Determinants of Successful CD8⁺ T-Cell Adoptive Immunotherapy for Large Established Tumors in Mice

Christopher A. Klebanoff, Luca Gattinoni, Douglas C. Palmer, Pawel Muranski, Yun Ji, Christian S. Hinrichs, Zachary A. Borman, Sid P. Kerkar, Christopher D. Scott, Steven E. Finkelstein, Steven A. Rosenberg, and Nicholas P. Restifo

Abstract

Purpose: Adoptive cell transfer (ACT) of tumor infiltrating or genetically engineered T cells can cause durable responses in patients with metastatic cancer. Multiple clinically modifiable parameters can comprise this therapy, including cell dose and phenotype, in vivo antigen restimulation, and common gamma-chain (γc) cytokine support. However, the relative contributions of each of these individual components to the magnitude of the antitumor response have yet to be quantified.

Experimental Design: To systematically and quantitatively appraise each of these variables, we employed the Pmel-1 mouse model treating large, established B16 melanoma tumors. In addition to cell dose and magnitude of in vivo antigen restimulation, we also evaluated the relative efficacy of central memory (T_CM), effector memory (T_EM), and stem cell memory (T_SCM) subsets on the strength of tumor regression as well as the dose and type of clinically available γc cytokines, including IL-2, IL-7, IL-15, and IL-21.

Results: We found that cell dose, T-cell differentiation status, and viral vaccine titer each were correlated strongly and significantly with the magnitude of tumor regression. Surprisingly, although the total number of IL-2 doses was correlated with tumor regression, no significant benefit to prolonged (>6 doses) administration was observed. Moreover, the specific type and dose of γc cytokine only moderately correlated with response.

Conclusion: Collectively, these findings elucidate some of the key determinants of successful ACT immunotherapy for the treatment of cancer in mice and further show that γc cytokines offer a similar ability to effectively drive antitumor T-cell function in vivo.

Introduction

Adoptive cell transfer (ACT), the infusion of ex vivo expanded antitumor or antiviral lymphocytes to patients, represents a potent therapy for certain advanced solid tumors (1–3), relapsed hematologic malignancies (4), and posttransplant viral-induced lymphoproliferative disorders (5). Once the purview of highly specialized research centers, multiple technical and conceptual innovations in recent years have allowed this promising approach to cancer therapy to extend to a wider range of institutions and patients. For example, the methodology used to generate tumor infiltrating lymphocytes (TIL) has been greatly simplified by using unselected and minimally cultured T cells as the infusion product (6, 7). Importantly, the ability of TIL to mediate objective cancer regression in roughly 50% of metastatic melanoma patients when combined with prior host conditioning using lymphocyte-depleting chemotherapy (8) has now been shown at multiple institutions outside of the intramural NIH (6, 9). Moreover, with the recent advent of clinical grade retroand lenti-viral vectors capable of genetically redirecting the reactivity of autologous peripheral lymphocytes toward tumor or viral-associated antigens by introduction of exogenous T-cell receptors (TCR; refs. 10–13) or chimeric antigen receptors (14–18), adoptive T-cell immunotherapy can potentially be deployed more widely and easily. Thus, there has been a notable increase in the number of phase I and II clinical trials using TIL or genetically redirected T cells initiated throughout the world in recent years (3).

ACT allows for the precise manipulation of multiple variables that may impact treatment efficacy. Previous work in humans has correlated in vivo persistence of transferred T cells (18, 19), telomere length (19), the reexpression of CD27 after IL-2 withdrawal (19), and lympho-depleting host conditioning (20, 21) with cancer regression. In addition to host preconditioning before cell transfer...
Translational Relevance

Simplification of the methods used to generate tumor-infiltrating lymphocytes combined with the ability to genetically redirect T cell reactivity toward tumor-associated antigens has led to an increased number of phase I/II adoptive cell transfer (ACT) trials in recent years. Optimizing the design and execution of future ACT protocols will require a detailed understanding of how different parameters related to cellular therapies might impact treatment outcomes. Using the Pmel-1 CD8+ T-cell receptor transgenic model, a realistic murine model for the treatment of established melanomas targeting the shared self/tumor-antigen gp100, we systematically and quantitatively assessed the relative contributions of multiple parameters potentially amenable to clinical manipulation toward treatment efficacy. Specifically, we evaluated the impact of intensifying in vivo antigenic restimulation with a viral vaccine, cell dose and differentiation status, and duration and type of cytokine support following cell transfer. Collectively, these findings provide for the rationale design of ACT-based immunotherapy trials.

(22–24), preclinical experiments in mice have identified additional parameters associated with treatment outcome, including the dose (23, 25) and differentiation status of transferred T cells (25–28), reactivation of transferred T cells at the time of cell infusion (29–31), and the efficiency of TCR transduction for gene-modified T cells (23). Although each of these factors may incrementally improve T-cell therapies, knowledge of which factors contribute most greatly to tumor regression is essential for prioritizing future clinical development. It is for this reason that we sought to systematically and quantitatively assess the impact of multiple variables potentially amenable to clinical manipulation to improve future adoptive T cell-based immunotherapy protocols.

Specifically, we evaluated the impact of the strength of in vivo Ag restimulation with a recombinant viral vaccine, T-cell dose and differentiation status, and schedule, dose, and type of exogenous common gamma chain (γc) cytokine support on the magnitude of tumor regression. As the donor source of T cells, we used cells derived from the Pmel-1 TCR transgenic mouse whose CD8+ T cells have specificity for a D8-restricted epitope derived from the shared self/tumor-antigen (Ag) gp100 (29). Our findings showed that not all parameters evaluated contributed equally to the magnitude of the antitumor response mediated by adoptively transferred CD8+ T cells. Indeed, intensity of in vivo Ag restimulation, T-cell differentiation status, and the absolute number of infected T cells were each strongly correlated with tumor regression. By contrast, the number, dose, and type of γc cytokine administered to support transferred T cells were each less strongly associated with treatment outcomes. Taken together, these results provide for the rationale prioritization, design, and execution of future T cell-based immunotherapy protocols in humans.

Materials and Methods

Mice and tumor lines

The Pmel-1 (The Jackson Laboratory) TCR transgenic mouse has been previously described (29). Female C57BL/6 mice (The Jackson Laboratory) were used at 6 to 12 weeks of age as recipient mice in all cell transfer experiments. B16 (H-2b), a spontaneous gp100+ murine melanoma cell line, was obtained from the National Cancer Institute (NCI) tumor repository and maintained in complete media (CM). All animal experiments were conducted with the approval of the NCI Animal Use and Care Committee.

Generation of CD8+ T cells for adoptive transfer

To generate Ag-specific CD8+ T cells for cell transfer, Pmel-1 splenocytes were isolated, pulsed with 1 μmol/L of human gp10025–33, and expanded for 1 week in the presence of CM supplemented with 4 ng/mL of recombinant human IL-2 (rhIL-2; Novartis). In some experiments, CD8+ T memory stem cells (TSCM), CD8+ T central memory cells (TCM), or CD8+ T effector memory cells (TEM) were generated as described previously (28). Briefly, naïve Pmel-1 CD8+ T cells were isolated from a single cell suspension of splenocytes after ACK lysis using a MACS negative selection kit (Miltenyi Biotec) and cells were activated with 2 μg/mL of plate bound anti-CD3, 1 μg/mL of soluble anti-CD28 (both from BD Biosciences), and expanded in 10 ng/mL of IL-2. TSCM and TCM cells were generated in the presence of 7 μmol/L of the GSK-3β inhibitor TWS119 (EMD Biosciences) dissolved in dimethyl sulfoxide (Sigma-Aldrich) whereas TEM cells were generated from separate cultures derived from an aliquot of the same single cell suspension in the presence of vehicle only. After 5 days in culture, cells were fractionated into CD44lowCD62Lhigh (TSCM), CD44highCD62Lhigh (TCM), and CD44highCD62Llow (TEM) populations to a purity of more than 98% using a FACSAria cell sorter (BD Biosciences).

Adoptive cell transfer, poxvirus vaccination, and γc cytokine administration

Unless otherwise indicated, mice (n ≥ 5 per group) were injected s.c. with 2 × 105 to 5 × 105 B16 melanoma cells. Ten to 14 days later, treated mice received i.v. injections of CD8+ T cells and indicated doses of 1 of 2 previously described (29) recombinant poxviruses encoding hgp100 (rPVhgp100): recombinant fowlpox virus expressing human gp100 (rFPhgp100) or recombinant vaccinia virus expressing hgp100 (rVhgp100). Previous work has shown that both poxviruses provide similar efficacy when used in tumor treatment experiments (29). Where indicated, treated mice received 500 cGy of sublethal irradiation prior to ACT to mimic the lymphodepletion done in our current clinical protocols (22). In addition to vaccination, all treated mice also received indicated doses of exogenous γc cytokine support with either rhIL-2 (Novartis), IL-7.
(Peprotech), IL-15 (Peprotech), or IL-21 (R&D Systems) by twice daily intraperitoneal (i.p.) injections. Except for rhIL-2, where dosing was varied between 1 to 14 total doses, all treated mice received 6 total doses of a γ, cytokine. All tumor measurements were done in a blinded fashion.

Statistics

The products of perpendicular tumor diameters were plotted as the mean ± SEM for each data point and tumor treatment graphs were compared by using the Wilcoxon rank sum test. Regression analysis of the slope of tumor regression as a function of different variables related to ACT was done as described previously (25, 32, 33) using a linear regression. Differences in animal survival were assessed using the log-rank test. All time points for a slope to be calculated. Differences in dual mice must have been evaluable for at least 3 separate minimum of 3 mice for each parameter analyzed. Indivi-

Results

Dose of adoptively transferred CD8\(^+\) T cells strongly correlates with tumor regression

In some clinical ACT trials, the absolute number of infused CD8\(^+\) T cells has retrospectively been correlated with the likelihood of obtaining an objective response consistent with standardized oncologic response criteria (6). This finding has not been uniformly found in all ACT trials, however (8, 20). Moreover, the generation and expansion of large numbers of T cells for infusion requires a significant investment in limiting resources such as allogeneic feeder cells and soluble OKT-3, laboratory and incubator space in GMP facilities, and technical personnel to care for these cells. For these reasons, we sought to determine the impact of the infusion dose of Ag-specific CD8\(^+\) T cells on the magnitude of tumor regression. We therefore adoptively transferred titrated doses of Pmel-1 CD8\(^+\) T cells into mice bearing established B16 melanoma tumors. Similar to current human cell transfer protocols (6–8, 20, 34), mice received \(\text{ex vivo}\) expanded self/tumor-reactive CD8\(^+\) T cells grown in IL-2. In addition to ACT, all treated mice received an identical, intermediate titer (10\(^7\) pfu) of rFPhgp100 vaccination and exogenous IL-2 and preconditioning with sublethal irradiation.

We found that infusion with as few as 10\(^4\) CD8\(^+\) T cells caused a significant delay in tumor growth compared with untreated control mice (Fig. 1A; \(P < 0.05\) for all treatment groups compared with untreated control). To gain further insight into the dose–response relationship between infusion cell dose and the magnitude of the antitumor response, we plotted the slope of tumor regression of treated mice as a function of cell dose on a semi-log plot. We found a significant linear correlation between the dose of infused antitumor CD8\(^+\) T cells and strength of tumor regression (Fig. 1B; \(r^2 = 0.71; P < 0.03\)). Finally, to assess whether these improvements in tumor regression with increased cell dose translated into extended survival, we plotted the survival of treated mice (Fig. 1C). We found a significant, stepwise improvement in animal survival as the total number of infused CD8\(^+\) T cells was increased (\(P < 0.05\)). A similar dose–response relationship between the numbers of infused antitumor CD8\(^+\) T cells and the strength of tumor regression and improvement in animal survival was also observed in mice that did not receive prior lymphodepletion (Supplementary Fig. S1A–C). These data
show that the dose of infused tumor-reactive CD8+ T-cells infused is strongly correlated with the magnitude of tumor regression.

**CD8+ T-cell differentiation status is inversely correlated with in vivo antitumor treatment efficacy**

Presently, it has been difficult to uncouple the processes of T-cell proliferation and expansion from differentiation and senescence such that prolonged ex vivo culture results in therapeutically less effective T-cells in both mice (25) and humans (35–37). As the variables of cell yield and differentiation status are at apparent odds with one another, we next aimed to quantify the extent to which T-cell differentiation status may impact in vivo treatment efficacy. Therefore, we next assessed the antitumor response mediated by adoptive transfer of a minimal number (4 × 10^6) of Ag-specific CD8+ T-cells possessing a defined differentiation status based on the pattern of expression for the surface molecules CD62L and CD44. Using these markers, mouse CD8+ T cells may be subdivided into defined antigen-experienced T-cell populations termed T-effector memory (T_Eff; CD62L<CD44<), T central memory (T_CM; CD62L<CD44>, and a recently described subset termed T stem cell memory (T_SCM; CD62L<CD44<; ref. 28), each of which has ontologies among human T cells (38). T-cell cultures were generated either in the presence of the glycan synthesis kinase-3β (GSK-3β) inhibitor TWS119, a pharmacologic agonist of the canonical Wnt/β-catenin pathway, and T-cell subsets were FACs-sorted to a purity of more than 98% prior to cell transfer. All treated mice received sublethal irradiation, vaccination with 10^7 pfu of rVVhgp100, and exogenous IL-2 support.

At this near-limiting dose of adoptively transferred T cells, we observed no significant delay in tumor growth in untreated mice compared with mice receiving T_CM CD8+ T cells (Fig. 2A; P > 0.05). By contrast, adoptive transfer of either T_CM or T_SCM CD8+ T cells caused a significant delay in tumor growth compared with both untreated controls and mice receiving T_Eff cells (Fig. 2A; P < 0.01 and < 0.05, respectively). Furthermore, T_SCM cells caused a more sustained reduction in tumor growth compared with T_CM cells (P < 0.05). When the slope of tumor regression was plotted as a function of T-cell differentiation status, we observed a significant linear correlation between these 2 parameters (Fig. 3B; r^2 = 0.72; P < 0.0001). This improvement in the control of tumor growth with transfer of progressively less-differentiated CD8+ T cells also translated into a significant enhancement in overall survival of treated animals (Fig. 2C; P < 0.02). These findings show that beyond the absolute number of adoptively transferred CD8+ T cells, T-cell differentiation status can also significantly impact the outcome of ACT therapy, even when limiting numbers of T cells are infused.

**Intensity of in vivo antigen restimulation is a critical determinant of the antitumor efficacy of adoptively transferred CD8+ T cells**

Antigen restimulation, either immediately prior to or directly following cell transfer, has been shown to significantly enhance the in vivo antitumor efficacy of adoptively transferred T cells in both preclinical (29–31, 39, 40), and

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**Figure 2.** CD8+ T-cell differentiation status is inversely correlated with in vivo antitumor treatment efficacy. A, sublethally irradiated B6 mice bearing 10-day established s.c. B16 tumors were left untreated as controls (●) or received 4 × 10^6 of FACS-sorted Pmel-1 CD8+ T-cells with the phenotype of T_SCM (□), T_CM (○), or T_EM (■) populations generated by in vitro priming of Pmel-1 splenocytes with 1 μmol/L hgp10025-33 in the presence of 7 μmol/L of TWS119 and 10 ng/mL of rhIL-2. T_SCM = CD44<CD62L<CD44<, T_CM = CD44<CD62L<CD44>, T_EM = CD44<CD62L<CD44<. All treated mice received rVVhgp100 vaccination (10^7 pfu) and exogenous rhIL-2 (36 μg/dose × 6 doses). Results are presented as the mean measurements from 5 mice per group (± SEM) and are representative of 3 independently conducted experiments. B, CD8+ T-cell differentiation status inversely is correlated with the magnitude of regression for established s.c. B16 melanoma tumors. The slope of tumor regression (mm^2/d) for individually treated mice is shown as a function of differentiation status of adoptively transferred CD8+ T-cells, as determined by classification into T_SCM, T_CM, or T_EM subsets. All mice received identical host preconditioning, rVVhgp100 vaccination, and exogenous rhIL-2. Linear regression analysis was done to determine the best line of fit and the P and the P were calculated. r^2 = 0.72; P < 0.001. C, survival curves of untreated mice and mice receiving, exogenous IL-2, rVVhgp100 vaccine, and adoptively transferred T_SCM, T_CM, or T_EM Pmel-1 CD8+ T-cells. * = P < 0.02, ** = P < 0.001 using log-rank statistics. Data shown was derived from 2 pooled independently conducted experiments.

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early phase clinical studies (18, 41). We therefore next sought to test the hypothesis that increasing the strength of in vivo Ag restimulation could improve the antitumor function of adoptively transferred CD8\(^+\) T cells. We infused mice bearing established B16 tumors with a dose (10\(^5\)) of IL-2 expanded Pmel-1 CD8\(^+\) T cells previously established to be noncurative in our model (25). To systematically modulate the intensity of Ag restimulation, treated mice were given titrated doses of a recombinant viral vaccine expressing an anchor-modified variant of gp100 (rFPhgp100) over a 4-log dose range. In addition, all treated mice received lymphodepletion with 500 cGy of irradiation prior to cell transfer and exogenous IL-2 to mimic current cell transfer protocols in patients (6, 7, 20).

We found that at or above a threshold vaccine titer of 10\(^6\) pfu, the in vivo antitumor function of transferred CD8\(^+\) T cells was significantly enhanced compared with nontreated control mice (Fig. 3A; \(P < 0.02\) for all groups). By contrast, there were no significant differences in the rate of tumor growth observed between control mice and mice that received ACT and vaccination with a rFPhgp100 titer of 10\(^5\) pfu or less (\(P > 0.05\)).

To gain further insight into the dose–response relationship between vaccine titer and the magnitude of the antitumor response, we next evaluated the slope of tumor regression for individually treated mice as a function of rFPhgp100 titer. We found that the titer of vaccine was strongly correlated with the slope of tumor regression for individual mice on a semi-log plot (Fig. 3B; \(r^2 = 0.78\); \(P < 0.001\)). To determine whether the magnitude of vaccine-enhanced tumor regression mediated by adoptively transferred CD8\(^+\) T cells correlated with improved animal survival, we plotted the survival of mice receiving titrated doses of vaccine in combination with ACT. Similar to the pattern of tumor regression, we saw a significant and progressive improvement in animal survival as the intensity of vaccination was increased (Fig. 3C; \(P < 0.01\)). A similar, although less prominent, trend of improved tumor regression and animal survival with a progressive increase in vaccine titer was also observed in mice that did not receive lymphodepletion prior to cell transfer (Supplementary Fig. 2A–C).

In a recent report, lethal toxicity from an uncontrolled cytokine storm was observed in mice receiving ACT of self/tumor-reactive CD8\(^+\) T cells followed by repeated vaccination with a synthetic long peptide (42) combined with IL-2 and imiquimod, a toll-like receptor 7 agonist (43). We therefore sought to determine whether mice treated with a single injection of the highest dose (10\(^8\) pfu) of a recombinant poxvirus vaccine we tested experienced similar toxicities. Treated mice displayed a moderate (<10\%) but significant loss of body weight in the first week compared with irradiated but otherwise untreated controls, but this weight loss entirely abated by the second week (Fig. 3D).

Importantly, no treated mice at any of dose levels evaluated acutely expired following treatment (Fig. 3C and Supplementary Fig. 2A–C).

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Importantly, no treated mice at any of dose levels evaluated acutely expired following treatment (Fig. 3C and Supplementary Fig. 2C).

Taken together, these data indicated that the strength of in vivo Ag restimulation provided by a recombinant viral vaccine is strongly and significantly correlated with the magnitude of antitumor treatment efficacy mediated by adoptively transferred CD8\(^+\) T cells in both lymphodepleted and lympho-replete hosts.

Number of IL-2 doses rapidly reaches an asymptotic dose–response relationship

Adaptively transferred T cells are supported with exogenous IL-2 following cell infusion in many current clinical cell transfer protocols (6, 20, 44).
single bolus agent, the absolute number of IL-2 doses received during the first cycle of therapy has been correlated with response to treatment in patients with metastatic melanoma (45). However, IL-2 can cause a number of significant dose-limiting toxicities, including hypotension, pulmonary edema, and neuro-cognitive changes. Moreover, exogenous IL-2 may also potentiate the suppressive activity and homeostasis of CD4+FOXP3+ T regulatory (Treg) cells by the nature of their constitutive expression of a component of the high affinity IL-2 receptor, CD25 (46, 47). Thus, we wished to establish whether there are an optimal number of IL-2 doses to administer following ACT (46, 47).

To address this question, we treated sublethally irradiated mice bearing established B16 melanoma tumors with adoptive transfer of 10^6 IL-2 expanded Pmel-1 CD8+ T cells, 10^7 pfu rFPhgp100 vaccine, and between 0 to 14 total doses of rhIL-2 administered as a 36 µg i.p. injection twice daily. We found a threshold effect for the total number of IL-2 doses such that the administration of 2 or more doses was associated with a significant delay in tumor growth compared with untreated controls (Fig. 4A; P < 0.02). By contrast, ACT and vaccination alone or ACT, vaccination and a single dose of IL-2 was insufficient to impact the rate of tumor growth (P > 0.05). Overall, we found a moderate but significant correlation between the total number of IL-2 doses administered and the magnitude of tumor regression (Fig. 4B; r^2 = 0.51; P < 0.0001 for the overall assessment of 0 to 14 doses). Surprisingly, however, we found no significant benefit to protracted IL-2 administration beyond 6 doses (Fig. 4B; r^2 = 0.06; P = 0.29 for the assessment of 6 to 14 doses). These data show that the benefit of exogenous IL-2 to support adoptively transferred T cells rapidly reaches an asymptotic dose–response relationship.

**IL-2, IL-7, IL-15, and IL-21 cause dose-dependent augmentation of in vivo antitumor treatment mediated by adoptively transferred CD8+ T cells**

Having established that 6 doses of exogenous IL-2 was optimal for antitumor treatment in the Pmel-1 model, we next sought to determine the relative impact of dose titrating 6 total doses of alternative γc-signaling cytokines. Specifically, we compared escalated doses of IL-7 (48, 49), IL-15 (50), and IL-21 (51, 52) to IL-2 as each of these cytokines are known to activate, expand, or promote the survival of T cells and all are either commercially available for human use or have entered into human clinical trials. Although each of the γc cytokines have overlapping functions by nature of their shared ability to activate common signal transducer and activator of transcription (Stat) family members, including Stat5, individual cytokines may possess unique or selective activities (53). We therefore adoptively transferred a noncurative dose (10^6) of in vitro activated, IL-2 expanded Pmel-1 CD8+ T cells into sublethally irradiated mice bearing established B16 melanoma tumors in combination with 10^7 pfu of rFPhgp100 vaccine. In addition, treated mice received exogenous γc cytokine support at a dose range in which other investigators observed immunologic activity (54, 55).

Similar to what we observed with respect to the total number of IL-2 injections, we found only a moderate...
correlation between the dose of any given γc cytokine and the magnitude of the antitumor response (Fig. 5; \( r^2 = 0.31\text{--}0.51; P < 0.001 \)). IL-15 had the weakest linear dose–response correlation (\( r^2 = 0.31 \)) while IL-21 had the strongest (\( r^2 = 0.51 \)). However, when the dose–response relationship was projected as a decay exponential curve, IL-15 was found to most rapidly reach a plateau in response compared with the other cytokines, followed by IL-2, suggesting that some cytokines can reach their maximal in vivo activity more rapidly than others. No overt evidence of cytokine-related toxicity was observed with any of the cytokines tested as the vast majority of mice treated at the highest dose levels remained alive beyond 2 weeks after the final cytokine dose (data not shown). Taken together, these data show that all γc cytokines are similarly capable of augmenting the in vivo antitumor function of adoptively transferred CD8⁺ T cells.

**Discussion**

In this study, we found that the parameters of CD8⁺ T-cell dose, the differentiation status of the input population of T cells, and the intensity of in vivo Ag restimulation were each independently strongly (\( r^2 > 0.7 \)) and significantly (\( P < 0.03 \)) correlated with the magnitude of tumor regression in our model. By contrast, the total number of IL-2 doses administered and the specific type and dose of γc cytokine used to support adoptively transferred T cells were only moderately (\( r^2 = 0.31\text{--}0.51 \)) correlated with the antitumor response. Our conclusions were based on data obtained using CD8⁺ T cells derived from a single TCR transgenic mouse (Pmel-1) that targets the shared melanocyte/melanoma differentiation antigen gp100, an Ag naturally expressed on the surface of the transplantable but genetically unmanipulated melanoma cell line B16. Despite these caveats, our findings suggest multiple parameters that may be optimized to improve the efficacy of future T cell-based adoptive immunotherapy clinical trials.

Recently, Rizzuto and colleagues proposed that a parabolic dose–response relationship exists between the number of adoptively transferred tumor-reactive CD8⁺ T cells and the magnitude of the antitumor response (56). This conclusion was based on experiments in which above a relatively small threshold number (10⁵) of infused naive self/tumor-reactive CD8⁺ T cells, diminished on-target therapeutic and autoimmune effects were observed. Using Ag experienced rather than naive T cells and a similar dynamic range of cell numbers, we found neither a plateau nor a decrease in the dose–response curve. On the contrary, we found a progressive improvement in the strength of tumor regression as the total number of adoptively T cells was increased. These findings held true regardless of whether or not the host received preconditioning with radiation. This apparent discrepancy between the behavior of naive and Ag experienced T cells targeting the same Ag may in part be explained by a greater sensitivity to Ag, differential requirement for costimulation, and faster recall kinetics of memory relative to naive CD8⁺ T cells (38). While these data, when viewed in isolation, would seem to suggest that infusion of the maximum possible number of T cells should be a goal of future T-cell transfer protocols, this approach is not without important potential tradeoffs. Thus far, it has been difficult to uncouple the processes of cell expansion and differentiation status such that the longer cells are expanded ex vivo, the more senescent they become (25, 36). Thus, we also assessed the impact of T-cell differentiation status on treatment outcomes while holding the number infused cells constant. We found a significant linear correlation between the differentiation status of infused T cells and the strength of tumor regression in the order TEM > TCM > TSCM (37). Importantly, when TEM cells
were transferred at near-limiting numbers ($10^4$ cells), we found no statistical difference in antitumor treatment efficacy compared with untreated control mice. Although not specifically evaluated in our current experiments, previous work in mice (25, 26) and humans (57) has shown that less differentiated T cells possess a number of desirable attributes relative to their more highly differentiated counterparts, including a greater proliferative capacity and ability to persist following cell transfer. In humans, the ability to persist has been positively correlated with response to ACT treatments (18, 19, 57). These findings, taken together with our observations about the impact of cell dose on treatment outcomes, show that the quality of transferred T cells, here assessed by surface phenotypes corresponding to different memory T-cell subsets, is as important to the efficacy of T-cell transfer therapy as is the absolute number of cells infused. For these reasons, methods to generate large numbers of relatively less differentiated T cells for adoptive immunotherapy remains a key clinical priority (58–60).

Our present findings again emphasize the potential benefit that providing Ag stimulation or a mimic for Ag stimulation to adoptively transferred T cells may provide to inducing maximal tumor regression (61). While Ag-specific vaccination in combination with ACT or infusion of tumor-reactive T cells coexpressing receptors for a chronic viral infection has been associated with augmented tumor regression in some patients (18, 41), a consistent benefit to adding a mono-specific vaccine with cell transfer therapy beyond cell transfer alone has yet to be observed (8). Several factors may account for this phenomenon, including the use of relatively weak immunogens, such as peptide vaccines with oil-in-water emulsions as adjuvant (8). For this reason, clinical protocols using recombinant viral vaccines encoding tumor-associated Ags in combination with adoptive transfer of TIL or TCR gene-engineered T cells are actively being pursued by our group. Alternatively, the use of high-intensity conditioning regimens prior to ACT (24, 33) or the "programming" of T cells at the time of cell infusion may (39) also provide stimulatory cues that mimic vaccination but in an Ag-independent manner.

IL-2 is often used to support the persistence, expansion, and activation of adoptively transferred T cells in many current clinical studies. However, as IL-2 possesses a number of limiting toxicities requiring close patient monitoring in an in-patient setting, and as this cytokine has the potential to expand Tregs and activate effector cells (46, 47), minimizing the amount of IL-2 administered would be a desirable goal if it did not negatively impact treatment outcomes. We therefore assessed the impact of varying the total number of IL-2 doses administered to support transferred T cells on tumor regression. We found that there was an overall moderate ($r^2 = 0.35$) correlation between the number of IL-2 doses administered and the antitumor response. Surprisingly, however, we found that the benefit of IL-2 administration reached an asymptotic dose–response relationship such that beyond 6 doses, there was no significant improvement in the antitumor response ($r^2 = 0.06$). These findings may offer some reassurance to providers caring for patients on study as the total number of IL-2 doses an individual patient receives is often dictated by physiology rather than a predetermined total number of doses.

Finally, we compared the impact of supporting adoptively transferred cells with different clinically available γc cytokines, including IL-7, IL-15, and IL-21, relative to IL-2. Although each of these cytokines signal in part through Stat5 based on their shared use of the γc receptor, each may in principle also deliver unique signals to responding T cells (53). We found that each γc cytokine was capable of augmenting tumor regression mediated by adoptively transferred T cells in a dose-dependent manner. Overall, no individual cytokine seemed to be capable of vastly improved strength of tumor regression compared with another. However, IL-15, and to a lesser extent IL-2, seemed to more rapidly reach a plateau in their dose–response curve compared with the other cytokines, such as IL-21, which maintained a much more linear relationship. This phenomenon may in part be due to the ability of these cytokines to be presented in trans to neighboring cells (62, 63). Trans presentation allows for cytokines, such as IL-15, to maintain a higher serum half-life and greater in vivo activity than would otherwise occur in the absence of this phenomenon (64). Indeed, a recent study evaluating the impact of daily injections of IL-15 to nonhuman primates found persistently elevated levels of plasma IL-15 and prolonged biologic activity (65).

In summary, we found that multiple parameters, including cell dose and differentiation status, strength of in vivo Ag restimulation, and the duration and type of γc cytokine support may each be adjusted to improve tumor treatment outcomes using adoptively transferred tumor-reactive CD8+ T cells. Although in this study, we systematically and quantitatively evaluated the impact of adjusting individual variables at a time, multiple variables may be adjusted concurrently in future ACT clinical trials in an effort to improve outcomes. For this reason, these findings provide several rational means of improving future T-cell adoptive immunotherapy protocols.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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