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Inflammation and TCR Signal Strength Determine the Breadth of the T Cell Response in a Bim-Dependent Manner

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Generating a diverse T cell memory population through vaccination is a promising strategy to overcome pathogen epitope variability and tolerance to tumor Ags. The effector and memory pool becomes broad in TCR diversity by recruiting high- and low-affinity T cells. We wanted to determine which factors dictate whether a memory T cell pool has a broad versus focused repertoire. We find that inflammation increases the magnitude of low- and high-affinity T cell responses equally well, arguing against a synergistic effect of TCR and inflammatory signals on T cell expansion. We dissect the differential effects of TCR signal strength and inflammation and demonstrate that they control effector T cell survival in a bim-dependent manner. Importantly, bim-dependent cell death is observed with a high Ag dose in the context of an inflammatory environment. Our data define the framework for the generation of a broad T cell memory pool to inform future vaccine design. The Journal of Immunology, 2014, 192: 200–205.

During the course of an infection, Ag-specific CD8 T cells become activated and acquire effector function, and a fraction of these cells differentiate into long-lived memory CD8 T cells (1–3). The generation of large numbers of high-affinity T cells during this process is generally considered a desirable and advantageous outcome, because it selects for the best responders. Recent studies strongly suggested a significant biological role for low-affinity T cells in creating a successful vaccination strategy. CD8 T cells activated by low-affinity ligands are recruited into the primary CD8 T cell response, as well as the ensuing CD8 memory T cell pool (4). T cell–mediated protection against pathogens that are capable of a high mutation rate may benefit from a repertoire of Ag-specific T cells that is very broad. A broad repertoire includes T cells that react only weakly to the initial priming Ag (low-affinity T cells) but may recognize subsequently mutated epitopes with high affinity. Recent evidence from an HIV vaccine trial showed that the mere presence of memory CD8 T cells was not sufficient to provide protection against HIV infection (5), and this lack of protection could potentially be attributed to insufficient potency or breadth of epitope recognition (6). Importantly, previous animal studies indeed underline a crucial role for TCR repertoire diversity to prevent viral escape mutants (7, 8). This protective benefit of a broad T cell repertoire is presumably true for all highly variable pathogens whether they are chronic infections, such as described in the aforementioned studies, or acute infections like influenza (9). Moreover, antitumor responses may depend on recruiting lower-affinity T cells, because higher-affinity T cells are often eliminated by tolerance mechanisms. The major focus in the cancer immunotherapy field is on generating high numbers of functional CTLs to eliminate tumor burden.

In the context of vaccine design, it is a major challenge to trigger potent T cell responses, and the parameters that allow for induction of T cell responses with a broad repertoire are still poorly understood. Historically, vaccines using live attenuated viruses, such as the smallpox, polio (Sabin), and yellow fever vaccine, have been highly successful and shown to prime long-lived, potent T cell responses (10–12). More recently, an SIV vaccine based on a recombinant rhesus CMV vector provided protection against highly pathogenic SIV (13). Two major differences between live and inactivated vaccines are the amount of inflammation and Ag dose elicited by each vaccine, with live vectors generating more potent inflammatory responses and presumably providing more Ag. It was established that TCR signal strength, Ag availability, and inflammation are the driving forces of the primary and memory T cell response (1–3, 14), but it is still unclear how these signals interact to shape the breadth of the T cell response and affect high- and low-affinity T cell responses (2, 14). Previous studies suggested that cytokine and TCR signals can synergize to mediate memory CD4 T cell survival (15). Whether comparable synergistic events take place in CD8 T cells is still unknown, but such an integration of different signals could enhance the selective outgrowth of the highest-affinity T cells and regulate affinity maturation of the memory T cell pool (16). Thus, gaining a more thorough understanding of the mechanisms involved in efficient priming and survival of low-affinity T cells is of broad general interest in the context of infectious disease, as well as cancer therapy.

The primary goal of this study was to address how inflammation, TCR signal strength, and Ag availability interact to shape the breadth and function of the effector and memory T cell response. We used an experimental system that gave us tight control over factors that can vary between different vaccine formulations or types of infection. This system allowed us demonstrate that
concurrent inflammation enhances effector T cell responses to the same extent, regardless of the strength of the TCR stimulus; inflammation and TCR signal strength control distinct and overlapping phenotypic and functional characteristics of the effector cell population; and bystander-mediated cell death is responsible for the loss of low-affinity effector cells under conditions of limited inflammation and Ag availability; however, these can be overcome with increased Ag availability and inflammation.

**Materials and Methods**

**Mice**

C57BL/6 (B6) and B6 congenic (CD45.1+ and Thy1.1+) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in specific pathogen–free conditions at the University of Washington and the Fred Hutchinson Cancer Research Center. OT-I TCR-transgenic mice congenic for CD45.1 or Thy1.1 and bim−/- OT-I mice were bred and maintained in the same facilities.

**Adoptive transfer and cell sorting**

Naïve CD44low OT-I T cells were isolated from lymph nodes by separation over a magnetic column (Miltenyi Biotec), as previously described (17). A total of 1  10^6 naïve OT-I T cells was transferred i.v. per recipient. This number was chosen based on a 10% take of transferred cells in a new host following adoptive transfer (17, 18) and a precursor frequency of Ag-specific, endogenous T cells that can range from <10^2 to >10^3 (19, 20).

**Dendritic cell isolation**

Dendritic cells (DCs) were expanded in B6 mice with an Flt3L-secreting cell line (B16-Flt3L). Ten to fourteen days after injection of the B16-Flt3L cells, CD11c+ cells were purified and pulsed with LPS (1 µg/ml) and peptide (1 µg/ml) in vitro for 1 h (37˚C) prior to transfer, as previously described (17). A total of 1  10^6 DCs was transferred i.v. per recipient.

**Infections**

Listeria monocytogenes was grown as previously described (17). For primary infections, mice were infected i.v. with 2  10^3 CFU L. monocytogenes. In some experiments, memory cells were boosted with a recombinant vesicular stomatitis virus (VSV) strain expressing the SIINFEKL (N4) epitope (22) (23), or a recombinant vaccinia (VAC) strain expressing the N4 epitope (22) (2 10^6 PFU, i.v., in each case) and were sacrificed 5–7 d later.

**Flow cytometry**

Recipient mice were sacrificed at the time points indicated. For intracellular staining, cells were prepared with the Cytofix/Cytoperm kit in the presence of brefeldin A (BD) and incubated or not with 100 nM N4 peptide for 4–5 h in RPMI 1640 supplemented with 10% FCS. Cells were analyzed using a FACSCanto (BD) and FlowJo software (TreeStar).

**Results**

**Inflammation enhances generation of CD8 effector cells**

We first addressed whether cytokine signals synergize with the strength of a TCR signal. For this purpose, we relied on the OT-I system to provide defined TCR specificity. OT-I T cells are activated by the high-affinity N4 peptide of chicken OVA presented by H-2Kb (23). A large number of altered peptide ligands with equal ability to bind to H-2Kb, but gradually reduced TCR affinity, have been described, which nevertheless all still activate OT-I T cells (4). We decided to use the low-affinity altered peptide ligand SIHQFEKL (Q4) for this study, because it elicits an OT-I T cell response that can still be easily measured, and it is well above the threshold of thymic negative selection (24, 25). To keep Ag dose and availability constant, we used a system in which Ag is provided by ex vivo–isolated (from B16-Flt3L–injected animals), LPS-activated, peptide-pulsed DCs. Inflammation is provided by a concurrent infection with wild-type (WT) L. monocytogenes that does not provide Ag for the OT-I T cells (17, 26). A small number of naive OT-I T cells was transferred into recipient mice together with high-affinity (N4) or low-affinity (Q4) Ag-pulsed DCs. In the presence of an accompanying L. monocytogenes infection, N4-pulsed DCs generated an ~2–3-fold greater OT-I effector population compared with the peptide-pulsed DC-only group in the blood (Fig. 1A) and spleen (Fig. 1B) on day 5. Interestingly, a similar enhancement by bystander Listeria infection was observed for Q4-stimulated OT-I T cells (Fig. 1A, 1B). We chose day 5 as a time point to examine OT-I T cell phenotype and function after the expansion stage was completed in both (N4 and Q4 primed) conditions (Fig. 1C) to allow for a meaningful comparison. Together, these data suggest that inflammation enhanced low- and high-affinity T cell responses equally and independently of ligand potency. It is important to note that CD4 T cell help is provided even in the absence of a bystander L. monocytogenes infection because DCs are exposed to Ag (e.g., FCS, enzymes) during the isolation process, which results in presentation of antigenic MHC class II–restricted epitopes (27, 28).

**Integrating inflammation and TCR signals to shape T cell function**

We demonstrated previously that priming high-affinity CD8 T cells in the context of minimal inflammation induces robust T cell expansion and IFN-γ production, but the cells lack granzyme expression and remain KLRG1high (26). We wanted to determine which aspects of T cell phenotype and function are regulated primarily by inflammatory stimuli versus TCR signal strength. We found that production of IFN-γ and granzyme B was enhanced by inflammation, whereas the affinity of the TCR ligand played only a limited role (Fig. 2A). In contrast, KLRG1 expression levels were dependent on both inflammation and TCR signal strength (Fig. 2A). Although inflammation was necessary to induce KLRG1
expression, a greater percentage of cells expressed KLRG1 when stimulated with a high-affinity ligand compared with an intermediate ligand (N4 versus Q4, \( \sim 2 \)-fold difference). OT-I T cells that were expanded in the context of systemic inflammation expressed less CD62L on day 5 postpriming, regardless of the nature of the ligand (Fig. 2B). Inflammation had very little impact on the ability of effector cells to produce IL-2, whereas priming with high-affinity ligand generated cells that produced more IL-2 (Fig. 2A). In contrast, expression of IL-2R\(_a\) (CD25) was directly correlated with both the extent of inflammation present and the strength of the TCR stimulus on day 5, when both N4 and Q4-primed OT-I T cells had finished expanding following peptide-pulsed DC immunization (Fig 1C). Together, these data show that TCR signal strength and inflammatory conditions exclusively control certain functional aspects of the T cell response, whereas other features are clearly the result of the integration of both signals.

**Bim-mediated cell death dictates the size of the effector pool**

We wanted to directly address the respective roles of TCR signal strength and inflammatory signals in regulating effector cell apoptosis and determining the magnitude of the T cell response. Using the same experimental approach as described in Fig. 1, we primed a 1:1 mix of WT and bim\(^{-/-}\) OT-I cells with high- or low-affinity ligand-pulsed DCs, with or without *L. monocytogenes* infection, and analyzed the magnitude of the OT-I response 5 d later (Fig. 3A). We found that bim-mediated T cell death does not play a role when high-affinity effector cells are primed in the context of an inflammatory environment (N4 + *L. monocytogenes*), whereas, in the absence of abundant inflammation, high-affinity T cells are lost in a bim-dependent manner (N4) (Fig. 3A). The ratio of bim\(^{-/-}\)/WT OT-I T cells is skewed toward bim\(^{-/-}\) cells when primed with Q4-pulsed DCs alone and, in contrast to N4 immunization, in this case inflammation was not sufficient to prevent bim-mediated cell death for low-affinity primed T cells (Q4 + *L. monocytogenes* compared with Q4 only). These data establish a central role for bim in regulating the breadth of the T cell repertoire and provide a mechanism for how TCR signal strength and inflammation integrate to determine T cell fate. When peptide-pulsed DCs are used for immunization, only a limited quantity of Ag is provided. This differs from a situation in which a pathogen expresses Ag, which is available at higher quantities for a longer period and with potentially wider tissue distribution of the Ag. Therefore, we next tested the effect of increasing Ag dose on bim-mediated cell death using the previously described recombinant strains LM–N4 and LM–Q4 to prime a 1:1 mix of WT/bim\(^{-/-}\) OT-I T cells (Fig. 3B). Strikingly, in contrast to immunization with peptide-pulsed DCs, we found that bim-mediated cell death did not occur following immunization with recombinant *L. monocytogenes* strains, even after low-affinity (LM–Q4) priming (Fig. 3B). To exclude the possibility that the different expansion and contraction kinetics of high- and low-affinity responders may skew interpretation of the results, we tracked WT and bim\(^{-/-}\) OT-I T cells on days 5 and 7 postpriming with LM–N4 and LM–Q4, confirming that bim-dependent cell death of effector cells during the expansion phase does not play a role in conditions of high-dose Ag and systemic inflammation (Fig. 3C). Together, these data show that bim-mediated cell death affects the size of the effector pool through TCR signal strength and inflammation-mediated signals, but this is overcome when low-affinity cells are primed in the context of a replicating pathogen (Fig. 3D).
Generating functional memory

We demonstrated previously that high-affinity CD8 T cells primed in the context of minimal inflammation give rise to effector cells that lack granzyme expression but that contract normally and give rise to fully functional memory T cells (26). Because inflammation and TCR signal strength both affected aspects of the CD8 effector phenotype (Fig. 2A), we next tested the ability of high- and low-affinity memory cells, primed with or without an accompanying L. monocytogenes (LM) infection, to respond to a recall response. Specifically, we wanted to address whether low-affinity memory T cells generated in the presence of limited inflammation are actually functional. OT-I T cells were primed with N4 or Q4-pulsed DCs, with or without L. monocytogenes, and rested for $30$ d. Mice were challenged with a recombinant V AC or VSV expressing OVA. This heterologous rechallenge strategy allowed us to directly address the recall ability of the memory OT-I T cells without engaging endogenous, Listeria-specific memory cells. This is important because pre-existing immunity to a vaccine vector can affect the ensuing memory response to the targeted Ags (29). Moreover, this secondary challenge elicits a comparable functional response from all memory T cells, regardless of the priming conditions, as measured by IFN-γ and granzyme B production (Fig. 4C).

**FIGURE 3.** TCR signal strength and inflammation dictate the magnitude of the primary response in a bim-dependent manner. A mix of $5 \times 10^3$ WT (Thy1.1+) and $5 \times 10^3$ bim$^{-/-}$ CD45.2 OT-I T cells was transferred into CD45.1+ congenic B6 hosts. (A) Mice were injected i.v. with $1 \times 10^6$ N4 or Q4 peptide–pulsed DCs, with or without an accompanying L. monocytogenes (LM) infection. Spleens were harvested at 5 d postpriming, and the ratio of bim$^{-/-}$/WT OT-I cells was determined. Horizontal lines represent the mean. (B) Mice were primed with LM-N4 or LM-Q4, and the number of WT and bim$^{-/-}$ OT-I cells in the spleen was determined on day 7 postinfection. (C) Mice were primed with LM-N4 or LM-Q4. The number of WT and bim$^{-/-}$ OT-I cells in the blood was determined on days 5 and 7 postinfection. Data shown are representative of two or three independently performed experiments, with a minimum of three mice/group. (D) Overview of the mechanisms dictating the composition of the T cell response. Low-affinity stimulated T cells are more susceptible to bim-mediated cell death, but this can be partially overcome by increasing inflammation. Increasing Ag availability in the context of inflammation can fully overcome the bim-mediated cell death and preserve low-affinity T cell responses. **p = 0.001–0.01, Student t test. ns, Not significant (p > 0.05).

**FIGURE 4.** Functional memory is generated even from low-affinity T cells primed in an environment with limited inflammation. More than 30 d after the primary response (as described in Fig. 1), mice from all four experimental groups were immunized with recombinant V AC expressing OVA, and T cell responses in the spleen (A) and lung (B) were determined 5 d later. (C) Memory cell function was assessed in an in vitro restimulation assay by measuring IFN-γ and granzyme B production. Data are representative of two independently performed experiments with a minimum of three mice/group. Similar data were obtained from two experiments using recombinant VSV expressing OVA. *p < 0.01, **p = 0.001–0.01, Student t test. ns, Not significant (p > 0.05).
**Discussion**

In the context of vaccine design, generating a broad memory T cell repertoire including high- and low-affinity responders is desirable to provide a diverse repertoire of responding memory T cells upon subsequent pathogen encounter. This is thought to be particularly beneficial for highly variable pathogens, such as HIV or influenza. Although the benefits of generating a broad memory T cell repertoire have been established, the parameters needed to achieve such a response remain poorly defined. We addressed how inflammatory signals, TCR signal strength (in this context defined as the functional avidity of a T cell for a given epitope), and Ag dose interact to shape the breadth and function of an effector and memory T cell response.

We show that inflammation provided by a bystander *L. monocytogenes* infection enhanced low- and high-affinity T cell responses equally and independently of ligand potency when keeping the Ag dose consistent (Fig. 1). TCR signal strength and inflammatory conditions exclusively control certain functional aspects of the T cell response, whereas other features are clearly the result of integrating both signals (Fig. 2). It is important to keep in mind that certain functional properties might not change between two ligands of different affinity if an affinity ceiling is reached (30). However, given the phenotypic and functional differences that we observed between N4 and Q4-prime OT-I T cells, our data argue against an affinity threshold model that equally affects all aspects of T cell function. This notion is particularly interesting when considering that at least certain features of the primary CD8 T cell response are programmed: brief exposure to high-affinity Ag is sufficient to initiate long-lasting T cell proliferation and induce effector function; however, prolonged exposure to Ag further enhances the magnitude of the response (17, 31–33).

Our study also defines the requirements for preserving low-affinity responders in a CD8 T cell response. Low TCR signal strength and limited inflammation led to impaired expansion, partially due to bim-dependent apoptosis of low-affinity responders (Fig. 3C). However, a high Ag dose is sufficient to overcome bim-dependent cell death, as shown by the use of recombinant *L. monocytogenes* strains compared with OT-I cells primed by peptide-pulsed DCs in the presence of an *L. monocytogenes* infection. We cannot formally exclude the possibility that there is a concurrent effect on cell division mediated by bim enhancing the difference in WT and bim<sup>−/−</sup> T cell accumulation, specifically in situations of low-affinity stimulation. WT and bim<sup>−/−</sup> OT-I T cells have comparable TCR surface expression levels, thus excluding differential sensitivity of these cells to TCR stimulation (34). An increase in epitope density was shown to regulate CD8 viability via the Nur77 family member Nor-1 (35). Nor-1 and bim were proposed to converge at the mitochondrial level to regulate cell fate in thymic selection (36), but it remains to be determined whether similar mechanisms are in place in effector cells to regulate apoptosis. The difference in cell accumulation in the LM–N4– and LM–Q4–infected animals could be due to differences in the number of cell divisions, as suggested by BrdU-incorporation experiments (4), without a significant role for bim-mediated apoptosis. Although we cannot measure the fold difference in Ag dose at different time points following immunization with peptide-pulsed DCs versus recombinant *L. monocytogenes*, it is important to consider that we are comparing limited versus replicating Ag scenarios. The number of infected cells in the spleen increases dramatically in the first days following *L. monocytogenes* infection (37) and Ag presentation persists for >10 d (38), supporting our notion that there is a higher Ag availability in the recombinant *L. monocytogenes* immunization scenario.

Following a secondary challenge, OT-I T cells from all four experimental groups expanded very robustly, but fold expansion across the different groups has to be interpreted carefully because of the different memory precursory frequencies in each group. Importantly, we show that functional memory cells can be generated, even in suboptimal conditions with a limited Ag dose, low-affinity TCR signal, and limited inflammation (Fig. 4). Together, our data provide mechanistic insight into the success of vaccines that are based on the use of live, replicating vectors and suggest that this approach is likely the most successful one to generate a broad CD8 memory pool.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


