memory maintenance [9, 10]. Overall, CD4 T cell help is required to generate functional CD8 T cell memory in response to some infections, including LM, although the nature of the CD4 help required is poorly defined. In contrast, some infectious agents have been shown to produce CD4-independent memory [11, 12], though the mechanism for this independence is also unknown. Given the clinical importance of robust CD8 memory in the face of failing CD4+ T cell responses, identifying methods capable of producing CD4-independent CD8+ T cell memory extends well beyond a purely intellectual pursuit. Overcoming the need for CD4 help would have a significant impact on the ability to immunize cancer and HIV/AIDS patients.

While we have long known the potency with which combined polyIC/antiCD40 (TLR/CD40) immunization elicits primary and secondary CD4 and CD8+ T cell responses [13–21], we show here that this vaccination strategy generates CD8+ T cell memory that is fully independent of the presence or participation of CD4+ T cells. This CD8+ T cell memory is protective against infectious challenge, and the memory cells elicited are functionally and phenotypically distinct from that generated by other forms of immunization that result in CD4-dependent CD8+ T cell memory. Further, we demonstrate that combined TLR/CD40 immunization produces protective central memory phenotype T cells even in the presence of high levels of Blimp-1, a transcription factor associated with terminal differentiation and clonal exhaustion [22]. Collectively, our data provide a comprehensive picture of the phenotype and function of CD4-independent CD8+ T cell memory elicited by combined TLR/CD40 vaccination.

Materials and methods

Mice

C57BL/6 mice aged 6–8 weeks were obtained from Jackson Laboratories or the National Cancer Institute (NCI). Mice expressing the congenic marker CD45.1 were also obtained from NCI (B6-Ly5.2/Cr, 01B96). MHC Class II^{-/-} mice (CII^{-/-}) were obtained either from Taconic Farms (B6.129-H2-Ab1^{tm1Gru}N12, ABBN12-M) or Jackson Laboratories (B6.129S2-H2^{dlAb1-Ea}/J, 003584). CD40^{-/-} mice were purchased from Jackson Laboratories (B6.129P2-CD40^{tm1Kik}/J, 002928). BAC transgenic mice expressing yellow fluorescent protein (YFP) under the control of the Blimp-1 promoter (Blimp-1-YFP) were a gift from Michel Nussenzweig at Rockefeller University [23]. The Blimp-1 YFP mice used in these studies were bred to be hemizygous for the Blimp-1 BAC reporter transgene. All experiments with mice were performed in accordance with National Jewish Health IACUC approval.

Immunizations

Where indicated, immunizations contained 50 µg of PolyIC (Amersham) and/or 50 µg of the agonistic anti-CD40 antibody FGK4.5 [19, 20]. These components were combined with antigen (see below) injected IV via tail vein. Primary immunizations were performed either with whole ovalbumin protein (Sigma) removed of LPS [24], at a concentration of 200 µg/mouse. Alternatively, B8R peptide (TSYKFESV) [25] was used at a concentration of 100 µg/mouse. Where indicated, CD4 depletion of WT mice was performed by the injection of 500 µg of the anti-CD4 antibody GK1.5 [26] four days prior to immunization followed by 200 µg of GK1.5 4 days after immunization.

Vaccinia challenge and plaque Assay

Where indicated, mice were challenged IV with 1×10^7 pfu of VV-WR. Five days after challenge, antigen-specific T cell responses were measured in the blood and spleen by tetramer stain. Viral titers in the ovaries of VV challenge mice were determined as previously described [27]. Briefly, ovaries were harvested from challenged mice, frozen at -80°C , thawed, and homogenized with a Dounce homogenizer. Equal volumes of homogenized ovary and trypsin without EDTA were then combined and sonicated in a water bath for 10–15 min. Samples were then serially diluted into 24-well plates containing BSC-1 cells. Plates were incubated for 2 days at 37°C . Following the 2 day incubation, media was removed and cells were washed with PBS and fixed with 0.5 ml 10 % buffered formalin per well. Plates were incubated for 5 min at RT. Formalin was then removed and plates were washed with PBS prior to the addition of 0.5 ml of 0.1 % gentian violet (Ricca Chemical Company) per well. Plates were then incubated at RT for 5 min. The crystal violet solution was removed and the plates were allowed to air dry. Viral titer in ovaries was determined by counting plaques.

Listeria monocytogenes challenge

Listeria expressing whole ovalbumin (LM-ova) protein under the control of the hyl promoter was a gift from Michael Bevan at the University of Washington [28]. LM-B8R, an ActA attenuated strain, expresses both the B8R peptide and whole ova protein and was a gift from Rodney Prell from Cerus Corporation. For a primary LM challenge, an overnight LM-ova culture was used to seed a 2–4 h culture in BHI medium. A dose of 2×10^3 colony-forming units (CFU) derived from the log-phase culture was diluted in sterile PBS, and a final volume of 200 µl per mouse was injected i.v. via tail vein. For secondary LM challenge, a dose of 2×10^5 CFU was prepared and injected IV in an identical manner to the primary immunization.

Adoptive transfer of memory cells

Two different versions of memory cell transfer were performed. First, we utilized the adoptive transfer of naïve or memory ovalbumin-specific T cells from congenically marked Vβ5 transgenic hosts. The Vβ5 transgenic mouse expresses only the TCRβ chain of the OT1 TCR (Vβ5) and requires the recombination of, and pairing with, endogenous TCRα chains. As a result, these mice have elevated frequencies of ova-specific T cells in the midst of a diverse repertoire of other specificities [29] but without some of the abnormalities associated with traditional TCR transgenic cells [30–32]. The Vβ5 transgenic has been bred onto the CD45.1+ congenic background; 2.5×10^5 negatively selected CD45.1 CD8+ Vβ5 cells were transferred into WT and MHC class II KO recipients. The following day mice were immunized with either PolyIC/CD40/ova or LM-ova. After 44 days, spleens were harvested, and following CD8 T cell negative selection, 7.5×10^5 antigen-specific cells were transferred into WT or MHC class II KO recipients. Twenty hours after transfer, mice were challenged with LM-ova, and four and a half days later, livers were harvested to determine

CFU. Alternatively, CD8 memory cells were harvested directly from CD45.2+ WT or class II KO mice immunized with either PolyIC/CD40/ova or LM-ova 48 days after immunization. Following CD8 negative selection, 3.5×10^5 CD8+ tetramer+ cells were transferred into CD45.1+ recipients. The following day mice were challenged with LM-ova. Four and a half days after challenge, the mice were killed and the expansion of transferred (CD45.2+) cells determined.

LM protection assay

Protective immunity was determined by bacterial counts in LM challenged mice previously immunized with the described vaccine formulations. Colony-forming units in the spleen 3–4 days after LM challenge were determined as previously described [33, 34]. Briefly, 3–4 days after LM challenge, spleens were homogenized in 0.2 % NP-40 in water and increasing dilutions plated on BHI-LB agar plates. Bacterial load was determined by colony counts 12–18 h later.

Staining and detection of antigen-specific CD8⁺ T cells

Seven days after primary challenge or 5 days after secondary challenge, spleen cells or PBLs were isolated, homogenized into single-cell suspensions as described previously [20]. Cells were plated in 96-well plates and stained with PE-conjugated K^b/SIINFEKL (Ovalbumin) or K^b/TSYKFESV (B8R) tetramer ([27] or Beckman Coulter) for 1 h at 37 °C. Unless otherwise noted, all antibodies were purchased from ebioscience or BioLegend. Antibodies against CD8, B220, CD44, KLRG1, and CD27 were added for an additional 30 min at 37 °C. For the analysis of transcription factor expression, the cells were fixed and permeabilized using the ebioscience FoxP3 fix/perm buffers according to the manufacturers' protocols. Cells were stained overnight with antibodies against Tbet (4B10, Santa Cruz) and Eomes (21mags8, ebioscience). The cells were washed and resuspended in FACS buffer for flow cytometric analysis. For cytokine assays, cells were incubated with $\pm$ antigenic peptide for 4 h at 37 °C in the presence of brefeldin A. Following the incubation period, cells were fixed, permeabilized, and stained for intracellular cytokines as described [20, 35].

Data were collected on a CyAn LX flow cytometer using Summit software (Dako Cytomation) and analyzed using FlowJo software (Tree Star). For analysis, the data were gated on CD8⁺ B220[−] events and analyzed for tetramer staining by the activation marker CD44, the antigen-specific cells being CD44^{high}.

Statistical analyses

Paired and unpaired statistical analyses were made between experimental populations or groups using Student's *t*-test with GraphPad Prism (GraphPad Software). All experiments were performed independently at least twice with a minimum of three mice per group.

Results

PolyIC/CD40 immunization generates CD8 memory T cells in the absence of CD4 T cells independent of direct CD40 stimulation

Immunization with a combination of PolyIC and an agonistic CD40 antibody (polyIC/ α CD40) generates a primary response in both wild type (WT) and CII^{−/−} (CD4 T cell deficient) mice (Fig. 1a, b). These data are consistent with previous results using model and tumor-associated antigens in WT [14, 17, 36] and CD4-deficient hosts [13, 36]. Also consistent with previous reports, the primary responses are equivalent between WT and CII^{−/−} mice, but there are fewer memory cells present in the CD4-deficient mice [6–8, 10]. However, in contrast to previous studies, the secondary response of the memory cells in the

CII^{-/-} is comparable in magnitude to the secondary response of the memory cells in the WT host (Fig. 1a, b). Indeed, given that their pre-boost levels are lower than in the WT host, the memory cells in the CD4-deficient mice undergo a greater fold expansion than their WT counterparts (Fig. 1c). This principle applies more broadly to vaccination with other innate/ α CD40 combinations since both IFN/ α CD40 and Pam3cys/ α CD40 combinations also promoted memory CD8⁺ T cell expansion in CD4-deficient hosts (data not shown). Thus, combined innate receptor/ α CD40 immunization generates memory CD8⁺ T cells with the capacity to expand rapidly following secondary challenge independent of CD4 T cell help.

Previous reports have suggested that activated CD8⁺ T cells express CD40 and that direct stimulation of CD8⁺ T cells through CD40 is necessary for their observed memory function [37]. Our use of an agonistic CD40 antibody required us to determine whether direct stimulation of the CD8⁺ T cells via CD40 was necessary for the observation of CD4 independence. The use of CD40^{-/-}: WT bone marrow chimeras demonstrated that WT and CD40^{-/-} antigen-specific CD8⁺ cells responded equivalently to polyIC/ α CD40 immunization in the presence or absence of CD4 (supplemental Fig 1). Thus, the CD4-independent CD8⁺ T cell memory elicited by combined polyIC/ α CD40 immunization is not mediated by CD40 expression on the CD8 T cells.

PolyIC/CD40 immunization generates protective CD4-independent CD8 T cell memory

A comparison of the polyIC/ α CD40 immunization to a known CD4-dependent immunization, an ovalbumin expressing strain of *Listeria monocytogenes* (LM-ova) [7, 10], was conducted to confirm the CD4 independence of the subunit vaccine (Fig. 2a). Although LM-ova and polyIC/ α CD40 differ in their ability to produce protective CD8 memory cells, the primary response to both is dependent on CD70 [15, 19, 20, 38]. During the primary response and maintenance of memory, polyIC/ α CD40 generates a greater percentage of antigen-specific cells than LM immunization in both the WT and CII^{-/-} (Fig. 2b). The difference in antigen-specific cells is most notable during the secondary responses of the CII^{-/-} mice where the response to LM challenge of the CD4-deficient host is hypo-responsive relative to the secondary response of the WT host primed with LM (Fig. 2c), consistent with previously published results [7, 10].

To assess the function of CD8 memory cells in the absence of CD4 cells, we immunized WT or CII^{-/-} mice with polyIC/ α CD40 using B8R as an antigen or a recombinant LM-B8R (Fig. 3a). B8R is the immunodominant epitope of vaccinia virus (VV) in C57BL/6 mice [25], allowing us to examine the generation of immune memory using vaccinia virus challenge as a secondary immunization. While WT mice immunized with LM-B8R are protected against secondary VV challenge, an LM prime cannot provide protection in the absence of CD4 T cells (Fig. 3b). Thus, despite the fact that the magnitude of the secondary T cell response in the CII^{-/-} host is reduced only threefold–fivefold as compared to the WT (Fig. 2), the degree of protective immunity is reduced by 6–8 logs (Fig. 3b). In contrast, polyIC/ α CD40 immunization can protect both a WT and CII^{-/-} mouse against secondary challenge. Collectively, the data indicate that combined polyIC/ α CD40 immunization produces protective CD8⁺ T cell immune memory in a CD4⁺ T cell-deficient environment. It is interesting to note that CD8 T cell memory in the absence of CD4 T cells is not irreversibly defective since combined polyIC/ α CD40 can also rescue the memory response in a CII^{-/-} host that was originally primed with LM (Supplemental Figure 2). Thus, combined polyIC/ α CD40 immunization can both program CD8⁺ T cell memory in the absence of CD4 T cells and rescue a CD8⁺ T cell memory response in a host primed with a CD4-dependent immunization.

CD8 T cells generated by polyIC/ α CD40 immunization are phenotypically distinct from those generated by LM

We next examined in more detail the phenotype and function of CD8⁺ T cells generated by either LM challenge or polyIC/ α CD40. WT and CII^{-/-} mice were given either form of immunization, and the antigen-specific CD8⁺ T cells were examined 7 (primary) and 45 (memory) days later. Compared to cells generated from LM challenge, cells produced by polyIC/ α CD40 immunization expressed high levels of CD27 and low levels of KLRG1 (Fig. 4a, b). This was true for antigen-specific cells in both WT and CII^{-/-} and at early or late time points after immunization. In addition to CD27, antigen-specific T cells following polyIC/ α CD40 immunization expressed high levels of CD127 and CCR7, but variable levels of CD62L (not shown). Further, polyIC/ α CD40 immunization generated cells that produce more IL-2 than their LM immunized counterparts even in the absence of CD4 T cells (Fig. 4c, d). This difference in IL-2 production became more pronounced during the memory phase where 25–30 % of the cells produced IL-2 and IFN γ after polyIC/ α CD40 immunization. These results indicate that polyIC/ α CD40 immunization produces cells with a largely “central memory” (CM) phenotype even in the absence of CD4 cells, while LM immunization produces cells with an “effector memory” (EM) phenotype [39–41].

T cells generated in a CII^{-/-} mouse by polyIC/ α CD40 immunization are more protective on a per cell basis to those generated by LM

PolyIC/ α CD40 induced a CD8⁺ T cell response that differs in both quantity and quality with respect to the response produced by LM challenge (Figs. 2, 4). That is, not only are the memory cells resulting from polyIC/ α CD40 strikingly different from LM-elicited memory cells, but there are also substantially more of them. It is, therefore, conceivable that the greater degree of protective immunity present in the CII^{-/-} host immunized by polyIC/ α CD40 is due more to a higher number of memory cells than to any intrinsic difference from cells generated by LM. To establish the protective capacity of polyIC/ α CD40- and LM-elicited memory on a per cell basis, we isolated memory cells from WT and CII^{-/-} mice primed with either polyIC/ α CD40 or LM-ova (see “Materials and methods”). Memory cells were then transferred in equal numbers into naive hosts that were then subsequently challenged with LM-ova. The degree of immune protection afforded by the transferred cells was determined by measuring the bacterial load in the recipient hosts 4 days after LM-ova challenge (Fig. 5a) [34]. Memory cells isolated from WT or CII^{-/-} hosts immunized with polyIC/ α CD40 fully protected the recipient mice (Fig. 5a), as were memory cells isolated from an LM primed WT host. However, memory cells from an LM primed CII^{-/-} host, while not completely defective, were substantially reduced in their capacity to protect the recipient host (Fig. 5a). In addition, the proliferation of the memory cells from the LM primed CII^{-/-} host was also substantially reduced compared to memory cells isolated from all other hosts and immunizations (Fig. 5b). Overall this indicates that cell intrinsic differences are responsible for the differential production of protective CD8⁺ T cell memory between polyIC/ α CD40 and LM immunization.

CD4-independent CD8⁺ T cell memory cells express low levels of Blimp-1 and sustained expression of Eomes

A reduction in the transcriptional repressor Blimp-1 produces CD8 memory T cells with a central memory phenotype [42–44], and depletion of CD4 cells leads to an increased expression of Blimp-1 [45]. In addition, long-term memory is associated with sustained expression of the transcription factor Eomes [39, 46–48]. We, therefore, examined memory cells within each host, derived from the different primary vaccinations, for their expression of Blimp-1 and Eomes using a Blimp-1-YFP reporter mouse [23] and intracellular staining, respectively. Three observations are noteworthy with respect to the expression of these

transcription factors in the memory cells. First, contrary to our original expectation, the expression of Blimp-1 during the primary response to combined polyIC/ α CD40 was similar if not higher than in response to LM (Fig. 6a, b). This high expression of Blimp-1 coincides with the extreme skewing of the phenotype of the cells toward a CM phenotype (Fig. 4), indicating that the expression of Blimp-1 is not causative in the CM phenotype observed during the primary response. Second, there was a reduction in the expression of Blimp-1 in the memory cells subsequent to polyIC/ α CD40 immunization in both WT and CII^{-/-} mice as compared to LM immunization in either host (Fig. 6b). Previous published data have shown that limiting Blimp-1 expression can rescue clonal exhaustion in a chronic viral infection model [44], and this is consistent with our observation that reduced Blimp-1 expression correlates with robust CD4-independent CD8⁺ T cell memory. Third, in addition to reduced Blimp-1 expression, memory cells competent to provide protective immunity (polyIC/ α CD40 in WT or CII^{-/-} and LM in WT) expressed significantly higher levels of Eomes (Fig. 6c). As elevated Eomes expression has been closely tied with effective long-term CD8⁺ T cell memory [39, 46–48], this phenotype again correlates with protective, CD4-independent CD8⁺ T cell memory.

Discussion

Our data indicate that CD4-independent protective CD8⁺ T cell memory cells (1) can be elicited in a vaccine setting, (2) possess phenotypic and functional attributes that make them superior to CD4-dependent CD8⁺ memory T cells on a per cell basis, and (3) display reduced Blimp-1 and sustained Eomes expression. While this form of vaccination was previously shown to elicit CD4-independent tumor-specific CD8⁺ T cell responses [36], the cellular and molecular characteristics of immune memory derived from this vaccination was not described. Our data represent the first comprehensive phenotypic and functional analysis of vaccine-elicited helper-independent CD8⁺ T cell memory.

PolyIC/ α CD40 immunization produces memory T cells that are intrinsically different than those produced by LM challenge. In the doses of antigen used in the present studies (100–200 μ g), this vaccination also produced an increased frequency of antigen-specific cells when compared to LM immunization. As previous data have shown that the frequency of memory cells is a primary contributor to effective protective immunity [49], it was feasible that the success of this vaccination in the CII^{-/-} host was due simply to its capacity to produce a larger number of T cells. However, using the adoptive transfer model system to equalize the number of memory cells, we demonstrated that T cells from a CII^{-/-} produced by combined polyIC/ α CD40 immunization provided better protective immunity than their LM-produced counterparts. Further, the cells produced by our vaccine also have vastly different potential for expansion, survival, phenotype, and functional capacity when compared to cells generated by an LM immunization. Thus, the difference between our vaccination and LM is at least partially due to T cell intrinsic factors. Global gene expression analysis is currently underway in both effector and memory cells resulting from each vaccination and from each host in order to determine the genes that may be responsible for these differences.

CM T cells have the unique capacity for long lived, self-renewing memory, as compared to its short-lived EM counterparts [40, 41, 50, 51]. As such, a strong argument can be made for the hypothesis that a vaccine capable of protecting the host against a systemic infectious challenge (such as for HepC or HIV) should produce as many central memory CD8 T cells as possible. Not coincidentally, the CD4-independent CD8⁺ T cell memory we describe here displays a largely, though not exclusively, CM phenotype, both phenotypically and functionally. Besides the KLRG1/CD27 profile, memory T cells derived from this vaccination show high expression of CCR7 and CD127, though low CD62L expression (not

shown). In particular, the increased production of IL-2 is most consistent with the documented self-renewing potential of CM cells. That said, this phenotype does not appear to compromise the capacity of the cells to also possess robust lytic activity [13, 14, 21]. Thus, to some degree, the CD8+ T cell elicited by our vaccination displays a somewhat hybrid phenotype/function similar to both CM and EM cells.

The observed course of Blimp-1 expression in our CD4-independent CD8+ memory cells is likely informative with regard to this combined CM/EM phenotype/function. Our data show that CD4-independent memory corresponds to low expression of Blimp-1 in the memory T cells, but high expression of Blimp-1 during the primary effector response. Previous reports have shown that limiting Blimp-1 expression has the capacity to rescue clonal exhaustion in a chronic viral infection [44]. Further, Blimp-1 is known to bind to, and negatively regulate, the IL-2 promoter [22, 52]. Thus, the CD4-independent long-term memory function and high IL-2 production in cells derived from combined polyIC/ α CD40 immunization are consistent with the observed late reduction in Blimp-1 expression. Similarly, the high Blimp-1 expression at the peak of the response is consistent with the observed robust lytic function at the peak of the primary response [13, 14, 21]. It is currently unclear how the memory cells can be derived from a primary pool of effector/memory cells with such prominent Blimp-1 expression since over expression of Blimp-1 is typically associated with terminal differentiation. It is possible that the expression of Eomes, which is strongly associated with differentiation of CD8+ T cells into long-term memory [39, 46–48], may be of significance in this regard. In contrast, Tbet expression is similar to Blimp-1 in its propensity to induce terminal differentiation and a reduction in memory cell formation [40, 47, 48]. Tbet expression is reduced in cells derived from combined polyIC/ α CD40 immunization as compared to those from LM challenge (not shown), but this applies to both WT and CII^{-/-} hosts and, therefore, does not segregate only to cells destined to produce functional memory in our model system. Since Blimp-1, Eomes and Tbet all operate as “goldilocks” factors, each producing some degree of memory/effector dysfunction when over/under expressed, the contributions of each to the formation of CD4-independent CD8+ T cell memory requires considerably more exploration.

Collectively, our data indicate that CD4-independent CD8+ T cell memory can be achieved using non-infectious methods of vaccination. Further, we demonstrate a phenotype and functionality of CD8+ T cell memory that can serve as a standard for screening other forms of vaccination for their production of long-lasting protective immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Combined polyIC/ α CD40 immunization generates CD8 T cell memory in the absence of CD4 T cells. Wild type (WT) and class II KO (ko) mice were immunized with combined PolyIC/ α CD40/ova as described in the “Materials and methods.” Sixty days later, the mice were challenged with LM-ova. Mice were bled at the indicated time points and the percent of CD8⁺ tetramer⁺ cells was determined. **a** Plots shown are gated on CD8⁺ cells. “Primary” indicates day 8 following immunization, “Memory” indicates day 47 post-immunization, and “Secondary” indicates 5 days following the day 60 LM challenge. **b** Percent of CD8⁺ tetramer⁺ cells over time in the peripheral blood. **c** Fold expansion of tetramer⁺ CD8 memory cells in the blood was calculated by dividing the percent tetramer⁺ cells in the peripheral blood post-LM-ova boost by the percent tetramer⁺ cells in the peripheral blood pre-boost. These data are representative of at least 3 experiments performed. Data points are from 3 to 4 mice per group. Error bars represent standard deviation

Fig. 2.

In contrast to LM challenge, combined polyI:C/ α CD40 immunization generates CD8 T cell memory in the absence of CD4 T cell help. **a** WT and CII KO mice were immunized with either PolyI:C/ α CD40/ova or LM ova as in Fig. 1. Mice were bled at multiple time points and the percent of CD8+ tetramer+ T cells determined. Representative flow cytometry results are shown (**b**). Plots are gated on all live, B220-, CD8+ cells. "Primary" represents day 8, and "Memory" day 29, post-immunization. **c** The percent of CD8+ tetramer+ cells in the spleen 5 days after challenge with LM-ova and the % max of the WT response. These data are representative of at least 4 experiments performed. Data points are from 3 to 4 mice per group. Error bars represent standard deviation

Fig. 3.

Combined polyIC/ α CD40 immunization mediates CD4-independent protective memory against pathogen challenge. WT and class II KO mice were immunized with either PolyIC/ α CD40/B8R or LM-B8R as described in the Materials and methods. Day 60 after immunization, mice were challenged IV with VV-WR. Five days after challenge mice, viral titers in the ovaries were determined by plaque assay as described in the “Materials and methods.” Data are expressed as plaque-forming units (PFU). These data are representative of 2 experiments performed. Data points are from 3 to 4 mice per group. *Error bars* represent standard deviation

Fig. 4.

Combined polyIC/ α CD40 immunization generates cells with central memory phenotype and function. WT (wt) and class II KO (ko) mice were immunized with PolyIC/ α CD40/ova or LM-ova. Representative flow cytometry results are shown. **a** Plots are gated on CD8+ tetramer+ cells. "Primary" represents day 8, and "Memory" day 29, after immunization. **b** Graphical representation of the percent of CD8+ tetramer+ cells expressing either CD27 or KLRG1. **c** Cells were surface stained, fixed, and then permeabilized to allow for intracellular cytokine staining. Representative flow cytometry data are shown. Plots shown are gated on CD8+ cells. Primary is day 8 and memory is day 30 following immunization. **d** The percent of IFN- γ producing cells that are also producing IL-2 primary and the memory time points. These data are representative of at least 3 experiments performed. Data points are from 3 to 4 mice per group. Error bars represent standard deviation

Fig. 5.

Memory CD8⁺ T cells elicited by combined polyIC/*a*CD40 immunization are superior in mediating protective immunity on a per cell basis as compared to memory cells elicited by LM; **a** 2.5×10^5 negatively selected CD8⁺ V β 5 cells were transferred into WT and class II KO recipients. The following day mice were immunized with either PolyIC/*a*CD40/ova or LM-ova. After 44 days, spleens were harvested, and following CD8 T cell negative selection, 7.5×10^5 antigen-specific cells were transferred into WT or class II KO recipients. Twenty hours after transfer, mice were challenged with LM-ova, and four and a half days later, livers were harvested to determine CFU. **p* value <0.05 for KO LM sample when compared to other experimental groups and naïve control, unpaired *t* test. **b** CD8 memory cells were harvested from WT and class II KO mice immunized with either PolyIC/*a*CD40/ova or LM-ova 48 days after immunization. Following CD8 negative selection, 3.5×10^5 CD8⁺ tetramer⁺ cells were transferred into CD45.1⁺ recipients. The following day mice were challenged with LM-ova. Four and a half days after challenge, the expansion of transferred (CD45.2⁺) cells determined. Total number of transferred antigen-specific cells in the spleen following challenge. **p* value <0.05 unpaired *t* test

Fig. 6.

Blimp-1 expression is reduced, and Eomes expression increased, in memory cells derived from polyIC/ α CD40 as compared to LM immunization. CD4 depleted or non-depleted Blimp-1-YFP mice were immunized with PolyIC/CD40/ova or LM-ova as described in Fig. 1. **a** Representative flow cytometry plots gated on CD8⁺ tetramer⁺ cells in the peripheral blood. Primary time point is 7 days, and memory 35 days, after immunization. **b** Blimp-1 YFP geometric mean fluorescence intensity (gMFI) for CD8⁺ tetramer⁺ cells over time in the peripheral blood of the cells from **a**. **c** WT and CII^{-/-} mice were immunized as in **A**. Thirty days later, peripheral blood was stained with tetramer, and gMFI of Eomes expression in CD8⁺ tetramer⁺ cells was determined. These data are representative of at least 2 experiments performed. Data points are from 3 to 4 mice per group. *Error bars* represent standard deviation