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IL-7 signals are crucial for the survival of naive and memory T cells, and the IL-7R is expressed on the surface of these cells. Following viral infection, the IL-7R is expressed on only a subset of effector CD8 T cells, and has been demonstrated to be important for the survival of these memory precursors. IL-7 message levels remain relatively constant during the T cell response to lymphocytic choriomeningitis virus, but a short-lived burst of GM-CSF is observed soon after infection. Retroviral expression of a chimeric GM-CSF/IL-7R, in which binding of GM-CSF by T cells leads to IL-7 signaling, allows for the delivery of an IL-7 signal in all effector T cells expressing the receptor. In mice infected with lymphocytic choriomeningitis virus, CD8 and CD4 T cells transduced with this chimeric receptor underwent an enhanced proliferative response compared with untransduced populations in the same host. Similarly, TCR transgenic CD8 cells expressing the chimeric receptor produced higher effector numbers during the peak of the T cell response to infection. Surprisingly, the enhanced proliferation did not lead to higher memory numbers, as the subsequent contraction phase was more pronounced in the transduced cell populations. These findings demonstrate that artificial IL-7 signaling during an infection leads to significantly increased Ag-specific effector T cell numbers, but does not result in increased numbers of memory progeny. The extent of contraction may be dictated by intrinsic factors related to the number of prior cell divisions.

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5 Abbreviations used in this paper: γc, common γ-chain; GMR, GM-CSF receptor; GP, glycoprotein; NP, nucleoprotein; HPRT, hypoxanthine phosphoribosyltransferase; huCD2, human CD2; LCMV, lymphocytic choriomeningitis virus; p-STAT, phosphorylated STAT.

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a chimeric GM-CSF/IL-7R drives activated T cells to proliferate even more robustly than control cells during viral infection. However, this prolific expansion of effector T cells does not result in the generation of a significantly larger memory cell pool.

Materials and Methods

Mice and infections

Six- to 8-wk-old female C57BL/6 mice were purchased from The Jackson Laboratory. Thy-1.1 P14 TCR transgenic mice were bred and maintained in specific pathogen-free facilities at the University of Washington. Lymphocytic choriomeningitis virus (LCMV)-Armstrong 53b was grown on baby hamster kidney cells and titered on Vero cells. Mice were injected i.p. with 2 x 10^5 PFU of virus. All animal studies were reviewed and approved by the University of Washington animal care committee.

Real-time quantitative RT-PCR

Spleens from B6 mice at indicated days postinfection were homogenized and tissue lysed in STAT-60 (Tel-Test). Total RNA was isolated and treated with DNase (Ambion), and cDNA was prepared using Moloney murine leukemia virus reverse transcriptase (Fermentas). The cDNA concentration and purity were determined by UV spectrometry, and cDNAs were normalized by TaqMan PCR (Applied Biosystems) for hypoxanthine phosphoribosyltransferase (HPRT), as described previously (28). Oligonucleotide primers and TaqMan probes were designed to detect the following transcripts: HPRT (5'-TGGAAAAGATGTCGGTGGTGA-3', 5'-AGCTTGCAACCTTAACCATTTTG-3'), IL-7 (5'-AAAAATCTGCTTCTCTGGTATCT-3', 5'-GTTCATTATCTGGCGCAATCATCA-3'), IL-7R (5'-ATTATGGGTTGGTACAGGCGG-3', 5'-GGTCAATTTCGCGGAATCATC-3'), IL-7R (5'-CTCCCCGGCACAGCTATCCGTCGTCTG-3'), and GM-CSF (5'-GCTGATCACAAAGACGCCCTGGA-3', 5'-GGCGGTCTGCACTGACATGTTA-3'). Using the TaqMan Universal Master Mix (Applied Biosystems), real-time RT-PCR was conducted on an ABI Prism 7700 Sequence Detector (Applied Biosystems) and data were analyzed using Sequence Detector software (Applied Biosystems).

Construction of retroviral vectors containing chimeric receptors

The extracellular domain of the GM-CSFRβ joined to the transmembrane and intracellular domains of the IL-7Rα was cloned into the Mig R1 retrovirus so that resulting expression vector encodes a bicistronic transcript for chimeric GM-CSF receptor (GMR)α/β/7Rα chain and GFP separated by an internal ribosomal entry sequence. The full-length cDNA for chimeric GMRα/β was provided by P. Greenberg (University of Washington, Seattle, WA) and cloned into the MigR2 retrovirus so that the resulting expression vector encodes a bicistronic transcript for GMRα/β and huCD2 separated by an internal ribosomal entry site x. A third construct containing both chains of the chimeric receptor separated by the previously described self-cleaving 2A linker peptide (29) was cloned into MigR1. Transient retroviral transfection of the Phoenix ecotropic packaging cell line was performed similar to protocols previously described (30). Cells were incubated for 24–48 h at 37°C, and then analyzed for expression of GFP and GM-CSFRβ (MigR1 transfection), or surface human CD2 (huCD2) (MigR2 transfection). MigR2-transfected cells were further analyzed for expression of the chimeric GMRα/β by Western blot using a rabbit polyclonal Ab specific for the intracellular domain of the murine γc (Santa Cruz Biotechnology).

Generation of bone marrow chimeric mice

Bone marrow stem cells were prepared, as described previously (30). Retroviral supernatants were prepared from transfected Phoenix cell lines initially cultured overnight at 37°C and then incubated for 24–48 h at 32°C. Retrovirus supernatant was added to bone marrow cells with 4 μg/ml polybrene in six-well culture dishes and spun at 2500 rpm for 1.5 h at 37°C. Following centrifugation, cells were incubated at 37°C overnight and the transduction was repeated the following day. On the fourth day, 2 x 10^6 cells were injected into lethally irradiated (1000 rad) host mice maintained on antibiotic water. Irradiated mice were infected with LCMV 8 wk after bone marrow reconstitution. In P14 TCR transgenic mice experiments, CD8+ T cells were purified with a CD8+ T cell isolation kit (Miltenyi Biotec), followed by AutoMACS magnetic bead separation, and 10^7 cells were adoptively transferred to congenic recipient mice before LCMV infection.

Intracellular staining and flow cytometry

Following in vitro stimulation of freshly isolated splenocytes in serum-free medium containing 20 ng/ml mouse rGM-CSF (R&D Systems) or 20 ng/ml mouse rIL-7 (R&D Systems) for 1 h at 37°C, intracellular phosphorylated STAT5 (p-STAT5) staining was performed. Cultured cells were stained for surface markers, fixed using a 2% paraformaldehyde solution at 37°C for 15 min, treated with ice-cold methanol while gently vortexing, and permeabilized using a Fix and Perm kit, according to manufacturer’s protocol (Caltag Laboratories). Intracellular p-STAT5 staining was performed at room temperature for 1 h. Following in vitro stimulation with or without peptides (0.1 μM glycoprotein (GP)33–41, 0.1 μM nucleoprotein (NP)396–404, or 1 μM GP33-41), for 5 h at 37°C, intracellular IFN-γ staining was performed in accordance with the manufacturer’s protocol (BD Pharmingen), as previously described (23). Stained cells were analyzed on a FACSCalibur (BD Biosciences).

Results

GM-CSF levels escalate during LCMV infection, while IL-7 levels remain constant

Following LCMV-Armstrong infection of mice, viral titers in the spleen peak at day 3 postinfection, and only minimal levels are detected by 7 days postinfection. Because soluble IL-7 and GM-CSF are difficult to detect in the serum, real-time quantitative RT-PCR was used to measure cytokine message levels in the spleen during the infection. IL-7 levels remained relatively constant during the infection. In contrast, the steady state level of GM-CSF message increased dramatically very soon after LCMV infection, peaking at day 3 postinfection (Fig. 1). Between 3 and 7 days postinfection, the amount of GM-CSF message returned to baseline levels, correlating with viral clearance and subsiding inflammation.

Construction of expression vectors encoding a chimeric GM-CSF/IL-7R

We constructed a chimeric GM-CSF/IL-7R cDNA by fusing the extracellular domain of GM-CSFRβ with the transmembrane and intracellular domains of the IL-7Rα, and cloned this receptor into the MigR1 expression vector (Fig. 2A). The complementary chain
consisting of the extracellular domain of GM-CSFRα fused to the transmembrane and intracellular domains of the γc was cloned into the MigR2 retrovirus (Fig. 2A). Both chains of the chimeric receptor were also cloned into the MigR1 retrovirus, with the GMRβ/7Rα and GMRα/γc separated by a T2A linker peptide (Fig. 2A). The picornavirus-derived, self-cleaving 2A peptide allows for the efficient translation of multiple cistrons via a ribosomal skip mechanism (29). This chimeric GM-CSF/IL-7R system allows for the efficient translation of multiple cistrons via a ribosomal skip mechanism (29). This chimeric GM-CSF/IL-7R system allows for the efficient translation of multiple cistrons via a ribosomal skip mechanism (29).

We assessed expression of the retroviral constructs by transient transfection of Phoenix cells. Transfection with GMRβ/7Rα in MigR1 resulted in GFP expression in almost 50% of the cells (Fig. 3A). The GFP⁺-transfected cells stained at the surface with an Ab specific for the extracellular domain of murine GM-CSFRβ (Fig. 3B), demonstrating expression of this chain of the chimeric receptor. Cells transfected with the control MigR1 vector expressed GFP, but these cells did not stain for GM-CSFRβ (Fig. 3B). Transfection with GMRα/γc in the MigR2 vector resulted in huCD2 staining on >90% of the Phoenix cells (Fig. 3C). Because an Ab to detect the extracellular domain of murine GM-CSFRα by flow cytometry is not available, we demonstrated expression of this second chain of the chimeric receptor by Western blot analysis of transfected cell lysates. Using an Ab against the intracellular domain of murine γc, we detected a ~60-kDa band in cells transfected with GMRα/γc in MigR2, which was absent in cells transfected with an empty MigR2 vector (Fig. 3D).

**Phosphorylation of STAT5 during following stimulation of transduced CD8 T cells from bone marrow chimeric mice**

Bone marrow chimeric mice were generated by reconstituting lethally irradiated mice with hemopoietic stem cells transfected with both retroviral constructs (GMRβ/7Rα in MigR1 and GMRα/γc in MigR2). Eight weeks after reconstitution, ~15% of CD8 and 20% of CD4 T cells in the spleen expressed both GFP and huCD2 (Fig. 4A), with slight variation from mouse to mouse. Similar percentages of doubly transduced T cells were detected in the blood and peripheral lymph nodes (data not shown).

**We generated a second set of bone marrow chimeric mice by reconstituting irradiated recipient B6 mice with stem cells from congenic (Thy-1.1⁺), TCR transgenic P14 donor mice transduced either with the chimeric receptor/T2A construct in MigR1 or with the control MigR1 vector encoding GFP only.** From the experimental group, we obtained TCR transgenic CD8 T cells expressing both chains of the receptor and GFP; these P14 cells were present at ~5% in the spleen. Within the same mouse, P14 cells that did not express GFP served as a control population, and were present in the spleen at ~14% (Fig. 4B). Analysis of surface CD62L and IL-7Rα levels on naive CD8 T cells revealed no gross phenotypic differences between the GFP⁺ and GFP⁻ populations, although there is a suggestion that the chimeric receptor-transduced cells do express lower levels of the endogenous IL-7R (Fig. 4C). This might imply that basal levels of GM-CSF are enough to provide IL-7 signals.

To determine whether or not the GM-CSF/IL-7 chimeric receptor on GFP⁺ CD8 T cells could signal, naive splenocytes from bone marrow chimeric P14 mice were cultured in the presence of GM-CSF, IL-7, or medium alone, followed by intracellular staining for p-STAT5. Ex vivo splenocytes subjected to intracellular staining did not reveal any changes in p-STAT5 levels compared with isotype IgG controls (data not shown). After 1 h in vitro in the presence of IL-7, both the chimeric receptor-expressing GFP⁺ and...
untransduced GFP/H11002 CD8 T cell populations showed shifts in p-STAT5 staining (Fig. 4D). As expected, neither GFP/H11001 nor GFP/H11002 CD8 T cells incubated with medium alone exhibited any changes in p-STAT5 levels (Fig. 4D). In the presence of GM-CSF, however, only GFP/H11001 CD8 T cells expressing the chimeric receptor, and not GFP/H11002 CD8 T cells, showed a shift in p-STAT5 expression (Fig. 4D), demonstrating the specificity of the chimeric receptor for GM-CSF, and its ability to translate ligand binding into a productive IL-7 signal.

**FIGURE 4.** Expression of functional receptor in T cells from bone marrow chimeric mice. A, Bone marrow chimeric mice were generated containing cells expressing both chains of the chimeric receptor using MigR1 and MigR2 vectors. CD8 and CD4 T cells from bone marrow chimeric mice were analyzed for dual expression of GFP and huCD2. B, TCR transgenic bone marrow chimeric mice were generated containing cells expressing both chains of the chimeric receptor in the MigR1 vector alone. CD8 T cells from TCR transgenic bone marrow chimeric mice were analyzed for GFP expression. C, TCR transgenic CD8 T cells expressing the chimeric receptor were stained with Abs to CD8, Thy-1.1, and either CD62L or IL-7Rα. Plots shown are gated on CD8+Thy-1.1 cells and analyzed for GFP expression along with CD62L (left plot) or IL-7Rα (right plot) by flow cytometry. D, TCR transgenic CD8 T cells were analyzed after incubation for 1 h in the presence of GM-CSF, IL-7, or medium alone. Cells were stained for CD8, Thy-1.1, and p-STAT5. Thy-1.1+ P14 cells that were GFP+ (bold line) and GFP+ (dotted line) were analyzed for expression of intracellular p-STAT5 by flow cytometry. An isotype matched IgG Ab (filled gray) was included in staining protocols as a control.

the untransduced GFP+ CD8 T cell populations showed shifts in p-STAT5 staining (Fig. 4D). As expected, neither GFP+ nor GFP+ CD8 T cells incubated with medium alone exhibited any changes in p-STAT5 levels (Fig. 4D). In the presence of GM-CSF, however, only GFP+ CD8 T cells expressing the chimeric receptor, and not GFP+ CD8 T cells, showed a shift in p-STAT5 expression (Fig. 4D), demonstrating the specificity of the chimeric receptor for GM-CSF, and its ability to translate ligand binding into a productive IL-7 signal.

T cells expressing a chimeric GM-CSF/IL-7R undergo greater expansion following infection, but do not produce higher memory cell numbers

We asked whether the delivery of enhanced IL-7 signals to activated T cells would program them to become memory cells in greater numbers. In bone marrow chimeric mice with an endogenous, polyclonal TCR repertoire, we compared Ag-specific CD8 and CD4 T cell responses in chimeric receptor-transduced (GFP+ huCD2+) vs untransduced (GFP+ huCD2+) T cell populations in the same animal by measuring intracellular IFN-γ production after peptide stimulation at 8, 15, and 60 days postinfection with LCMV. Interestingly, we observed a greater number of effector CD8 T cells generated at the peak of the response from the chimeric receptor-transduced compared with the untransduced populations in the same mice. The expansion of transduced GP33+ and NP396-specific CD8 T cells was ~3- and ~7-fold greater than the untransduced Ag-specific effector populations in the same mouse (Fig. 5A). Similarly, the difference in expansion between chimeric receptor-transduced and untransduced GP61-specific CD4 T cells

**FIGURE 5.** LCMV-specific CD8 and CD4 T cell responses in bone marrow chimeric mice. Bone marrow chimeric mice were infected with LCMV, and 8, 14, and 60 days later, T cell responses were measured by intracellular IFN-γ staining. A, GP33+ and NP396-specific CD8, and B, GP61-specific CD4 T cell percentages within the untransduced (GFP+ huCD2+) and transduced (GFP+ huCD2+) populations were determined. The plots show percentages of IFN-γ-producing cells normalized to the total transduced or untransduced CD8 or CD4 T cell populations. The data shown are an average of three to five mice per group at each time point.
was ~5-fold (Fig. 5B). The elevated numbers of transduced effector T cells were not maintained during the contraction phase of the response, however. On the contrary, the more prolific expansion of the transduced T cells was followed by a more pronounced contraction phase, resulting in comparable or only slightly elevated memory numbers compared with untransduced cells (Fig. 5).

We also compared the response of chimeric receptor-transduced (GFP\(^+\)) and untransduced (GFP\(^-\)) TCR transgenic P14 T cell populations after transfer to secondary recipients that were subsequently infected with LCMV. To rule out any contributing effects of retroviral components other than the receptor inserts, we made two sets of bone marrow radiation chimeras: bone marrow chimeric mice were prepared using P14 stem cells transduced with the control MigR1 vector (empty vector) encoding only GFP and were studied in parallel with bone marrow radiation chimeras prepared with retrovirus encoding the GM-CSF/IL-7R. Naive P14 cells from both sets of mice were purified using magnetic beads, and 10\(^5\) cells were adoptively transferred to recipient B6 mice. At the start of the experiment, the ratio of transduced to untransduced P14 cells from mice containing the GM-CSF/IL-7R was roughly 1:3, while the ratio in mice containing the empty vector was closer to 1:9 (Fig. 6A). Following LCMV infection of the adoptive secondary hosts, a more robust expansion was observed in P14 cells expressing the chimeric receptor compared with P14 cells in the same mice that did not contain the GM-CSF/IL-7R such that by day 8 postinfection, the ratio of transduced to untransduced cells approached 1:1 (Fig. 6A). Similar to the pronounced contraction observed with the polyclonal, endogenous T cells expressing the chimeric receptor, the transduced effector P14 cells underwent greater apoptosis than their untransduced counterparts during the contraction phase. The 1:1 ratio of transduced to untransduced cells on day 8 fell to roughly 1:1.5 on day 14 postinfection and 1:2 by day 60 postinfection (Fig. 6A).

The more pronounced expansion and contraction of transduced compared with untransduced cells were not observed in the P14 cell populations derived from mice containing the empty vector expressing only GFP, suggesting that the retroviral components alone did not play a role (Fig. 6A). When we calculated the fold expansion based on absolute numbers of P14 cells measured in each of the groups, the data confirm the 3-fold greater expansion of effector T cells expressing the chimeric receptor compared with untransduced cells or cells transduced with the empty vector (Fig. 6B). However, the more robust expansion of effector T cells receiving enhanced IL-7 signals via the chimeric receptor did not translate into higher memory cell numbers.

At day 60 postinfection, we gated on CD8\(^+\)Thy-1.1\(^+\) cells and examined the surface phenotype of chimeric receptor-transduced (GFP\(^+\)) and untransduced (GFP\(^-\)) P14 memory cells. Both populations were uniformly CD44\(^+\) and CD122\(^+\), and 63 and 66%, respectively, were CD62L\(^-\) (data not shown). Thus, there was no obvious difference in the phenotype of the transduced vs untransduced memory cells in the same mouse.

**Discussion**

IL-7 has been shown to be vital for the survival and maintenance of resting naive and memory T cell populations. IL-7 signals have not been thought to promote the growth of effector cells, but rather to provide a signal that memory cell precursors need to differentiate into long-lived memory cells. Therefore, by harnessing the availability of environmental as well as autocrine GM-CSF during effector T cell generation and redirecting GM-CSF ligation into an IL-7 signal in T cells, we did not expect to find a more robust expansion in transduced T cells at the peak of the T cell response. We rather expected that the chimeric receptor would provide the necessary signal required for effector T cells to differentiate into memory cells after viral clearance. To our surprise, augmented IL-7 signals directly affected the expansion of effectors, leading to 3- to 7-fold higher cell numbers in the chimeric receptor-transduced T cells on day 8 after infection, but did not provide the survival signals necessary for the effectors to become memory cells (Figs. 5 and 6).

Down-regulation of the IL-7R on rapidly expanding effector T cells may be a mechanism to safeguard against uncontrolled proliferation of activated T cells. By restricting access to a limiting resource such as IL-7, this mechanism ensures a controlled proliferative response. We showed that the level of IL-7 message in lymphoid organs remains relatively fixed during an immune response when effector T cell numbers inflate (Fig. 1). Down-regulation of the receptor on activated T cells will also prevent a depletion of existing naive and memory T cells competing for the same survival factor. In accord with this latter idea, a recent study has suggested that naive T cells receiving a signal through the IL-7R respond by down-modulating the receptor, so as to not exhaust the available IL-7 supply in the environment (31). Interestingly, the slightly lower expression of endogenous IL-7R\(\alpha\) on GFP\(^+\) compared with GFP\(^-\) cells in naive T cell populations in our system suggests that even basal levels of GM-CSF can have an effect on naive T cell populations (Fig. 4C). Presumably, the basal levels of GM-CSF in a naive host are not produced by the resting T cells themselves. Activated T cells produce GM-CSF (26), but it

![FIGURE 6.](http://www.jimmunol.org/) TCR transgenic CD8 T cell response to infection following adoptive transfer. A. A total of 10\(^9\) purified CD8 T cells from TCR transgenic bone marrow chimeric mice was adoptively transferred into recipient mice and immunized with LCMV. P14 cells containing the chimeric receptor or an empty vector (as a control) were analyzed at the indicated days postinfection (PI) by gating on GFP\(^-\) cells that are Thy-1.1\(^+\) and GFP\(^+\). B. The fold expansion of GFP\(^-\) and GFP\(^+\) P14 cells from mice generated with the chimeric receptor or an empty vector is graphed for each time point. The data shown are an average of three to five mice per group at each time point.
remains to be determined which are the main cell types contributing to the rapid rise in environmental GM-CSF levels immediately following infection during the proliferative expansion of effector T cells.

From our studies, it is clear that enhanced delivery of an IL-7 signal is not sufficient for effector cells to generate long-lived memory cells. What possibilities could explain such a finding? First, our real-time RT-PCR data suggest that the burst of GM-CSF production wanes before the peak of effector T cell expansion (Fig. 1). Loss of an IL-7 survival signal from either the chimeric receptor or the endogenous IL-7R itself could be responsible for the rapid decline seen in late effector/early memory T cell numbers following LCMV clearance. Continued delivery of higher levels of IL-7 signals late during the effector stage may be required to maintain the effector T cell numbers into the memory phase.

Secondly, although the chimeric receptor results in production of larger numbers of effector cells, the receptor may also cause them to undergo apoptosis more rapidly during the contraction phase. Could the IL-7 signal, which is tightly regulated on the majority of lymphocytes through controlled expression of IL-7R, be overly robust or prolonged in late effector T cells, driving these effectors to abort their regular programming into memory cells? Although counterintuitive, the paradigm of signal strength modulating cellular responses is present throughout immunology, and will need to be tested in future studies in which exogenous IL-7 or GM-CSF is provided to transduced T cells at later stages of T cell expansion. Alternatively, blocking the IL-7 signal at various stages of effector T cell development would allow one to pinpoint the kinetics of the requirement for IL-7 signals.

Finally, the finding that the enhanced expansion of T cells expressing the chimeric receptor results in a pronounced contraction phase suggests the notion that greater expansion of Ag-specific T cells itself results in greater contraction. Could the Ag-activated T cells themselves be “counting” the number of divisions they undergo in order that they contract accordingly? This would imply that expansion and contraction levels are more cell intrinsic than they are dependent on levels of environmental survival or differentiation factors. Such a cell-intrinsic mechanism would ensure that a constant number of memory T cells would be produced following an infection regardless of the total effector T cell population. In this scenario, even when we artificially boost the effector T cell numbers via signals from the chimeric receptor, the immune system would “know” to eliminate an excess number of progeny. Future studies are required to define the mechanisms governing expansion and contraction of T cells during infection and the immune system’s ability to control the balance between naive and memory cells—in short, how homeostasis is achieved.

Disclosures

The authors have no financial conflict of interest.

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