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Generation of Tumor-specific T Lymphocytes Using Dendritic Cell/Tumor Fusions and Anti-CD3/CD28

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Summary

Adoptive immunotherapy with tumor-specific T cells represents a promising treatment strategy for patients with malignancy. However, the efficacy of T-cell therapy has been limited by the ability to expand tumor-reactive cells with an activated phenotype that effectively target malignant cells. We have developed an anticancer vaccine in which patient-derived tumor cells are fused with autologous dendritic cells (DCs), such that a wide array of tumor antigens are presented in the context of DC-mediated costimulation. In this study, we demonstrate that DC/tumor fusions induce T cells that react with tumor and are dramatically expanded by subsequent ligation of the CD3/CD28 costimulatory complex. These T cells exhibit a predominantly activated phenotype as manifested by an increase in the percentage of cells expressing CD69 and interferon γ . In addition, the T cells upregulate granzyme B expression and are highly effective in lysing autologous tumor targets. Targeting of tumor-specific antigen was demonstrated by the expansion of T cells with specificity for the MUC1 tetramer. Stimulation with anti-CD3/CD28 followed by DC/tumor fusions or either agent alone failed to result in a similar expansion of tumor-reactive T cells. Consistent with these findings, spectratyping analysis demonstrates selective expansion of T-cell clones as manifested by considerable skewing of the V β repertoire following sequential stimulation with DC/tumor fusions and anti-CD3/CD28. Gene expression analysis was notable for the upregulation of inflammatory pathways. These findings indicate that stimulation with DC/tumor fusions provides a unique platform for subsequent expansion with anti-CD3/CD28 in adoptive T-cell therapy of cancer.

Keywords

dendritic cell tumor fusion vaccine; adoptive immunotherapy; educated T cell

Tumor cells express unique antigens that are potentially recognized by the host T-cell repertoire. However, tumor cells evade host immunity because antigen is presented in the absence of costimulation, and tumor cells express inhibitory cytokines that suppress native antigen-presenting and effector cell populations.^{1,2} A key element in this immunosuppressive milieu is the increased presence of regulatory T cells that are found in the tumor bed, draining

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lymph nodes, and circulation of patients with malignancy.^{3,4} A promising area of investigation is the development of cancer vaccines that reverse tumor-associated anergy and stimulate effector cells to recognize and eliminate malignant cells.

Dendritic cells (DCs) are potent antigen-presenting cells that prominently express costimulatory molecules and are uniquely capable of inducing primary immune responses.^{5,6} We have developed a DC-based cancer vaccine in which tumor cells are fused to autologous DCs. DC/tumor fusion cells present a broad array of tumor antigens in the context of DC-mediated costimulation. In diverse animal models, vaccination with DC/tumor results in the eradication of established disease.^{7,8} In clinical trials, vaccination induces antitumor immunity in a majority of patients; however, clinical responses were seen in only a subset of patients.^{9,10} Minimizing the influence of tumor-mediated immune suppression, including that of regulatory T cells, is likely crucial to augment the efficacy of the fusion cell vaccine.

Cancer vaccine therapy relies on the ability of a vaccine to stimulate tumor-specific T-cell responses in vivo. Although this approach has promise, effector cell dysfunction in patients with malignancy limits vaccine efficacy. In addition, regulatory T cells may prevent response to active immunization in patients with malignancy. This provides a strong rationale for examining the ex vivo use of vaccines to generate functionally active T cells. In adoptive T-cell transfer, one can seek to modulate the number of regulatory T cells, and transfer an antigen-specific population of effector cells.^{11–13} Studies in patients with metastatic melanoma have shown that the transfer of autologous melanoma-reactive tumor-infiltrating lymphocytes (TILs) following lymphodepletion results in sustained clinical responses.^{14,15} These studies have shown that adoptive transfer of tumor-reactive T cells following removal of tumor suppressor cells induces tumor regression in 50% of patients with advanced disease.¹⁶ The use of TILs is limited, however, to a small number of tumors types from which they are obtainable. Therefore, using T cells that have been expanded ex vivo by tumor vaccines for adoptive immunotherapy remains a focus of interest.

Ligation of CD3/CD28 provides a powerful antigen-independent stimulus mediated by the T-cell receptor/costimulatory complex resulting in the activation of signaling pathways including NFκB.^{17–19} This process delivers a strong activation and proliferation signal which induces T-cell expansion and enhances complexity of the T-cell repertoire in patients with HIV and malignancy.^{17,20} T cells expanded ex vivo with anti-CD3/CD28 have been explored as a potential strategy to reverse tumor-associated cellular immune dysfunction. However, exposure to anti-CD3/CD28 alone may expand activated or suppressor cells dependent on the associated cytokine milieu.²¹ We hypothesized that DC/tumor fusions would provide a unique platform for anti-CD3/CD28-mediated expansion by selectively stimulating activated T cells against tumor-associated antigens. As such, sequential stimulation with fusions and anti-CD3/CD28 potentially allows for the generation of significant yields of tumor-reactive T cells while minimizing the presence of regulatory T cells in the expanded population.

Earlier we have demonstrated that DC/tumor fusions stimulate tumor-reactive T cells with the capacity to lyse autologous tumor targets.^{22,23} Here we sought to examine whether the addition of antigen-independent stimulation through ligation of the CD3/CD28 complex further amplifies the antitumor response. Earlier studies have demonstrated that exposure to anti-CD3/CD28 restores the complexity of the T-cell repertoire. We, therefore, hypothesized that primary exposure to anti-CD3/CD28 might enhance the capacity of DC/tumor fusions to expand tumor-reactive clones. In contrast, secondary exposure to anti-CD3/CD28 following fusion-mediated stimulation could result in the more specific expansion of activated, tumor-reactive cells. Therefore, we examined whether the sequence of fusion and anti-CD3/CD28 stimulation impacts the nature of the subsequent T-cell response.

In this study, we have examined the phenotypic and functional characteristics of T cells that have undergone sequential stimulation with DC/tumor fusions and anti-CD3/CD28 in both solid tumor and hematological malignancy models. We demonstrate that fusion-mediated stimulation of tumor-reactive T cells followed by anti-CD3/CD28 expansion provides a uniquely synergistic effect in dramatically expanding antitumor T cells with an activated phenotype.

MATERIALS AND METHODS

Generation of DC, Tumor, and DC/Tumor Fusion Cell Preparations

Peripheral blood mononuclear cells (PBMCs) were isolated from leukopaks obtained from normal donors by Histopaque –1077 density gradient centrifugation. PBMCs were suspended at $1 \times 10^6/\text{mL}$ in RPMI 1640 complete medium and plated in 5mL aliquots in 6-well tissue culture plates and incubated for 2 hours at 37°C in a humidified 5% CO₂ incubator. The monocyte-enriched adherent fraction was cultured in RPMI 1640 complete medium containing granulocyte macrophage colony-stimulating factor (GM-CSF) (1000 U/mL) and interleukin-4 (IL-4) (1000 U/mL) for 5 days to generate immature DCs. The DCs were matured by culturing for an additional 48 hours in the presence of tumor necrosis factor α (25 ng/mL). The RCC cell line, RCC786, was maintained in RPMI 1640 culture media. Myeloid leukemia cells were obtained from bone marrow aspirates or peripheral blood collections and myeloma cells were obtained from bone marrow aspirates as per an institutionally approved protocol. Leukemia cells and myeloma cells were isolated by ficoll density centrifugation and cultured with RPMI 1640 complete medium. DCs and tumor cells underwent phenotypic analysis by flow cytometry and immunohistochemistry as outlined below. To generate fusion cells, tumor cells were mixed with DCs at ratios of 1:1 to 1:3 (dependent on cell yields) and washed 3 times in serum-free RPMI 1640 culture media. After the final wash, the cell pellet was resuspended in 1mL of 50% polyethylene glycol (PEG) solution. After 2 minutes at room temperature, the PEG solution was progressively diluted and cells were washed twice with serum free media. The DC-tumor fusion cells were cultured in RPMI complete media in the presence of GM-CSF. DC/tumor fusions were quantified by determining the percentage of cells that expressed unique DC and tumor antigens by immunohistochemical analysis.

Analysis of DC, Tumor, and Fusion Cell Preparations by Immunohistochemical Analysis

DC, tumor, and fusion cell preparations underwent immunocytochemical analysis to assess the presence of tumor-associated antigens and DC-associated costimulatory and maturation markers. RCC cells underwent staining with primary murine monoclonal antibodies against MUC1 (PharMingen, San Diego, CA), cytokeratin (Boehringer Mannheim, Indianapolis, IN), and CAM (Becton Dickson, San Jose, CA). Myeloid leukemia cells were stained for CD34, CD117, and MUC1. The absence of DC markers was confirmed as outlined below. DC preparations were stained for HLA-DR, CD80, CD83, or CD86 (PharMingen) and an isotype-matched negative control for 60 minutes. The cells were incubated with a biotinylated F(ab')₂ fragment of horse anti-mouse IgG (Vector Laboratories) for 45 minutes, washed twice with phosphate-buffered saline, and incubated for 30 minutes with ABC (avidin-biotin complex) reagent solutions followed by AEC (3 amino-9-ethyl carbazole) solution (Vector Laboratories). In the fusion cell preparations, detection of tumor-associated antigens with the ABC reagents was followed by staining for DC-associated markers with the ABC-AP (alkaline phosphatase) kit (Vector Laboratories). Slides were washed, fixed in 2% paraformaldehyde, and analyzed using an Olympus AX70 microscope. Fusion cells were quantified by determining the percentage of cells that coexpressed unique DC and tumor antigens.

Flow Cytometric Analysis

DC, tumor, and fusion cell preparations were studied by flow cytometric analysis for expression of the antigens outlined above. Cells were incubated with the indicated primary mAb or a matching isotype control for 30 minutes at 4°C. Bound primary murine monoclonal antibodies were detected with a secondary affinity purified fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Chemicom Intl, Temecula, CA) followed by fixation in 2% paraformaldehyde. For bidimensional flow cytometry, cells were incubated with antibody directed against tumor-associated antigens (RCC: MUC1, CAM, or cytokeratin; AML: CD34, CD117, or MUC1), FITC-conjugated secondary antibody and antibody directed against DR or CD86 conjugated with phycoerythrin (PE). Analysis was performed on the fluorescence-activated cell sorting (FACS) Calibur flow cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson).

T-cell Stimulation and Expansion With DC/Tumor Fusions and/or Anti-CD3/CD28

Nonadherent PBMCs were isolated from the leukopak collection used for DC generation and cultured at a density of $1 \times 10^6/\text{mL}$ in RPMI complete media in the presence of 10U/mL IL-2. T cells were isolated by nylon wool separation. T cells were exposed to the immobilized monoclonal antibodies, anti-CD3 (clone-UCHT1; Pharmingen) and anti-CD28 (clone-CD28.2; Pharmingen; CD3i/CD28i). Twenty-four-well nontissue culture-treated plates (Falcon, Fisher) were coated with each of the antibodies (1 $\mu\text{g}/\text{mL}$ in phosphate-buffered saline) and left overnight at 37°C. T cells were (1) cultured on the anti-CD3/CD28-coated plates for 48 hours; (2) cocultured with fusion cells for 5 days at a fusion to T-cell ratio of 1:10; (3) cocultured with fusion cells and followed by anti-CD3/CD28-coated plates for 48 hours; or (4) cultured with anti-CD3/CD28 for 48 hours followed by stimulation with fusions for 5 days. Following stimulation, T cells underwent phenotypic analysis as outlined below.

Proliferation of Stimulated T-cell Populations

Following stimulation, as outlined above, T cells were harvested and proliferation was determined by incorporation of [^3H]-thymidine (1 $\mu\text{Ci}/\text{well}$; 37 kBq; NEN-DuPont, Boston, MA) added to each well 18 hours before the end of the culture period. Thereafter, the cells were harvested onto glass fiber filter paper (Wallac Oy, Turku, Finland) using an automated TOMTEC harvester (Mach II, Hamden CT), dried, placed, and sealed in BetaPlate sample bag (Wallac) with 10mL of ScintiVerse (Fisher Scientific, Fair Lawn, NJ). Cell bound radioactivity was counted in a liquid scintillation counter (Wallac, 1205 Betaplate). Data are expressed as stimulation index (SI) determined by calculating the ratio of [^3H]-thymidine incorporation (mean of triplicates) over background [^3H]-thymidine incorporation (mean of triplicates) of the unstimulated T-cell population.

Phenotypic Characterization of Stimulated T-cell Populations

T cells stimulated by fusion cells, anti-CD3/CD28, or sequential stimulation with fusions and anti-CD3/CD28 underwent phenotypic analysis by multichannel flow cytometry to assess for the presence activated [(CD69, interferon gamma ($\text{IFN}\gamma$)] or regulatory (Foxp3, IL-10) T cells. T-cell preparations were stained for FITC-conjugated CD4, cychrome-conjugated CD25, and PE-conjugated CD69 (PharMingen). Cells were then permeabilized by incubation in Cytofix/Cytoperm plus (containing formaldehyde and saponin) (PharMingen) and stained with PE-conjugated anti-FoxP3 (Caltag, Burlingame, CA). Alternatively, cells were pulsed with GolgiStop (1 $\mu\text{g}/\text{mL}$; PharMingen) for 3 to 4 hours at 37°C and then permeabilized by incubation in Cytofix/Cytoperm plus for 30 minutes at 4°C followed by 2 washes in Perm/Wash solution (Pharmingen). Cells were then incubated with PE-conjugated $\text{IFN}\gamma$, IL-10, or a matched isotype control antibody for 30 minutes, washed twice in $1 \times \text{Perm/Wash}$ solution

and fixed in 2% paraformaldehyde. Labeled cells were analyzed by flow cytometry using FACScan (Becton Dickinson) and CellQuest Program.

Assessment of Tumor-specific Immune Responses by Binding to the MUC1 Tetramer and Cytolytic Capacity by Granzyme B Expression

Antigen-specific MUC1⁺CD8⁺ T cells were identified using PE-labeled HLA-A*0201+iTA_gTM MHC class I human tetramer complexes composed of 4 HLA MHC class I molecules each bound to MUC1-specific epitopes M1.2 (MUC112-20) LLLLTVLTV (Beckman Coulter, Fullerton, CA). A control PE-labeled tetramer was used in parallel. T cells stimulated by anti-CD3/CD28, fusions, or sequential exposure to anti-CD3/CD28 and fusions were incubated with the MUC1 or control tetramer and then stained with FITC-conjugated CD8 antibody. Cells were washed and analyzed by bidimensional FACS analysis. Cytolytic capacity of the stimulated T-cell populations was assessed by staining with FITC-conjugated anti-CD8 and PE-conjugated anti-granzyme B. A total of 3×10⁵ events were collected for final analysis. Similarly, nonadherent unstimulated cells were analyzed in parallel.

CTL Response Following Stimulation With DC/Myeloma Fusions Alone or in Combination With Anti-CD3CD28

DC/myeloma fusion cells were generated and cocultured with autologous T cells at a ratio of 1:10 for 7 to 10 days. DC/myeloma fusions generated with DC autologous to T-cell effectors or autologous tumor cells were used as target cells in a standard 5-hour ⁵¹Cr-release assay. Target cells (2×10⁴ cells/well) were incubated with ⁵¹Chromium (NEN-DuPont) for 1 hour at 37°C followed by repeated washes. ⁵¹Cr release was quantified following 5 hours coculture of effector and target cell populations at a ratio of 30:1 or 10:1. Percentage cytotoxicity was calculated using mean of triplicates by a standard assay as follows: % specific cytotoxicity=[(sample counts – spontaneous counts)/(maximum counts – spontaneous counts)]×100.

Statistical Analysis

Assessment of statistical significance was determined using a paired Wilcoxon signed-rank test.

Spectratype Analysis

Total RNA was isolated from stimulated lymphocytes using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions and cDNA was prepared, as described previously.²⁴ Semi-nested polymerase chain reaction (PCR) was performed using a panel of human Vb sense oligoprimers and 2Cb antisense oligoprimers; the second Cb being fluorescently labeled. The PCR products were run on an ABI 3130 capillary gel system (Perkin Elmer-Applied Biosystems, Foster City, CA) at the Molecular Resource Facility of The University of Medicine and Dentistry of New Jersey. Spectratype analysis was performed using the GeneMapper (version 3.7) program (PE-Applied Biosystems) which allows direct comparison of the Vb T-cell repertoire from each of the T-cell stimulation conditions (T cells stimulated by fusions, T cells stimulated by anti-CD3/CD28, T cells stimulated by anti-CD3/CD28 followed by fusion, and T cells stimulated sequentially by fusion followed by anti-CD3/CD28). In brief, the software program identified each histogram peak by their PCR-size length and determined the area under each peak. The percent area under the corresponding peaks between the control unstimulated cells and the stimulated samples was determined by direct comparison as described previously.²⁴ A peak within the Vb family spectratype histograms of the stimulated samples was considered to be skewed if the area under the peak was ≥2-fold the area of the corresponding peak in the Vb family spectratype histogram of the control sample.²⁴

Differential Gene Expression and Pathway Analysis

Total RNA was extracted from stimulated lymphocytes using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Gene expression profiles were determined using human HG-U133 Gene Chip (Affymetrix). Genes that were differentially expressed (fold change cutoff of 2) between T cells stimulated sequentially by fusions and anti-CD3/CD28, T cells stimulated by anti-CD3/CD28 followed by fusions, T cells stimulated by fusions alone, and T cells stimulated by anti-CD3/CD28 alone, were identified. For further mechanistic insights, we analyzed 279 predefined pathways (Biocarta database, NCI) for differential expression among the 4 aforementioned classes. Pathway differential expression was assessed using the functional scoring method implemented in the BRB-Array Tools software (Version 3.6, Biometrics Research Branch, National Cancer Institute).²⁵ Statistical significance was set at $P < 0.05$.

RESULTS

Phenotypic Characterization of DC/RCC Fusions

DCs were generated from adherent mononuclear cells isolated from leukopak collections obtained from normal donors. DCs were cultured with GM-CSF and IL-4 for 5 days and then underwent maturation by exposure to tumor necrosis factor α for 48 to 72 hours. DCs exhibited a characteristic phenotype with high levels of expression of costimulatory (CD80, CD86) and maturation markers (CD83) (Fig. 1A shows DC expression of CD86). RCC cells expressed MUC1, CAM, and cytokeratin in the absence of DC-associated markers (Fig. 1B shows RCC expression of CAM). Following coculture of tumor and DCs in the presence of PEG, fusion cells demonstrated dual expression of DC-derived costimulatory molecules (CD80, 86) and tumor-derived antigens (Cytokeratin/CAM) (Fig. 1C shows fusion cell coexpression of CD86 and CAM).

Stimulation of T-cell Proliferation

The goal of the study was to determine the optimal approach to generating tumor-reactive lymphocytes while minimizing the presence of nonspecific T cells. As such, a relatively brief period of exposure to anti-CD3/CD28 was chosen to facilitate the selective expansion of vaccine-educated T cells. DC/RCC fusions were cocultured with autologous T cells for 5 days and then exposed to tissue culture plates coated with anti-CD3/CD28 for 48 hours. Alternatively, T cells were first cultured on anti-CD3/CD28-coated plates and then stimulated with DC/RCC fusions for 5 days. As a control, T cells were cultured with anti-CD3/CD28 or DC/RCC fusions alone. T-cell proliferation was determined by incorporation of tritiated thymidine following overnight pulsing (Fig. 2). Exposure to DC/RCC fusions or anti-CD3/CD28 alone did not result in significant T-cell proliferation, with SIs of 0.9 and 1.0, respectively ($N=9$). In contrast, stimulation with DC/RCC fusions followed by exposure to CD3/CD28 resulted in a dramatic and synergistic increase in T-cell proliferation with an SI of 13.2 ($P=0.03$ compared with stimulation with fusions alone). Exposure to anti-CD3/CD28 before fusion cell stimulation did not induce T-cell proliferation (SI: 1.0). Of note, more prolonged stimulation with DC/tumor fusion cells for up to 15 days did not augment T-cell proliferative response (SI: 0.9, $n=2$). In contrast, prolonged exposure to anti-CD3/CD28 alone for 14 days resulted in a 25-fold expansion of T cells ($n=3$).

Stimulation of Activated Compared With Regulatory T Cells

We subsequently examined whether combined stimulation with DC/RCC fusions and anti-CD3/CD28 results in the preferential expansion of T cells exhibiting an activated phenotype. We assessed the characteristics of T cells stimulated by DC/RCC fusions, anti-CD3/CD28 or sequential stimulation with these agents (Figs. 3A–C). In 11 experiments, a modest increase

in CD4⁺/CD25⁺ was observed following stimulation with DC/RCC fusions alone. The mean percentage of CD4⁺ cells that express CD25 increased from 7.5% to 12.6%. Prolonged stimulation with DC/tumor fusion cells for up to 10 days did not increase the expansion of CD4⁺/CD25⁺-expressing T cells (10%, n=2). Similarly, following stimulation of T cells with anti-CD3/CD28 alone, 16% of the CD4⁺ cells demonstrated coexpression of CD25. In contrast, following sequential stimulation with DC/RCC fusions and anti-CD3/CD28, a dramatic expansion of CD4⁺/CD25⁺ T cells was observed, with 64.3% of CD4⁺ T cells coexpressing CD25 ($P=0.001$, 0.02 , and 0.002 compared with unstimulated T cells, T cells stimulated by fusions; and T cells stimulated by anti-CD3/CD28 alone, respectively). The sequence of exposure was critical in that stimulation by anti-CD3/CD28 followed by fusion cells resulted in only 18.4% of CD4⁺ cells expressing CD25⁺ ($P=0.008$ compared with stimulation with fusion followed by anti-CD3/CD28).

To further define the nature of the CD4/CD25⁺ cells, we performed multichannel flow cytometric analysis to determine whether the CD4/CD25 population expressed markers of activation or suppression (Fig. 3C). CD4/CD25⁺ T cells were isolated by FACS gating and expression of CD69 was determined. Stimulation of T cells with DC/RCC fusions or anti-CD3/CD28 alone resulted in a 5-fold and 6-fold increase, respectively, in the percentage of activated T cells, defined as cells expressing CD4/CD25/CD69. Remarkably, a 42-fold increase in the percentage of CD4/CD25/CD69⁺ cells was observed following sequential stimulation with fusion cells and anti-CD3/CD28, demonstrating a statistically significant increase as compared with anti-CD3/CD28 alone ($P=0.01$), fusion cells alone ($P=0.05$) or after anti-CD3/CD28 expansion followed by stimulation with fusions (9-fold increase, $P=0.02$).

Similarly, we examined the effect of combined stimulation of DC/RCC fusions and anti-CD3/CD28 on expansion of regulatory T cells as defined by cells coexpressing of CD4, CD25, and FOXP3 (Fig. 3C). In 9 experiments, the combination of stimulation with the DC/tumor fusion vaccine followed by expansion with anti-CD3/CD28 resulted in a 15-fold expansion of regulatory T cells which was statistically greater than that observed following stimulation with fusions alone (1.9-fold; $P=0.008$), anti-CD3/CD28 alone (1.7-fold; $P=0.004$), or sequential stimulation with anti-CD3/CD28 and fusions (3.4-fold; $P=0.03$). These data suggest that sequential stimulation with DC/RCC fusions synergistically induces T-cell proliferation and expansion of activated T cells far in excess to that observed with DC/RCC or anti-CD3/CD28 alone. In addition, this result was uniquely observed when T cells were first stimulated with the DC/RCC fusions, suggesting that DC-mediated antigen-specific stimulation is necessary before the antigen-independent expansion created by ligation of the anti-CD3/CD28 complex. Of note, sequential stimulation with DC/RCC fusions and anti-CD3/CD28 also resulted in some expansion of regulatory T cells, however, to a far lesser degree than the expansion-activated T-cell populations that was observed.

Functional Characteristics of Stimulated T-cell Populations

To further characterize the functional characteristics of the T-cell populations, we assessed the intracellular expression of TH-1 and TH-2 cytokines by T cells that had been stimulated with fusion cells, anti-CD3/CD28, or their combination. In 8 serial studies, intracellular expression of IFN γ was observed in 0.5% of the unstimulated CD4⁺ T-cell population. Following stimulation with anti-CD3/CD28 or DC/RCC fusions alone, the mean percentage of IFN γ -expressing T cells rose to 1.7% and 1.8%, respectively (Fig. 4A). In contrast, sequential stimulation with DC/RCC fusions and anti-CD3/CD28 resulted in statistically significant increases in mean levels of IFN γ -expressing cells (4.7%, $P=0.05$ compared with stimulation with anti-CD3/CD28 or fusions, respectively) representing a 10.5-fold increase as compared with unstimulated T cells ($P=0.008$) (Fig. 4A). Stimulation of T cells with anti-CD3/CD28 alone resulted in an increase of the percent of CD4⁺ T cells demonstrating intracellular

expression of IL-4 from 1.0% to 2.4%. In contrast, exposure to DC/RCC fusions alone or sequential stimulation with DC/RCC fusions and anti-CD3/CD28 did not result in an increase in IL-4 expression (0.9% and 0.6%, respectively). Mean intracellular expression of IL-10 increased from 0.9 to 3.4% following stimulation with DC/RCC fusions and anti-CD3/CD28. In comparison, no increase in IL-10 expression was observed following stimulation with DC/RCC fusions alone. These data suggest that sequential stimulation with DC/RCC fusions and anti-CD3/CD28 induces the expansion of activated effector cells expressing IFN γ with a relatively more modest increase in T cells expressing IL-10.

Although stimulation of T cells with anti-CD3/CD28 alone for 48 hours did not result in significant T-cell expansion, prolonged stimulation with anti-CD3/CD28 results in the dramatic expansion of T-cell populations. As indicated above, following stimulation with anti-CD3/CD28 alone for 14 days, a 25-fold increase in T cells was observed (n=3). However, in contrast to sequential stimulation with DC/tumor fusion cells and anti-CD3/CD28, prolonged stimulation with anti-CD3/CD28 alone preferentially expands suppressor T-cell populations. 49% of the expanded T-cell population coexpress CD4 and CD25 following stimulation with anti-CD3/CD28 for 14 days. Unlike sequential stimulation with DC/tumor fusion and anti-CD3/CD28 where the expanded T-cell population predominantly expresses markers of activation, following prolonged stimulation with anti-CD3/CD28, 20% of the expanded CD4⁺/CD25⁺ T-cell population coexpress FOXP3, whereas only 4.6% coexpress CD69 (n=3). In concert with these findings, mean intracellular secretion of IL-10 increased to 13% following stimulation with anti-CD3/CD28 alone for 14 days (n=2).

Expansion of Tumor-reactive T Cells

To determine if sequential stimulation with DC/RCC fusion and anti-CD3/CD28 resulted in the selective expansion of tumor-reactive lymphocytes, we examined whether T cells specific for the tumor-associated antigen, MUC1, were increased following expansion (Fig. 4B). DCs and T cells were isolated from HLA -A2.1 donors for this analysis. In contrast to stimulation with fusions or anti-CD3/CD28 alone, sequential stimulation with DC/RCC fusions followed by anti-CD3/CD28 resulted in a dramatic increase in MUC1 tetramer⁺ cells ($P=0.02$ and 0.004 as compared with stimulation with fusions or anti-CD3/CD28 alone, respectively). These data suggest that initial exposure to an antigen-specific stimulus with the DC/RCC was crucial for the subsequent expansion of tumor-reactive T cells using anti-CD3/CD28.

Stimulation With DC/RCC Fusions and Anti-CD3/CD28 Results in the Expansion of T Cells With Cytolytic Capacity

We subsequently examined whether T cells stimulated by DC/RCC fusions and anti-CD3/CD28 demonstrate cytolytic capacity as evidenced by expression of granzyme B. Expression of granzyme B is upregulated in activated cytolytic CD8⁺ T cells that kill target cells via perforin-mediated killing. Following sequential stimulation with DC/RCC fusions and anti-CD3/CD28, the percentage of CD8⁺ cells expressing granzyme B increased from 0.5% to 8%. In contrast, stimulation with anti-CD3/CD28 alone, DC/RCC fusion cells alone, and anti-CD3/CD28 followed by DC/RCC fusions resulted in a minimal increase in granzyme B-positive cells (1%, 0.7% and 1.1%, respectively). In addition, following sequential stimulation with DC/tumor fusion cells and anti-CD3/CD28, IFN γ secretion by CD8⁺ T cells increased from 2% to 11.7% (n=4). These data suggest that sequential stimulation with DC/RCC fusions and anti-CD3/CD28 is uniquely effective in expanding functionally potent cytotoxic T lymphocytes (CTLs).

Stimulation With DC/AML and DC/Myeloma Fusions and Anti-CD3/CD28

To determine whether these results were reproducible in a hematologic malignancy model using patient-derived tumor cells, we examined the phenotypic characteristics of T cells

undergoing sequential stimulation with DC/tumor fusions using patient-derived acute myeloid leukemia samples and anti-CD3/CD28. Myeloid leukemia cells were obtained from peripheral blood or bone marrow in patients with high levels of circulating disease. Tumor cells were fused with DCs generated from normal leukopak collections. DC/AML fusions were quantified as determined by the percentage of cells that coexpressed antigens unique to the DC (CD86) and myeloid leukemia (CD117-ckit ligand, CD34, and/or MUC1). Mean fusion efficiency was 28% of the total cell population. Exposure to fusion cells followed by anti-CD3/CD28 resulted in the synergistic proliferation of T cells that was greater than that seen with fusions alone, anti-CD3/CD28 alone, or stimulation with anti-CD3/CD28 followed by DC/tumor fusion cells. A rise in CD4⁺/CD25⁺ cells was observed following stimulation with DC/AML fusions followed by anti-CD3/CD28 (Fig. 5A). In addition, an increased number of cells expressed IFN γ . In three experiments, stimulation with fusions followed by anti-CD3/CD28 resulted in a 25-fold mean increase in IFN γ secretion, compared with a 5-fold and 2-fold increase following stimulation with anti-CD3/CD28 and fusions alone, respectively. Of note, a rise in the percent of Foxp3⁺ cells was not observed. Sequential stimulation with DC/AML fusions and anti-CD3/CD28 induced higher levels of T-cell expression of granzyme B as compared with stimulation with fusion cells alone or anti-CD3/CD28 (Fig. 5B).

We subsequently examined whether T cells stimulated by fusions and anti-CD3/CD28 demonstrate the capacity to lyse autologous tumor targets. The expanded T-cell population exhibited significant levels of target-specific killing, as demonstrated by the lysis of autologous tumor or semi-autologous fusion targets. Autologous tumor was obtained from bone marrow aspirates of patients with multiple myeloma. Mean CTL lysis for effector:T-cell ratio of 30:1 was 29% and 57% for T cells stimulated with fusions alone and fusions followed by antiCD3/C28, respectively (N=3). Similar to the results observed in the RCC model, these data demonstrate that in a hematologic malignancy model using patient-derived cells, stimulation with DC/tumor fusions followed by exposure anti-CD3/CD28 resulted in significant increase in activated T cells with cytolytic capacity.

CDR3-Size Spectratyping Analysis

CD3R-size spectratype analysis was used to determine whether sequential stimulation with DC/tumor fusions and anti-CD3/CD28 results in the expansion of a clonal T-cell population. RNA was extracted from stimulated T-cell populations for spectratype analysis. In 2 experiments, the results for the resolvable V β families indicate that T cells undergoing sequential stimulation with fusion followed by antiCD3/CD28 demonstrate greater skewing compared with stimulation with either CD3/CD28 alone or fusions alone. An example of a representative V β family from T cells stimulated by CD3/CD28 alone, fusions alone, and sequentially by fusion followed by CD3/CD28 is shown (Fig. 6). Overall, most of the V β spectratype histograms following stimulation with fusions or anti-CD3/CD28 alone exhibit a Gaussian-like distribution of a large heterogeneous pool of CDR3-size lengths that one would expect in a broad polyclonally expanded T-cell repertoire. In contrast, greater V β family skewing is seen when T cells undergo sequential stimulation with DC/tumor fusion cells and anti-CD3/CD28, consistent with the expansion of a defined clonal population.

Differential Gene Expression and Pathway Analysis

Gene expression profiling was performed to determine if the T-cell phenotypic differences observed following sequential stimulation with DC/tumor fusions and anti-CD3/CD28 correlate with unique patterns of gene expression. We demonstrated that gene expression changes were most amplified in this population. Applying a fold-change cutoff of 2, compared with stimulation with DC/tumor fusions alone, 897 genes were identified to be differentially expressed in T cells stimulated sequentially by DC/tumor fusions and anti-CD3/CD28. In contrast, only 124 genes were shown to be differentially expressed in T cells stimulated first

by anti-CD3/CD28 followed by DC/tumor fusions (Fig. 7, left). Similarly, compared with stimulation with anti-CD3/CD28 alone, sequential stimulation by DC/tumor fusions and anti-CD3/CD28 resulted in 945 genes being differentially expressed, whereas the reverse sequence (stimulation by anti-CD3/CD28 followed by DC/tumor fusion) resulted in differential expression of only 53 genes (Fig. 7, right). Stimulation with anti-CD3/CD28 alone for 48 hours resulted in differential expression of 314 genes compared with unstimulated T cells. The data demonstrate that stimulation of T cells with DC/tumor fusions followed by anti-CD3/CD28 results in a unique pattern of gene expression with significant change from that observed following stimulation with fusions, anti-CD3/CD28, or anti-CD3/CD28 expansion followed by fusion stimulation.

Interrogation of the data demonstrated upregulation of pathways associated with regulation of Th1 and Th2 development and differentiation in T cells sequentially stimulated by fusions followed by anti-CD3/CD28 as compared with T cells stimulated by anti-CD3/CD28 alone (Table 1) and DC/tumor fusions alone (Table 2) (functional scoring method $P=0.005$ and 0.008 , respectively). Indicative of these changes, an increase in IFN γ gene expression was observed in T cells undergoing sequential stimulation. In contrast, these pathways were not differentially expressed when T cells were first nonspecifically expanded by anti-CD3/CD28 followed by fusion stimulation. Furthermore, upregulation of genes associated with 4-1BB-dependent immune response were also significantly upregulated in T cells sequentially stimulated by fusions followed by anti-CD3/CD28 compared with T cells stimulated by DC/tumor fusions alone (functional scoring method $P=0.009$) (Table 2). In contrast, these pathways were not differentially expressed when T cells were first nonspecifically expanded by anti-CD3/CD28 followed by fusion stimulation.

DISCUSSION

DC/tumor fusions present a broad array of tumor antigens in the context of DC-mediated costimulation and are highly effective in generating antitumor immunity. Vaccination with fusion cells results in the eradication of metastatic disease in animal models.^{7,8,26} In clinical studies, a majority of patients demonstrate evidence of antitumor immunity, but clinical responses have been observed in only a subset of patients.^{9,10} An important factor-limiting clinical responses to active vaccination strategies is effector cell dysfunction in patients with malignancy. The use of cancer vaccines to stimulate educated T-cell populations *ex vivo* for use as an adoptive immunotherapy may thus augment the clinical efficacy of tumor vaccines.

Ligation of the T-cell costimulatory/receptor complex with antibodies targeting CD3/CD28 delivers a potent antigen-independent signal that stimulates T cells through the activation of the NF- κ B pathway and promotes significantly higher levels of proliferation as compared with stimulation via CD3 alone.^{17–19,27,28} However, the effect of anti-CD3/CD28 stimulation on T-cell phenotype is complex and results in diverse and contradictory effects dependent on the model being examined. Exposure to anti-CD3/CD28 promotes the expansion of activated or suppressor T cells dependent on the nature of the immunologic milieu.²¹ For example, stimulation with anti-CD3/CD28 and IL-15 results in the expansion of regulatory T cells that demonstrate an inhibitory phenotype.²⁹ In a graft versus host disease model, polarization toward a Th1 or Th2 phenotype following anti-CD3/CD28 stimulation is determined by cytokine exposure.²¹ CD4⁺ cells cocultured with anti-CD3/CD28, IL-4, and IL-2 secrete increased levels of IL-4 and IL-10. In contrast, in an animal model, exposure of antigen-specific T cells to anti-CD3/CD28 resulted in the expansion of memory effector cells that expressed IFN γ upon exposure to antigen and were protective against tumor challenge.³⁰

In this study, we examined the phenotypic characteristics of T cells that had undergone stimulation with DC/tumor fusions followed by expansion with anti-CD3/CD28. We

hypothesized that primary stimulation with the fusion cells would elicit a broad antigen-specific response that would be amplified by the subsequent activation of anti-CD3/CD28-mediated signaling. The fusion cell stimulation would provide an immunologic platform that would favor expansion of activated rather than inhibitory T cells following exposure to anti-CD3/CD28. In an effort to selectively expand vaccine-educated lymphocytes and minimize nonselective expansion, we limited the exposure to anti-CD3/CD28 to 48 hours. In a RCC model, we demonstrated that sequential stimulation with DC/RCC fusions followed by anti-CD3/CD28 resulted in the relatively selective expansion of activated T cells as manifested by significantly increased yields of CD4/CD25⁺ cells that express CD69 and IFN γ . Fusion-stimulated T cells that had undergone anti-CD3/CD28 expansion demonstrated a marked increase in MUC1-reactive T-cell clones, suggesting that tumor-reactive clones that were primed during culture with the fusion cells were subsequently being expanded. In addition, as a measure of cytolytic capacity, T cells stimulated by DC/RCC fusions and anti-CD3/CD28 demonstrated increased levels of granzyme B expression, in excess of that observed following stimulation with fusion cells or anti-CD3/CD28 alone. We demonstrated that while prolonged exposure to anti-CD3/CD28 alone expands suppressor T-cell populations, and a 48 hours exposure to anti-CD3/CD28 alone does not result in significant T-cell expansion, the sequential stimulation of T cells with DC/tumor fusions and anti-CD3/CD28 results in the dramatic expansion of activated tumor-reactive T-cell populations.

These findings were confirmed in a hematological malignancy model using fusions generated with patient-derived myeloid leukemia and myeloma cells. Importantly, T cells sequentially stimulated by fusions followed by anti-CD3/CD28 were uniquely effective at killing patient-derived tumor cells in a CTL assay. In spectratyping analysis, T cells undergoing sequential stimulation with fusion cells and anti-CD3/CD28 demonstrate greater skewing of CDR3-size usage in the T-cell receptor as compared with T cells stimulated by fusions or anti-CD3/CD28 alone consistent with the expansion of a defined clonal population. Interestingly, gene expression analysis demonstrated that sequential stimulation with DC/tumor fusions and anti-CD3/CD28 results in the greatest impact on gene expression patterns with a unique effect on pathways-associated expression of Th1/Th2 cytokines and 4-1BB-mediated immune response. Moreover, IFN γ gene expression was greater with fusions followed by anti-CD3/CD28 stimulation compared with anti-CD3/CD28 stimulation alone, consistent with our findings in functional studies.

Earlier studies have demonstrated that the infusion of anti-CD3/CD28 expanded T cells restores the diversity of the immunologic repertoire in patients with HIV, boosting levels of immune competence.³¹ Phase 1 trials have also examined the capacity of anti-CD3/CD28-stimulated T cells to augment antitumor immunity in patients with malignancy. In a phase 1 study, 26 patients with metastatic renal cancer were treated with 2 infusions of T cells activated and expanded by beads with immobilized CD3 and CD28 (Xcyte system) at a mean of 21×10^9 cells.³² Two patients demonstrated evidence of regression of bone metastases. In another study, administration of up to 1×10^8 CD3/CD28-activated donor T cells resulted in complete remissions without excessive rates of graft-versus-host disease in patients with hematological malignancies who had relapsed following allogeneic transplantation.³³ In a recent study, administration of anti-CD3/CD28-expanded T cells enhanced the capacity of patients to respond to a pneumococcal vaccine following autologous transplantation.³⁴ These studies support the hypothesis that primary exposure to anti-CD3/CD28 will broaden the number of tumor-reactive clones in the T-cell repertoire and enhance the capacity of to demonstrate a meaningful response to vaccination. However, in this study, stimulation with anti-CD3/CD28 followed by DC/RCC fusions did not result in the expansion of tumor-reactive T cells. The data suggest that primary anti-CD3/CD28 exposure may inhibit the capacity of T cells to respond to a tumor vaccine. In a recently reported study, investigators demonstrated ex vivo expansion of Her2/neu and CMV-positive T-cell clones using peptides followed by anti-CD3/

CD28 exposure. Similar to our findings, the resultant T-cell population was activated, without the significant presence of regulatory T cells. Expansion of antigen-specific T cells was most pronounced in patients who had previously undergone in vivo vaccination.³⁵

In an earlier report, depletion of regulatory T cells with chemotherapy followed by the infusion of ex vivo expanded TILs resulted in disease regression in 50% of patients with melanoma.¹⁶ The applicability of this model is limited to disease settings in which TILs are available for expansion. Alternatively, generation of tumor-reactive lymphocytes may be more effectively accomplished through ex vivo exposure to the DC/tumor fusion vaccine in the presence of signals that promote T-cell activation and expansion. We have demonstrated that DC/tumor fusion vaccines stimulate a mixed response of activated and regulatory T cells. Sequential stimulation of T cells with DC/tumor fusions and anti-CD3/CD28 did result in a modest increase in cells expressing IL-10 and Foxp3, suggesting that some expansion of inhibitory populations occurred. Strategies to deplete regulatory T-cell populations before ex vivo stimulation, or concomitant in vivo depletion of regulatory T-cell populations may be required to further augment the activity of educated T-cell populations.

In summary, we demonstrate that sequential stimulation with DC/tumor fusions followed by anti-CD3/CD28 results in the dramatic expansion of tumor-reactive lymphocytes with a predominant-activated phenotype. As such, this strategy provides an ideal platform for adoptive immunotherapy. Of note, a more modest expansion of T cells with an inhibitory phenotype was also observed. Approaches to further deplete regulatory T cells in the expanded population might further enhance efficacy.

References

1. Speiser DE, Miranda R, Zakarian A, et al. Self antigens expressed by solid tumors Do not efficiently stimulate naive or activated T cells: implications for immunotherapy. *J Exp Med* 1997;186:645–653. [PubMed: 9271580]
2. Gabrilovich DI, Corak J, Ciernik IF, et al. Decreased antigen presentation by dendritic cells in patients with breast cancer. *Clin Cancer Res* 1997;3:483–490. [PubMed: 9815709]
3. von Boehmer H. Mechanisms of suppression by suppressor T cells. *Nat Immunol* 2005;6:338–344. [PubMed: 15785759]
4. Liyanage UK, Moore TT, Joo HG, et al. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adeno-carcinoma. *J Immunol* 2002;169:2756–2761. [PubMed: 12193750]
5. Avigan D. Dendritic cells: development, function and potential use for cancer immunotherapy. *Blood Rev* 1999;13:51–64. [PubMed: 10225609]
6. O'Neill DW, Adams S, Bhardwaj N. Manipulating dendritic cell biology for the active immunotherapy of cancer. *Blood* 2004;104:2235–2246. [PubMed: 15231572]
7. Gong J, Chen D, Kashiwaba M, et al. Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. *Nat Med* 1997;3:558–561. [PubMed: 9142127]
8. Gong J, Chen D, Kashiwaba M, et al. Reversal of tolerance to human MUC1 antigen in MUC1 transgenic mice immunized with fusions of dendritic and carcinoma cells. *Proc Natl Acad Sci USA* 1998;95:6279–6283. [PubMed: 9600956]
9. Avigan D, Vasir B, Gong J, et al. Fusion cell vaccination of patients with metastatic breast and renal cancer induces immunological and clinical responses. *Clin Cancer Res* 2004;10:4699–4708. [PubMed: 15269142]
10. Avigan D, George DJ, Kanto PW, et al. Interim safety and efficacy results from a phase I/II study of vaccination with electrofused allogeneic dendritic cells/autologous tumor-derived cells in patients with stage IV renal cell carcinoma. *J Clin Oncol ASCO Annu Meet Proc* 2004;22:169.
11. June CH. Adoptive T cell therapy for cancer in the clinic. *J Clin Invest* 2007;117:1466–1476. [PubMed: 17549249]

12. June CH. Principles of adoptive T cell cancer therapy. *J Clin Invest* 2007;117:1204–1212. [PubMed: 17476350]
13. Knutson KL, Wagner W, Disis ML. Adoptive T cell therapy of solid cancers. *Cancer Immunol Immunother* 2006;55:96–103. [PubMed: 15891880]
14. Zhou J, Dudley ME, Rosenberg SA, et al. Persistence of multiple tumor-specific T-cell clones is associated with complete tumor regression in a melanoma patient receiving adoptive cell transfer therapy. *J Immunother* 2005;28:53–62. [PubMed: 15614045]
15. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002;298:850–854. [PubMed: 12242449]
16. Robbins PF, Dudley ME, Wunderlich J, et al. Cutting edge: persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy. *J Immunol* 2004;173:7125–7130. [PubMed: 15585832]
17. Bonyhadi M, Frohlich M, Rasmussen A, et al. In vitro engagement of CD3 and CD28 corrects T cell defects in chronic lymphocytic leukemia. *J Immunol* 2005;174:2366–2375. [PubMed: 15699173]
18. Wang D, Matsumoto R, You Y, et al. CD3/CD28 costimulation- induced NF-kappaB activation is mediated by recruitment of protein kinase C-theta, Bcl10, and IkappaB kinase beta to the immunological synapse through CARMA1. *Mol Cell Biol* 2004;24:164–171. [PubMed: 14673152]
19. Herndon TM, Shan XC, Tsokos GC, et al. ZAP-70 and SLP-76 regulate protein kinase C-theta and NF-kappa B activation in response to engagement of CD3 and CD28. *J Immunol* 2001;166:5654–5664. [PubMed: 11313406]
20. Kalamasz D, Long SA, Taniguchi R, et al. Optimization of human T-cell expansion ex vivo using magnetic beads conjugated with anti-CD3 and Anti-CD28 antibodies. *J Immunother* 2004;27:405–418. [PubMed: 15314550]
21. Jung U, Foley JE, Erdmann AA, et al. CD3/CD28-costimulated T1 and T2 subsets: differential in vivo allosensitization generates distinct GVT and GVHD effects. *Blood* 2003;102:3439–3446. [PubMed: 12855580]
22. Gong J, Avigan D, Chen D, et al. Activation of antitumor cytotoxic T lymphocytes by fusions of human dendritic cells and breast carcinoma cells. *Proc Natl Acad Sci USA* 2000;97:2715–2718. [PubMed: 10688917]
23. Gong J, Nikrui N, Chen D, et al. Fusions of human ovarian carcinoma cells with autologous or allogeneic dendritic cells induce antitumor immunity. *J Immunol* 2000;165:1705–1711. [PubMed: 10903782]
24. Friedman TM, Goldgirsh K, Berger SA, et al. Overlap between in vitro donor antihost and in vivo posttransplantation TCR Vbeta use: a new paradigm for designer allogeneic blood and marrow transplantation. *Blood* 2008;112:3517–3525. [PubMed: 18541718]
25. Pavlidis P, Qin J, Arango V, et al. Using the gene ontology for microarray data mining: a comparison of methods and application to age effects in human prefrontal cortex. *Neurochem Res* 2004;29:1213–1222. [PubMed: 15176478]
26. Lespagnard L, Mettens P, Verheyden AM, et al. Dendritic cells fused with mastocytoma cells elicit therapeutic antitumor immunity. *Int J Cancer* 1998;76:250–258. [PubMed: 9537588]
27. Khoshnan A, Bae D, Tindell CA, et al. The physical association of protein kinase C theta with a lipid raft-associated inhibitor of kappa B factor kinase (IKK) complex plays a role in the activation of the NF-kappa B cascade by TCR and CD28. *J Immunol* 2000;165:6933–6940. [PubMed: 11120819]
28. Yamada-Ohnishi Y, Azuma H, Urushibara N, et al. Cytotoxic difference of T cells expanded with anti-CD3 monoclonal antibody in the presence and absence of anti-CD 28 monoclonal antibody. *Stem Cells Dev* 2004;13:315–322. [PubMed: 15186727]
29. Lin SJ, Cheng PJ, Hsiao SS. Effect of interleukin-15 on effector and regulatory function of anti-CD3/anti-CD28-stimulated CD4(+) T cells. *Bone Marrow Transplant* 2006;37:881–887. [PubMed: 16565741]
30. Hughes DP, Baskar D, Urban FF, et al. Fate and function of anti-CD3/CD28-activated T cells following adoptive transfer: IL-2 promotes development of anti-tumor memory T cells in vivo. *Cytotherapy* 2005;7:396–407. [PubMed: 16236629]

31. Bernstein WB, Cox JH, Aronson NE, et al. Immune reconstitution following autologous transfers of CD3/CD28 stimulated CD4(+) T cells to HIV-infected persons. *Clin Immunol* 2004;111:262–274. [PubMed: 15183147]
32. Thompson JA, Figlin RA, Sifri-Steele C, et al. A phase I trial of CD3/CD28-activated T cells (Xcellerated T cells) and interleukin-2 in patients with metastatic renal cell carcinoma. *Clin Cancer Res* 2003;9:3562–3570.1. [PubMed: 14506142]
33. Porter DL, Levine BL, Bunin N, et al. A phase 1 trial of donor lymphocyte infusions expanded and activated ex vivo via CD3/CD28 costimulation. *Blood* 2006;107:1325–1331. [PubMed: 16269610]
34. Rapoport AP, Stadtmauer EA, Aqui N, et al. Restoration of immunity in lymphopenic individuals with cancer by vaccination and adoptive T-cell transfer. *Nat Med* 2005;11:1230–1237. [PubMed: 16227990]
35. Dang Y, Knutson KL, Goodell V, et al. Tumor antigen-specific T-cell expansion is greatly facilitated by in vivo priming. *Clin Cancer Res* 2007;13:1883–1891. [PubMed: 17363545]

FIGURE 1.

Immunohistochemical staining of DC, tumor, and fusion cells. DCs were generated from adherent mononuclear cells isolated from leukopak collections obtained from normal donors. A, DCs were cultured with GM-CSF and IL-4 for 5 days and then underwent maturation by exposure to TNF α for 48 to 72 hours. DC preparations underwent immunohistochemical analysis for expression of costimulatory molecules. DC expression of CD86 (blue) is shown ($\times 60$). B, RCC 786 cells were cultured in RPMI 1640 complete medium and underwent immunohistochemical analysis for expression of the tumor-associated antigens cytokeratin and CAM. Tumor expression of CAM (red) is shown ($\times 60$). C, Fusion cells were generated by coculture of DCs and RCC 786 cells in the presence of polyethylene glycol. Fusion cell preparations underwent immunohistochemical analysis for coexpression of the DC-derived costimulatory molecule CD86 (blue) and tumor-associated antigen CAM (red). DC indicates dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; TNF, tumor necrosis factor.

FIGURE 2.

T-cell proliferation T cells were (1) cocultured with fusion cells for 7 days at a fusion to T-cell ratio of 1:10; (2) cultured on the anti-CD3/CD28-coated plates for 48 hours; (3) cocultured with fusion cells for 5 days followed by anti-CD3/CD28-coated plates for 48 hours; or (4) cultured with anti-CD3/CD28 for 48 hours followed by stimulation with fusion cells for 5 days. Following stimulation, T-cell proliferation was measured by uptake of tritiated thymidine following an overnight pulse. Results are expressed as a stimulation index (T-cell proliferation following coculture/proliferation of unstimulated T cells). Mean values of 9 experiments, with associated standard error of the means are presented.

FIGURE 3.

Stimulation of activated as compared with regulatory T-cell populations. A, T cells stimulated by fusion cells, anti-CD3/CD28, or sequential stimulation with fusions and anti-CD3/CD28 underwent phenotypic analysis by multichannel flow cytometry to assess for the percentage of CD4⁺ cells that coexpress CD25. T-cell preparations were stained for FITC-conjugated CD4 and cychrome-conjugated CD25. A representative example is shown. Upper panel demonstrates the percentage of T cells expressing CD4. Lower panel demonstrates the percentage of cells in the CD4⁺ gate that coexpress CD25. B, Stimulated T-cell populations were stained with FITC-conjugated CD4 and cychrome-conjugated CD25 to determine the percentage of CD4⁺ cells expressing CD25. Mean values of 11 experiments with associated standard error of the means is shown. C, Stimulated T-cell preparations were stained for FITC-conjugated CD4, cychrome-conjugated CD25, and PE-conjugated CD69. Alternatively, cells were stained for CD4/CD25, permeabilized, and incubated with PE-conjugated Foxp3 or a matched isotype control antibody. CD4/CD25⁺ T cells were isolated by FACS gating and expression of CD69 and Foxp3 was determined. Results are presented as the fold increase in activated or regulatory T cells compared with unstimulated T-cell populations. Mean values of 9 experiments are presented. FACS indicates fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

FIGURE 4.

A, IFN γ production by stimulated T-cell populations. A, Stimulated T-cell preparations were stained for FITC-conjugated CD4. Cells were then washed, permeabilized, and incubated with PE-conjugated anti-human IFN γ or a matched isotype control antibody. Intracellular expression of IFN γ was determined by flow cytometric analysis. Mean values of 8 experiments are presented, with associated standard error of the means. B, Expansion of MUC1 tetramer-positive T cells. HLA*0201⁺ autologous nonadherent cells were cocultured with fusion cells, anti-CD3/CD28, fusions followed by anti-CD3/CD28 followed by fusion cells, and anti-CD3/CD28 followed by fusions cells. The cells were harvested and analyzed for MUC1⁺CD8⁺ T cells using the MUC1-specific PE-conjugated tetramers or a control tetramer and using the

appropriate CD8⁺ T-cell gating. The percent CD8⁺ cells binding the MUC1 tetramer (after subtraction of nonspecific binding to a control tetramer) is presented. A representative example is shown. FITC indicates fluorescein isothiocyanate; IFN γ , interferon gamma; PE, phycoerythrin.

FIGURE 5.

Phenotype and function of T cell sequentially stimulated by DC/tumor fusions and anti-CD3/CD28 in a hematologic malignancy model. A, T cells stimulated by fusion cells, anti-CD3/CD28, or sequential stimulation with fusions and anti-CD3/CD28 underwent phenotypic analysis by multichannel flow cytometry to assess for coexpression of CD4 and CD25. Stimulated T-cell populations were stained with FITC-conjugated CD4 and cychrome-conjugated CD25 to determine the percentage of dually expressing cells. A representative example is shown. B, T cells were cocultured with DC/AML fusion cells, anti-CD3/CD28, DC/AML fusion cells followed by anti-CD3/CD28, and anti-CD3/CD28 followed by DC/AML fusion cells. Cells were stained with FITC-conjugated CD8 antibodies, fixed and permeabilized, incubated with PE-conjugated granzyme B antibody or matching isotype control and analyzed by flow cytometry. A representative example is shown. DC indicates dendritic cell; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

FIGURE 6.

CDR3-size spectratype analysis. This analysis was used to determine whether sequential stimulation with DC/tumor fusion cells and anti-CD3/CD28 results in the expansion of a clonal T-cell population. An example of a representative V β family is shown. DC indicates dendritic cell.

FIGURE 7.

Differential gene expression analysis. Scatterplots demonstrate differentially expressed genes between (A) T cells stimulated by DC/tumor fusion cells followed by anti-CD3/CD28 compared with T cells stimulated by DC/tumor fusion cells alone. B, T cells stimulated by anti-CD3/CD28 followed by DC/tumor fusion cells compared with T cells stimulated by DC/tumor fusion cells alone. C, T cells stimulated by DC/tumor fusion cells followed by anti-CD3/CD28 compared with T cells stimulated by anti-CD3/CD28. D, T cells stimulated by anti-CD3/CD28 followed by DC/tumor fusion cells compared with T cells stimulated by anti-CD3/CD28 alone. Reference lines denote 2-fold expression differences between different classes.

TABLE 1

Pathway Analysis: Genes Upregulated in T Cells Stimulated by Fusions followed by Anti-CD3/CD28 Compared With T Cells Stimulated by Anti-CD3/CD28 Alone

Pathway	Pathway (<i>P</i>) [*]	Upregulated Genes
Dendritic cells in regulating Th1 and Th2 development	0.005	Interferon γ Colony stimulating factor 2 CD7 molecule Tumor necrosis factor receptor superfamily 5 (CD40)
Th1/Th2 differentiation	0.008	Interleukin-2 receptor α Interleukin-4 receptor Major histocompatibility complex, class II, DR β 1 Interleukin-12 receptor, β 2

* Functional scoring test.

TABLE 2

Pathway Analysis: Genes Upregulated in T Cells Stimulated by Fusions Followed by Anti-CD3/CD28 Compared With T Cells Stimulated by Fusions Alone

Pathway	Pathway (<i>P</i>) [*]	Upregulated Genes
The 4-1BB-dependent immune response	0.009 [*]	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α Tumor necrosis factor receptor superfamily, member 9 TNF receptor-associated factor 2 Nuclear factor of κ light polypeptide gene enhancer in B-cells 1

^{*} Functional scoring test.