IL-15 Transpresentation Augments CD8\(^+\) T Cell Activation and Is Required for Optimal Recall Responses by Central Memory CD8\(^+\) T Cells

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IL-15 Transpresentation Augments CD8+ T Cell Activation and Is Required for Optimal Recall Responses by Central Memory CD8+ T Cells

Andy I. Kokaji, Deanna L. Hockley, and Kevin P. Kane

Although the adaptive immune system has a remarkable ability to mount rapid recall responses to previously encountered pathogens, the cellular and molecular signals necessary for memory CD8+ T cell reactivation are poorly defined. IL-15 plays a critical role in memory CD8+ T cell survival; however, whether IL-15 is also involved in memory CD8+ T cell reactivation is presently unclear. Using artificial Ag-presenting surfaces prepared on cell-sized microspheres, we specifically addressed the role of IL-15 transpresentation on mouse CD8+ T cell activation in the complete absence of additional stimulatory signals. In this study we demonstrate that transpresented IL-15 is significantly more effective than soluble IL-15 in augmenting anti-CD3ε-induced proliferation and effector molecule expression by CD8+ T cells. Importantly, IL-15 transpresentation and TCR ligation by anti-CD3ε or peptide MHC complexes exhibited synergism in stimulating CD8+ T cell responses. In agreement with previous studies, we found that transpresented IL-15 preferentially stimulated memory phenotype CD8+ T cells; however, in pursuing this further, we found that central memory (T\text{CM}) and effector memory (T\text{EM}) CD8+ T cells responded differentially to transpresented IL-15. T\text{CM} CD8+ T cells undergo Ag-independent proliferation in response to transpresented IL-15 alone, whereas T\text{EM} CD8+ T cells are relatively unresponsive to transpresented IL-15. Furthermore, upon Ag-specific stimulation, T\text{CM} CD8+ T cell responses are enhanced by IL-15 transpresentation, whereas T\text{EM} CD8+ T cell responses are only slightly affected, both in vitro and in vivo. Thus, our findings distinguish the role of IL-15 transpresentation in the stimulation of distinct memory CD8+ T cell subsets, and they also have implications for ex vivo reactivation and expansion of Ag-experienced CD8+ T cells for immunotherapeutic approaches.

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Interleukin-15 (IL-15) is a member of the four α-helix family of cytokines and has diverse roles in lymphocyte development, homeostasis, and activation (1-3). Both IL-15- and IL-15 receptor α- (IL-15Rα)-deficient mice have similar phenotypes consisting of severe defects in the development of NK, NK T cells, and intraepithelial lymphocytes, as well as a lack of peripheral memory phenotype (MP) CD8+ T cells (4, 5). Despite these defects, the initiation of primary CD8+ T cell responses against lymphocytic choriomeningitis virus (LCMV) occurs in mice lacking IL-15 or IL-15Rα expression; however, the long-term maintenance and homeostatic proliferation of memory CD8+ T cells is critically dependent on IL-15-derived signals (4-8). Consistent with these findings, in vivo administration or transgenic overexpression of IL-15 results in enhanced proliferation and increased numbers of MP CD8+ T cells (9-11).

Since the identification of IL-15, a significant amount of data supporting its role as a T cell activator has accumulated due to structural and functional similarities to IL-2 (12, 13). In vitro studies have shown that high concentrations of IL-15 can induce cellular proliferation and transcription of effector molecules such as IFN-γ and granzyme B (grB) (14, 15). Additionally, soluble IL-15 can augment anti-CD3ε-induced activation and cytokine production of both mouse and human CD8+ T cells (16-19). Whether IL-15 synergizes with, or acts independently of, TCR stimulation is presently unclear; however, stimulation of MP CD8+ T cells by IL-15 or anti-CD3ε induces remarkably similar gene expression patterns, as identified by cDNA microarray analysis (20).

It was initially thought that IL-15 mediated its effects upon binding to a heterotrimeric receptor complex composed of IL-15Rα, CD122 (IL-2/15Rβ), and CD132 (common γ-chain) (21, 22). It was later revealed that CD8+ T cells did not require IL-15Rα expression and could respond to IL-15 when expressing only CD122 and CD132 (23). Upon further analysis, coordinated expression of IL-15Rα and IL-15 by bone marrow-derived cells was found to be crucial for IL-15-mediated effects on CD8+ T cells in vivo (23-26). Unlike other soluble cytokines that induce signals upon binding to their respective receptors, IL-15 bound to its specific high-affinity IL-15Rα-chain can be retained on the cell surface and presented in trans to neighboring cells expressing only CD122 and CD132 (27). Due to the extremely high affinity of the IL-15 and IL-15Rα interaction (Kd = 38 pM), it has been suggested that the receptor–cytokine complex may act as a membrane-bound stimulatory molecule in a contact-dependent manner (28). Taken together, these findings suggest that a bone marrow-derived cell capable of expressing both IL-15Rα and IL-15 could...
IL-15 TRANSPRESENTATION ENHANCES MEMORY CD8 T CELL RESPONSES

regulate memory CD8 T cell responses. Because dendritic cells (DC) can express both IL-15Rα and IL-15 following activation, they are likely candidates involved in IL-15 transpresentation (29). In support of this concept, DC-derived IL-15 is essential for the induction of delayed-type hypersensitivity responses in mice (30).

Requirements for the initiation of primary CD8 T cell responses have been well documented; however, the events required for the initiation of recall responses by memory CD8 T cells are less well defined. Similar to naive CD8 T cells, recall responses by memory CD8 T cells to previously encountered Ags require the presence of DCs (31). Cell-sized microspheres have been successfully used to investigate the role of immobilized protein ligands on lymphocyte activation and/or adhesion (32–37). Substitution of microspheres for DCs allows for the precise control of the constellation and density of ligands displayed to a responding CD8 T cell. Using microspheres as a platform to generate Ag-presenting surfaces, we sought to determine the specific role of IL-15 transpresentation in the reactivation of MP and ex vivo Ag-specific memory CD8 T cells. Because DCs themselves respond to IL-15 through enhanced survival, up-regulation of costimulatory molecules, and production of effector cytokines, microspheres allow a focus on the direct effects of IL-15 transpresentation on the responding CD8 T cells in the absence of other stimulatory signals (29, 38–40).

In the present study, we demonstrate that transpresented IL-15 in combination with a TCR stimulus provided by either anti-CD3ε or peptide MHC complexes (pMHC) was significantly more effective at inducing proliferation and up-regulating IFN-γ and grB expression by CD8 T cells than was soluble IL-15. Additionally, coinoculation of anti-CD3ε and transpresented IL-15 were more effective than either anti-CD3ε or transpresented IL-15 alone, or anti-CD3ε and transpresented IL-15 provided on two separate surfaces. In agreement with previous studies, we found that transpresented IL-15 preferentially stimulated MP CD8 T cells; however, in pursuing this further, we found that central memory (T CM) CD8 T cells were more responsive to IL-15 transpresentation than were effector memory (T EM) CD8 T cells in vitro. Following Ag-specific pMHC stimulation, T CM CD8 T cells were also more dependent on transpresented IL-15 than were T EM CD8 T cells for the induction of grB and proliferation in vitro. Upon examination in vivo, LCMV-specific T CM CD8 T cells were further sorted into naive, T CM, and T EM populations based on CD8 expression. Purity of each population was typically >90% CD3ε CD8ε as determined by flow cytometric analysis (data not shown). For CFSE labeling, CD8 T cells were washed with 0.1% BSA (Sigma-Aldrich) and resuspended in culture medium. In some instances, 1 × 10^7 microspheres were incubated with 100 ng of peptide in 100 μl of 5 × 10^5 cells/ml in 0.1% BSA/PBS containing a final concentration of 2 or 5 μM CFSE for 5 min at 37°C, followed by washing with 2% FBS (HyClone) in PBS. In some instances, negatively enriched, CFSE-labeled CD8 T cells were further sorted into naive, T CM, and T EM populations based on CD8ε, CD44, and CD26L expression. Purity of each population was typically >95% following sorting with a BD FACSAria (BD Biosciences).

Microsphere preparation

Microsphere constructs were prepared by incubating 1 × 10^7 5-μm sulfate-modified polystyrene microspheres (Invitrogen) with 0.1–1 μg of various proteins at 4°C with rotation for 15 min in PBS. Unbound sites on the microspheres were blocked with the addition of 1% BSA/PBS followed by an additional 30 min incubation at 4°C with rotation. Microspheres were washed with 0.1% BSA/PBS and resuspended in culture medium. For peptide loading of immobilized H-2Db/Ig, 1 × 10^7 microspheres were incubated with 20 μg of peptide in 100 μl PBS for 1 h at 37°C, washed extensively with 0.1% BSA/PBS, and resuspended in culture medium. In some instances, 1 × 10^6 microspheres were incubated with 100 ng of recombinant mouse IL-15 overnight at 4°C with rotation and washed extensively before use with 0.1% BSA/PBS. Unless otherwise stated, 1 μg of IL-15Rα/Fc was immobilized alone or in combination with 0.1 μg of anti-CD3ε or 1 μg of H-2Dβ/Ig onto 1 × 10^6 microspheres. Density of immobilized proteins or transpresented IL-15 on microspheres was analyzed by flow cytometry using ligand-specific Abs.

CD8 T cell stimulation with microspheres

Two hundred fifty thousand CD8 T cells or sorted CD8 T cell populations were cultured with 0.5 × 10^6 microspheres in 96-well flat-bottom culture plates (Corning) in a final volume of 0.25 ml. Culture medium consisted of RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.05 mM 2-ME (Invitrogen). Cultures were incubated at 37°C with 5% CO₂ and harvested at the indicated time points for flow cytometric analysis. Where indicated, IL-15 was added at a final concentration of 0–100 ng/ml at the start of culture.

CFSE dilution and intracellular cytokine analysis

Brefeldin A (Invitrogen) was added to cultures at a final concentration of 1 μg/ml for 4 h before harvest. CFSE-labeled CD8 T cells were harvested and stained with fluorochrome-conjugated mAbs against cell-surface markers, fixed and permeabilized with BD Cytofix and Cytoperm buffers (BD Biosciences), and counterstained with fluorochrome-conjugated anti-IFN-γ and anti-grB mAbs. All staining and fixation steps were performed at 4°C for 15 min. Flow cytometric acquisition was performed using a BD
Results

IL-15 augmentation of anti-CD3e-induced CD8\(^+\) T cell activation is more effective in the presence of IL-15Ra/Fc

Although exogenous IL-15 has been shown to augment T cell responses following TCR stimulation (16–19), to our knowledge no direct comparison of soluble IL-15 vs transpresented IL-15 has been examined in the presence of a TCR stimulus. Additionally, previous reports have used total mouse splenocyte preparations or human PBMCs for the measurement of CD8\(^+\) T cell responses, which may include cells expressing IL-15Ra and hence capable of transpresenting IL-15. Therefore, we sought to directly compare soluble IL-15 vs transpresented IL-15 in augmenting anti-CD3e-induced activation of purified C57BL/6 CD8\(^+\) T cells. To address this issue we used a 5-µm microsphere platform to control and manipulate the stimulatory signals that evoke CD8\(^+\) T cells would receive. Microspheres were prepared with either anti-CD3e or IL-15Ra/Fc immobilized alone or coimmobilized together and analyzed by flow cytometry (Fig. 1A). The density of anti-CD3e could be preserved when IL-15Ra/Fc was coimmobilized, thereby maintaining microspheres offering equivalent TCR stimulatory signals with or without IL-15Ra/Fc. To potentially boost sensitivity for the detection of IL-15-mediated stimulation, anti-CD3e was immobilized at a density that induced suboptimal T cell proliferation (data not shown). Once prepared, the various microsphere constructs were incubated with CD8\(^+\) T cells in the presence of increasing concentrations of soluble IL-15. In the absence of immobilized IL-15Ra/Fc, soluble IL-15 would act directly on the responding CD8\(^+\) T cells. In contrast, IL-15 in the presence of IL-15Ra/Fc could be transpresented by the immobilized receptor due to the high affinity of the IL-15Ra/Fc for IL-15 (28). CD8\(^+\) T cells were cultured with the various microsphere constructs for 24 or 48 h and analyzed for expression of intracellular IFN-γ and grB, or for cell division detected by CFSE dilution.

Following culture of CD8\(^+\) T cells with BSA or IL-15Ra/Fc microspheres in the absence of soluble IL-15, no induction of IFN-γ, grB, or proliferation was noted (Fig. 1B). Minimal induction of CD8\(^+\) T cell responses was also detected in the absence of soluble IL-15 following stimulation with either anti-CD3e or anti-CD3e and IL-15Ra/Fc microspheres, thus confirming the suboptimal density of immobilized anti-CD3e. Upon addition of increasing concentrations of soluble IL-15 to BSA or IL-15Ra/Fc microsphere cultures, minimal increases in intracellular IFN-γ and grB expression and proliferation were detected in the responding CD8\(^+\) T cells (Fig. 1B). In comparison, soluble IL-15 could augment anti-CD3e-induced IFN-γ and grB expression, as well as cellular proliferation (Fig. 1B). However, in the presence of coimmobilized anti-CD3e and IL-15Ra/Fc, IFN-γ and grB expression and proliferation of responding CD8\(^+\) T cells were induced to substantially higher levels at all IL-15 concentrations examined (Fig. 1B). These findings demonstrate that approximately 5-fold higher concentrations of soluble IL-15 are required to induce similar levels of proliferation and IFN-γ and grB production with immobilized anti-CD3e, compared with when IL-15Ra/Fc is coimmobilized with anti-CD3e.

Synergism of IL-15 transpresentation and anti-CD3e on CD8\(^+\) T cell stimulation

To investigate the direct ability of transpresented IL-15 to augment anti-CD3e-induced proliferation and cytokine production in light of our preceding findings, microspheres were prepared with coimmobilized anti-CD3e and IL-15Ra/Fc in the presence or absence of preloaded IL-15. To ensure that IL-15 available in the culture was transpresented in the context of IL-15Ra/Fc, microspheres were incubated overnight with IL-15 and subsequently washed extensively to remove any soluble unbound IL-15. To verify that IL-15 was indeed transpresented by our microsphere constructs, they were stained and analyzed by flow cytometry (Fig. 2, A and B). The microspheres allowed for the precise titration of transpresented IL-15, as demonstrated by the tight correlation between the density of immobilized IL-15Ra/Fc and bound IL-15 shown as individual histograms (Fig. 2A, middle and right panels), or as

Adoptive transfers

Negatively enriched CD8\(^+\) T cells from B6129SF2/J mice infected i.p. with LCMV-Armstrong 40 days prior were CFSE labeled and sorted into naive, TcR, and TcM populations, as previously described. A half million of each T cell population was transferred by i.v. injection into naive B6129SF2/J or B6129X1il15ratm1Ama/J mice. The following day, recipient mice were infected i.p. with LCMV-Armstrong as previously described. Four days after infection, recipient mice were euthanized and spleens and lymph nodes were harvested. Single-cell suspensions of spleen and lymph node samples were prepared and stained with anti-CD8α and analyzed for CFSE dilution.

FIGURE 1. Soluble IL-15 is more effective at augmenting anti-CD3e-induced CD8\(^+\) T cell stimulation in the presence of IL-15Ra/Fc. A, Immobilization of anti-CD3e and IL-15Ra/Fc onto cell-sized microspheres. FACS histograms of microsphere constructs with immobilized anti-CD3e, IL-15Ra/Fc, or both. Immobilized anti-CD3e was detected by a FITC-conjugated goat anti-hamster IgG. IL-15Ra/Fc and IL-15 were detected using polyclonal goat anti-IL-15Ra or IL-15, followed by an R-PE-conjugated donkey anti-goat IgG F(ab‘)\(_2\). Shaded histograms represent staining of BSA microspheres with the indicated Abs. B, Soluble IL-15 in the presence of immobilized IL-15Ra/Fc augments anti-CD3e activation of CD8\(^+\) T cells. Negatively enriched, CFSE-labeled C57BL/6 CD8\(^+\) T cells were cultured with the indicated microsphere constructs in the presence of soluble IL-15 at a final concentration of 0–100 ng/ml. At the indicated time points, cells were analyzed for intracellular IFN-γ and grB and CFSE dilution by flow cytometry. Data represent means of triplicate samples ± SEM. Thirty thousand gated events were acquired per sample.
CD8^+ T cell responses occur when IL-15 and anti-CD3e are presented on two separate surfaces, while copresentation of IL-15 and anti-CD3e provides optimal stimulation

We next determined whether IL-15 transpresentation could augment an anti-CD3e stimulus provided on a separate surface, or whether the two signals must be presented on the same surface. To this end, microspheres were prepared with immobilized IL-15Ra/Fc alone (Ra) or in combination with anti-CD3e in the presence or absence of preloaded IL-15 (2C11Ra and 2C11Ra15, respectively). BSA-blocked microspheres served as a negative control. Anti-CD3e was immobilized at a suboptimal density as previously demonstrated (Figs. 1 and 2), and IL-15Ra/Fc was immobilized at a high density to provide optimal IL-15 stimulation. The various microspheres were cultured individually or in combination with CFSE-labeled C57BL/6 CD8^+ T cells for 24 and 48 h and subsequently analyzed for proliferation and intracellular IFN-γ and grB production by flow cytometry. When CD8^+ T cells were cultured with each microsphere construct individually, BSA and Ra microspheres had no effect on any of the responses examined (Fig. 3). Ra15 microsphere stimulation of CD8^+ T cells resulted in no IFN-γ production and a low level of grB expression and proliferation. 2C11Ra microspheres induced a low level of proliferation and IFN-γ and grB expression, while maximal responses were generated by 2C11Ra15 microspheres. Combined stimulation of CD8^+ T cells with 2C11Ra and BSA or Ra microspheres resulted in no augmentation of responses compared with 2C11Ra microspheres alone. Interestingly, the combination of Ra15 and 2C11Ra microspheres led to significantly increased IFN-γ and grB production, as well as enhanced proliferation compared with when either was cultured alone. However, the responses were consistently lower than those induced by 2C11Ra15 microspheres. Taken together, our findings suggest that IL-15 transpresentation can substantially augment TCR stimulation even when presented on a separate surface. Furthermore, to optimally enhance anti-CD3e-mediated responses, transpresented IL-15 should be codisplayed on the same surface.
CD8+ T cell responses are augmented when transpresented IL-15 and anti-CD3e are presented on separate surfaces, and copresentation of transpresented IL-15 and anti-CD3e provides optimal CD8+ T cell stimulation. Negatively enriched, CFSE-labeled C57BL/6 CD8+ T cells were cultured with various microsphere constructs alone or in combination for 24 and 48 h. Two hundred fifty thousand CD8+ T cells were cultured with 0.5 × 10^6 of each indicated microsphere construct (total 0.5–1 × 10^6 microspheres). The various microsphere constructs used are abbreviated as follows: IL-15Ro/Fc (R15), IL-15Ro/Fc:IL-15 (Ro15), 145-2C11 + IL-15Ro/Fc (2C11Ro), and 145-2C11 + IL-15Ro/Fc:IL-15 (2C11Ro15); a plus sign indicates the combination of two different microsphere constructs in the culture (e.g., 2C11Ro microspheres + BSA microspheres (2C11Ro + BSA)). At 24 and 48 h of culture, the cells were harvested and analyzed by flow cytometry for intracellular IFN-γ and grB, and for CFSE dilution. Data represent means of triplicate samples ± SEM. More than 40,000 gated events were acquired per sample. One-way ANOVA analysis with Bonferroni’s multiple comparison test were performed with GraphPad Prism software (***, p < 0.001; **, p < 0.01).

**FIGURE 3.** CD8+ T cell responses are augmented when transpresented IL-15 and anti-CD3e are presented on separate surfaces, and copresentation of transpresented IL-15 and anti-CD3e provides optimal CD8+ T cell stimulation. Negatively enriched, CFSE-labeled C57BL/6 CD8+ T cells were cultured with various microsphere constructs alone or in combination for 24 and 48 h. Two hundred fifty thousand CD8+ T cells were cultured with 0.5 × 10^6 of each indicated microsphere construct (total 0.5–1 × 10^6 microspheres). The various microsphere constructs used are abbreviated as follows: IL-15Ro/Fc (R15), IL-15Ro/Fc:IL-15 (Ro15), 145-2C11 + IL-15Ro/Fc (2C11Ro), and 145-2C11 + IL-15Ro/Fc:IL-15 (2C11Ro15); a plus sign indicates the combination of two different microsphere constructs in the culture (e.g., 2C11Ro microspheres + BSA microspheres (2C11Ro + BSA)). At 24 and 48 h of culture, the cells were harvested and analyzed by flow cytometry for intracellular IFN-γ and grB, and for CFSE dilution. Data represent means of triplicate samples ± SEM. More than 40,000 gated events were acquired per sample. One-way ANOVA analysis with Bonferroni’s multiple comparison test were performed with GraphPad Prism software (***, p < 0.001; **, p < 0.01).

**IL-15 transpresentation enhances ex vivo LCMV-specific memory CD8+ T cell responses**

Thus far we have addressed the ability of IL-15 transpresentation to augment anti-CD3e-induced activation of CD8+ T cells from unimmunized C57BL/6 mice. Physiological CD8+ T cell activation occurs only upon TCR recognition of cognate peptide Ag presented by class I MHC, whereas anti-CD3e stimulation results in the polyclonal activation of responding CD8+ T cells. Additionally, unimmunized mice are populated with both naive and MP CD8+ T cells specific for self or environmental Ags. Therefore, our preceding results using anti-CD3e as a TCR stimulus did not distinguish whether the responding cells were naive or Ag-specific memory CD8+ T cells. To approach this question, particularly the capacity of IL-15 transpresentation to influence Ag-specific memory CD8+ T cell restimulation, various microsphere constructs were prepared with immobilized recombinant class I MHC fusion proteins (H-2Dβ/Ig) together with IL-15Ro/Fc. Following immobilization, microspheres with coimmobilized H-2Dβ/Ig and IL-15Ro/Fc were pulsed with either LCMV gp33 or control influenza NP366 peptides in the presence or absence of IL-15. To investigate the stimulatory capacity of the various microspheres on naive and Ag-specific MP CD8+ T cells, C57BL/6 mice were infected with LCMV-Armstrong and allowed sufficient time to generate a population of LCMV-specific memory CD8+ T cells. At least 40 days postinfection, CD8+ T cells were negatively enriched from the spleen and lymph nodes of the LCMV-immune C57BL/6 mice, labeled with CFSE, and sorted into naive (CD44low) and MP (CD44high) CD8+ T cell populations. CFSE-labeled naive and MP CD8+ T cells were cultured with the various microsphere constructs and analyzed at the indicated time points for proliferation and expression of intracellular IFN-γ and grB.

Using this approach, we found that CD44low naive phenotype CD8+ T cells did not undergo proliferation or express IFN-γ or grB when cultured with any of the microspheres examined (Fig. 4). The lack of responsiveness by the CD44low naive CD8+ T cells may have been due to the low frequency of gp33 and NP366-specific CD8+ T cells in the sorted populations. However, because naive CD8+ T cells did not respond to IL-15 transpresenting microspheres regardless of the peptide Ag, it suggested that naive CD8+ T cells are unresponsive to transpresented IL-15 alone. Our findings with naive CD8+ T cells are consistent with the well-established concept that primary activation and expansion of naive CD8+ T cells require stimulation through other costimulatory receptors in addition to TCR stimulation (42–44).

In contrast to naive CD44low CD8+ T cells, analysis of IFN-γ production by CD44high MP CD8+ T cells revealed differential

**FIGURE 4.** IL-15 transpresentation enhances ex vivo LCMV-specific memory CD8+ T cell responses. CD8+ T cells were negatively enriched from the spleen and lymph nodes of C57BL/6 mice previously infected i.p. with LCMV-Armstrong (>40 days postinfection). CD8+ T cells were labeled with CFSE, stained for CD8α, CD44low and CD44high populations, and sorted into naive (CD44low) and MP (CD44high) CD8+ T cell populations. CFSE-labeled naive and MP CD8+ T cells were cultured with the various microsphere constructs and analyzed at the indicated time points for proliferation and expression of intracellular IFN-γ and grB. Using this approach, we found that CD44low naive phenotype CD8+ T cells did not undergo proliferation or express IFN-γ or grB when cultured with any of the microspheres examined (Fig. 4). The lack of responsiveness by the CD44low naive CD8+ T cells may have been due to the low frequency of gp33 and NP366-specific CD8+ T cells in the sorted populations. However, because naive CD8+ T cells did not respond to IL-15 transpresenting microspheres regardless of the peptide Ag, it suggested that naive CD8+ T cells are unresponsive to transpresented IL-15 alone. Our findings with naive CD8+ T cells are consistent with the well-established concept that primary activation and expansion of naive CD8+ T cells require stimulation through other costimulatory receptors in addition to TCR stimulation (42–44).

In contrast to naive CD44low CD8+ T cells, analysis of IFN-γ production by CD44high MP CD8+ T cells revealed differential
responsiveness upon culture with the various microspheres. Stimulation of CD4^{high} MP CD8^{+} T cells from LCMV-immune mice with influenza NP366 peptide-pulsed microspheres (NP366/Rx) induced no detectable level of IFN-γ expression (Fig. 4A). Pre-loading of IL-15 onto NP366/Rx microspheres (NP366/Rx15) also had no effect on IFN-γ production. Taken together, the data demonstrated that neither nonspecific pMHC complexes nor IL-15 transpresentation in the absence of specific peptide Ag can induce IFN-γ expression by CD4^{high} MP CD8^{+} T cells. In contrast, when LCMV gp33 peptide was presented by H-2D^{b}/Ig in the absence of IL-15 (gp33/Ro), a low percentage of IFN-γ-producing cells was detectable, confirming the presence of LCMV gp33-specific memory CD8^{+} T cells in the CD4^{high} population. However, IL-15 preloading onto gp33/Ro microspheres (gp33/Ro15) greatly augmented IFN-γ expression by CD4^{high} MP CD8^{+} T cells in a synergistic manner, as suggested by the 3-fold increase in the percentage of IFN-γ-expressing cells.

In addition to IFN-γ, sorted CD8^{+} T cells cultured with the various microspheres were analyzed for intracellular grB expression (Fig. 4B). CD4^{high} MP CD8^{+} T cells stimulated with NP366/Rx microspheres were found to not express grB, whereas a small percentage of CD4^{high} grB-positive CD8^{+} T cells were present following stimulation with NP366/Rx15 microspheres. The ability of NP366/Rx15 to induce grB was in line with our previous results demonstrating that transpresented IL-15 alone can induce a low level of grB expression in the absence of a TCR stimulus (Figs. 2 and 3). Ag-specific stimulation of CD4^{high} MP CD8^{+} T cells with gp33/Ro beads resulted in a low level of grB expression similar to NP366/Rx15 microspheres. However, gp33/Ro15 microsphere stimulation of CD4^{high} MP CD8^{+} T cells induced production of grB in a significant percentage of cells far beyond that generated by NP366/Rx15 or gp33/Ro microspheres. The percentage of grB expressing cells was comparable to the percentage of IFN-γ producing cells following stimulation with gp33/Ro15 microspheres (Fig. 4, A and B). Taken together, these findings suggest synergy between pMHC and transpresented IL-15 in induction of grB by Ag-specific memory CD8^{+} T cells.

Following 48 h of culture with the various microsphere constructs, proliferation of the sorted CD8^{+} T cell subsets was analyzed by CFSE dilution (Fig. 4C). At 24 h of culture, no cellular proliferation was induced by any of the microsphere constructs (data not shown). Consistent with the IFN-γ and grB expression analyses, NP366/Rx microspheres did not induce any cell division following culture. In contrast to IFN-γ, but similar to grB expression, NP366/Rx15 as well as gp33/Ro microspheres induced significant levels of proliferation by CD4^{high} MP CD8^{+} T cells from LCMV-immune mice. However, following stimulation with gp33/Ro15 microspheres, a doubling in the percentage of divided cells was observed such that ~50% of the cells had undergone cell division.

Taken together, our results demonstrate that IL-15 transpresentation alone or in combination with pMHC primarily stimulates CD4^{high} MP CD8^{+} T cell responses. Multiple Ag-specific memory CD8^{+} T cell responses to pMHC complexes were substantially augmented by transpresented IL-15, including IFN-γ and grB production and possibly proliferation. In the cases of IFN-γ and grB production, the dramatic differences in response levels to Ag or IL-15 transpresentation alone compared with when they are combined suggest that Ag and IL-15 transpresentation synergize in stimulating LCMV-specific memory CD8^{+} T cells. Furthermore, because CD4^{low} naive CD8^{+} T cell responses were not induced or enhanced by transpresented IL-15, it suggests that the responses seen following anti-CD3ε stimulation in Figs. 1–3 may have been the result of stimulation of the endogenous MP CD8^{+} T cells present in the unimmunized mice.

**Differentiation of TEM and TCM CD8^{+} T cells to transpresented IL-15**

Because CD4^{high} MP CD8^{+} T cells can be further divided into TCM and TEM CD8^{+} T cell populations based on the expression of CD62L, we next sought to determine whether TCM and TEM CD8^{+} T cells have differing responses to transpresented IL-15 following pMHC stimulation. Unfortunately, CD62L is rapidly shed from the cell surface following TCR stimulation through cleavage by the TNF-α-converting enzyme, thereby making analysis of stimulated populations of bulk or CD4^{high} CD8^{+} T cells difficult (45–47). Therefore, CFSE-labeled CD8^{+} T cells from LCMV-immune C57BL/6 mice were sorted into naïve, TEM, or TCM populations based on CD8α, CD44, and CD62L expression to >95% purity (Fig. 5A). To examine Ag-specific responses to pMHC complexes, the sorted CD8^{+} T cell populations were stimulated with peptide-pulsed microspheres codisplaying H-2D^{b}/Ig and IL-15/Ro/Fc in the absence (NP366/Rx or gp33/Ro) or presence of transpresented IL-15 (NP366/Rx15 or gp33/Ro15). Following culture with microspheres, sorted T cell populations were analyzed for CFSE dilution, tetramer binding, and intracellular grB expression by flow cytometry.

Following 72 h of culture, naive CD8^{+} T cells did not proliferate in response to any of the microsphere constructs examined (data not shown). We also found that TEM CD8^{+} T cells underwent very little, if any, proliferation following stimulation with either NP366/Ro or NP366/Rx15 microspheres (Fig. 5B). Following Ag-specific stimulation with gp33/Ro or gp33/Ro15 microspheres, TEM CD8^{+} T cells were induced to undergo several rounds of division. Tetramer analysis of the TEM CD8^{+} T cells that proliferated in response to gp33/Ro or gp33/Ro15 stimulation revealed that a similar proportion of TEM CD8^{+} T cells stained with the gp33 tetramer after the same number of cell divisions (Fig. 5B). A significant amount of Ag-nonspecific division was noted following stimulation with gp33 peptide-pulsed microspheres, as evidenced by proliferating CD8^{+} T cells that did not stain with either gp33 or control NP366 tetramers. During an active immune response against LCMV in vivo, a large percentage of CD8^{+} T cells undergo bystander activation and are not specific for LCMV epitopes (48, 49). This may also be the case following stimulation with gp33 peptide-pulsed microspheres, whereby a proportion of the proliferating CD8^{+} T cells may be specific for environmental Ags or other non-gp33 LCMV epitopes. Regardless, a strong Ag-specific response was detected by gp33 tetramer staining in the responding TEM CD8^{+} T cell population and was only slightly enhanced by IL-15 transpresentation (e.g., for cells that underwent four or more divisions).

Similar to TEM, sorted TCM CD8^{+} T cells also did not proliferate in response to NP366/Ro microspheres (Fig. 5C). Interestingly, whereas NP366/Ro15 microspheres were unable to induce proliferation of TEM CD8^{+} T cells, they did induce proliferation in 22% of TCM CD8^{+} T cells (Fig. 5, B and C). Tetramer analysis demonstrated that TCM CD8^{+} T cells that underwent one to three rounds of cell division when cultured with NP366/Rx15 did not stain positive for either gp33 or NP366 tetramers, suggesting that transpresented IL-15 alone induced Ag-independent proliferation of TCM CD8^{+} T cells (Fig. 5C). Upon stimulation of TCM CD8^{+} T cells with gp33/Ro microspheres, a low level of proliferation was found that was substantially reduced compared with TEM CD8^{+} T cells cultured with the same gp33/Ro microspheres (Fig. 5, B and C). Additionally, regardless of the division number, a
dependence of Ag-stimulated TCM CD8+ T cells with the gp33 tetramer. When the TCM CD8+ T cell response seen when TEM CD8+ T cells were stimulated with NP366/Rα, NP366/Rα15, gp33/Rα, or gp33/Rα15 microspheres for 72 h. Microsphere-stimulated CD8+ T cell populations were harvested and stained with H-2Db/gp33 or H-2Db/NP366 tetramers and mAbs against CD8α, CD62L, and CD44. CFSE dilution and tetramer staining was analyzed by flow cytometry. CFSE division markers represent cells that have undergone 1–3 divisions and 4+ divisions (right to left). The percentage of divided cells within each division group is indicated above each marker gate (upper panels). Middle and lower panels, Open histograms represent staining with H-2Db/gp33 tetramers; shaded histograms represent staining with H-2Db/NP366 tetramers. One representative experiment of three is shown. D, Histograms represent intracellular grB staining of the TEM and TCM CD8+ T cell populations cultured with the microsphere constructs described in B and C. Open histograms correspond to staining with anti-grB; shaded histograms represent staining with a mouse IgG1 isotype control. Overton subtraction was used to calculate the percentage of H-2Db/gp33 tetramer and grB-positive populations. For all flow cytometric analysis, >30,000 CD8+ T cell events were acquired.

FIGURE 5. Differential responsiveness of TCM and TEM CD8+ T cells to transpresented IL-15 in the presence and/or absence of Ag stimulation. CD8+ T cells were negatively enriched from C57BL/6 mice previously infected with LCMV-Armstrong (40 days postinfection). A, Enriched CD8+ T cells were labeled with CFSE, stained for CD8α, CD44, and CD62L, and subsequently sorted by flow cytometry into naive, TCM, and TEM populations. Purified (B) TEM and (C) TCM CD8+ T cells were cultured with NP366/Rα, NP366/Rα15, gp33/Rα, or gp33/Rα15 microspheres for 72 h. Microsphere-stimulated CD8+ T cells responded by expressing grB. Consistent with our previous findings (Fig. 4), gp33/Rα15 stimulation augmented grB expression of both TEM and TCM CD8+ T cells (Fig. 5D). Thus, the presence of transpresented IL-15 substantially augments the normally minimal TCM CD8+ T cell grB response to pMHC, whereas TEM CD8+ T cells mount an effective grB response to pMHC stimulation alone. Interestingly, IL-15 transpresentation alone could induce grB expression in TEM CD8+ T cells but was unable to induce Ag-independent proliferation (Fig. 5, B and D). In contrast, TCM CD8+ T cells responded by both proliferating and expressing grB following transpresented IL-15 stimulation alone (Fig. 5, C and D). Taken together, TCM CD8+ T cells appear to be more dependent on transpresented IL-15 than are TEM CD8+ cells for the induction of cellular proliferation and grB expression.

TCM CD8+ T cells require host IL-15Rα expression for optimal proliferative responses in vivo

Our findings thus far suggest that TCM CD8+ T cells require IL-15 transpresentation for optimal proliferative responses in vitro; therefore, we next wanted to determine whether this was also occurring in vivo following LCMV infection. To address this issue we generated LCMV-specific memory CD8+ T cells in B6 × 129 mice by i.p. infection with LCMV-Armstrong (Fig. 6A). Forty
days after infection, CD8$^+$ T cells were negatively enriched from the spleen and lymph nodes, labeled with CFSE, and sorted into naïve, T_CM, and T_EM populations. Equivalent numbers of each CD8$^+$ T cell population were then adoptively transferred into naïve B6 $\times$ 129 or B6 $\times$ 129 IL-15Rα$^{-/-}$ mice by i.v. injection. Twenty-four hours after transfer, recipient mice were infected with LCMV-Armstrong i.p. Four days postinfection, spleens and lymph nodes were harvested and analyzed by flow cytometry. Because our analysis was limited to CFSE dilution profiles of the adoptively transferred naïve, T_CM, and T_EM, CD8$^+$ T cells in the (B) spleen and (C) lymph nodes following LCMV infection. Bar graphs represent the percentage divided of each adoptively transferred population from 3 mice (±SEM).

**Discussion**

In recent years, significant progress has been made in understanding the role of IL-15 in diverse aspects of CD8$^+$ T cell function. Following its initial cloning and characterization, several studies demonstrated the ability of IL-15 to induce CD8$^+$ T cell activation (14, 50). However, these analyses were limited to the expression of early activation markers and secretion of cytokines by unfractionated spleen and PBMC populations (16–19). Furthermore, transpresentation of IL-15 by the high-affinity IL-15Rα-chain has only recently been discovered and therefore was not taken into account during these early studies (27). Evidence now suggests that IL-15 transpresentation is the primary physiological mechanism of IL-15 function in vivo and requires the coordinated expression of both IL-15Rα and IL-15 by DCs or a similar hematopoietic cell type (24). Recent studies have also demonstrated that in vivo administration of soluble IL-15Rα/Fc complexed with IL-15 is able to induce hyperagonistic proliferation of memory CD8$^+$ T cells (28, 51, 52). The proliferation seen in these studies was in the complete absence of TCR stimulation and required extremely high non-physiological serum concentrations of IL-15. Thus, despite the vast numbers of studies conducted on this intriguing cytokine, there have been limited data regarding the contribution of IL-15 transpresentation to TCR-induced activation of CD8$^+$ T cells. Our approach using microspheres allowed us to specifically examine the role of IL-15 transpresentation in augmenting TCR-induced activation of CD8$^+$ T cells in the absence of other stimulatory signals.

Ligand immobilization on microspheres as single ligands,
or as combinations, can be performed in such a manner so as to result in physiological ligand densities similar to those observed on DCs. Therefore, microspheres offered the ideal platform to specifically address the role of IL-15 transpresentation on TCR stimulation-induced CD8⁺ T cell activation.

In this report, we have demonstrated that transpresented IL-15 is significantly more effective than soluble IL-15 at equivalent concentrations in augmenting anti-CD3ε-induced CD8⁺ T cell proliferation and effector molecule expression. It remains to be determined whether transpresented IL-15 is more effective than soluble IL-15 at augmenting Ag-specific CD8⁺ T cell responses; however, in this study our emphasis was on IL-15 transpresentation because it appears to be the primary mechanism of IL-15 action in vivo (23–26). Furthermore, IL-15Rα-deficient mice are capable of producing IL-15. Therefore, if soluble IL-15 was as effective as transpresented IL-15 in augmenting Ag-specific responses, we may not have seen such dramatic reductions in the proliferation of responding CD8⁺ T cells in IL-15Rα-deficient mice that are incapable of transpresenting IL-15. However, it would be interesting to examine Ag-specific responses in mice deficient in both IL-15Rα and IL-15 to determine the contribution of soluble IL-15 to Ag-specific recall responses. Nevertheless, our results demonstrate that transpresentation is significantly more effective than soluble IL-15 at augmenting anti-CD3ε-induced CD8⁺ T cell activation, and upon titration of the density of transpresented IL-15, a clear dose-dependent augmentation of anti-CD3ε-induced activation was evident.

Further examination of the role of IL-15 transpresentation in CD8⁺ T cell responses revealed that transpresented IL-15 synergizes with TCR stimulation to augment CD8⁺ T cell responses. Synergism was particularly apparent following CD8⁺ T cell stimulation with anti-CD3ε and transpresented IL-15, whereby the combined effect of the two stimuli was far beyond the additive effects of the individual stimuli for each of the responses examined. Upon pMHC stimulation of CD4⁺CD45⁺MP CD8⁺ T cells, the synergistic relationship applied only to IFN-γ and grB expression, whereas the combined effect on proliferation appeared to be additive rather than synergistic. However, when CD4⁺CD45⁺MP CD8⁺ T cells were further divided into TCM and TEM populations, it was apparent that pMHC and IL-15 transpresentation synergize to enhance proliferation of Ag-specific TCM CD8⁺ T cells. In contrast, the proliferative response of Ag-specific TEM CD8⁺ T cells was not significantly enhanced by transpresented IL-15 following pMHC stimulation in vitro. Taken together, our findings indicate that IL-15 transpresentation augments TCR-stimulated MP and Ag-specific memory CD8⁺ T cell activation in a synergistic manner. Finally, our observation that naive CD44low CD8⁺ T cells were unresponsive to transpresented IL-15 suggests that transpresented IL-15 may not play a major role during the primary activation of naive CD8⁺ T cells; however, our experiments have not formally ruled out this possibility. Interestingly, IL-15 and anti-CD3ε have been shown to induce highly similar gene expression patterns in MP CD8⁺ T cells; however, the combined effects of IL-15 and anti-CD3ε on gene expression were not examined (20). It would therefore be interesting to determine whether combined IL-15 and anti-CD3ε or pMHC stimulation alters the expression pattern of genes not affected by either stimulus alone, or whether there is a corresponding enhancement or reduction in the shared gene transcripts.

Our demonstration that IL-15 transpresentation could augment anti-CD3ε immobilized on a separate bead surface suggests several interesting possibilities. Our results showed that transpresented IL-15 alone resulted in a low level of proliferation and grB expression by MP CD8⁺ T cells, but not by naive CD8⁺ T cells. Serial encounter of CD8⁺ T cells with activated DCs would likely result in more frequent interactions with transpresented IL-15 rather than MHC presenting a specific antigenic peptide. Therefore, transpresented IL-15 may provide a low level of stimulation to responding MP CD8⁺ T cells and, upon TCR stimulation, MP CD8⁺ T cells would be poised to mount a robust recall response. IL-15 transpresentation may therefore serve a dual purpose by providing necessary survival signals and preparing MP CD8⁺ T cells for enhanced Ag-specific activation. This may especially be the case for TCM CD8⁺ T cells that are highly responsive to transpresented IL-15 and are also enriched in lymph nodes where they would regularly interact with activated DCs. A second intriguing possibility is that activated DCs transpresenting IL-15 may provide signals to nearby MP CD8⁺ T cells receiving TCR stimulation from a separate DC. This situation would require a CD8⁺ T cell to maintain multiple contacts with separate DCs, which could occur during DC and T cell aggregation (53). Because IL-15 transpresentation can augment TCR stimulation provided on a separate surface, the synergism between IL-15 transpresentation and TCR stimulation presented on the same surface or cell could not simply be due to increased adhesion that results in enhanced or prolonged interactions with a TCR ligand. If this were the case, IL-15 transpresentation could only enhance responses when copresented with a TCR stimulus. Taken together, the ability of IL-15 transpresentation to act as an independent stimulator of MP CD8⁺ T cell responses and also function in combination with TCR stimulation suggest that IL-15 plays a complex role in CD8⁺ T cell immune function.

Based on tissue localization, functional characteristics, and cell-surface markers, memory T cells have been broadly divided into TCM and TEM populations (54, 55). We have shown in this report that TCM and TEM CD8⁺ T cells have differential responsiveness to transpresented IL-15. TCM CD8⁺ T cells undergo limited cell division following stimulation with transpresented IL-15 alone, whereas TEM CD8⁺ T cells are relatively unresponsive to transpresented IL-15 in the absence of Ag. In our study, TEM and TCM CD8⁺ T cells have similar proliferative capacities following optimal stimulation, so that the lack of TEM CD8⁺ T cell proliferation following transpresented IL-15 stimulation alone does not correlate with an intrinsic lower proliferative ability. Furthermore, similar to TCM CD8⁺ T cells, TEM CD8⁺ T cells can be induced to express grB following transpresented IL-15 stimulation alone. Taken together, these results suggest that TCM and TEM CD8⁺ T cells have different responsiveness to transpresented IL-15. With regard to TCR stimulation, TEM CD8⁺ T cells vigorously proliferate and express grB following TCR stimulation alone, whereas TCM CD8⁺ T cells have negligible responses following Ag pMHC stimulation alone. However, if transpresented IL-15 is provided together with Ag pMHC stimulation, TCM CD8⁺ T cell responses are equivalent to the responses mediated by TEM CD8⁺ T cells. In striking contrast, TEM CD8⁺ T cell responses were only slightly enhanced when transpresented IL-15 was provided together with Ag pMHC stimulation. These findings were further supported by our in vivo adoptive transfer experiments. TCM CD8⁺ T cells from LCMV-immune mice adoptively transferred into IL-15Rα-deficient mice had minimal proliferative responses following LCMV challenge; in contrast, TEM CD8⁺ T cell proliferation was reduced but not absent in IL-15Rα-deficient mice. Taken together, our results suggest that TEM CD8⁺ T cells require only cognate pMHC stimulation to initiate recall responses to a previously encountered pathogen, whereas TCM CD8⁺ T cells are more dependent on transpresented IL-15 for optimal responses both in vitro and in vivo. It has been suggested that because TEM CD8⁺ T cells are prevalent in nonlymphoid tissue, they may require less stringent
activation criteria to facilitate rapid responses upon Ag reexposure, whereas T_{CM} CD8 T cells that reside primarily in lymphoid tissues may require interaction with DCs to initiate their full activation. In support of this concept, in vivo DC depletion experiments have revealed that T_{CM} CD8 T cells are more dependent on DCs than are T_{EM} CD8 T cells following vesicular stomatitis virus infection, although both mounted equivalent responses in the presence of DCs (31). Taken together, our findings may provide a clue as to how the division of labor between T_{CM} and T_{EM} CD8 T cells may be controlled by differential activation requirements. Therefore, in addition to differences attributable to tissue localization, activation requirements may play an important role in determining the contribution of T_{CM} and T_{EM} CD8 T cells to secondary recall responses upon pathogen reencounter. T_{EM} CD8 T cells may provide a first line of defense because they can respond in a rapid manner due to their reduced activation requirements (e.g., a TCR stimulus in the absence of IL-15 transpresentation) and localization within peripheral tissues, whereas T_{CM} CD8 T cell activation requires additional signals provided by activated DCs. Type I IFNs and TLR ligands are required for the coordinated up-regulation of IL-15 and IL-15Rs by DCs and may therefore regulate T_{EM} CD8 T cell responses such that they occur only during times of inflammation (24–26, 29). Migrated of activated DCs transpresenting IL-15 together with an appropriate TCR ligand could function to regulate recall responses by activating Ag-specific T_{CM} CD8 T cells only during situations when T_{EM} CD8 T cell responses are unable to control the infection.

Finally, our findings may have significant implications for the use of artificial cell surfaces for the propagation of memory CD8 T cells for immunotherapy (56–58). Our results clearly define a role of transpresented IL-15 in the reactivation of memory CD8 T cells, especially for T_{CM} CD8 T cells. Therefore, IL-15 transpresentation may provide utility in immunotherapeutic strategies aimed at enhancing Ag-specific memory CD8 T cell reactivation, particularly ex vivo expansion of memory CD8 T cells for adoptive immunotherapeutic approaches.

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Disclosures

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