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Article Vaccines

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Adjuvant IL-7 or IL-15 overcomes immunodominance and improves survival of the CD8+ memory cell pool

Fraia Melchionda,[1,2] Terry J. Fry,[1] Matthew J. Milliron,[1] Melissa A. McKirdy,[1] Yutaka Tagaya,[3] and Crystal L. Mackall[1]

[1]Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland, USA. [2]Department of Pediatric Hematology-Oncology, S. Orsola Hospital, University of Bologna, Bologna, Italy. [3]Metabolism Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland, USA.

Current models of T cell memory implicate a critical role for IL-7 in the effector-to-memory transition, raising the possibility that IL-7 therapy might enhance vaccine responses. IL-7 has not been studied, to our knowledge, before now for adjuvant activity. We administered recombinant human IL-7 (rhIL-7) to mice during immunization against the male antigen HY and compared these results with those obtained from mice immunized with rhIL-2 and rhIL-15. Administration of rhIL-7 or rhIL-15, but not rhIL-2, increased effector cells directed against these dominant antigens and dramatically enhanced CD8+ effectors to subdominant antigens. The mechanisms by which the cytokines augmented effector pool generation were multifactorial and included rhIL-7–mediated costimulation and rhIL-15–mediated augmentation of the proliferative burst. The contraction phase of the antigen-specific response was exaggerated in cytokine-treated mice; however, CD8+ memory pools in rhIL-7– or rhIL-15–treated groups demonstrated superior long-term survival resulting in quantitative advantages that remained long after the cytokines were discontinued, as demonstrated by improved survival after challenge with an HY-expressing tumor undertaken several weeks after cytokine cessation. These results confirm the adjuvant activity of rhIL-15 and demonstrate that rhIL-7 also serves as a potent vaccine adjuvant that broadens immunity by augmenting responses to subdominant antigens and improving the survival of the CD8+ T cell memory pool.

Introduction

Immunization is the central therapeutic maneuver that immunology has to offer. Whereas vaccines designed to induce B cell memory to prevent acute infections are highly effective, vaccines that aim to induce and maintain high level T cell responses for chronic infections such as HIV and cancer have had limited success (1, 2). The challenges of immunization for cancer and chronic infection are manifold and include ongoing mutation of target antigens (3), anergy and/or suppression due to chronic antigen overload (4), and the large size of the target pool, which requires dramatic T cell population expansion for therapeutic benefit (5). Thus, new approaches for increasing the size and broadening the diversity of effector and memory pools generated after immunization are needed to improve the prospect of generating an effective preventative vaccine for HIV and to enhance the effectiveness of immunotherapy for chronic infections and cancer. Extensive progress has been made in understanding the mechanisms regulating effector-to-memory cell transition and in identifying factors that maintain the memory T cell pool. Currently, paradigms hold that memory CD8+ T cells represent highly "fit" cells derived from the effector T cell population (6–9), thus leading to the prediction that therapies which augment T cell effector pools will also augment T cell memory pools (10, 11). Several recent insights into the biology of IL-7 suggest that IL-7 might serve as an effective vaccine adjuvant. First, IL-7 receptor-α is expressed on the majority of resting, naive CD8+ T cells, and in lymphopenic hosts, IL-7 signaling recruits T cells specific for low-affinity antigens into the proliferative pool (12, 13). Second, IL-7 therapy in T cell–replete hosts induces widespread T cell cycling (14). Because CD8+ effector T cells undergo programmed proliferation upon receipt of TCR stimulation above a critical threshold (8, 11, 15), IL-7–mediated cycling could broaden immune responses by enabling threshold-level TCR stimulation to cells already recruited into the cell cycle. Third, recent data demonstrating that IL-7 signaling (16) is critical for T cell transition from the effector to the memory pool suggest that IL-7 therapy could increase the fraction of effectors selected to enter the memory pool. Fourth, like other common γ receptor chain (γc) cytokines, IL-7 prevents programmed cell death (17) and thus IL-7 therapy could diminish the magnitude of contraction that follows antigen-specific activation. Finally, like IL-15 (18), IL-7 can directly modulate the survival and cycling of established memory cell populations (12, 19–21), raising the possibility that IL-7 therapy could directly expand the memory pool. Despite extensive evidence implicating IL-7 as a critical player in the biology of the T cell immune response, relatively little information exists regarding its effectiveness as a vaccine adjuvant (22–25). Our study here investigates whether systemic administration of recombinant human IL-7 (rhIL-7) modulates immunity generated in response to a DC-based vaccine and compares the effects of rhIL-7 to those of rhIL-2 and rhIL-15, 2 well studied γc cytokine adjuvants (26–30).

Results

Cytokine therapy induces widespread T cell population expansion and rhIL-15 induces long-term alterations in CD8 subset composition. We first evaluated the effects of rhIL-7 (5 μg/d), rhIL-15 (5 μg/d), or rhIL-2

Nonstandard abbreviations used: γc, common γ receptor chain; ELISPOT, enzyme-linked immunospot; IL-15Tg, transgenic for IL-15; MFI, mean fluorescence intensity; NCI, National Cancer Institute; rh, recombinant human.

Conflict of interest: The authors have declared that no conflict of interest exists.

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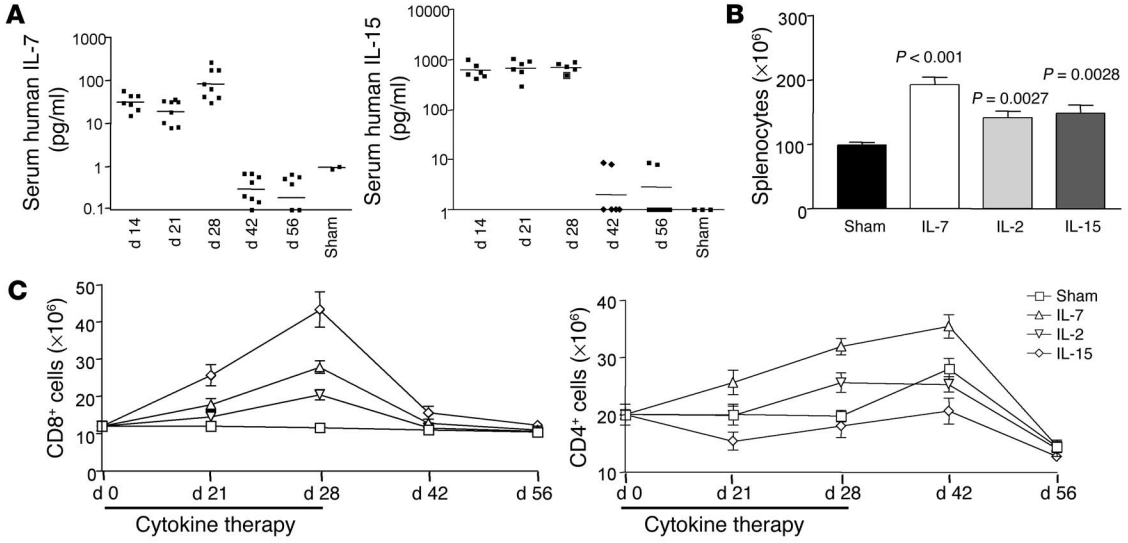

Figure 1

The γc cytokines expand splenic T cell populations. (**A**–**C**) C57BL/6 mice received rhIL-2 ($n = 9$), rhIL-7 ($n = 9$), rhIL-15 ($n = 9$), or carrier alone ($n = 9$) daily i.p. from day 0 (d 0) to day 27 as described in Methods. Data from 1 representative experiment are shown; results were confirmed in 3 separate experiments. (**A**) Serum levels of hIL-7 (left panel) and hIL-15 (right panel) were measured 24 hours after injection. Levels were stable throughout therapy and returned to baseline upon cessation of therapy. (**B**) All cytokine groups showed significant increases in splenocyte number on day 28. (**C**) $CD8^+$ ($P < 0.0001$) and $CD4^+$ ($P < 0.0001$) splenocyte populations were expanded equally (1.5- to 2.0-fold) by rhIL-7, with persistent $CD4^+$ expansion for 14 days despite serum clearance of the cytokine. $CD8^+$ cell populations were expanded (4-fold; $P < 0.0001$) but $CD4^+$ T cell numbers were reduced by 30% by rhIL-15. Increases of less than 2-fold were induced by rhIL-2 in $CD8^+$ ($P < 0.0001$) and $CD4^+$ ($P = 0.008$) splenocytes.

(25,000 IU units/d) on non–antigen-specific T cells. Neutralizing antibodies were not induced to rhIL-7 or rhIL-15, as serum cytokine levels remained stable throughout treatment (Figure 1A). The levels of rhIL-7 were in the range found in T cell–depleted humans, in whom IL-7 plays a central role in homeostatic proliferation (31–33), and the levels of rhIL-15 were similar those present in mice transgenic for human IL-15 (*hIL-15Tg* mice) (34), which show physiological effects. Due to a short $t_{1/2}$ (35), IL-2 levels were undetectable within 24 hours, but the dose of rhIL-2 used in the studies here is in the range that has biological activity in humans (36). All cytokines increased splenocytes on day 28 (Figure 1B), with rhIL-7 expanding both $CD8^+$ and $CD4^+$ T cell populations, as reported previously (Figure 1C) (37). We and others have reported previously that rhIL-7 expands T cell populations in vivo by inducing widespread cell cycling and diminishing cell death with little if any effect on thymopoiesis (14, 32, 38). Although rhIL-15 expanded $CD8^+$ splenocyte populations (4.0-fold), it induced a reciprocal decrease in $CD4^+$ splenocytes (Figure 1C). Modest increases (less than 2.0-fold) in both $CD4^+$ and $CD8^+$ splenocytes were induced by rhIL-2 (Figure 1C). Similar changes were also observed in the lymph nodes and peripheral blood of cytokine-treated animals.

After cessation of cytokine therapy, circulating levels of rhIL-7 and rhIL-15 returned to baseline within 14 days (Figure 1A). Although the recycling and "trans-presentation" capacity of IL-15 (39) raised the possibility that bioactive rhIL-15 might remain available despite normalization of circulating levels, this was not the case, as $CD8^+$ T cell normalization coincided with the decline in circulating hIL-15 levels (Figure 1C). After cessation of rhIL-7, $CD8^+$ T cell numbers normalized coincident with declines in circulating human IL-7 (hIL-7 levels, whereas $CD4^+$ splenocyte population expansion persisted for at least 14 days (Figure 1C), demonstrating that compared with $CD8^+$ cells, $CD4^+$ T cells either respond to lower levels of IL-7 or are less susceptible to apoptosis induced by "relative" cytokine "starvation." Both rhIL-7 and rhIL-15 also increased the number of Mac-1^+ and Gr-1^+ cells in the spleen, presumably reflecting expansion of macrophage and/or myeloid populations (data not shown).

IL-7 induces cycling of naive and memory populations (12, 13, 40), whereas IL-15 has been implicated mainly in cycling of memory $CD8^+$ T cells (18, 19). We evaluated the effects of rhIL-7 and rhIL-15 on the number and frequency of activated/memory $CD8^+$ cells using CD122 and CD44, markers expressed on activated and memory but not naive $CD8^+$ T cells. Both agents increased $CD8^+CD122^+$ (Figure 2A) and $CD8^+CD44^{hi}$ (Figure 2C) populations, but rhIL-15 preferentially expanded activated/memory $CD8^+$ T cell populations, as $CD8^+$ T cell populations in the spleens of rhIL-15–treated mice on day 28 were composed nearly entirely of $CD8^+CD122^+$ and $CD8^+CD44^{hi}$ cells (Figure 2, B and D). In contrast, rhIL-7 expanded both naive and memory/activated $CD8^+$ T cell populations, leaving the subset proportions unchanged (Figure 2, B and D). Despite normalization of total $CD8^+$ numbers by day 42 (Figure 1C), rhIL-15–treated mice continued to show a preponderance of activated/memory $CD8^+$ T cells on day 120 (Figure 2, B and D). Interestingly, rhIL-15 therapy also increased the frequency of $CD8^-CD122^+$ cells, which were essentially exclusively $CD4^+$, thus demonstrating a clear biological effect of rhIL-15 on $CD4^+$ populations as well (see Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI200523134DS1).

Both rhIL-7 and rhIL-15 induced larger than expected short-term T cell population expansion, but it was possible that cells remaining after cytokine-induced expansion would be prematurely

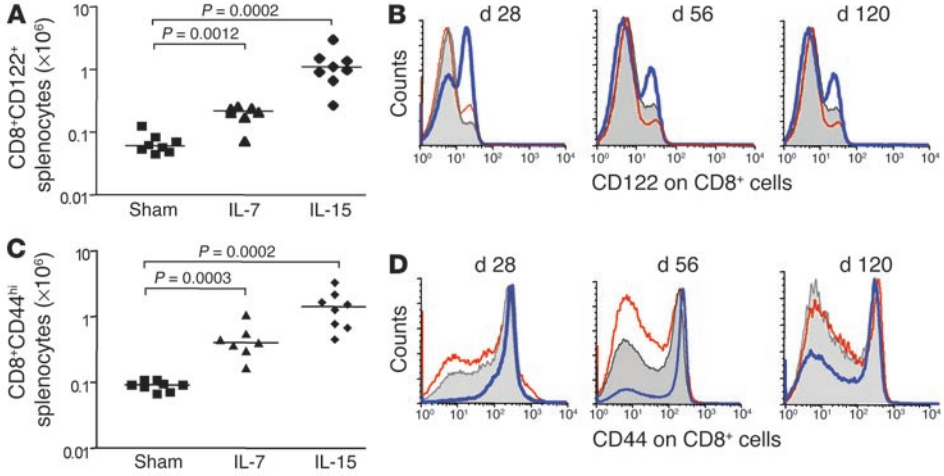

Figure 2

The cytokine rhIL-7 expands both naive and memory/activated CD8+ T cell populations, whereas rhIL-15 preferentially expands memory/activated CD8+ T cell populations. (**A**–**D**) Mice received rhIL-7 (n = 7), rhIL-15 (n = 8), or carrier alone (n = 8), as described in Figure 1. Data from 1 representative experiment are shown; results were confirmed in 3 separate experiments. (**A** and **C**) CD8+CD122+ and CD8+CD44+ splenic T cells are increased in both rhIL-7– and rhIL-15–treated animals on day 28. (**B** and **D**) Mice treated with rhIL-7 (red lines) show no difference compared with sham-treated mice (gray filled histograms) in the frequency of CD8+ cells that were CD122+ or CD44+ at any time point studied. The increased frequency of CD8+CD122+ and CD8+CD44+ T cells in rhIL-15–treated mice (blue line) on day 28 persists through day 120.

senescent/exhausted and potentially less able to expand their populations after subsequent antigenic encounter. To assess this, we adoptively transferred T cells from cytokine-treated animals into SCID mice in order to assess their capacity for homeostatic peripheral expansion, which comprises proliferation of both naive and memory populations via high- and low-affinity TCR interactions (41–43). T cell numbers in the lymph nodes and spleens of SCID mice 28 days after transfer were essentially equal, demonstrating that inocula from sham-treated or cytokine-treated mice expand equally in a lymphopenic environment (Figure 3, A and B). Therefore, T cell populations that persist after rhIL-7 or rhIL-15 therapy show no obvious defects in subsequent proliferative capacity.

Both rhIL-7 and rhIL-15 dramatically increase the effector cell pool and mitigate immunodominance. To examine the effects of the cytokines on the generation of antigen specific effector T cells, we administered cytokines from day 0 to day 27 to female mice immunized against the male minor histocompatibility antigen complex (HY) (male DCs on day 0; male splenocytes on day 14). On day 28, rhIL-7 and rhIL-15 recipients had significantly more cells binding tetramers bearing the immunodominant MHC class I–binding peptide (Uty) than did sham- or rhIL-2–treated mice (Figure 4, A and B). To confirm these findings and to assess function of the antigen-specific cells, we measured IFN-γ production in response to Uty. Compared with immunized controls, rhIL-7– and rhIL-15–treated mice showed dramatic increases and rhIL-2–treated animals showed modest increases in the number of Uty-specific IFN-γ–producing cells (Figure 4C, left panel). The entire activated/memory cell pool was expanded by rhIL-7 and rhIL-15 (Figure 2, A and C), and bystander activation is enhanced by rhIL-15 (18), raising the possibility that the increased number of antigen-specific splenocytes did not reflect preferential expansion of antigen-specific cells. However, rhIL-7 and rhIL-15 also significantly increased the frequency of CD8+ cells producing IFN-γ in response to Uty (Figure 4C, right panel), whereas no significant increase in the frequency of Uty-specific effectors occurred with rhIL-2. Therefore, antigen-specific cell populations are preferentially expanded when rhIL-7 or rhIL-15 is administrated at the time of immunization, increasing the size of the antigen-specific effector pool generated against a dominant antigen. Interestingly, we observed no additive or synergistic effects when both rhIL-7 and rhIL-15 were administered together (Figure 4C), raising the possibility of some overlap in their mechanism of action.

To investigate the mechanisms by which rhIL-7 and rhIL-15 preferentially expand antigen-specific effector populations after immunization, we considered the possibility that they enhance the magnitude of the proliferative burst after TCR stimulation. To test this, we immunized cytokine- and sham-treated groups on day 0 and "boosted" them on day 14, then on day 26, we adoptively transferred CFSE-labeled CD8+ T cells from each group into congenic hosts and continued their respective cytokine therapy for 96 hours before assessing CFSE staining. As shown in Figure

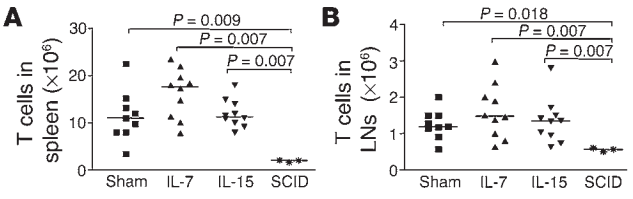

Figure 3

T cell populations from cytokine-treated and control mice undergo equivalent homeostatic expansion. (**A** and **B**) LN T cells (3×10^6) harvested on day 120 after therapy with carrier, rhIL-7, or rhIL-15 as described in Figure 1 were adoptively transferred into lymphopenic SCID recipients. At 22 days after transfer, splenocytes (**A**) and LN cells (**B**) in SCID mice were enumerated. Significant differences between control SCID mice versus recipients of adoptive transfer from the various groups are shown. There were no significant differences in the degrees of expansion observed using inocula harvested from rhIL-7–, rhIL-15–, or sham-treated mice.

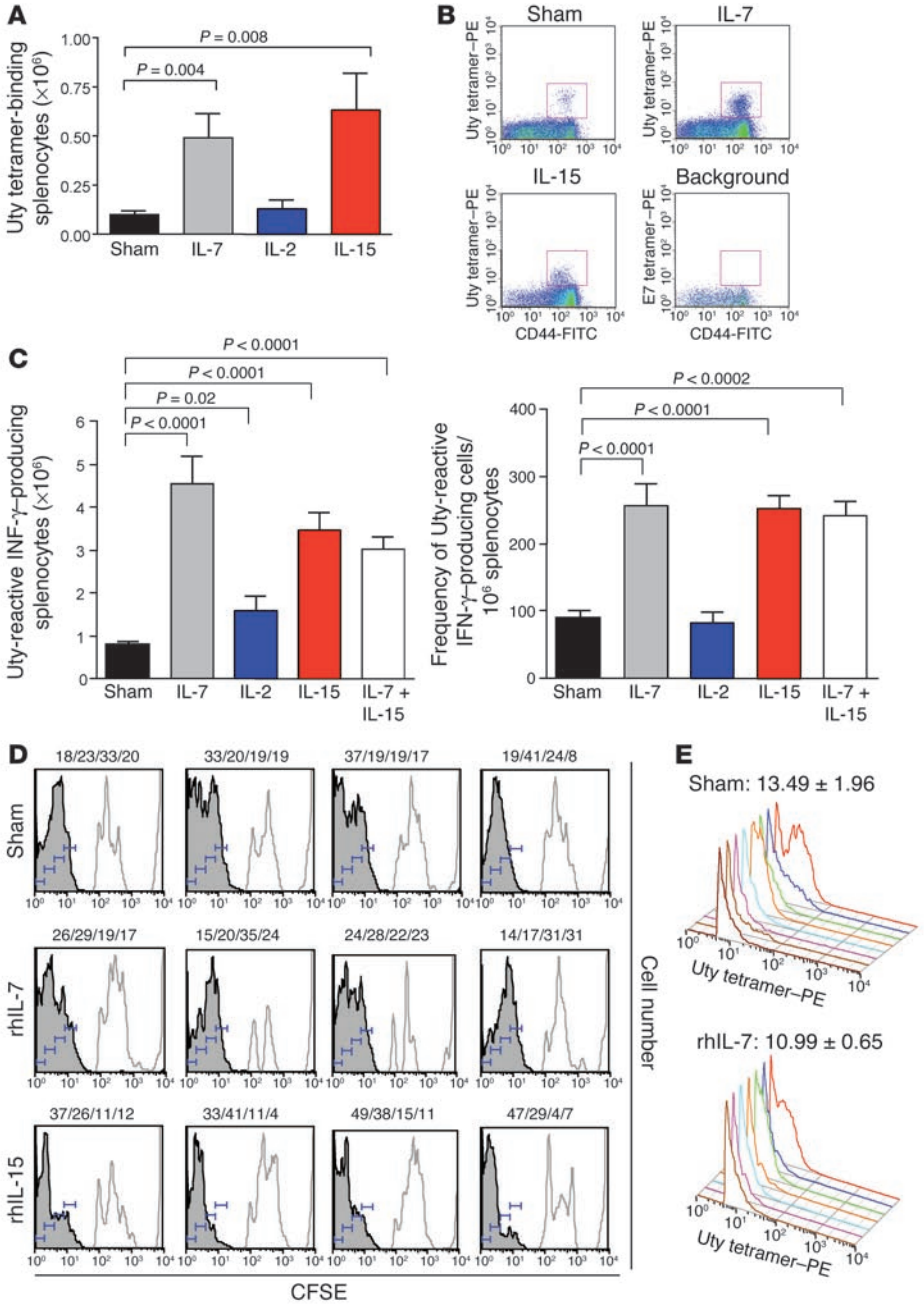

Figure 4

Both rhIL-7 and rhIL-15 increase CD8⁺ effectors specific for the immunodominant antigen. (**A**–**E**) Mice were treated as described in Figure 1. Uty responses on day 28 are shown from 3 pooled experiments with $n = 25$ (sham, rhIL-7, and rhIL-15) and $n = 17$ (rhIL-2). (**A**) Splenocytes binding Uty tetramers, with binding to irrelevant E7 tetramers subtracted. (**B**) FACS plots illustrating Uty tetramer binding in representative sham-, rhIL-7–, and rhIL-15–treated mice and E7 tetramer background staining. (**C**) Absolute number (left) and frequency (right) of splenocytes producing IFN-γ after in vitro stimulation with Uty peptide. (**D**) LN cells from cytokine- or sham-treated immunized mice were harvested on day 26, labeled with CFSE, and transferred to congenic recipients, and cytokine therapy was continued for 96 hours. CFSE expression on antigen-specific cells (shaded histograms) and non–antigen-specific cells (white histograms) are shown from 4 representative animals per group. Blue brackets on each histogram designate gates used to generate the percentages shown above each panel. Mean percents in the far left gates were 20 ± 2.4 for rhIL-7 versus 39 ± 3.5 for rhIL-15 versus 26.7 ± 4.8 for sham ($P = 0.008$, rhIL-15 versus sham; $n = 6$ per group). (**E**) Day 28 tetramer-binding cells from sham- and cytokine-treated mice were analyzed for MFI. Each colored histogram displays tetramer binding on gated CD8⁺CD44⁺ cells from individual mice within each group. RhIL-7–treated animals had significantly reduced tetramer binding, as assessed by MFI, compared to sham-treated animals ($P = 0.04$).

4D, rhIL-15–treated animals showed significantly increased CFSE dilution compared with that of controls and rhIL-7–treated animals, suggesting that a substantial element of the adjuvant effect of rhIL-15 involves an increase in the size of the proliferative burst after activation. In contrast, the CFSE dilution profile was not significantly different in control versus rhIL-7–treated mice.

A possible explanation for the adjuvant effect of rhIL-7 on Uty responses could relate to its capacity to costimulate for T cell activation, thus potentially rendering weak peptide-TCR interactions sufficient to induce the CD8 proliferative burst (12, 13, 42). If so, we hypothesized that a greater proportion of the effector cells generated under the influence of IL-7 may have lower affinity for cognate peptide which could result in a lower mean fluorescence intensity (MFI) following tetramer binding. Indeed, we observed significantly lower tetramer binding, assessed by MFI, in rhIL-7–treated animals than in immunized controls (Figure 4E), a finding consistent with the idea of a role for IL-7–mediated co-stimulation in its adjuvant effect. Despite the lower tetramer binding, antigen-specific cells generated under the influence of rhIL-7 or rhIL-15 showed no difference in activation marker expression (CD122, CD44, or Ly6C) compared with that of immunized controls (data not shown). In contrast, similar to the results shown in Figure 2, rhIL-7– and rhIL-15–treated animals showed increased CD122 expression on non–antigen-specific cells (those not binding tetramers): CD122 MFI of tetramer-nonbinding CD8⁺ cells in sham recipients, 13.7 ± 0.38, versus rhIL-7 recipients, 17.0 ± 0.51 ($P = 0.0006$), versus rhIL-15 recipients, 24.3 ± 1.02 ($P = 0.0002$). Together, the results suggest that IL-7 therapy compensates for weak peptide-TCR interactions, resulting in an increased number of cells that receive a signal capable of inducing a proliferative burst after T cell activation. Nonetheless, similar levels of activation apparently result in antigen-specific cells in both groups, as no differences in activation marker expression on antigen-specific cells were observed for rhIL-7 versus control.

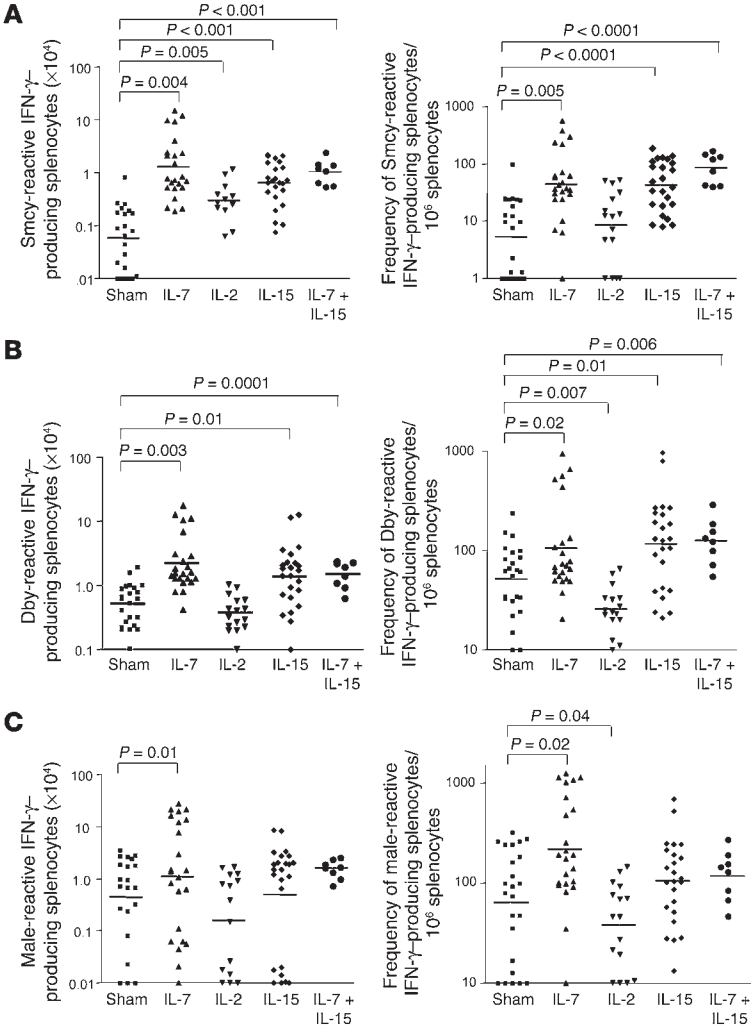

Figure 5
Both rhIL-7 and rhIL-15 increase effectors specific for subdominant and MHC class II–restricted antigens. (**A**–**C**) Results were pooled from the groups of animals described in Figure 4. Splenocytes were analyzed for IFN-γ production using ELISPOT. Data are shown as absolute number of responding splenocytes (left panels) and frequency of responding splenocytes (right panels) to the antigen designated on the *y* axis. (**A** and **B**) Net IFN-γ–producing cells were calculated by enumeration of the ELISPOT IFN-γ response to the Smcy peptide (**A**) and Dby peptide (**B**) and subtraction of the number of cells responding to the control peptides, as described in Methods. (**C**) Net male-reactive cells were calculated by enumeration of responses to male splenocytes and subtraction of responses to female splenocytes. *P* values are shown where results for sham- and cytokine-treated mice are significantly different.

If IL-7–mediated costimulation renders subthreshold peptide-TCR interactions sufficient for cells to undergo a proliferative burst, and if rhIL-15 enhances the size of the proliferative burst, one would predict that these cytokines would increase the breadth of the effector pool by augmenting responses to subdominant antigens. To study this, we evaluated the effects of the cytokines on responses to the peptide Smcy, which are known to be subdominant to Uty responses in C57BL/6 mice responding to HY. Similar to the results obtained with Uty, statistically significant increases in the number of splenocytes producing IFN-γ in response to Smcy were seen with all cytokines, but the results were most marked with rhIL-7 and rhIL-15 and there was no further advantage when the 2 agents were given together (Figure 5A, left panel). Both rhIL-7 and rhIL-15 also significantly increased the number of cells binding an Smcy-specific tetramer (data not shown). Furthermore, both rhIL-7 and rhIL-15, but not rhIL-2, preferentially expanded Smcy-reactive cell populations, resulting in an increased frequency of splenocytes producing IFN-γ in response to the Smcy peptide (Figure 5A, right panel). Smcy responses were enhanced 5- to 10-fold, which was substantially greater than the approximate 3-fold increase for Uty induced by rhIL-7 and rhIL-15. Indeed, the immune response generated against the subdominant antigen in cytokine-treated animals equaled the response generated to the dominant antigen in non–cytokine-treated animals. These results illustrate that rhIL-7 and rhIL-15 each increase the frequency of immunodominant effectors generated after immunization and that these agents also broaden immunity by enhancing the responses to a subdominant antigen. Indeed, those factors that render Smcy subdominant in C57BL/6 mice are largely overcome by provision of supraphysiological levels of rhIL-7 or rhIL-15 at the time of immunization.

With regard to $CD4^+$ effectors, rhIL-7, rhIL-15, and rhIL-7 plus rhIL-15 significantly increased the absolute number and frequency of $CD4^+$ cells responding to the immunodominant Dby peptide (Figure 5B). RhIL-7–mediated enhancement of $CD4^+$ effectors is perhaps not surprising, given that IL-7 ably signals and expands $CD4^+$ cell populations. In contrast, rhIL-15 has been demonstrated to be relatively $CD8^+$-selective in its effects (27, 44), and we observed diminished total $CD4^+$ splenocytes in rhIL-15–treated mice (Figure 1C), yet rhIL-15 clearly increased the absolute number and frequency of $CD4^+$ effectors. We found that rhIL-2 actually diminished the frequency of $CD4^+$ cells responding to Dby in this system ($P = 0.04$; Figure 5B). When responses to the entire HY complex were evaluated by enumeration of responses to male splenocytes, only rhIL-7 induced a significant increase, whereas rhIL-2 decreased responses to the full antigen complex (Figure 5C).

Effector pool expansion by rhIL-7 and rhIL-15 results in long-term expansion of the antigen-specific memory pool. The results presented above demonstrate that both rhIL-7 and rhIL-15 expand and broaden antigen-specific T cell effector pools generated after DC-based immunization, but do not address whether this results in long-term increases in the memory pool. In an effort to increase the chance that vaccine induced increases might be maintained through cytokine withdrawal, we administered booster immunizations on day 28 and day 42 in all groups in subsequent experiments. On day 56, when the contraction associated with cytokine withdrawal was complete, rhIL-7–treated animals continued to harbor increased frequencies of $CD8^+$ cells responding to both dominant and subdominant antigens, but the magnitude of the increase was reduced compared with that seen on day 28 (Supplemental Figure 2). Moreover, the beneficial effects of rhIL-15 alone were lost on day 56, and while the recipients of combined rhIL-7 plus rhIL-15 continued to demonstrate benefits at this time point,

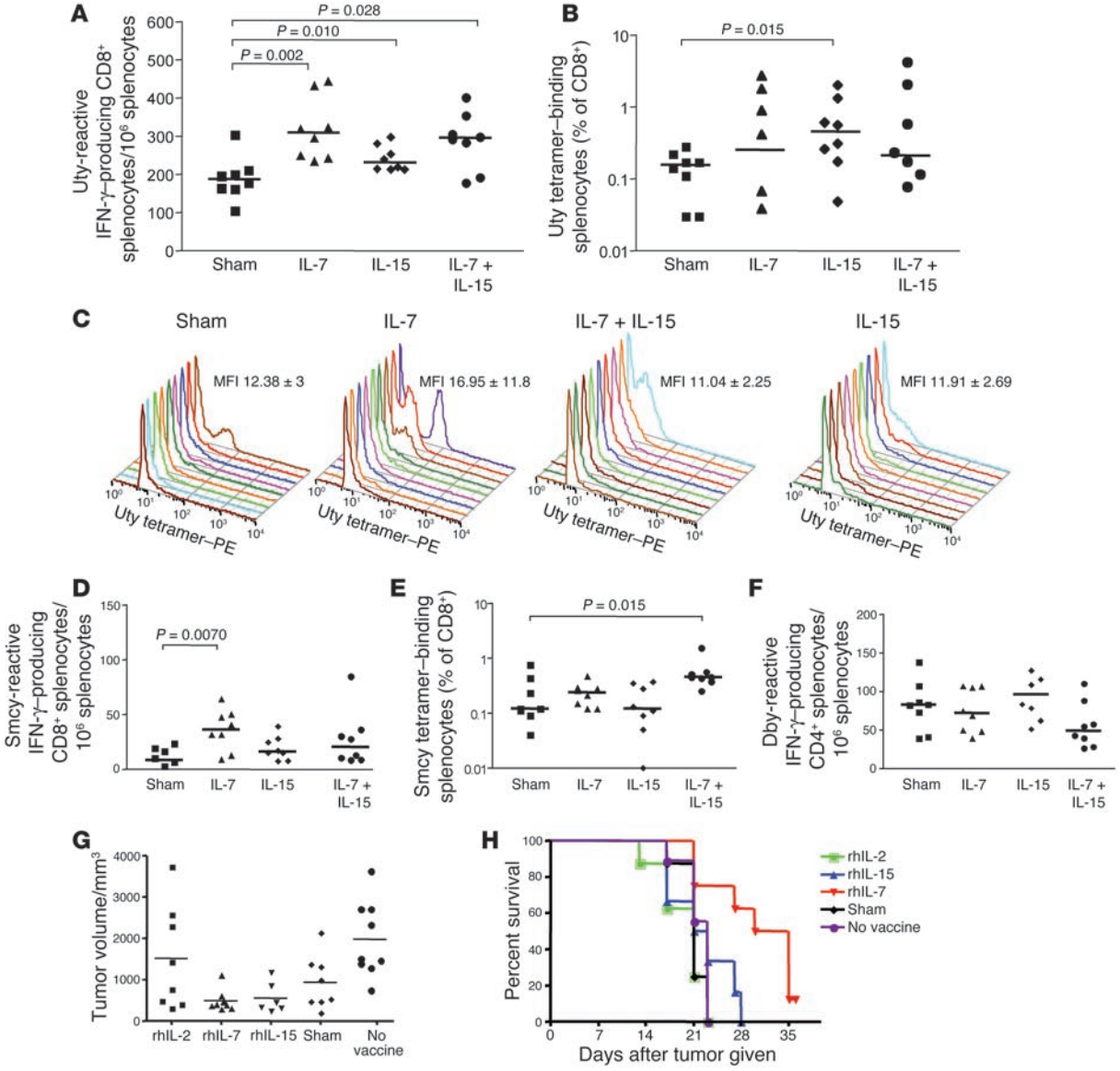

Figure 6

Administration of rhIL-7 or rhIL-15 with immunization results in long-term expansion of the memory pool. (**A**–**F**) Animals (n = 8 per group) were immunized (days 0, 14, 28, and 42) and treated with cytokines or carrier from days 0–27, then allowed to rest until day 120. Frequency of splenocytes producing IFN-γ in response to Uty (**A**) and binding Uty tetramers (**B**). (**C**) Uty tetramer–binding cells were electronically gated as described in Figure 4D. Each panel shows colored histograms from 9 individual mice in each group. No significant differences in tetramer binding, as measured by MFI, were seen between groups. Frequency of splenocytes producing IFN-γ in response to Smcy (**D**) and binding Smcy tetramers (**E**). (**F**) Frequency of cells producing IFN-γ in response to Dby. (**G** and **H**) Animals were immunized on day 0 and treated with cytokines from days 0–27. On day 60, groups were challenged with 3×10^6 MB49 tumor cells, then were euthanized when tumor progression met NCI Animal Care and Use guidelines. (**G**) Mean tumor volume 21 days after tumor challenge. (**H**) Overall survival is shown. There is significantly prolonged survival for rhIL-7 compared with all groups (versus vaccine alone, P = 0.0029; versus IL-2, P = 0.002; versus no vaccine, P = 0.008; versus rhIL-15, P = 0.02).

the effects were substantially reduced compared with the benefits observed on day 28. These results suggested that cytokine-mediated enhancements might be short lived and that the gains might not survive the effector-to-memory cell transition.

To address potential effects of cytokine therapy, as administered here, on the memory pool, we evaluated responses on day 120, a time point when the memory pool was firmly established, as it was 3 months since cytokines were discontinued and 11 weeks since the last vaccine. Remarkably, groups that had received rhIL-7, rhIL-15, or rhIL-7 plus rhIL-15 showed significant increases in the frequency of memory cells producing IFN-γ in response to the immunodominant CD8 epitope (Figure 6A). Because splenocyte numbers were equivalent among groups at this time point, significant changes in frequency also resulted in significant changes in the total number of responding cells (data not shown). Only rhIL-15 significantly increased the frequency of cells binding the

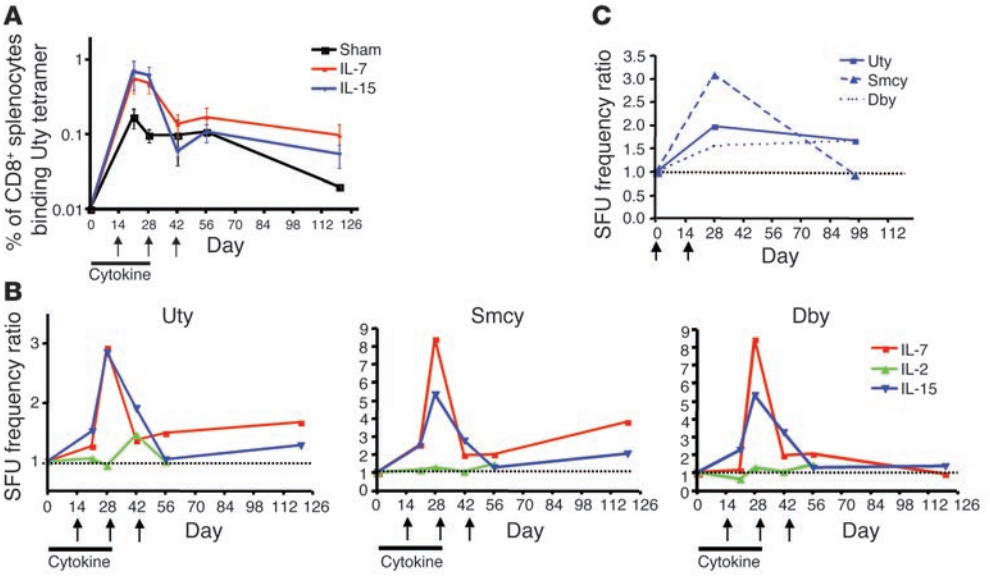

Figure 7

Mice treated with rhIL-7 or rhIL-15 show exaggerated contraction of the effector pool but improved survival of the memory pool. (**A**–**C**) Animals received cytokines as described in Figure 1 (horizontal black bars), were immunized as described in Methods (arrows), and then were sacrificed for analysis at day 21 (n = 16), day 28 (n = 24), day 42 (n = 9), day 56 (n = 24), and day 120 (n = 8). Data are pooled from 2 to 3 experiments. (**A**) The frequency of Uty-specific cells in cytokine-treated groups dramatically drops from day 28 to day 42, indicating a greater death rate for antigen-specific than for non–antigen-specific cells during cytokine withdrawal. Compared with controls, effectors generated with cytokines show a steeper slope, indicating increased contraction from day 28 to day 42, but a diminished slope from day 56 to day 120, indicating improved survival. (**B**) Ratio of spot-forming unit (SFU) frequencies in cytokine-treated versus sham-treated animals is shown as the SFU frequency ratio. Therapy with rhIL-7 and rhIL-15 expands the effector pools, which undergo exaggerated contraction from day 28 to day 42, resulting in a downward slope. From day 56 onward, antigen-specific CD8+ cells in rhIL-7– and rhIL-15–treated hosts have improved survival, resulting in an upward slope. Survival of CD4+ memory cells is not improved in cytokine-treated groups. (**C**) IL-15Tg mice (6 per time point) and littermate controls (9 per time point) were immunized on day 0 and day 14. Data represent ratio of the mean frequency of IFN-γ producers/10^6 splenocytes in IL-15Tg mice to that in control mice at designated time points.

Uty tetramers at this time point (Figure 6B). Unlike the results on day 28, the MFI of the tetramer-binding cells in the memory pool was not diminished in rhIL-7–treated mice compared with immunized controls, consistent with the notion that transition to the memory pool does not occur via indiscriminate maturation but instead involves selection of cells with adequate TCR-binding affinities (Figure 6C). Only rhIL-7 significantly increased the frequency of cells responding to the subdominant antigen Smcy, as determined by enzyme-linked immunospot (ELISPOT) assay (Figure 6D), and only rhIL-7 plus rhIL-15 significantly increased the frequency of cells binding Smcy tetramers (Figure 6E). In contrast to those sustained benefits on CD8+ responses, rhIL-7, rhIL-15, and rhIL-7 plus rhIL-15 had no effect on the size of the CD4+ memory pool in this model system (Figure 6F).

In order to determine whether numerical increases in the memory pool observed in vitro translate into improve immunity in vivo, we challenged immunized mice which received cytokines and immunized controls with an HY-expressing tumor, MB49 (45). In these experiments, animals were immunized on day 0 and did not receive subsequent boosts prior to tumor challenge. All groups challenged in the midst of the effector response (day 16) showed approximately 50% survival with no difference between cytokine-treated and immunized control groups. However, when animals were challenged with tumor after establishment of the memory pool (day 60), we saw diminished tumor volume in rhIL-7– and rhIL-15–treated groups and significant improvement in overall survival in rhIL-7–treated mice compared with that of mice given vaccine alone (P = 0.0029), rhIL-15 (P = 0.02), or rhIL-2 (P = 0.002) (Figure 6, G and H). Therefore, rhIL-7–induced augmentation of the memory pool translates into improved tumor immunity, a result that confirms the relevance of the in vitro results already described. Interestingly, this in vivo test also suggested that rhIL-7 was superior to rhIL-15 in augmenting memory cell responses.

Together the results demonstrate that immunization undertaken under the influence of rhIL-7 and rhIL-15 augments the size and function of the CD8+ memory pool. However, the diminishing magnitude of the effect over time raised the possibility that antigen-specific memory cells in cytokine-treated hosts might manifest diminished survival, in which case the memory pool might not be stably expanded. To address this, we compared survival of antigen-specific cells in the various groups over time. Figure 7A, which plots the frequency of cells binding Uty tetramers in Immunized, cytokine-treated mice versus immunized controls over time, demonstrates the cytokine-induced expansion from day 0 to day 28 followed by contraction between day 28 and day 56 in both rhIL-7– and rhIL-15–treated animals. Because the graph plots the frequency of tetramer-binding cells rather than absolute numbers, the contraction in cytokine-treated groups cannot be attributed to the global, nonspecific death seen in non–antigen-specific CD8+ populations during cytokine withdrawal (Figure 1, C and D). Instead, this demonstrates that antigen-specific cells in both rhIL-7– and rhIL-15–treated groups sustain a greater death rate during cytokine withdrawal (days 28–56) than do nonspecifically expanded populations and a more precipitous decline than that seen during physiological contraction after activation, which occurred in immunized mice that did not receive cytokine therapy. In contrast, from day 56 to day 120, antigen-specific cells in cytokine-treated animals showed a slower decline in frequency than that of controls, demonstrating that once the memory pool is established and cytokine withdrawal is complete, memory cell populations generated under the influence of the γc cytokines rhIL-7 and rhIL-15 have improved survival.

To further compare survival of antigen-specific cells in immunized controls versus cytokine-treated groups, we plotted the ratio

of the frequency of IFN-γ–producing cells in each group over time for each of the HY antigens studied (Figure 7B). The upward slope from day 0 to day 28 followed by a downward slope between days 28 and 56 again demonstrated marked cytokine-induced expansion followed by a more marked contraction of antigen-specific cell populations in cytokine recipients compared with that of immunized controls during cytokine withdrawal. Importantly, however, between day 56 and day 120, the number of antigen-specific $CD8^+$ cells responding to dominant and subdominant antigens in cytokine-treated hosts showed an upward slope, indicating enhanced survival compared with that of cells generated in immunized controls. This is in contrast to the $CD4^+$ memory pool, in which previous rhIL-7 or rhIL-15 therapy conferred no survival benefit in the long term. Together, these results illustrate that γc cytokines administered during the expansion phase induce dramatic increases in the effector pool and that this advantage is subsequently minimized by an exaggerated contraction phase. Despite the exaggerated contraction, the $CD8^+$ memory pool but not the $CD4^+$ memory pool generated under the influence of rhIL-7 or rhIL-15 enjoys improved long-term survival resulting in a stably augmented memory pool.

In order to test whether "cytokine withdrawal" was fully responsible for the exaggerated contraction that abrogated much of the quantitative effects noted in the effector pool, we compared effector and memory cell pools in normal mice versus *hIL-15Tg* mice, a model wherein supraphysiological levels of rhIL-15 were maintained throughout the effector-to-memory transition and effects due to cytokine withdrawal would not occur. As shown in Figure 7C, *hIL-15Tg* mice showed increased numbers of both CD4 and CD8 cells responding to immunodominant and subdominant epitopes similar to that observed in mice treated with rhIL-15. Furthermore, at least for the subdominant pool for which the effect of rhIL-15 was most significant, the magnitude of the increase was substantially reduced over time, despite continued high levels of rhIL-15. These data demonstrate that, on a per-cell basis, the effector cell pool generated under the influence of the γc cytokine is less fit, when compared to an effector pool generated in the absence of cytokine, to enter the memory cell pool. Given all the data reported above, we favor the interpretation that the enhanced contraction observed in cytokine-treated hosts from day 28 to day 56 is probably due to a combination of death induced by cytokine withdrawal as well as some vetting of the cytokine-augmented effector pool so that only cells with appropriate fitness enter the memory pool.

Discussion

Hope for long-term control of the global HIV epidemic ultimately depends upon development of a potent, T cell–based, preventative vaccine. Similarly, the remarkable progress made in identifying tumor antigens can be translated into effective therapy only if strategies can be developed to augment T cell responses to weak tumor antigens (46). Identification of the critical role for T cell costimulation and technological advances in isolating DC populations to optimize T cell costimulation fueled high hopes for the success of T cell–based vaccines for chronic infection and cancer. Thus far, however, the clinical impact of DC-based vaccines remains limited (47). DC vaccines themselves and/or the administration of local adjuvants to recruit DCs can reliably induce measurable immune responses to weak antigens, but the magnitude and duration of the responses are generally not sufficient to eradicate established tumors or to provide the broad-based immunity required for response to ever-changing viral quasispecies. Therefore, new approaches for enhancing T cell responses generated after immunization are critically needed.

A consistent theme from recent studies of immune effector and memory cell biology is the critical roles of γc cytokines in general and of IL-7 and IL-15 in particular (9, 11, 16, 19, 48). Although γc cytokines are not required for effector cell generation when optimal costimulation is provided, they provide potent metabolic activation and survival signals that enhance survival of naive and memory T cells (13, 17, 44) and play critical roles in the transition from $CD8^+$ effector cell to $CD8^+$ memory cell (16). Thus, as γc cytokine–mediated costimulation can augment T cell activation after suboptimal TCR triggering, it can be reasonably hypothesized that γc cytokines may be clinically useful for enhancing responses to T cell–based vaccines.

Several previous studies have demonstrated that IL-15 can increase the size of the effector pool generated in response to infection or immunization (25, 28, 29, 49), and in at least 1 study, memory pool expansion was also observed (28). Our studies here, however, are the first to our knowledge to demonstrate that IL-7 is a potent vaccine adjuvant that is at least as effective as and in some cases more effective than (Figure 5D and Figure 6, C and H) rhIL-15. Our study also illustrates the dramatic benefit of either rhIL-7 or rhIL-15 on vaccine responses toward subdominant antigens. Remarkably, coadministration of rhIL-7 or rhIL-15 at the time of immunization largely overcomes those factors that naturally limit the immunodominance of Smcy in C57BL/6 mice, converting this antigen from one that induced marginally detectable responses to one that was nearly as strong as Uty, the dominant antigen. Because the factors responsible for the consistent hierarchical arrangement of the immune response magnitude called "immunodominance" are multiple and are as-yet poorly understood (reviewed in ref. 50), an explanation of the mechanisms through which rhIL-7 and rhIL-15 overcome immunodominance must remain somewhat speculative. That said, it appears likely that the changes in immunodominance observed in the effector pool in our study probably occur as a result of effects of rhIL-7 and rhIL-15 that are already well known. With regard to IL-7, it is clear from previous work that IL-7–mediated costimulation can compensate for weak TCR signaling such as that induced by suboptimal anti-CD3 and that in the setting of homeostatic peripheral expansion, IL-7 works largely through inducing naive T cells to proliferate in response to antigens with low affinity for the TCR (12, 13). These well recognized properties of IL-7 are indeed likely to result in augmented responses to subdominant antigens in the vaccine setting. Furthermore, evidence in other models has demonstrated that the effects of rhIL-7 observed here are likely to be generally applicable to antigen responses in other settings. For example, we recently observed that IL-7 augments the number of tumor-reactive T cells responding to subdominant tumor antigens in tumor-bearing hosts (51), and we have previously reported that rhIL-7 lowers the threshold for graft-versus-host disease, thus implicating IL-7 in the augmentation of responses to histocompatibility antigens (52). Furthermore, we have observed that rhIL-7 increases immune responses in C3H.SW mice toward subdominant male antigens when they are challenged concomitantly with cells that coexpress immunodominant B6dom1 and subdominant male antigens (unpublished observations) (53). Therefore, the ability of IL-7 therapy to augment immune responses in general, and to augment responses to subdominant antigens

in particular, appears to be a general property of rhIL-7 that is observed in several model systems.

With regard to rhIL-15, previous work has demonstrated that this cytokine can potently augment antigen-independent cycling of activated/memory populations (18–20), and the CFSE data shown here demonstrated increased cycling of cells undergoing the proliferative burst. Interestingly, mathematical modeling of the effect that changes in early effector response magnitude would have on immunodominance suggests that even small changes in the frequency of cells responding to antigen (as may be induced by IL-7) or in the size of the proliferative burst as could be induced by rhIL-15 would result in substantial diminution of the immunodominance hierarchy (50, 54). Thus, it is reasonable to hypothesize that transient effects on effector cell population expansion that could be explained as a result of already defined biological effects of these agents would result in the mitigation of immunodominance by rhIL-7 and/or rhIL-15 observed here. The overall similarity of the effects of the 2 agents and the lack of additive or synergistic effects when rhIL-7 and rhIL-15 were administered together suggest some overlap in their mechanisms of action, which has already been noted for rhIL-7 and rhIL-15 (21).

Augmentation of the size of the effector pool was not unexpected, as the cytokines were provided at the time of immunization. However, it was more difficult to predict how an effector pool generated under the influence of rhIL-7 or rhIL-15 would fare during the critical transition to the memory pool, especially when such a transition occurs in the midst of cytokine withdrawal. Indeed, after the dramatically expanded effector phase in cytokine-treated animals, we observed an exaggerated contraction phase, probably due to a combination of cytokine withdrawal and diminished fitness for the effector-to-memory transition. Despite the exaggerated contraction, benefits remained present in the memory pool (Figures 6 and 7). Indeed, the temporal studies illustrated that once the memory cell pool was established, cells generated under the influence of cytokines actually showed improved rather than diminished survival over time (Figure 7). Therefore, immunizing under the influence of γc cytokines both overcomes immunodominance and results in an augmented memory pool, a result that provides proof-of-principle for the basic themes that have arisen in memory cell biology (9, 48). Furthermore, as augmentation of the size of the memory pool resulted in improved tumor resistance long after the cytokines were stopped, these results demonstrate the clinically relevant principle that long-term immunological benefit can result from short-term cytokine therapy administered at the time of immunization. These studies also clearly demonstrate fundamental differences between CD4 and CD8 memory, as both rhIL-7 and rhIL-15 increase the size of the CD4 effector pool but this does not translate into an increased $CD4^+$ memory pool. Clearly, further studies are needed to identify optimal approaches for modulating CD4 memory.

In summary, new approaches for augmenting immunization-induced T cell responses are needed to enhance the effectiveness of vaccines for cancer, HIV, and other chronic infections. Previous clinical application of γc cytokines as vaccine adjuvants has been limited to IL-2 and has yielded disappointing results. Our results here have demonstrated that both rhIL-7 and rhIL-15 are superior vaccine adjuvants to rhIL-2 and that either agent can substantially modulate the size of both the effector and the long-term memory pool, with the most potent effect on subdominant antigens, which are at a competitive disadvantage. As rhIL-7 is currently undergoing early-phase clinical trials (55), these data provide the basis and rationale for testing coadministration of rhIL-7 with cancer and viral antigen vaccines wherein increased size and breadth of the T cell response is predicted to improve therapeutic efficacy.

Methods

Mice and cell populations. Mice were C57BL/6Ncr females (B6/Ly5.1) or, where indicated, C57BL/6Ly5.2 and were purchased from the Animal Production Unit of the National Cancer Institute. The *hIL-15Tg* mice were provided by Y. Tagaya. All experiments were approved by the Animal Care and Use Committee of the National Cancer Institute (NCI). Day 0 occurred between 8 and 12 weeks of age. HY-immunized mice received 1×10^5 male DCs i.p. on day 0, followed by 5×10^6 male splenocytes i.p. on day 14. Spleen samples were enriched for DCs (50–70% $CD11c^+$ MHC class II–positive) after plate adherence and incubation with 1 ng/ml GM-CSF and 0.1 μg/ml IL-4 (Peprotech Inc.) as described previously (56).

Cytokines. Cytokines were lyophilized, reconstituted with sterile water, and resuspended in buffer containing 5% sucrose and 0.1% HSA in PBS. The rhIL-7 (5 μg/d) was supplied by Cytheris Inc.; rhIL-15 (5 μg/d) was purchased from Peprotech Inc.; and rhIL-2 (25,000 IU/d) was acquired from the NCI repository. Cytokines were administered as daily i.p. injections from day 0 to day 27.

Tetramer and ELISPOT analysis. Tetramers were produced by the Tetramer Facility of the National Institute of Allergy and Infectious Diseases (NIAID) using standard techniques. The tetramers comprised 4 MHC class I $H\text{-}2D^b$ molecules bound to the immunodominant HY peptide Uty (WMHHNMDLI), the subdominant peptide Smcy (KCSRNRQYL), or a control E7 peptide (RAHYNIVTF). Flow cytometry–based enumeration was performed using a FACSCalibur (BD) equipped with CellQuest software and was analyzed using FlowJo software (Tree Star Inc.). Splenocytes were treated with anti-FcγIII/II (clone 2.4G2), then were stained at 4°C for 20 minutes with anti-CD44–FITC, anti-CD4–tricolor, and anti-CD8–allophycocyanin (Caltag Laboratories Inc. and Pharmingen Inc.) and washed in FACS buffer (HBSS with 0.2% human serum albumin and 0.1% sodium azide). Cells were incubated with tetramers in the dark at room temperature for 1 hour, washed, resuspended, and then analyzed. With light scatter and propidium iodide extrusion (Sigma-Aldrich), the live $CD8^+$ lymphocyte population was gated upon and analyzed for CD44 and tetramer expression (Uty, Smcy, or E7). The percent of Uty tetramer– or Smcy tetramer–binding cells was determined by enumeration of $CD8^+CD44^{hi}$ tetramer-positive cells and subtraction of those binding E7 tetramer within the same subset. Tetramer-binding cells were not observed in the $CD4^+$ or $CD8^+CD44^{lo}$ subsets during these experiments. Background staining for the E7 tetramer never exceeded 0.3% of $CD8^+$ T cells.

Immunospot M200 plates (Cellular Technology Inc.) with a high protein-binding membrane were coated overnight at 4°C with 2.5 μg/ml mouse IFN-γ capture mAb (clone R4-6A2; Pharmingen Inc.). Plates were blocked with 1% BSA (Sigma-Aldrich) in PBS and were washed. Freshly isolated splenocytes were resuspended in HL-1 serum-free media (BioWhittaker Inc.) supplemented with 1% penicillin/streptomycin/L-glutamine and were plated at a density of 1×10^6/well in triplicate. Stimulator cells were freshly isolated splenocytes and consisted of male or female splenocytes alone or pulsed with synthetic $H\text{-}2D^b$–binding peptides (Uty, Smcy, or E7 with the sequences noted above) or $I\text{-}A^b$–binding peptides (Ddy HY immunodominant peptide [NAGFNSNRANSSRSS] and T. cruzi peptide [SHNFTLVASVIIEEA]). Peptide pulsing was performed by incubation of 1×10^7 splenocytes with 10 μM of each peptide in serum-free RPMI 1640 medium for 2 hours at 37°C in 5% CO_2. Cells were plated a an effector/stimulator ratio of 1:1 and were incubated for 24 hours at 37°C in 5% CO_2. After incubation, cells were removed and plates were washed 4 times with PBS

plus 0.05% Tween 20. A detection mAb, anti–IFN-γ (clone biotin-XMG1.2; Pharmingen Inc.), was added at a concentration of 1 μg/ml, then plates were incubated overnight at 4°C and then were washed and then developed for 2 hours with streptavidin-alkaline phosphatase (DAKO Inc.) at a 1:2000 dilution, then plates were washed and spots were colored for 5–10 minutes with 5-bromo-4-chloro-3-indolyl phosphate membrane phosphatase substrate (Kirkegaard and Perry Labs Inc.). Finally, plates were washed once with distilled H_2O and were allowed to dry at room temperature. Image analysis of ELISPOT plates was performed with a Series 1 Immunospot Satellite Analyzer (Cellular Technology Inc.). Briefly, digitized images were analyzed for the presence of areas in which the color density exceeded that of the background by a preset factor based on the comparison of control wells and experimental wells. The number of specific spots was calculated by subtracting the average number of spots in specific control wells from the average number of spots formed in each experimental well.

Tumor challenge. MB49 was grown to confluence and was harvested by trypsin digestion, and a single-cell suspension was prepared. Animals were immunized with male DCs on day 0 and were treated with cytokines for 28 days as described in Methods. In 1 set of experiments, 4×10^6 MB49 cells were injected subcutaneously on day 16 and in a second group of experiments, 3×10^6 cells were injected subcutaneously on day 60. Tumors were measured thrice weekly and animals were humanely euthanized according to NCI Animal Care and Use guidelines (with a maximal tumor dimension of 2 cm, tumor ulceration, or severe morbidity due to tumor growth). The experiments were approved by the NCI Animal Care and Use Committee (Bethesda, Maryland, USA).

Statistical analysis. Statistical tests were performed using GraphPad Prism version 4.0a for Macintosh (GraphPad Software Inc.). Significant differences for comparisons of 2 groups were determined by the 2-tailed unpaired Student's *t* test. *P* values were considered significant when less than 0.05. Survival curves were analyzed using a Wilcoxon log-rank test.

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Address correspondence to: Crystal L. Mackall, Building 10, Room 13N240, 10 Center Drive, MSC 1928, Bethesda, Maryland 20892, USA. Phone: (301) 402-5940; Fax: (301) 402-0575; E-mail: cm35c@nih.gov.

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