Failed Adoptive Immunotherapy with Tumor-Specific T Cells: Reversal with Low-Dose Interleukin 15 but not Low-Dose Interleukin 2

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ABSTRACT
Adoptive immunotherapy with tumor-specific T cells has emerged as a valid approach for prevention or treatment of diseases, such as melanoma and EBV-associated lymphoma. As interleukin (IL) 15 promotes survival of CD8+ memory CTLs, we hypothesized that it could be used to enhance antitumor immunity in vivo through the maintenance of adoptively transferred memory CTL. To test this, we treated mice bearing P1A+ tumors with adoptively transferred T cells possessing a transgenic Vα8+ T-cell receptor specific for the P1A tumor antigen (called PICTL). Mice were then randomized to receive daily low-dose IL-15 (0.5 μg/day) or PBS. Mice receiving the transgenic PICTL and IL-15 experienced a significantly delayed tumor relapse or complete tumor regression (P < 0.002 compared with PBS), with a striking persistence of the CD8+ Vα8+ PICTL compared with mice receiving the CD8+ Vα8+ PICTL and PBS vehicle (26.3 versus 5.1% P < 10−5). Animals exhibiting complete tumor regression had a significant population of CD8+ Vα8+ PICTL (46%) that persisted with IL-15 treatment until 140 days after adoptive transfer and successfully defended them against tumor rechallenge without IL-15. Low-dose IL-2 afforded no protection over vehicle and resulted in lower percentages of T cells with an activated memory phenotype, lower Bcl-2 expression, and lower ex vivo antitumor cytotoxicity compared with mice treated with IL-15. Collectively, the data support the notion that exogenous low-dose IL-15 therapy can enhance and even reverse the limited efficacy of adoptively transferred tumor-specific T-cell therapy and may do so in a fashion that is superior and distinct from exogenous IL-2 therapy.

INTRODUCTION
Adoptive immunotherapy with tumor-specific T cells has been developed clinically for several human cancers, including malignant melanoma and EBV-associated lymphoma, and it has potential applications for other immunogenic malignancies expressing novel tumor antigens (1). After successful identification of tumor antigens, antigen-specific T cells can be generated ex vivo and delivered to patients as either a therapy for existing disease or prevention strategy for patients at high risk for disease recurrence. The addition of growth factors that promote T-cell activation, proliferation, and tumor killing may augment clinical outcomes for adoptive immunotherapy. For >20 years, interleukin (IL) 2 has been used in the treatment of malignant melanoma and more recently added as a component of adoptive T-cell immunotherapy (1, 2). Although high-dose IL-2 stimulates activation and proliferation of T cells, it can be associated with toxicity and stimulate activation-induced cell death of T cells (3–6). In contrast, IL-15, a related T-cell growth factor, protects T cells against activation-induced cell death and promotes homeostatic main-

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Adoptive Transfer. P1CTL transgenic females (10–14 weeks of age) were used as donors for adoptive immunotherapy. Donor mice were euthanized with CO₂ inhalation. Spleens and lymph nodes were harvested from each mouse and then homogenized into single cell suspensions with cell strainers and syringe plungers. The splenocytes and lymphocytes were pooled and centrifuged, followed by standard red blood cell lysis. We adoptively transferred whole mononuclear lymphocyte suspensions containing 5 × 10⁶ CD8⁺ P1CTL (determined by flow cytometry) into tumor bearing rag2⁻ ⁻/⁻ mice. All of the adoptively transferred CD8⁺ CTLs were Vo8⁺ P1CTL transgenic T cells. The engraftment of J558 tumor cells in rag2⁻ ⁻/⁻ mice followed by adoptive transfer was performed in four separate experiments.

Cytokines. Cytokines were diluted in filtered PBS containing 0.05% human albumin, and frozen aliquots were thawed each day for sterile injection. Animals were dosed with 0.5 µg of human IL-15 (Amgen, Inc., Thousand Oaks, CA), 0.5 µg of human IL-2 (proleukin, Chiron Corp., Emeryville, CA), or an equal volume of PBS vehicle by i.p. injection twice a day for 1 week after adoptive transfer. Thereafter, animals received a single daily injection of IL-15 or IL-2 (0.5 µg/dose) or PBS for 180 days.

**In vivo Proliferation Assays.** Mice were given three i.p. injections of 0.4 mL bromodeoxyuridine (BrdUrd) solution (Zymed Laboratories, Inc., San Francisco, CA) over a 9-day period after adoptive transfer of pooled splenocytes and lymph nodes from P1CTL transgenic mice on days 6, 8, 10, 12, and 14. On day 15 after adoptive transfer, spleens were harvested and analyzed for incorporation of BrdUrd via intracellular flow cytometry (described below). Pooled splenocytes and lymph nodes were labeled with carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Inc., Eugene, OR). Cells (40 to 50 × 10⁶) were labeled in 1 mmol/L carboxyfluorescein diacetate succinimidyl ester solution for 20 minutes at 37°C. Cells were washed, resuspended in 10 mL of RPMI 1640 with 10% FBS, and incubated for 30 minutes at 37°C. Cells (4 to 5 million CD8⁺ T cells/mouse) were washed again in serum-free RPMI 1640 and then injected into mice via tail vein. After 3 days, spleen and blood cells were harvested, stained with antimouse CD8, and analyzed for carboxyfluorescein diacetate succinimidyl ester fluorescence (FL-1) via flow cytometry.

Flow Cytometry. Peripheral blood lymphocytes or splenocytes were stained with fluorophore-conjugated monoclonal antibodies (mAbs) recognizing the following surface antigens: CD8 (adenomatous polyposis coli or PerCP), CD62L (adenomatous polyposis coli), Vo8.3 TCR (phycoerythrin), CD44 (FITC), CD4 (PerCP), and CD43 (FITC). All of the mAbs were purchased from BD Pharmingen (San Diego, CA). Nonspecific binding of mAbs was blocked by preincubating samples with unconjugated goat IgG (Sigma, St. Louis, MO). Cells were stained with conjugated mAbs on ice for 30 minutes. Cells were washed once with 3 mL of PBS and then fixed in 0.1% formalin and analyzed using a Becton-Dickinson FACSCalibur flow cytometer.

Fig. 1. Adoptive immunotherapy with single agent low-dose IL-15 maintains tumor regression in vivo. A, pharmacokinetic analysis of plasma human IL-15 (after a single i.p. injection of 0.5 µg of recombinant human IL-15) demonstrates peak plasma concentration of 4771 pg/mL at 1 hour and the persistence of cytokine for as long as 12 hours. In B, BALB/c rag2⁻ ⁻/⁻ mice were inoculated s.c. with 5 × 10⁶ P1A-expressing J558Neo tumor cells. When tumors reached a mean diameter of 8–10 mm, mice were adoptively transferred (black arrow), via i.p. injection, with pooled spleen and lymph node cells containing 5 × 10⁶ CD8⁺ Vo8⁺ P1CTL, along with either IL-15 (○, 0.5 µg/day) or PBS vehicle (■). Mean tumor size for each group is graphed with SE bars. The administration of IL-15 resulted in significant delay in tumor relapse (P < 0.002) compared with PBS vehicle. Data shown are representative of three independent experiments with identical results. C, growth curve demonstrating unchecked J558 tumor progression in rag2⁻ ⁻/⁻ mice receiving no adoptive transfer. Line, a single mouse.
Intracellular staining was performed with hamster antimouse Bcl-2 phycoerythrin, hamster isotype control phycoerythrin, or hamster anti-BrdUrd FITC with DNase (BD Pharmingen). Intracellular staining was performed using an intracellular flow cytometry kit (BD Pharmingen) as described (21). Briefly, cells were stained for CD8 and Vα8.3 TCR surface markers, washed, and then fixed with the Cytofix/CytoPerm reagent. For BrdUrd staining, cells also were fixed using Cytofix/Cytoperm Plus before staining. Cells were preincubated with unconjugated goat IgG (Sigma) to block nonspecific antibody binding. Data were analyzed with CellQuest (Becton-Dickinson) and WinMDI (Scripps Research Institute, La Jolla, CA) software.

Chromium Release Cytotoxicity Assay. Assays were performed as described (22). Briefly, splenocytes were RBC lysed and cultured at effector:target ratios of 10:1, 20:1, 40:1, and 80:1. PIA-expressing J558 tumor cells were used as targets (10,000 cells/well). Specific lysis was expressed as a percentage with the following formula: sample release – minimum release/maximum release – minimum release.

Statistical Analysis. An exact Wilcoxon rank-sum test was performed to test for a difference between the percentage of peripheral blood P1CTL and time of tumor relapse in mice receiving adoptive transfers of T cells and cytokines. Each outcome was tested separately. Student’s t test was used to interpret Bcl-2 expression. Cytotoxicity data were analyzed using two-way ANOVA.

RESULTS

Adaptive Immunotherapy with Single Agent Low-Dose IL-15 Maintains Tumor Regression In vivo. We performed a pharmacokinetic analysis after a single i.p. injection of human IL-15 (0.5 μg) and found peak plasma levels of 4771 ± 670 pg/mL occurring at 1 hour. IL-15 persisted as long as 12 hours after injection with an estimated half-life of 1.15 hours (Fig. 1A). We next inoculated immunodeficient rag2−/− mice with the PIA-expressing J558 tumor cell line, and when tumors reached a mean diameter of 10 mm, mice were treated with a single adoptive transfer of pooled spleen and lymph node cells from P1CTL transgenic mice. These mice were randomized to receive daily low-dose recombinant human IL-15 as a single agent or PBS vehicle control. Adoptive transfer with vehicle treatment resulted in initial tumor regression, followed by stabilization of tumor size, and then subsequent tumor relapse around day 35 (Fig. 1B). Relapsing tumors in vehicle-treated mice expanded unchecked to a diameter of 25 to 30 mm, at which time mice were sacrificed. In contrast, administration of exogenous IL-15 after adoptive transfer significantly prolonged tumor remission compared with vehicle treatment (Fig. 1B; day 50, P < 0.002). IL-15 treatment of control mice that did not receive adoptive transfer of T cells did not result in inhibition of tumor growth (Fig. 1C).

Single Agent Low-Dose IL-15 Therapy Maintains Tumor-Specific Memory CD8+ T Cells In vivo. We sought to determine whether provision of IL-15 could sustain or increase the proportion of adoptively transferred P1CTLs in tumor-bearing mice. As a means to identify tumor-specific T cells in vivo, we used the Vα8 TCR chain expressed on transgenic tumor-specific T cells. Twenty-one days after adoptive transfer of P1CTL, mice receiving IL-15 treatment had significantly greater CD8+ Vα8+ P1CTLs in peripheral blood compared with vehicle-treated mice (Fig. 2A, day 50, P < 0.002). IL-15 treatment of control mice that did not receive adoptive transfer of T cells did not result in inhibition of tumor growth (Fig. 1C).

IL-15 Mediates Complete Tumor Regression and Long-Term Maintenance of Tumor-Specific Memory CD8+ T Cells. In one experiment, 50% (3 of 6) of mice receiving IL-15 treatment until day 180 completely rejected their tumors and had no evidence of tumor as of 217 days after adoptive transfer of P1CTL (Fig. 3A). Nine months after adoptive immunotherapy and in the absence of IL-15 treatment, tumor-free mice were resistant to rechallenge with J558 tumor cells compared with naïve rag2−/− mice that developed palpable tumors by day 24 (data not shown). The tumor-free survival of these mice permitted analysis of long-term persistence of blood P1CTL. At 140 days after adoptive transfer, each mouse demonstrated a substantial (mean 46%) population of CD8+ Vα8+ P1CTLs (Fig. 3B). However, compared with day 21, P1CTLs now exhibited a CD44high CD62Lhigh central memory phenotype (Fig. 3C, refs. 23, 24).

Low-Dose IL-15 Does Not Enhance In vivo Proliferation of P1CTL during T-Cell Priming. To evaluate the effects of IL-15 on in vivo T-cell proliferation during the early priming phase, pooled spleen and lymph node cells from P1CTL transgenic mice were labeled with carboxyfluorescein diacetate succinimidyl ester dye and adoptively transferred into tumor-bearing mice. Three days after adoptive transfer, analysis of carboxyfluorescein diacetate succinimidyl ester content demonstrated up to seven rounds of CD8+ Vα8+ P1CTL proliferation in both spleen and peripheral blood compartments (Fig. 4A). CD8+ Vα8+ P1CTL proliferation was similar in mice treated with exogenous IL-15 or vehicle control (Fig. 4A). Furthermore, there was no difference in CD8+ Vα8+ P1CTL incorporation of BrdUrd in mice treated with either IL-15 or vehicle control at day 15 after adoptive transfer (Fig. 4B).

Exogenous IL-15 but not IL-2 Delays Tumor Relapse After Adoptive Transfer with Tumor-Specific T Cells. Immunodeficient mice with J558 tumors were treated with a single adoptive transfer of pooled spleen and lymph node cells from P1CTL transgenic mice and randomized to receive daily low-dose IL-15, low-dose IL-2, or vehicle control treatments. Tumors in mice receiving T cells and vehicle control or IL-2 treatment relapsed around day 35, whereas regression...
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was sustained in mice receiving IL-15 (Fig. 5). IL-2 treatment of mice that did not receive an adoptive transfer did not result in inhibition of tumor growth (data not shown).

Low-Dose IL-15 Promotes T-Cell Expression of Bcl-2 and Effector Function In vivo. There were no significant differences in the percentage of CD8+ Vo8+ P1CTLs found in blood of mice treated with either IL-15 or IL-2 (Fig. 6A), yet there was a significant difference in the fraction of these cells exhibiting a CD62Llow or effector memory phenotype (Fig. 6B; 91 versus 61%, P < 0.002). Furthermore, splenocytes from mice treated with IL-15 displayed greater ex vivo cytolytic activity (57%) against J558 tumors than splenocytes from mice treated with PBS vehicle (24%) or IL-2 (40%; Fig. 6C; IL-15 versus IL-2, P < 0.01; IL-15 versus PBS vehicle, P < 0.01, n = 3 mice per group). Lastly, IL-15 treatment resulted in increased Bcl-2 expression in blood CD8+ Vo8+ P1CTL compared with IL-2 treatment [Fig. 6D; mean fluorescent intensity (MFI) 263 versus MFI 176, P < 0.0005, n = 4 mice per group].

DISCUSSION

Systemic administration of IL-15 to thymectomized mice selectively stimulates proliferation of CD44high CD122high CD8+ memory T cells in lymph nodes (17). In addition, transgenic overexpression of IL-15 selectively expands memory CD8+ T cells and natural killer cells (18). In this study, we present in vivo data on the use of low-dose IL-15 with adoptive immunotherapy of tumor-specific T cells in a mouse tumor model that is ultimately refractory to adoptive cellular immunotherapy alone. Although Sarma et al. (20) reported previously similar adoptive therapy studies in a wild-type background, more recent work (25) and the experiments presented here have used rag2−/− mice, where it is possible that T-cell effector function was enhanced via an initial boost for homeostatic T-cell proliferation attributable to a lack of suppressor or regulatory T cells in a lymphopenic environment. To our knowledge, this study demonstrates for the first time that exogenous low-dose IL-15 but not IL-2 mediates a significant improvement in long-term tumor regression and survival time in a tumor model that involves the adoptive transfer of tumor-specific T cells. Furthermore, the antitumor efficacy of exogenous low-dose IL-15 correlated with the successful in vivo maintenance of tumor-specific memory CTLs.

Maintenance of CD8+ memory T cells by IL-15 in vivo occurs via two mechanisms. The first mechanism is through stimulation of slow homeostatic proliferation. Bai et al. (26) demonstrated that the majority of T-cell priming and proliferation in the P1A transgenic T-cell tumor model occurs in lymphoid tissues via direct priming by tumor or via cross priming by host lymphoid tissues. Expansion of T cells in the spleen occurred between 3 and 5 days after adoptive transfer. In our experiments, we did not observe a difference in the number of divisions undergone by day 3 or in BrdUrd incorporation at day 15 in mice treated with IL-15 or vehicle control. Because the percentage of peripheral blood P1CTLs at 21 and 140 days after adoptive transfer increased (mean of 25% and 46%, respectively), it is possible that this

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increase was a result of non-CD8 T-cell death, leading to a relative increase in CD8 percentages or a slow rate of proliferation induced by IL-15. Alternatively, IL-15 could have promoted the differentiation of P1CTL into longer-lasting memory T cells.

A second mechanism through which IL-15 maintains memory CD8+ T cells is by the promotion of T-cell survival factors, including the antiapoptotic protein Bcl-2 (27). In our in vivo studies, we found that IL-15 treatment results in an increase in T-cell Bcl-2 expression compared with IL-2 controls. Thus, it is possible that IL-15 promoted survival of activated tumor-specific T cells after priming and tumor regression, instead of or in addition to their proliferation. There is ample evidence that IL-15 supports Bcl-2 expression in antigen-specific memory T cells both in vitro and in vivo (27, 28). Furthermore, there is evidence that IL-15 can prevent IL-2-stimulated activation-induced cell death (7). Low levels of IL-15 (0.05 to 6 ng/mL) protected purified memory CD8+ T cells from apoptosis in vitro but failed to stimulate proliferation (27). The concentrations that were sufficient to promote in vitro survival coincided with our pharmacokinetic data demonstrating peak plasma levels of 4.7 ng/mL. Thus, administration of exogenous low-dose IL-15 may have promoted tumor-specific T-cell survival via an increase in Bcl-2 expression.

IL-15 treatment maintained P1CTLs that displayed the phenotype of effector memory T cells (T_EM, CD44^high CD62L^low) 3 weeks after adoptive transfer. However, 17 weeks after adoptive transfer, the majority of P1CTL were central memory T cells (T_CM, CD44^high CD62L^high; refs. 23, 24). This progression toward T_CM is consistent with data from Wherry et al. (29), who reported that CD8+ memory T-cell development is a linear continuum: naïve T > effector T > T_EM > T_CM, as demonstrated by their adoptive transfer of sorted T_CM and T_EM LCMV-specific T cells. Manjunath et al. (30) have reported previously the generation of T_CM cultured with IL-15 (20 ng/mL) after peptide or anti-CD3 stimulation of CD8+ T cells. T_CM displayed less cytoxicity but persisted longer in vivo compared with T_EM that were highly cytotoxic (30). Supporting our data, Bai et al. (26) have already reported that P1CTLs from transgenic mice are indeed competent for lysis. IL-15 reportedly up-regulates gene expression of effector molecules, such as perforin and granzyme B (31, 32). In our experiments, the maintenance of a largely T_CM P1CTL

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**Fig. 6.** P1CTL assessment after adoptive transfer and treatment with IL-15, IL-2, or PBS. In A, analysis of the percentage of blood CD8^+ Vγ8^+ P1CTL 30 days after adoptive transfer demonstrated a significant increase of P1CTL in mice treated with IL-15 compared with PBS control treatment, yet no difference between mice treated with IL-15 and IL-2. In B, CD8^+ Vγ8^+ P1CTL were analyzed for CD62L^low (black bar) or CD62L^high (gray bar) expression. Only exogenous administration of IL-15 resulted in a significantly greater percentage of CD62L^low, compared with either vehicle control or IL-2 administration. All T cells expressed CD44^high (data not shown). In C, 3 weeks after adoptive transfer, splenocytes were harvested and assayed for ex vivo cytolytic activity against J558 tumor cells (n = 3 mice per group). IL-15 treatment resulted in significantly greater cytolytic activity (57%) than PBS (24%), IL-2 (40%), and vehicle control (0%). In D, Bcl-2 expression was analyzed by intracellular flow cytometry on CD8+ gated blood cells from mice treated with either IL-15 or IL-2 (D, mean fluorescent intensity (MFI) 263 versus MFI 176; P = 0.0005, n = 4 mice per group).
correlated with antitumor efficacy in vivo, implying that despite its biased differentiation away from TEM cytotoxic phenotypes, preservation of long-term T-cell memory with IL-15 has therapeutic potential.

Recently, Klebanoff et al. (33) compared high-dose IL-15 and IL-2 therapy for enhancement of in vivo antitumor activity of tumor-specific T cells. Transgenic pmel-1–specific T cells were stimulated in vitro with pmel-1–derived peptide, cultured in IL-2 or IL-15 for 1 week, and then adoptively transferred into wild-type mice bearing murine B16 melanoma tumors (33). Additional treatment of these mice with exogenous high-dose IL-2 or IL-15 (12 to 108 μg, twice daily for 6 days) resulted in a significant delay in tumor growth (12 versus 29 days) compared with control (no cytokine treatment in vivo); there was no significant difference observed between treatment with exogenous IL-2 and IL-15. Interestingly, Kobayashi et al. (34) demonstrated distinct kinetics for IL-15 and IL-2, where IL-15 displayed faster clearance from the blood and greater accumulation in bone, spleen, and kidney tissues. Although both tumor models used transgenic T cells against tumor antigens, our data show, for the first time, that effective tumor regression could be achieved with low-dose IL-15 but not IL-2 and without requirement for peptide stimulation in vitro and peptide vaccination in vivo.

In summary, our data show that IL-15 promotes the long-term maintenance of tumor-specific memory T cells and can maintain and, in some cases, enhance tumor regression in vivo when cellular therapy alone is unsuccessful. Current strategies for adoptive immunotherapy with tumor-specific T cells, therefore, may benefit from the addition of recombinant IL-15 protein or gene therapy (35). In addition to providing ligands for costimulatory receptors (25), IL-15 also could be applied as an adjuvant in cancer vaccine strategies using tumor antigens that aim to expand tumor-specific T cells in vivo. Because IL-15 stimulates proinflammatory cytokine production by natural killer cells and recent evidence suggests that it also can promote maturation of antigen-presenting cells (36, 37), IL-15 may have multiple effects on the enhancement of cell-mediated antitumor efficacy that could be exploited clinically.

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