Complete Regression of Experimental Solid Tumors by Combination LEC/chTNT-3 Immunotherapy and CD25+ T-Cell Depletion

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ABSTRACT

LEC/chTNT-3, a chemokine fusion protein generated previously in our laboratory, produces a 40–60% reduction in well-established solid tumors of the BALB/c mouse. In this study, CD25+ T-cell depletion was used in combination with LEC/chTNT-3 treatment to enhance the therapeutic value of this approach. In two tumor models (Colon 26 and RENCA), this combination immunotherapy produced complete regression of established s.c. tumors after 5 consecutive days of i.v. treatment. To show that targeted LEC is critical to these results, similar combination studies were performed with chTNT-3/chemokine fusion proteins consisting of human interleukin 2, murine IFN-γ, and murine granulocyte macrophage colony-stimulating factor using identical treatment regimens. These studies showed no significant improvement indicating that combination therapy with anti-CD25+ antisera requires LEC localization to tumor to produce complete regression. To study the mechanism of this remarkable response, immunotherapeutic studies were repeated in knockout mice and showed that successful treatment with CD25+ depletion was dependent on the presence of IFN-γ but not perforin. Other studies using real-time PCR, ex vivo proliferation, and intracellular cytokine staining with lymphocytes from tumor draining lymph nodes, suggested that this combination treatment was associated with increased T-helper 1 cytokine expression, enhanced T-cell activation, and increased IFN-γ production by T cells. Rechallenge experiments showed that combination LEC/chTNT-3 treatment and CD25+ depletion produced long-lasting memory cells capable of preventing re-engraftment of the same but not different tumor cell lines. These studies suggest that LEC/monoclonal antibody fusion proteins, when used in combination with CD25+ T-cell depletion, is a viable method of immunotherapy for the treatment of solid tumors.

INTRODUCTION

T-reg.1 or suppressor T cells have been described for >20 years (1–3), but only recently have their roles in autoimmune disease and cancer been recognized. CD4+CD25+ cells constitutively express CD25 (IL-2 receptor α chain) on their surface and constitute 5–10% of CD4+ T cells in humans and rodents. Although a number of studies on CD4+CD25+ T-cell subsets have appeared in the literature, most have focused on their role in autoimmune diseases. Recent studies indicate that there may be two mechanisms regarding the function of T-reg., one that is cell contact-dependent, and the other that is cytokine (IL-4 and IL-10) -dependent (4). Most recently, it was found that the cytotoxic T lymphocyte-associated antigen 4, which is constitutively expressed on CD4+CD25+ T cells, also plays a key role in T cell-mediated dominant immunological self-tolerance (5). Another suppressive cell population, CD8−suppressive T cells, has been shown to be related to oral tolerance (6, 7), but these cells have only been shown to suppress local intestinal immune function (8). Finally, CD8+CD25+ T cells have been identified recently in human thymus (9). Although these cells appear to share a similar phenotype and regulatory functions as CD4+CD25+ T cells, their role in peripheral tissues remains to be demonstrated.

It is clear that T-reg. is essential in immune system homeostasis (10–12), and the manipulation of its function should have potential therapeutic effects in the clinic (13). Specifically, the enhancement of T-reg. may be beneficial for autoimmune diseases, and the removal of T-reg. should result in increased immune responses that can facilitate the induction or augmentation of tumor immunity (14–16). Indeed, recent studies have demonstrated that in vivo injection of anti-CD25 antibody caused the regression of leukemia and solid tumors in animal models (14, 17). In most of these studies, CD4+ or CD4+CD25+ T-cell depletion was tumor suppressive, but T-reg. depletion alone resulted in either incomplete tumor reduction or a delay in the growth of well-established tumor implants. Combination therapy seems to be more effective as exemplified by studies performed by Shimizu et al. (14), which revealed a synergistic effect of cytotoxic T lymphocyte-associated antigen 4 blockage and the suppression of CD4+CD25+ T cells in tumor therapy.

In the current investigation, a new combination treatment is proposed, which uses the depletion of CD4+CD25+ T-reg. and the use of a chemokine/mAb fusion protein to attract and activate the immune response at the tumor site. It has been shown previously that there is a strong correlation between the infiltration of lymphocytes into tumor sites and increased survival, suggesting that cytotoxic T-cells contribute to tumor remission (18). Methods to increase the penetration of lymphocytes into the tumor microenvironment would be very beneficial for active immunotherapy. To accomplish this, chemokine/antibody fusion proteins are logical candidates due to the chemotactrant properties of their chemokine moiety on different populations of lymphocytes and dendritic cells by receptor-ligand interactions (19–21).

LEC/chTNT-3 is a fusion protein generated in our laboratory (22), which was genetically engineered to link the liver expression chemokine (LEC, also named HCC-4, NCC-4, and CCL-16; Ref. 23) to mAb chTNT-3, which targets the necrotic regions of tumors (24). We demonstrated previously that LEC/chTNT-3 attracts different subpopulations of lymphocytes including CD4+ and CD8+ T cells, neutrophils, dendritic cells, and B cells (22). Although dramatic lymphocyte migration has been observed in the tumors of treated mice, LEC/chTNT-3 treatment alone resulted in a modest 40–50% reduction of tumor growth. We hypothesize that the infiltrated lymphocytes might be suppressed by normal regulatory pathways. To test this hypothesis, we combined the use of LEC/chTNT-3 with methods to deplete T-reg. in an effort to optimize the immunotherapy of experimental solid tumors of the mouse.

MATERIALS AND METHODS

Antibodies and Cell Lines

Hybridomas, including rat antimouse L3T4 (anti-CD4) mAb GK1.5, anti-lyt-2.3 (anti-CD8) mAb 2.43, and anti-IL-2 (anti-CD25) receptor mAb 7D4...
and PC61 were purchased from American Type Culture Collection (Manassas, VA). To obtain sufficient quantities of reagents, hybridoma cells were grown in Integra CL 1000 culture chambers (IBS Integra Biosciences, Wallisellen, Switzerland) and purified by ammonium sulfate precipitation following by Q-Sepharose ion-exchange chromatography (Bio-Rad Laboratories, Hercules, CA). Anti-asialo GM1 (anti-NK) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The Colon 26 murine colon carcinoma and the RENCA murine renal carcinoma were obtained from the American Type Culture Collection. The MAD109, a murine lung carcinoma, was purchased from the National Cancer Institute (Frederick, MD).

**Animals**

Six-week-old female BALB/c mice were obtained from Harlan Sprague Dawley (Indianapolis, IN). Perforin and IFN-γ knockout mice with H-2d backgrounds were generously provide by Dr. Stephen Stohlman (Department of Neurology, University of Southern California Keck School of Medicine, Los Angeles, CA; Ref. 25).

**Depletion of Lymphocytes Subsets in Vivo**

Antibodies were administered 1 day before tumor implantation for CD25⁺ T-cell depletion studies or 6 days after tumor implantation for the depletion of the other T-cell subsets. For CD4⁺, CD8⁺, and CD25⁺ T-cell depletion, 0.5 mg of anti-CD4 antibody (GK1.5), anti-CD8 antibody (2.43), or anti-CD25 (PC61) were injected i.p. in a 1 ml inoculum, and repeated every 5 days. For NK cell depletion, 0.35 mg of anti-asialo GM1 was injected i.p. every 7 days. Depletion of specific T-cell subsets was confirmed by flow cytometric analysis of splenocytes using normal mice (data not shown). Each of these studies was repeated two or three times, and each group contained 5 mice each.

**Immunotherapy Studies**

Groups (n = 5) of 6-week-old female BALB/c mice were injected s.c. in the left flank with a 0.2 ml inoculum containing ~10⁵ Colon 26, RENCA, or MAD109 cells under a University Animal Care Committee-approved protocol. Treatments were started when tumors reached ~0.5 cm in diameter. Groups of tumor-bearing mice (with or without lymphocyte subset depletion) were treated i.v. with a 0.1 ml inoculum containing LEC/chTNT-3 (20 µg) or control chTNT-3 (20 µg). In addition, LEC/chTNT-3 was replaced with three other fusion proteins developed in our laboratory, including chTNT-3/IL-2 (26), chTNT-3/IFN-γ (27), muTNF-3/mu-granulocyte macrophage stimulating factor using the same dosage level. All of the groups were treated daily twice, 5 days, and tumor growth was monitored every other day by caliper measurement in three dimensions. Tumor volumes were calculated by the formula: length × width × height. The results were expressed as the mean ± SD, and the significance levels (P values) were determined using the Wilcoxon rank-sum test. Each of these studies was repeated twice.

**Rechallenge Experiments**

One month (n = 7) and 6 months (n = 10) after the completion of treatment, tumor-free mice from previous studies and control naïve mice (n = 5) were challenged with 10⁷ cells of Colon 26 and MAD109 or Colon 26 and RENCA in the left and right flanks, respectively. The injection sites were observed for 3–4 weeks. To study the presence of CD4⁺ CD25⁺ T cells in different groups, TDLNs were removed, and T-reg. were stained with PE-anti-CD25 and FITC-anti-CD4 for FACS analysis.

**Mechanistic Studies**

**Infiltration of Lymphocytes by Flow Cytometric Analysis**

Tumors from control and treated mice were aseptically removed on days 9, 15, and 20 after tumor implantation and manually cut into 2–3-mm pieces in a culture Petri dish. The small tissue fragments were then digested with 0.01% DNase, 0.01% hyaluronidase, and 0.1% collagenase (all from Sigma Chemical Co.) in RPMI 1640 for 2–3 h at 37°C with continuous stirring. The resulting single cell suspensions were then washed twice with 0.1% FCS in PBS and stained by standard flow cytometry methods. To detect subpopulations of lymphocytes infiltrating these tissues, the following conjugated antibodies were used for FACS: PE-anti-CD4, FITC-anti-CD8, PE-anti-PMN, FITC-anti-CD25, APC-anti-CD11b, FITC-anti-CD11c, and FITC-Pan NK (BD Biosciences Pharmingen, San Diego, CA). Experiments were repeated three times, and the most representative data are shown below.

**Intracellular IFN-γ Production.** TDLNs from control and treated mice were removed from mice on days 15 and 20 after tumor implantation. Single cell suspensions were obtained as described above, and 2 × 10⁶ viable cells/well were plated into a 24-well plate. Intracellular IFN-γ production was performed by first stimulating the cells for 4 h in complete RPMI 1640 containing 10 ng/ml PMA (Sigma, Aldrich) and 1000 ng/ml of ionomycin (Sigma) in the presence of GolgiStop (BD Pharmingen). Cells were then washed, and mouse Fc receptors were blocked with 1 µg Fe Blocking antibody (CD16/CD32) per 10⁷ cells in 100 µl of Staining Buffer (1% fetal bovine serum in PBS) for 15 min at 4°C. Cells were then stained with a PE-conjugated anti-CD3e antibody for 30 min at 4°C, fixed/permeabilized with 100 µl Cytofix/Cytoperm (BD Pharmingen) for 15 min at 4°C, and washed with 300 µl of Perm/Wash (BD Pharmingen). The fixed cells were then resuspended in 50 µl Perm/Wash containing of FITC-conjugated anti-IFN-γ antibody (BD Pharmingen) for 30 min at 4°C in the dark. Cells were washed and resuspended in FACS buffer, and the intracellular production of IFN-γ was analyzed by FACS.

**Knockout Mouse Immunotherapy Studies.** For the perforin knockout mouse studies, 10⁶ Colon 26 cells were implanted on day 1 in the left flank, and mice were depleted of CD4⁺ T cells on day 6 with 0.5 mg/mouse of GK1.5, which was repeated every 5 days. Treatment began on day 7 when tumors reached 0.5 cm in diameter. Mice were divided into three groups and treated with: (a) PBS; (b) chTNT-3 (20 µg/mouse); or (c) LEC/chTNT-3 (20 µg/mouse) i.v. with a 0.1-ml inoculum. All of the groups were treated daily for 5 days, and tumor volumes were monitored every other day. For the IFN-γ knockout mouse studies, Colon 26 tumor-bearing mice were divided into four groups and treated for 5 consecutive days i.v. with: (a) control chTNT-3 (20 µg/mouse); (b) LEC/chTNT-3 (20 µg/mouse); (c) CD25⁺ depletion and control chTNT-3 (20 µg/mouse); or (d) CD25⁺ depletion and LEC/chTNT-3 (20 µg/mouse). Tumor volumes were monitored by caliper as described above.

**T-Cell Proliferation Assay.** The proliferation of T cells was measured by a modified flow cytometry method. Briefly, TDLNs from control and treated mice were removed from tumor-bearing mice on days 15 and 20 after tumor implantation. Single cell suspensions obtained by mincing the lymph nodes in a Petri dish were labeled with CFSE (Molecular Probes, Eugene, OR) with the following modification (28). Briefly, cells were washed with PBS twice, resuspended in PBS containing 1–5 µM of CFSE, incubated at 37°C for 5–10 min, and the reaction stopped by addition of 1 ml of pretwarmed 10% FBS in PBS. 100 000 cells were plated into a 24-well plate. Intracellular IFN-γ was detected by FACS on day 3, 7, and 10 after treatment. The results were expressed as the mean ± SD, and the significance levels (P values) were determined using the Wilcoxon rank-sum test. Each of these studies was repeated twice.

**Detection of Cytokines by Real-Time PCR.** Tumors were removed at days 9, 12, and 15 after tumor implantation for real-time PCR analysis of infiltrating lymphocytes. For these studies, single cell suspensions of tumors were incubated in tissue culture flasks containing RPMI 1640 and 10% FCS for 3 h at 37°C to separate nonattached lymphocytes from tumor cells. Primers for selected cytokine were designed by software provided by the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Briefly, total lymphocyte RNA was extracted by Trizol (Life Technologies, Inc., Rockville, MD) following by treated with DNase I (Ambion, Inc, Austin, TX) to eliminate possible genomic DNA contamination, and 1 µg of total RNA was reverse transcribed into cDNA using a SuperScript cDNA synthesis kit (Life Technologies). The remaining DNA was removed by a DNA-free kit (Ambion, Inc.) according to the manufacturer’s protocol. The real-time PCR reaction mixture (20 µl reaction) consisted of 5 µl of cDNA, 10 µl of SYBR Green Master Mix (Applied Biosystems), 2 µl of primers (3.3 µM), and 1 µl
of water. The PCR reaction was performed for 30 cycles, and the quantity of cytokines (IL-2, IL-10, IL-4, IFN-γ, transforming growth factor β1, and TNF-α) was detected by the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

RESULTS

Combination LEC/chTNT-3 Immunotherapy and T-Cell Subset Depletion in Colon 26 Tumor-Bearing Mice

NK cells, and CD4+ and CD8+ T cells have proved necessary for tumor immunotherapy. Our previous data have shown that LEC/chTNT-3 treatment can dramatically increase the infiltration of different subpopulations of lymphocytes into tumor sites, including CD4+ and CD8+ T cells, PMN cells, dendritic cells, and B cells (22). To determine the relative roles of NK, CD4+, and CD8+ T-cell subsets in LEC/chTNT-3 immunotherapy, each of these populations was first depleted starting 1 day before the initiation of LEC/chTNT-3 immunotherapy. As shown in Fig. 1A, the depletion of NK and CD8+ T cells destroyed the antitumor effects of LEC/chTNT-3 providing supporting data for the immunotherapeutic role of these subsets in LEC-induced immunotherapy. In contrast with these results, CD4+ T-cell depletion when combined with LEC/chTNT-3 treatment caused complete tumor remission by 17–19 days (Fig. 1B), and these mice remained tumor-free 6 months later. CD4+ T-cell depletion combined with control antibody chTNT-3 produced an ~50% reduction in tumor growth compared with mice treated with chTNT-3 alone, demonstrating the importance of this T-cell subset in the suppression of an effective immune response to tumor.

Combination chTNT-3/Cytokine Fusion Protein Immunotherapy and CD4+ Depletion in Colon 26 Tumor-Bearing Mice

As shown in Table 1, substitution of LEC/chTNT-3 with three different chTNT-3/cytokine fusion proteins when used in combination with CD4+ depletion did not produce complete remissions in the Colon 26 tumor model. The only fusion protein that showed some enhancement with CD4+ depletion was chTNT-3/IL-2, which doubled its effectiveness in these studies.

Combination LEC/chTNT-3 Immunotherapy and CD25+ T-Cell Depletion in Colon 26-Bearing Mice

To determine whether CD25+ T cells were responsible for the above effects seen with CD4+ depletion, CD25+ T cells were depleted by PC61 (a rat antimouse CD25 antibody). As above, depletion was performed 1 day before tumor implantation and repeated every 5

Fig. 1. Combination LEC/chTNT-3 immunotherapy and CD4+, CD8+, and NK cell depletion on the growth of Colon 26 tumors. Six-week-old female BALB/c mice were implanted with 10⁷ Colon 26 s.c. and divided into eight groups (n = 4). When the tumors reached 0.5 cm in diameter, groups of mice were depleted of CD4+ T cells by anti-CD4 (GK1.5), CD8+ T cells by anti-CD8 (2.43), or NK cells by antiasialo GM1. One day later, each group was treated i.v. with either chTNT-3 (20 μg/mouse) or LEC/chTNT-3 (20 μg/mouse) with or without T-cell subset or NK cell depletion. A, growth curves of tumors. B, gross appearance of tumors on day 23.
days. FACS analysis of spleens removed from these mice demonstrated essentially complete depletion of this small subpopulation of CD4+ cells (data not shown). The depletion of CD25+ T-cells treated with control antibody dramatically decreased the tumor growth by 70% and 60% in Colon 26 (Fig. 2A) and RENCA (Fig. 2B) models, respectively. These results were similar to that seen with LEC/chTNT-3 treatment alone. However, the combination of LEC/chTNT-3 immunotherapy and CD25+ depletion caused complete remission of these well-established tumors for up to 6 months.

Tumor Rechallenge Experiments

Tumor-free mice from previous treatments (LEC/chTNT-3 and CD25+ depletion) and naïve mice were implanted with Colon 26 and MAD109 on contralateral flanks. Two weeks after implantation, all of the naïve mice were found to have solid tumors growing on their left (Colon 26) and right (MAD109) flanks (Fig. 3A), whereas the tumor-free mice 60 days after previous tumor implantation only had tumors growing on the right flank (MAD109; Fig. 3B). Caliper measurements of these tumors showed that their size was reduced by 50% compared with those growing in naïve mice. Additional studies with tumor-free mice rechallenged after 6 months, however, showed similar implantation of Colon 26 as naïve mice. To determine whether the presence of CD4+CD25+ T-cells in TDLNs correlated with the ability of tumor to take in these mice, the percentage of T-reg. was measured by FACS in each of these groups of mice. The data showed that CD4+CD25+ T cells in naïve mice constituted 9.7% of total T cells (Fig. 3C), whereas in 2–3-month tumor-regressed mice, they constituted only 5% of total T cells (Fig. 3D). By contrast, T-reg. constituted 13% of total T cells in 5–6-month tumor-regressed mice (Fig. 3E), which may explain the failure of these mice to reject tumor.

Mechanistic Studies

Infiltration of Lymphocytes Subpopulation into Tumor Sites Studied by FACS. To determine the role of lymphocyte and dendritic cell infiltration in the tumor microenvironment, single cell suspensions of tumors removed on day 15 after tumor implantation were stained with antibody markers for FACS analysis. As shown in Fig. 4A, the percentage of infiltrating CD8+ T cells in the LEC/chTNT-3-treated mice (with or without CD25+ depletion) showed a greater number of infiltrating CD8+ T cells (1.5% and 2.5%, respectively) than their control groups (0.6% and 1.04%, respectively). CD25+ depletion itself, however, did not increase the actual number of infiltrating CD8+ T cells. A similar phenomenon was also observed for infiltrating dendritic cells that were identified by dual staining with anti-CD11c and anti-CD11b antibodies. As shown in Fig. 4B, a greater CD11b+ and CD11c+ double-positive dendritic cell infiltration was found in the LEC/chTNT-3-treated groups (with and without CD25+ depletion) compared with control-treated mice (14.12% and 11.67%, respectively, compared with 7.98% and 5.08%, respectively).

Intracellular Cytokine Production. IFN-γ is an important cytokine for cancer therapy, and it is also a marker for T-cell activation. T cells from TDLNs were collected and stained with anti-CD3ε, and their intracellular IFN-γ levels were analyzed. As shown in Fig. 5, intracellular FACS analysis showed that in the chTNT-3-treated mice, the IFN-γ producing T cells comprised 10.9% of the total T-cell population, whereas IFN-γ producing T cells comprised 15.7% of the total T-cell population in LEC/chTNT-3-treated mice. However, in those mice in which the CD25+ T cells were depleted, combination therapy with LEC/chTNT-3 increased the number of IFN-γ-producing T cells to 25.6% as compared with 17.9% in control-treated mice.

IFN-γ Knockout Mouse Studies. The increased production of IFN-γ in CD25+ T-cell-depleted mice shown above suggests that this cytokine may play a key role in the antitumor effects seen in the experimental therapy groups. To test this hypothesis, IFN-γ knockout mice were implanted with Colon 26 tumor cells s.c. LEC/chTNT-3 immunotherapy with and without CD25+ depletion was performed as describe above. As shown in Fig. 6A, LEC/chTNT-3 still reduced tumor growth to ~40% of that seen in the control-treated mice suggesting that this cytokine is not essential for LEC immunotherapy. By contrast, combination therapy with CD25+ depletion resulted in a similar growth curve, indicating that IFN-γ is critical for complete

### Table 1 Combination cytokine or chemokine fusion protein immunotherapy and T-cell subset depletion in the treatment of colon 26 carcinoma

<table>
<thead>
<tr>
<th>Immunotherapy</th>
<th>T-cell subset depletion</th>
<th>% tumor reduction (Day 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chTNT-3 (control)</td>
<td>—</td>
<td>0%</td>
</tr>
<tr>
<td>LEC/chTNT-3</td>
<td>CD4+ depletion</td>
<td>33%</td>
</tr>
<tr>
<td>LEC/chTNT-3</td>
<td>CD4+ depletion</td>
<td>100%</td>
</tr>
<tr>
<td>LEC/chTNT-3</td>
<td>CD3- IL-2</td>
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<td>CD3- IFN-γ</td>
<td>33%</td>
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<td>10%</td>
</tr>
<tr>
<td>LEC/chTNT-3</td>
<td>CD3- IFN-α</td>
<td>33%</td>
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*a Antibodies and fusion proteins (20 μg/dose) were injected i.v. for 5 consecutive days after tumors reached 0.5 cm in diameter.
*b CD4+ depletion (0.5 mg/dose of GK1.5) was performed i.p. 1 day before the initiation of immunotherapy treatment and repeated every 5 days.
regression when CD25⁺ depletion is used with LEC/chTNT-3 immunotherapy.

**Perforin Knockout Mouse Studies.** Perforin is a downstream factor critical for NK and CD8⁺ T-cell function. To test whether the combination of CD4⁺ depletion and LEC/chTNT-3 treatment enhanced NK and CD8⁺ T-cell function via cell to cell contact using the perforin pathway, perforin-knock out mice were used in place of normal BALB/c mice. For these studies, Colon 26-bearing perforin-knockout mice were treated with anti-CD4 antibody 1 day before the initiation of treatment with LEC/chTNT-3 or control antibody as described above. As shown in Fig. 6B, CD4⁺ depletion performed in combination with LEC/chTNT-3 treatment still reduced tumor growth by 80% as compared with control groups. These results are consistent with the notion that perforin-mediated tumor lysis is not a major pathway for CD4⁺ and presumably CD25⁺-mediated immunotherapy in this tumor model.

**T-Cell Proliferation Assays.** To confirm whether CD25⁺ depletion activates T cells, a tumor-specific T-cell proliferation assay was performed using the vital dye, CFSE. As shown in Figs. 7A for TDLNs and Fig. 7B for splenocytes, incubation with Colon 26 tumor lysate increased T-cell proliferation significantly in those mice treated with CD25⁺ depletion compared with controls. As shown in Fig. 7A for TDLNs, both the chTNT-3- and LEC/chTNT-3-treated groups by 20 h had minimal T-cell proliferation (1.6% and 3.0%, respectively). By contrast, CD25⁺ depletion (with and without LEC/chTNT-3 immunotherapy) showed dramatic induction of T-cell proliferation (20% and 42%, respectively). Moreover, combination therapy showed a marked shift to the left indicating an additional dilution of CFSE indicative of additional generations of dividing T cells. Similar results were also observed after 50 h of incubation with tumor lysate (data not shown). Interestingly, when splenocytes were incubated with tumor lysate as shown in Fig. 7B, T-cell proliferation was only observed in the CD25⁺-depleted group and not in the group treated with combination therapy. These results can be interpreted to mean that activated T cells in the spleens of mice receiving combination therapy migrated to TDLNs due to the presence of LEC/chTNT-3 in tumor.

**Cytokine Expression Analyzed by Real-Time PCR.** The mRNA expression of Th1 (IL-2, IFN-γ, and TNF-α) and Th2 (IL-4, IL-10, and transforming growth factor β) cytokines in tumor infiltrating lymphocytes was detected by real-time PCR. To compare cytokine expression in the various tumor samples, the mRNA levels were standardized against that found in the control antibody-treated group to form an index of expression. As shown in Fig. 8, both Th1 and Th2 cytokines were found to have the highest expression in the LEC/chTNT-3-treated groups. In contrast, LEC/chTNT-3 and CD25⁺ depletion combination therapy induced higher Th1 expression (IFN-γ = 1.86, IL-2 = 2.7, and TNF-α = 2.2) than those of the control group (IFN-γ = 1.0, IL-2 = 1.9, and TNF-α = 1.6) and less Th2 cytokine expression.

**DISCUSSION**

Immunotherapy as a modality for the treatment of cancer is an exciting approach, which is beginning to show promise in the clinic (29). Because individual cytokines with the exception of IL-2 (30–32) have not been especially effective in the elimination of established tumors, combinations of these reagents are now being investigated in the form of vaccines and fusion proteins (33–35). Fusion proteins have the advantage of targeting cytokines into the tumor microenvironment thereby enabling higher concentrations in the tumor and reducing systemic toxicity (36). More recently, with the understanding that tumor infiltration of lymphoid cells may be predictive of active tumor immunotherapy (18), chemokines are now actively being investigated as promising reagents due to their function as chemotactic
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agents. In many regards, chemokine fusion proteins are ideal therapeutics because they target the chemoattractant into the tumor, thereby facilitating the intratumoral infiltration of immune cells. To investigate the therapeutic potential of this approach, Challita-Eid et al. (37) and our laboratory (Li et al.; Ref. 22) have generated RANTES and LEC fusion proteins, respectively, using different tumor targeting mAbs. Although the RANTES fusion protein was not tested in vivo (37), our studies with LEC/CHNT-3 showed marked reduction in tumor size recently in three experimental solid tumor models of the BALB/c mouse (22). In the present study, we now show that complete remission can be obtained when LEC/CHNT-3 immunotherapy is used in combination with either CD4⁺ or CD4⁺CD25⁺ T-cell depletion. Treatment with control antibody chTNT-3 and CD25⁺ T-cell depletion produced only a 20% remission rate in Colon 26-bearing mice and a 0% remission rate in RENCA-bearing mice. Combinations of CD4⁺ depletion and other cytokine fusion proteins such as those consisting of IL-2, TNFα, and IFN-γ, were next tested to determine whether these treatments had synergistic effects on tumor growth. As shown in Table 1, two of the three fusion proteins, chTNT-3/IL-2 and chTNT-3/TNFα, showed some improvement with CD4⁺ depletion, but these results were not as dramatic as those with LEC/CHNT-3, suggesting that complete remission required LEC/CHNT-3 localization to tumor. One difference between the LEC fusion protein and the cytokine fusion proteins is the location of the cytokine moiety on the antibody molecule. As described in “Materials and Methods” above, LEC was attached to the NH₂ terminus near the variable region of the heavy chain, whereas the other cytokines were attached to the COOH terminus distal to the Fc region. These fusion proteins were constructed in this manner to ensure that the active binding sites of these cytokines or chemokines were distal to the linkage site enabling them to bind to their respective receptors. Construction in the reverse manner might compromise the full activity of the chemokine or cytokine making it difficult to determine whether this difference may be in some way responsible for observed results. Finally, it should also be noted that studies conducted by Golgher et al. (38) showed a much higher frequency of Colon 26 tumor rejection (70%) by CD25⁺ T-cell depletion alone, a finding that may be explained by their use of a much smaller inoculum of tumor cells compared with our studies (5 × 10⁷/mouse versus 10⁷ cells tumor cells) 1 day after the initiation of T-cell depletion.

T-reg. was first identified by Hill et al. (2), who showed that the administration of anti-CD4 antibody significantly increased the antitumor effects by mAb therapy, radiation therapy, and chemotherapy in various tumor models (3). Sakaguchi et al. (39) later showed that the CD25⁺ subset of CD4⁺ was responsible for the immunoregulation of the antitumor response in immunocompetent mice. Our studies verify these results and show for the first time that the combination of a chemokine fusion protein and CD25⁺ depletion has the potential to produce complete remissions in well-established solid tumors. Rechallenge experiments in naïve and cured mice showed that memory was intact for up to 3 months after completion of immunotherapy and that the return of CD25⁺ T-reg. coincided with the loss of tumor recognition as shown by successful implantation of tumor at 6 months. During CD25⁺ depletion, we found that the immune response was both tumor-specific and nonspecific because implanted MAD109 tumors in the contralateral flank were found to grow much slower than those implanted in naïve controls. These results were consistent with those of Murakami et al. and Shevach et al. (40, 41) who showed that the presence of CD4⁺CD25⁺ T cells inhibits the proliferation of CD8⁺ memory cells in the C57BL/6 mouse model.

The suppressive mechanisms mediated through CD4⁺CD25⁺ T cells still remain unknown. Our data using perforin knockout mice indicate that combination therapy was not associated with perforin

Fig. 5. FACS analysis of intracellular IFN-γ expression in TDLNs. Single cell suspensions of TDLNs were obtained 20 days after tumor implantation from treated and control groups of mice. The cells were stimulated with 10 ng/ml of PMA and 100 ng/ml of ionomycin in the presence of GolgiStop for 4–6 h before being double stained with PE-anti-CD3ε and FITC-anti-IFN-γ for FACS analysis.
because the combination of CD4$^+$ T-cell depletion and LEC/chTNT-3 treatment still resulted in an 80% tumor reduction as compared with control groups. Because previous studies showed that the depletion of CD4$^+$ (or CD4$^+$ CD25$^+$) T cells could dramatically induce CD8$^+$ T-cell penetration into the tumor sites in a B16 melanoma model (42), the infiltration of different populations of lymphocytes was also measured. Flow cytometry data of intratumoral lymphoid cells, however, did not support these findings. Although treatment with LEC/chTNT-3 consistently induced greater lymphocyte infiltration compared with control antibody-treated groups, CD25$^+$ T-cell depletion was not found to induce a greater number of infiltrating CD8$^+$ T cells or CD11b$^+$CD11c$^+$ dendritic cells, suggesting that tumor regression is not the result of the number of infiltrating cells but the activation status of these lymphocytes.

In view of the results of the perforin experiments described above, and the observation that the tumors appeared necrotic and not apoptotic after LEC/chTNT-3 immunotherapy (22), it was next decided to study the effects of cytokine-mediated pathways as the primary mechanism of combination immunotherapy. Real-time PCR studies were performed to quantitate the expression of Th1- and Th2-associated cytokines in tumor infiltrating lymphocytes removed from control and experimentally treated mice. These studies showed that LEC/chTNT-3 increased both Th1- and Th2-associated cytokine expression in tumor infiltrating lymphocytes. Combination therapy with LEC/chTNT-3 and CD25$^+$ depletion, however, showed a selective increase in Th1 cytokine expression compared with CD25$^+$ depletion alone. As shown by other investigators using knockout mice (43, 44), soluble mediators such as IL-4 and IL-10 have not been implicated in mechanism of action of T-reg. More likely, IFN-γ is probably a key factor in T-cell activation induced by CD4$^+$CD25$^+$ T-cell depletion. Two different lines of evidence are presented to show the importance of IFN-γ in the induction of active antitumor immunity by CD25$^+$ depletion. First, intracellular IFN-γ staining showed that depletion of
T-reg. could significantly reduce the production of IFN-γ-producing T cells in TDNLs. Secondly, studies performed in IFN-γ knockout mice showed that the therapeutic effects of CD25+ depletion no longer enhanced LEC/chTNT-3 immunotherapy. In contrast, LEC/chTNT-3 immunotherapy alone was only slightly affected in IFN-γ−/− mice. By contrast, LEC/chTNT-3 immunotherapy restored the finding that CD4+ T cell proliferation in the absence of T-reg. could significantly induce the production of IFN-γ in those mice receiving CD25−/− depletion. Thus, depletion of T-reg. may be useful for the immunotherapy or combination therapy with CD25−/−/H11001 (Suppl. 1) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4 (J. Exp. Med., 192: 303–310, 2000).


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