Transient Enhanced IL-2R Signaling Early during Priming Rapidly Amplifies Development of Functional CD8+ T Effector-Memory Cells

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Transient Enhanced IL-2R Signaling Early during Priming Rapidly Amplifies Development of Functional CD8$^+$ T Effector-Memory Cells

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Much is known concerning the cellular and molecular basis for CD8$^+$ T memory immune responses. Nevertheless, conditions that selectively support memory generation have remained elusive. In this study, we show that an immunization regimen that delivers TCR signals through a defined antigenic peptide, inflammatory signals through LPS, and growth and differentiation signals through the IL-2R initially favors Ag-specific CD8$^+$ T cells to develop rapidly and substantially into T effector-memory cells by TCR transgenic OVA-specific OT-I CD8$^+$ T cells. Amplified CD8$^+$ T memory development depends upon a critical frequency of Ag-specific T cells and direct responsiveness to IL-2. A homologous prime-boost immunization protocol with transiently enhanced IL-2R signaling in normal mice led to persistent polyclonal Ag-specific CD8$^+$ T cells that supported protective immunity to Listeria monocytogenes. These results identify a general approach for amplified T memory development that may be useful to optimize vaccines aimed at generating robust cell-mediated immunity.

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(CD122<sup>−/−</sup>) mice have been previously described (30) and were crossed to OT-I mice to yield OT-I CD122<sup>−/−</sup> mice. Animal studies were approved by the Institutional Animal Care and Use Committee at the University of Miami.

Adoptive transfer and peptide immunizations

OT-I T cells were purified (typically ∼95%) by magnetic-based positive selection using anti-CD8 beads (Miltenyi Biotec). For adoptive transfer, naive OT-I T cells (CD45.2) were injected i.v. through the tail vein into naive CD45.1 CD8<sup>+</sup> OT-I recipients. Unless otherwise indicated, mice were immunized by i.v. injection in the tail vein with OVA<sub>257-264</sub> (10 μg) (AnaSpec, San Jose, CA) and LPS (10 μg) (E. coli 055:B5; Sigma, St. Louis, MO). Twenty-four hours after immunization, the indicated mice received i.p. IL-2 IC in 100 μl HBSS. IL-2 IC was prepared by mixing 1.5 μg mouse IL-2 (eBioscience) and 15 μg Jes-6.1A12 mAb to mouse IL-2 (eBioscience) in 18 μl per injection for 15 min at room temperature followed by addition of HBSS.

FACS analysis

Flow cytometry for cell surface molecules, intracellular cytokines, and p-STAT5 and p-S6 were performed as previously described (7, 31). DimerX was purchased from BD Biosciences and loaded with OVA<sub>257-264</sub> according to the manufacturer’s instructions such that 0.33 μg OVA<sub>257-264</sub> to 0.5 μg of DimerX was added to ∼1 × 10<sup>6</sup> cells. Typically, 100,000–300,000 events were collected per sample. Intracellular cytokine production was performed after the culture of spleen cells (2.5 × 10<sup>5</sup> cells/well) from immunized mice in 24-well plates for 5 h with OVA<sub>257-264</sub> (0.1 nM) for OT-I, (100 nM for polyclonal T cells) in the presence of brefeldin A. For p-STAT5 and p-S6, spleens cells were cultured (2.5 × 10<sup>5</sup> cells/well) in 24-well plates in medium for 30 min and stimulated with mouse IL-2 (10 ng/ml) and IL-15 (10 ng/ml) for 15 min prior to p-STAT5 and p-S6 staining. To examine signals in vivo, immunized mice were euthanized, and spleen suspensions were immediately prepared in RPMI 1640 medium containing 5% FCS and 1.5% formaldehyde. These cells were incubated at 37°C for 10 min and then permeabilized with ice-cold methanol and stained for p-STAT5, p-S6, and relevant surface markers.

Gene expression analysis

Total RNA was isolated using TRizol and further purified with RNeasy Minikit (Qiagen). RNA quantity and quality was evaluated using an Agilent 2100 BioAnalyzer. A single round of linear RNA probe amplification and labeling was performed using the NuGEN Ovation Pico WTA system, WT-Ovation Exon Module, and Encore Biotin Module (NuGEN, San Carlos, CA). Gene expression was assessed using Affymetrix Mouse Gene ST 1.0 arrays at the Microarray and Gene Expression Core within the John P. Hussman Institute for Human Genomics at the University of Miami. Image analysis was performed using the Affymetrix Command Console Software.

Bacterial infections and determination of CFU

Recombinant Listeria monocytogenes that express OVA<sub>34-387</sub> (LM-OVA) (32), kindly provided by H. Chen (University of Pennsylvania, Philadelphia, PA), was grown using brain heart infusion (BHI) broth. Log-phase growing (OD<sub>560</sub> of 0.2) LM-OVA was diluted in PBS to 5 × 25 × 10<sup>7</sup> CFU for primary responses or to 5 × 10<sup>8</sup> CFU for challenging previously immunized mice and injected i.v. in the tail vein. The actual number of bacteria injected was confirmed by growth on BHI agar plates. Three days postinfection, the spleen and liver were processed using an 0.2-μm screen in 0.05% Triton X-100, and CFUs were determined by serial dilutions after incubation for 18 h at 37°C on BHI agar plates.

Statistical analysis

Data were analyzed using Prism 5.0 software. All data were analyzed by unpaired Student t test except for protection from Listeria infections where the Mann–Whitney U test was used. The p values are shown for statistically significant differences.

Results

IL-2R signaling in amplified CD8<sup>+</sup> T memory development

Mice were immunized to induce optimal, but transient, TCR, inflammatory, and IL-2R signaling. Initially, we examined CD8<sup>+</sup> T memory development by TCR transgenic OVA-specific MHC class I-restricted CD8<sup>+</sup> OT-I T cells. OT-I T cells (CD45.2) were adoptively transferred into wild-type (WT) congenic CD45.1-recipient mice, which facilitated identification of donor OT-I T cells. One day after the OT-I cell transfer, recipient mice were immunized with OVA<sub>257-264</sub> to induce TCR signaling and LPS to provide an inflammatory signal. IL-2 in the form of a single application of an IL-2 IC that targets the high-affinity IL-2R was administered 20–24 h after Ag to coincide with expression of the Ag-induced high-affinity IL-2R and to take advantage of the improved pharmacokinetics of IL-2 IC (14, 15).

Immunization with MHC class I binding peptides is a direct approach to drive an exogenous Ag into the class I presentation pathway. Immunization with peptides provides a convenient means for transient Ag, but the short half-life of peptides in vivo has generally resulted in disappointing immune responses. However, peptide immunization with OVA<sub>257-264</sub> and transient inflammation induced by LPS favored a rapid, large production of persistent CD8<sup>+</sup> T cells when IL-2R signaling was enhanced by IL2-IC (Fig. 1A, left). In the absence of IL2-IC, the magnitude of the primary response to LM-OVA was comparable to that elicited by OVA/LPS (Fig. 1A). Importantly, IL-2 IC did not substantially amplify the memory response to LM-OVA (Fig. 1A, right), suggesting that IL-2-amplified CD8<sup>+</sup> T memory is specific for immunization with OVA peptide/LPS. Immunization with OVA/LPS did not support production of CD8<sup>+</sup> Klrg1<sup>+</sup> short-lived Teff cells, whereas these cells dominated the OT-I response to LM-OVA (Fig. 1B). This trend was not influenced by IL2-IC, indicating that the production of CD8<sup>+</sup> Klrg1<sup>+</sup> OT-I cells was primarily due to the nature of the Ag and inflammatory signals and represented conditions that do not support an amplified T memory response.

When compared with administration of IL2-IC 1 d after OVA<sub>257-264</sub> and LPS priming, the application of a lower amount of IL2-IC at this interval or the simultaneous immunization with OVA/LPS and IL2-IC did not support increased persistent OT-I T cells (Fig. 1C). Multiple applications of IL2-IC substantially increased the primary response but only modestly increased persistent OT-I cells compared with a single application of IL2-IC 24 h after immunization (Fig. 1C). In addition, a similar level of OT-I memory cells was obtained when IL2-IC was applied 1 and 3 d, but not 5 d, after priming (Fig. 1D). Thus, early after a primary immunization, the Ag-activated T cells showed a window of responsiveness to IL2-IC to promote increased persistent OT-I memory T cells, where Ag-dependent activation must precede that of IL-2. On the basis of these results, in the remaining experiments in this report we always applied IL2-IC 24 h after immunization with OVA/LPS.

IL2-IC using Jes-6.1, which targets the high-affinity IL-2R, also caused a transient increased in spleen cellularity (Fig. 1E). This effect in part represents a substantial transient increased in CD8<sup>+</sup> T cells due to IL2-IC–driven expansion of OVA/LPS activated OT-I T cells (Fig. 1F), and an unexpected transient increase in CD4<sup>+</sup> Foxp3<sup>+</sup> Tregs (Fig. 1G). Collectively, these experiments show that targeting the high-affinity IL-2R for a brief period of time is sufficient to selectively support substantial expansion of not only Tregs but also Ag-reactive CD8<sup>+</sup> T cells, the latter of which persist for an extended period of time.

IL2-IC promotes rapid development of functional TEM cells

IL2-IC caused the CD8<sup>+</sup> T compartment to be dominated by OT-I cells 3–30 d postimmunization (Fig. 2A). This effect is primarily due to enhanced expansion (Fig. 2A, day 4–5) as the level of contraction was similar with and without IL2-IC (Fig. 2B). The overall effect of IL2-IC resulted in a 40- to 70-fold increase in the absolute number of OT-I memory cells by 10 and 30 d postpriming, respectively. At 4 d postpriming, IL2-IC favored production of OT-I
Teff cells (CD62Llo, CD127lo), but these cells substantially decreased by 10 and 30 d postimmunization (Fig. 2C). In contrast, mainly TEM OT-I cells (CD62Llo, CD127hi) were seen 10 and 30 d postimmunization with a minor population of T central-memory (TCM) cells (CD62Lhi, CD127hi) (Fig. 2C). Importantly, as assessed 15 d after immunization for mice receiving IL2-IC, only OT-I...
T cells developed into CD44hi CD62Llo CD127hi TEM cells (84.0 ± 1.0%; mean ± SE) as the remaining recipient-derived CD8⁺ T cells were mostly naive CD44lo/bright, CD62Lhi, and CD127hi cells (69.0 ± 2.4%; mean ± SE) (Fig. 2D). High expression of Ly-6C, which also marks CD8⁺ memory cells (33), was noted by day 10 postpriming, consistent with a rapid development of CD8⁺ T memory (data not shown). At 30 d postpriming, these memory cells were widely distributed in other lymphoid tissues, including the mesenteric lymph node, peripheral lymph node, and Peyer’s patch, and nonlymphoid tissues, including the bone marrow and the lamina propria of the small intestine (Fig. 2E). Functional analyses of splenic OT-I cells indicated that at 30 d after priming, IL-2-IC led to equivalent high production of IFN-γ, TNF-α, and IL-2 (Fig. 2F). Thus, IL-2-IC rapidly amplified the development of highly functional TEM cells.

Amplified TEM development leads to TCM cells

When induced in response to infection, CD8⁺ TEM cells persist for a relatively short period of time but appear to give rise to long-lasting TCM cells (3). Similarly, the high level of OT-I TEM cells found on day 30 postimmunization with IL-2Rα decreased when mice were examined for up to 334 d (Fig. 3A). For 10 mice followed for ≥110 d, the majority (n = 7) contained a substantial fraction of OT-I memory cells (at least 1% of the total CD8⁺ T cells or ~2 × 10⁵ memory cells, assuming 20 × 10⁶ CD8⁺ T cells/mouse), and these cells were typically >40% TCM cells (Fig. 3B). For seven mice followed for 334 d, OT-I memory cells were found in five mice, and they persisted at levels similar to that found on day 110 postimmunization (Fig. 3A). When these seven mice were challenged on day 334 with LM-OVA, the five mice with detectable OT-I memory cells each generated a recall response, as the proportion of OT-I cells increased 3- to 5-fold in the spleen (Fig. 3C) compared with the prechallenged levels in the PBL (Fig. 3A). An anti-LM-OVA protective response was also found based on a substantial reduction in the number of CFUs in the spleen that was proportional to the fraction of recall OT-I T cells (Fig. 3C). Collectively, these data indicate that a peptide-based immunization scheme with IL-2Rα initially favors high T cells (Fig. 3C). Collectively, these data indicate that a peptide-based immunization scheme with IL-2-IC initially favors high T cells (Fig. 3C). Collectively, these data indicate that a peptide-based immunization scheme with IL-2-IC initially favors high T cells (Fig. 3C). Collectively, these data indicate that a peptide-based immunization scheme with IL-2-IC initially favors high T cells (Fig. 3C).

IL-2R- and IL-15R signaling for TEM development

Given the striking effect of IL-2-IC on TEM production, we assessed the capacity of IL-2-IC to alter IL-2R- or IL-15R-dependent signaling by the Ag-responsive OT-I T cells. Our past work showed that in response to OVA257–264 and LPS by OT-I T cells without IL-2-IC, IL-2Rα is very transiently expressed and easily detected 24–48 h postimmunization, whereas IL-2Rβ, γc, and IL-15R remained expressed for at least 30 d (7). In this study, IL-2Rα was similarly expressed by OT-I after immunization without IL-2-IC (Fig. 4A, left). However, for OT-I T cells immunized in the presence of IL-2-IC, IL-2Rα was detected for an extended period of time, albeit at a somewhat lower level after day 10 (Fig. 4A, right). This result is consistent with the known role of IL-2 to upregulate expression of IL-2Rα (34) and indicates that the large increase in IL-2Rα expression by IL-2-IC is transient. IL-2- and IL-15- and IL-15- and IL-15-dependent signaling activates the STAT5 and PI3K/Akt pathways through the shared use of IL-2Rβ and γc by their receptors (35, 36). Activation of these pathways by Ag-activated OT-I T cells in the presence or absence of IL-2-IC in vivo or ex vivo after restimulation with IL-2 or IL-15 was assessed by measuring p-STAT5 and p-S6 (downstream of the PI3K/Akt) by phospho-flow analysis. p-STAT5, but not p-S6, increased when OT-I cells were cultured with IL-2 ex vivo for 15 min (Fig. 4B, left) and 60 min (data not shown). This effect was long-lasting only for the mice that were immunized with IL-2-IC, consistent with expression of IL-2Rα. This finding suggests that IL-2–dependent activation of STAT5 is primarily responsible for amplified CD8⁺ T memory development. Essentially identical responsiveness to IL-15 was noted for OT-I T cells immunized with and without IL-2-IC, with only p-STAT5 readily detected (Fig. 4B, right). Immunization with IL-2-IC increased the potential of OT-I T cells to respond to IL-2, which may facilitate T memory development and survival, while not altering responsiveness to IL-15. In contrast, p-STAT5 and p-S6 activation by OT-I T cells in vivo was observed, but only between days 2 and 4 postimmunization with IL-2-IC (Fig. 4C). The effect on p-S6 likely reflects TCR and/or costimulatory signaling because culture of these OT-I cells with IL-2 did not lead to increased p-S6 levels (Fig. 4B). Collectively, these experiments are consistent with the main contribution of IL-2-IC in amplified TEM development by inducing strong transient IL-2R- and IL-15R-dependent signaling by Ag-responsive T cells early after priming. We cannot, however, rule out that the increased potential of OT-I cells to activate STAT5 after immunization with IL-2-IC may result in weak IL-2R signaling that is difficult to detect in vivo but still contributes to maintain these TCM cells. The expansion and development of TEM cells depended upon a direct effect of IL-2-IC on the OT-I cells because the response by IL-2Rβ⁻/⁻ (CD122⁻/⁻) OT-I cells with IL-2-IC resembled that of WT OT-I cells immunized without IL-2-IC (Fig. 5A). We also tested the possible contribution of IL-15 in amplified development of TEM cells in the presence of IL-2-IC because IL-15R signaling overlaps with IL-2R due to sharing of IL-2Rβ and γc; because IL-2 and IL-15 induce similar levels of STAT5 in IL-2-IC–treated OVA/LPS-primed OT-I T cells (Fig. 4B); and because IL-15 is important for the persistence of CD8⁺ T memory cells (37). For these experiments, WT CD45.1 OT-I T cells were transferred into CD45.2 IL-15⁻/⁻ recipients. In the absence of IL-15, time-course experiments revealed that OT-I T cells in PBL initially expanded to high numbers but were found at significantly lower levels 30 d

**Figure 3.** Persistence of IL-2-IC–driven CD8⁺ T memory cells. OT-I T cells (5 × 10⁵) were transferred into CD45.1 B6 mice and then immunized with OVA257–264 LPS, and IL-2-IC. (A and B) At the indicated days, (A) the proportion of total and (B) the relative representation of TCM by OT-I T cells in the PBL were determined. (C) On day 334, mice were infected with 5 × 10⁵ CFU LM-OVA, and 3 d later the CFUs and donor OT-I T cells in the spleen were determined. Each point represents an individual mouse.
postimmunization (Fig. 5B). The relative development of TEM cells, however, was not affected by the absence of IL-15, whereas substantially lower Teff cells were detected (Fig. 5C). Analysis of the spleen at 30 d postimmunization confirmed this lower proportion of OT-I T cells in IL-15<sup>-/-</sup> recipients (Fig. 5D). This lower level is likely due to a role for IL-15 in the homeostasis of the persistent OT-I T cells because in the absence of IL-15, expression of Bcl-2 and the proliferative marker Ki67 was significantly lower (Fig. 5D). Thus, transient and relatively high IL-2R signaling by the responding Ag-specific T cells is required for the development of amplified TEM development while IL-15 supports their survival.

**IL-2–dependent gene expression profile and TEM development**

To begin to explore the molecular mechanisms that contribute to amplified TEM production, genome-wide transcriptional profiling was performed for OT-I cells immunized in the presence or absence of IL2-IC near the peak of p-STAT5 activation and OT-I expansion and after contraction by OT-I T cells without IL2-IC (i.e., day 3 and 5 postimmunization). Comparison across these groups revealed 1990 Affymetrix targets that varied by at least 2-fold between these samples. Euclidean clustering of these differentially expressed genes revealed three clades, with transcripts from day 3 OT-I cells with IL2-IC in a distinct clade, indicating that IL-2 exerted its most distinctive influence on gene expression at this time (Fig. 6A). Gene enrichment analyses (GEA) annotated 1089 of 1990 Affymetrix targets (54.7%) into five Gene Ontology functional classifications with z scores from 4.0 to 17.2. GEA after pairwise analysis revealed that immunization with IL2-IC increased transcripts for metabolism and cell cycle but downregulated transcripts for cell death and immune system processes (Fig. 6B).
Genes listed within metabolism were also highly enriched in transcripts related to the mitochondrion, and 80.4% of these 97 unique targets were overexpressed selectively on day 3 with IL2-IC, suggesting that mitochondria-dependent functions were enhanced by IL-2R signaling. Transcripts for histone modification were selectively enriched on day 3 after priming, suggesting that IL-2 may regulate chromatin remodeling to favor TEM development. Even though individual transcripts between groups varied, similar trends in GEA (downregulated: metabolism, cell cycle, histone modification; upregulated: cell death, immune system processes) were noted when comparing gene expression day 3 versus day 5 postimmunization irrespective of administering IL2-IC. This pattern corresponds to gene expression as Ag-activated T cells contract, but T memory cells persist.

Considering expression of selected transcripts (Fig. 6C), OT-I cells expressed a gene profile of TEM cells by day 5 after immunization with IL2-IC ([Se]low, CD62L, Ccr7lo, Cd44hi, IL7rihi (CD127), Cd27hi, Ly6clow, Gzmbhi, Prf1hi, IFNghi). This result is consistent with FACS analysis of these cells, further demonstrating the rapidity of TEM development. The development of IL2-IC–amplified TEM cells is likely due in part to low expression of inhibitory receptors (Cdl4 and Lagg5) and proapoptotic molecules (Fas and Bcl2l11(bim)) selectively on day 3 postimmunization. The accumulation of IL2-IC–driven TEM cells is also reflected by increased expression of transcripts related to cell cycle progression (Cdc34, Nudc, Cdkn3, Birc5, Mki67) as noted day 5 postpriming. IL-2IC caused several transcriptional regulators associated with T cells (Prdm1 (Blimp-1) and Irf4) and TEM (Eomes and Id2) cells to increase but other factors (Id3, Tcft7) supporting long-lived TEM cells to decrease. Other transcriptional regulators related to T effector (Tcf3, Tbx21, Gata3) and T memory (Bcl6) cells were not substantially altered. As suggested by other studies (29, 38–41), the relative levels of Blimp-1, Eomes, and Id3 and perhaps Irf4 may contribute to preferred TEM development by this immunization scheme.

A prime-boost regimen with IL2-IC supports TEM development by a low number of OT-I

The number of Ag-specific T cells to an individual epitope has been estimated to be between 50 and 1000 cells within a mouse (42). We have determined that after naive OT-I T cells are transferred into normal recipient mice, ~5% of the cells initially engraft and are available to respond to an antigenic challenge (see Fig. 2A, cell number on day 0). Thus, preferred TEM development might reflect a response to Ag and IL2-IC by a high number (~2.5 × 10^4 cells) of naive OT-I cells. Indeed, when lower numbers of OT-I T cells (0.1 × 10^3 to 10^3) were transferred into CD45.1 B6 mice and immunized in the presence of IL2-IC, a low primary response was detected on day 8 postimmunization, and the cells readily contracted to low levels (Fig. 7A). Nevertheless, successful priming was confirmed based on the proportion and number of OT-I T cells detected in the spleen after these mice were rechallenged with OVA254–264 and LPS (Fig. 7B). This finding indicates that amplified CD8^+ TEM development in the primary response (Figs. 1, 2) depends on a relatively high frequency of naive OT-I T cells.

Because the low number of naive OT-I T cells increased after immunization with IL2-IC, we tested whether a homologous prime-boost immunization strategy would suffice for the required high OT-I frequency for the accumulation of TEM cells. WT recipient mice were adoptively transferred with 1000–2000 naive OT-I T cells (estimated 50–100 engrafted naive OT-I T cells) and then were immunized and boosted 1 and 14 d later with OVA257–264 and LPS with or without IL2-IC. Marked expansion and minimal contraction of OT-I T cells were noted only after the boost with the IL2-IC (Fig. 7C), and these cells were mostly TEM cells (Fig. 7D). Thus, a low number of Ag-specific CD8^+ T cells are driven

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**FIGURE 6.** Gene expression by OT-I T cells immunized in the presence and absence of IL2-IC. Differential expressed genes from triplicate biological independent replicates were selected based on a 2-fold cutoff using one-way ANOVA (p < 0.05) after applying the Benjamini and Hochberg correction for false positives. (A) Euclidean clustering of sample relatedness based on differentially expressed genes. (B) Z-score analysis of GEA groups of differentially expressed genes based on the indicated pairwise analysis. Z score ≤ 2 was considered to represent significant gene enrichment within a given category. (C) Selected individual differentially expressed genes. All genes shown differed by at least 2-fold except those underlined, which are <2-fold.
T cells was enumerated using OVA257–264 bound to H-2Kb:Ig in the presence or absence of IL2-IC. The frequency of OVA-specific CD8+ T cells in the PBL were derived from three experiments each containing three mice/group. (A) OT-I T cells were enumerated in the spleen 3 d later. Data (mean ± SE) are derived from one experiment containing three mice/group. (C) OT-I T cells were enumerated in the spleen 3 d later. Data (mean ± SE) are derived from one experiment containing three mice/group. (D) Thirty days after the initial immunization, the distributions of Teff, TCM, and TEM based on expression of CD62L and CD127 were enumerated for the persistent OT-I T cells in the spleen. Data (C, D) (mean ± SE) are derived from three experiments each containing three mice/group.

FIGURE 7. Memory development by low numbers of Ag-specific naive T cells. CD45.1-B6 mice received the indicated number of naive OT-I T cells were immunized with OVA257–264, LPS, and IL2-IC. (A) OT-I T cells were enumerated in the PBL at the indicated time. (B) At 30 d postimmunization, mice were rechallenged with OVA257–264 and LPS, and OT-I T cells were enumerated in the spleen 3 d later. Data (mean ± SE) are derived from one experiment containing three mice/group. (C) CD45.1-B6 mice received 2000 OT-I T cells and were primed and boosted with OVA257–264 (10 μg) and LPS (10 μg) with and without IL2-IC as indicated. (D) Thirty days after the initial immunization, the distributions of Teff, TCM, and TEM based on expression of CD62L and CD127 were enumerated for the persistent OT-I T cells in the spleen. Data (C, D) (mean ± SE) are derived from three experiments each containing three mice/group.

A prime-boost regimen with IL2-IC supports immunity to LM-OVA in normal mice

To test the utility of this approach for immune protection by polyclonal T cells, normal mice without OT-I T cells were vaccinated with a prime-boost regimen with OVA257–264 and LPS in the presence or absence of IL2-IC. The frequency of OVA-specific T cells was enumerated using OVA257–264 bound to H-2Kβ-Ig fusion protein (OVA-DimerX) (Fig. 8A). When examined 5 and 19 d after the boost (i.e., days 18 and 33 after the primary immunization), 8.3 and 3.8% of the CD8+ T cells in the PBL were OVA257–264 specific (Fig. 8B). When considering the more numerous OVA-specific CD8+ T cells in the PBL from mice receiving IL2-IC, initially most were Teff cells just after the boost, but the dominant populations was TEM cells 19 d after the boost (Fig. 8C). Analysis of various tissues revealed a significant preference for OVA-specific CD8+ T cells in the spleen, lung, and mesenteric lymph node after the prime and boost with IL2-IC, with greatest numbers of such cells in the spleen and lung (Fig. 8D). Moreover, a similar frequency and preference for OVA-specific CD8+ T cells were found in the spleen when enumerated as IFN-γ+ cells after stimulation with OVA257–264 in vitro (Fig. 8E). Thus, these data indicate that IL2-IC readily supports production of a substantial number of OVA-specific polyclonal CD8+ T cells that preferentially persist as TEM cells.

To test the functional activity of these OVA-specific CD8+ T cells, at 30 and 60 d after the primary immunization (15 and 45 d after the boost), other mice were infected with a lethal dose of LM-OVA, which requires a CD8+ T cell response for protection (43). When examined 3 d postinfection, LM-OVA CFUs were reduced by 103- to 104-fold in the liver (Fig. 9A) and spleen (data not shown) for only those mice that received the homologous prime-boost vaccination with OVA257–264 and IL2-IC. A similar protective effect was found for mice that received a lower dose of OVA257–264 and IL2-IC for the primary and booster injections (Fig. 9B). This protective response was Ag-dependent because no protection was noted for mice that received only LPS and IL2-IC on days 1 and 14 (Fig. 9C). The frequency of OVA-specific CD8+ T cells in the spleen after challenge with LM-OVA (Fig. 9D) was similar to that detected in the PBL just prior to the LM-OVA challenge (Fig. 8B). For the IL2-IC–treated mice, the large majority of these were TEM cells (Fig. 9E). Thus, this immunization protocol that supports amplified TEM development provides potent Ag-dependent protective immunity when applied to normal mice.

Discussion

This study demonstrates that an immune response that is initially dominated by Teff cells yields an IL2-2-dependent amplified T memory response by a strategy that limits TCR, inflammatory, and IL-2R signaling after priming and booster injections. This finding is consistent with the notion that development of Ag-activated CD8+ T cells into memory cells can be optimized when receiving proper instructive signals. These conditions were met by immunization with a class I binding peptide, LPS, and transient application of IL2-IC. By targeting the high-affinity IL-2R for a relatively short time after priming, three beneficial activities occurred for Ag-reactive CD8+ T cells, enhanced clonal expansion, largely uniform and rapid differentiation to TEM cells, and persistence of highly functional memory cells. For OT-I T cells, memory cells persisted at least for ~1 y that led to an increased representation of TCM cells.

In an infection, Ag load and the associated inflammatory increase until immune clearance. Persistent antigenic and inflammatory signals and endogenous IL-2, likely through CD4+ T cells, are known to favor production of terminally differentiated short-lived CD8+ Klrg1+ Teff cells (8, 44). These conditions appear to subvert amplified TEM development, as CD8+ TEM development was not strongly favored for mice infected with LM-OVA and treated with IL2-IC. Indeed, the lack of CD8+ Klrg1+ short-lived Teff cells after immunization with OVA257–264, LPS, and IL2-IC provide further support that antigenic and inflammatory signals are limited by this immunization scheme.

Our data indicate that the sequence of administration of IL2-IC is critical to promote amplified CD8+ T memory development. Even though the IL2-IC we used has a half-life of ~72 h (15), the simultaneous immunization with OVA257–264, LPS, and IL2-IC did not support amplified CD8+ T memory. Presumably, high early IL-2R signaling by IL2-IC aborts the process by which IL-2R signaling supports large numbers of TEM cells, most likely by altering the sequence of expression of a critical set of genes. However, a single application of a relatively high amount of IL2-IC 24 h after Ag immunization was sufficient to support substantial amplified TEM production that was only marginally improved by increased application of IL2-IC. Our data indicate that there is a window of time, ~1–3 d after immunization, where Ag-activated CD8+ T cells are receptive to IL2-IC to amplify TEM cells.

Past work by others has also shown that IL2-IC using Jes-6.1 also increased the primary response by OVA254–264-stimulated OT-I T cells, but a large expansion of Ag-specific memory cells did not occur (11). Our study found the same result when we used a similar low amount of IL2-IC. Thus, the level of IL-2 is another key variable for amplified T memory responses. In addition, enhanced IL-2R signaling primarily through stimulation of the intermediate-affinity IL-2R, composed of IL-2Rβ and γc, using...
a distinct IL-2-IC (IL-2/S4B6 mAb), increased the numbers of all memory phenotypic CD8\(^+\) T cells because IL-2RB and γc are expressed by most CD8\(^+\) T memory cells (11, 16, 17). Correspondingly, the augmented Ag-specific responses observed were not selective and were secondary to this generalized increase in memory cells. In marked contrast, an important advantage of targeting the high-affinity IL-2R with IL-2-IC is that essentially only the desired Ag-specific CD8\(^+\) T cells are driven into the memory pool.

IL-2 has been used with varied results to promote Teff and T memory responses (10, 12, 13, 45). Notably, application of IL-2 during the course of an immune response to viral infections did not lead to striking preferential development into CD8\(^+\) T memory cells (10, 13). Our findings provide several explanations that may account for the variable results in past studies. One point is that IL-2-dependent amplified CD8\(^+\) Teff and T memory responses did not strikingly occur in the context of LM-OVA infection, suggesting that an active infection represents a setting that does not readily support substantial amplified memory production due to more persistent Ag and inflammatory signals. The other point is that the improved pharmacokinetics of IL2-IC with its long half-life generates consistent and prolonged IL-2R signaling that is highly transient during most immune responses in vivo (7, 8). During immune responses to peptide Ag in vivo without application of IL-2, the selective and short duration of IL-2R signaling is difficult to replicate with free IL-2 with its very short half-life (30 min). Our findings raise the possibility that a selective and optimal enhancement of IL-2R signaling over 2–3 d in the context of peptide immunizations represent a unique set of conditions that yield potent cell-mediated Ag-specific CD8\(^+\) T cell immunity. However, this point will require more systemic testing of varied conditions of immunization, including other peptides and vaccine formulations.

Considering our results in the context of a physiological immune response, CD8\(^+\) TeM cells are predicted to be favored when they are in a microenvironment rich in IL-2 but low in Ag and inflammatory mediators. Moreover, there is a limited time for such TeM development to occur as expression of IL-2 and IL-2Rα are highly transient during most immune responses in vivo (7, 8). During immune responses to peptide Ag in vivo without application of IL-2, the selective and short duration of IL-2R expression and IL-2 production ensures development of TeM cells, but at limited numbers. Indeed, when IL-2R signaling is deliberately limited in this context, TEm but not TCM development is impaired (7). Thus, enhanced IL-2R signaling early after priming
or booster injections favors the development of CD8+ TEM memory cells.

Molecular analysis is consistent with transient IL-2R signaling in vivo primarily favoring activation of STAT5 in supporting amplified CD8+ T memory development. Minimal activation of p-S6, downstream of PI3K/Akt and mTOR, was seen when IL-2–treated OVA/LPS–primed OT-I were stimulated with IL-2 ex vivo. We believe this result is noteworthy because recent work indicates that lower mTOR signaling favors CD8+ T memory development (46, 47). Thus, IL-2-IC in the context of OVA/LPS intrinsically promotes intracellular signaling that favors CD8+ T memory. IL-2-IC substantially altered gene expression initially that favored growth, metabolism, and gene programming while reducing cell death. IL-2-IC increased many transcripts associated with the mitochondria suggesting that our regimen of IL-2-IC may lead to increased mitochondrial function that has been suggested to favor memory development (46, 48, 49). Another consequence of IL-2-IC is that the balance of key transcription factors, including Blimp-1, Irf4, Eomes, Id2, Id3, and Tcf7, are altered, which likely favors the expanding Ag-responsive CD8+ T cells to develop into TEM cells.

This immunization scheme used a defined class I binding peptide (i.e., OVA257–264) that does not activate Ag-specific CD4+ T cells. Even though the IL2-IC caused a transient increase in CD4+ Tregs, this approach should not yield an increase in Ag-specific natural or induced Tregs to a class I peptide. Importantly, the generalized transient increase in Tregs did not prevent a substantial immune response not only by OT-I T cells but also by non-TCR transgenic polyclonal OVA-specific CD8+ T cells. In addition, CD4+ T “help” was not provided for these CD8+ T cells responses. Other studies have shown that CD8+ T cells generate “helpless” immune responses and persistent memory cells, but their recall responses are impaired (50–52). CD4+ T help influences CD8+ T memory during the initial priming that in part is dependent upon IL-2R signaling (28). In this study, amplified TEM development by OT-I T cells was dependent on a relatively high frequency of naive OT-I T cells and IL2-IC. We speculate that the high number of Ag-specific CD8+ T cells in the context of IL2-IC provides their own “helper” signals that support amplified TEM memory development and effective recall responses. At this time, we have not defined the intrinsic “helper” signal associated with IL2-IC peptide–primed CD8+ T cells. Many studies have implicated CD4+ T cells in providing help for CD8–dependent T cell responses through licensing of dendritic cells and secretion of IL-2 (53). Lack of IL-2 readily explains the low memory responses by OT-I T cells at a high precursor frequency when mice were immunized with OVA257–264 in the absence of IL2-IC because enhancement of IL-2R signaling was sufficient for amplified CD8+ TEM cells. However, lack of IL-2 does not explain the inability to generate amplified TEM cells at a low precursor frequency of OT-I T cells because we supplied excess IL-2 in the form of IL2-IC. However, CD40L or other molecules such as OX40 or 4-1BB, which promote CD8 memory development and survival (54–56), remain potential candidates as each were increased as assessed by gene profiling of OT-I T cells that developed into amplified TEM cells (Fig. 6C and data not shown).

An important aspect of this study is that we have used the information obtained from OT-I T cells as a model to generate potent protective CD8+ T cell memory responses by polyclonal CD8+ T cells. Our ability to replicate amplified memory development for rare naive Ag-specific CD8+ T cells required the use of a homologous prime-boost immunization strategy where the initial priming increased the number of Ag-reactive CD8+ T cells to a critical number for TEM development. We readily detected a substantial fraction of Ag-specific T cells 1–3 wk after the boost in the PBL, spleen, and nonlymphoid tissues, and these cells persisted for at least 45 d after the boost as assessed by protection against a lethal challenge with LM-OVA. TEM cells were the main OVA-specific polyclonal T cells that persisted after the boost with IL2-IC. However, TEM cells did not strikingly dominate this memory response compared with OT-I T cells. This difference likely reflects heterogeneity by the responding polyclonal T cells. Nevertheless, the success of this homologous-primed boost immunization in generating protective CD8+ T cell responses after a lethal infectious challenge indicates that this approach represents a potentially general means to design vaccines where robust cell-mediated immune memory is a desired component. Prophylactic immunity to any infectious agent, including malaria, HIV/AIDS, and tuberculosis, may be possible to appropriate MHC class I epitopes by transiently increasing IL-2R signaling. Although peptide-based vaccines have been disappointing in various therapeutic settings, the transient enhancement of IL-2R signaling that amplifies CD8+ T memory responses offers a new approach to induce potent long-lasting immune responses in the context of infection or tumor immunotherapy.

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