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IL-7 Promotes CXCR3 Ligand-Dependent T Cell Antitumor Reactivity in Lung Cancer

Åsa Andersson,* Seok-Chul Yang,† Min Huang,*†§ Li Zhu,*†§ Upendra K. Kar,* Raj K. Batra,*†§ David Elashoff,* Robert M. Strieter,¶ Steven M. Dubinett,*†§ and Sherven Sharma2*†§

We are evaluating the immune enhancing activities of cytokines for their optimum utility in augmenting cellular immune responses against lung cancer. In this study, we evaluated the mechanism of antitumor responses following IL-7 administration to mice bearing established Lewis lung cancer. IL-7 decreased tumor burden with concomitant increases in the frequency of CD4 and CD8 T lymphocyte subsets, T cell activation markers CXCR3, CD69, and CD127low, effector memory T cells, and T cell cytolytic activity against parental tumor cells. Accompanying the antitumor responses were increases in IFN-γ, CXCL9, CXCL10, and IL-12. Individual neutralization of CD4, CD8 T lymphocytes, or the CXCR3 ligands CXCL9 and CXCL10 reversed the antitumor benefit of IL-7, indicating their importance for optimal responses in vivo. Furthermore, IL-7 decreased the tumor-induced apoptosis of T cells with subsequent decrease of the proapoptotic marker Bim. We assessed the impact of IL-7 treatment on regulatory T cells that negatively impact antitumor immune responses. IL-7 decreased regulatory T Foxp3 as well as cell suppressive activity with a reciprocal increase in SMAD7. These results indicate that IL-7 induces CXCR3 ligand-dependent T cell antitumor reactivity in lung cancer. The Journal of Immunology, 2009, 182: 6951–6958.

T lymphocyte activities are dysregulated in cancer patients (1). To restore host T lymphocyte responsiveness against lung cancer, we are evaluating cytokines with immune-enhancing activities. Intratumoral infiltration by relatively high numbers of T lymphocytes in lung cancer is associated with a better patient outcome (2, 3). In a recent study, Dieu-Nosjean et al. (4) have retrospectively identified ectopic lymph node (LN)3 or tertiary lymphoid structures within human non-small cell lung cancer specimens and demonstrated that there is a correlation of their cellular content with clinical outcome. The density of DC-Lamp+, mature dendritic cells within these structures is a predictor of long-term survival within their selected lung cancer patient population. The authors observation that a low density of tumor-infiltrating CD4+ and T-bet+ T lymphocytes are present in tumors poorly infiltrated by DC-Lamp+ mature dendritic cells provides additional supportive evidence for the prognostic importance of an adaptive immune reaction to a solid tumor (4).

Cytokines are crucial for the survival and proliferation of a broad range of T lymphocytes, resulting in the maintenance of a polyclonal naive T cell pool with the ability to respond to tumors. In this respect, IL-7 is an attractive cytokine because it can impact homeostatic T cell proliferation.

IL-7 is a 17.5-kDa cytokine produced by a variety of stromal cells, keratinocytes, dendritic cells, neurons, and endothelial cells and is essential for lymphopoiesis (5), T cell homeostasis (6–8), and maintenance (9, 10). IL-7 also promotes T cell cytolytic and innate responses (11, 12). The biological effects of IL-7 on target cells are mediated by binding to the high-affinity IL-7 receptor complex that is composed of the ligand binding IL-7 receptor α-chain and the common shared γ-chain (13). Naïve T cells express high levels of the IL-7 receptor CD127 and respond rapidly to IL-7 stimulation (14), making this molecule an attractive agent for restoration of T cell activities in tumor-bearing hosts.

In previous studies, we have shown that IL-7 treatment of lung cancer-bearing mice led to marked reduction in tumor burden with extensive lymphocytic infiltration of the tumors and enhanced survival. The antitumor responses were accompanied by the enhanced elaboration of IFN-γ and the IFN-γ-inducible CXCR3 targeting antiangiogenic chemokines CXCL9 and CXCL10. However the precise role of T cells, IFN-γ, CXCL9, and CXCL10 in IL-7-mediated antitumor reactivity was not determined in the earlier study (15). An understanding of the cellular and molecular mechanism of IL-7 mediated antitumor responses will enable the design of strategies for its optimum utility in tumor control.

In addition to a lack of maintenance of T cell activities in cancer-bearing hosts, tumor-induced immune suppression has been well documented in lung cancer and other malignancies (16). Tumor-reactive T cells accumulate in lung cancer tissues but fail to respond (17, 18), in part, because high proportions of lung cancer tumor-infiltrating lymphocytes are T regulatory (Treg) cells (19, 20). Treg cells actively down-regulate the activation and expansion of self-reactive lymphocytes (21). Thus, reducing the number of
Treg cells or abrogating their activity may induce effective tumor immunity in otherwise nonresponding hosts by activating tumor-specific as well as nonspecific effector cells (22–24). Recent clinical studies indicate that high levels of tumor infiltration by activated CD8+ T cells combined with a low number of Treg is a significant positive prognostic factor for patient survival in cancers (25–28).

In this study, we assessed the requirement for T cell subsets, IFN-γ, CXCCL9, and CXCCL10 in IL-7-mediated antitumor responses and the role of IL-7 on Treg activity in a murine model of lung cancer. Our findings indicate that IL-7 promotes CXCCL3 ligand-dependent T cell immune responses with a concomitant down-regulation of Treg cell activity in lung cancer.

**Materials and Methods**

**Reagents**

Fluorescent-conjugated flow cytometry anti-mouse Abs CD4, CD25, CD8, CD127, and CXCCL3, isotype-matched Abs, and an Annexin V Apoptosis kit were obtained from BD Pharmingen. Fluorescent-conjugated flow cytometry anti-mouse Abs Foxp3, anti-CD49b, anti-CD44, anti-CCR7 and recombinant murine IL-7 were from eBioscience. Alamar blue was obtained from BioSource International. ELISA Ab pairs for murine IFN-γ, CXCCL9, CXCCL10, and IL-12 were purchased from R&D Systems. Monoclonal anti-mouse IFN-γ, CD4, and CD8 (R4-462, TIB-207, and TIB-150 from American Type Culture Collection)-neutralizing Ab were purified from scid mice ascites using Ab purification kits from Millipore. The ascites were generated 3–4 wk after i.p. injection of 10^6 respective hybridoma cells per mouse. Recombinant goat anti-mouse IgG/CXCCL9 and anti-mouse IP-10/CXCCL10-specific antiserum were produced and characterized as previously described (29).

**Mice**

Pathogen-free C57BL6 mice (6–8 wk old; Harlan) and UBC-GFP/BL6 (The Jackson Laboratory) were maintained in the West Los Angeles Veterans Affairs Animal Research vivarium. The institution’s animal studies review board approved all studies.

**Cell culture**

The murine Lewis lung carcinoma (3LL, H-2a, also known as LLC, ATCC CRL-1642) from American Type Culture Collection was used in these studies. Cells were routinely cultured as monolayers in Corning T75-cm^2 tissue culture flasks in a humidified atmosphere containing 5% CO_2 in air. The culture medium contained RPMI 1640 (Irvine Scientific) supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mmol/L glutamine (JRH Biosciences). The cell line was Mycoplasma free and used up to the 10th passage before thawing frozen cells from liquid N2.

**Tumorigenesis model**

In brief, 1.5 × 10^7 3LL tumor cells were injected s.c. in the right suprascapular area of C57BL6 mice or UBC-GFP/BL6. UBC-GFP/BL6 mice express GFP in all cells and were used to determine the extent of lymphocyte infiltrates in the tumor. Mice bearing 5-day-old palpable tumors were treated with IL-7 (5 μg/dose) or normal saline (NS) daily via i.p. injections for the duration of the experiment or as stated. Tumor volumes were measured by monitoring two bisectional diameters of each tumor with calipers. Tumor volumes were calculated using the formula: \( V = \frac{4ab^2}{3} \), with \( a \) as the larger diameter and \( b \) as the smaller diameter.

To determine the role of CD4 T cells, CD8 T cells, IFN-γ, CXCCL9, or CXCCL10 in IL-7-mediated antitumor responses, the T cell subsets or cytokine-neutralizing Abs were treated individually with recombinant murine IL-7 for 5 days. Mice bearing 5-day-established tumors were treated with IL-7 as described above. Twenty-four hours before IL-7 treatment and then three times a week, mice were injected i.p. individually with 100 μg/dose of purified mAb: anti-CD4, anti-CD8, and anti-IFN-γ and 1 ml/dose of anti-IP-10/ CXCCL10 or anti-MIG/CXCCL9 or appropriate control Abs (goat IgG, rat IgG, and anti-mouse IgM) at equivalent doses for the duration of the experiment. In response to these neutralizing Abs, there was a significant reduction of the respective T cell subsets and cytokines (data not shown). Tumor volumes were assessed three times per week.

**Orthotopic 3LL model**

To determine the antitumor efficacy of IL-7 in an organ-specific manner, 3LL-GFP (10^7) tumor cells were inoculated in the left lung of mice via tracheal injection under mild anesthesia (ketamine; 33 mg/kg). The tumor cells were injected in 25 μl of saline with a 30-gauge needle. Seven days following tumor implantation, mice were treated daily with IL-7 via i.p. injections (5 μg/dose/mouse). Four weeks after tumor implantation, lungs were isolated to determine tumor burden in single-cell suspensions of lung tumor digests by using flow cytometry to quantify GFP-expressing 3LL cells. T cells in the lung, tumors, LN, and spleens were stained for cell surface markers CD3, CD4, CD8, and CXCCL3 and intracytoplasmic IFN-γ and evaluated by flow cytometry.

**Tumor tissue sectioning and immune fluorescence**

To determine the extent of lymphocytes infiltrating the tumors, UBC-GFP/BL6 mice bearing 5-day tumors were treated with IL-7 (5 μg/dose/day for 7 days). Non-necrotic tumors were isolated and frozen in OCT. The tissue section was sectioned to 5-μm thickness, fixed onto slides, and counterstained with 4,6-diamidino-2-phenylindole (DAPI) fixative. The slides were observed under a BX71 Olympus fluorescence microscope attached to a computerized digital camera. The images were acquired under ×10 and ×40 objectives using the Image Pro software.

**Flow cytometry**

Flow cytometry was performed on purified T cells from spleens following IL-7 (100 ng/ml for 48 h) stimulation in vitro or IL-7 (5 μg/dose/day for 7 days) treatment of 5-day-old tumor-bearing mice. For in vitro effects of IL-7, single-cell suspensions of T cells were stained for cell surface CD3, CD4, CD8, and CXCCL7 followed by flow cytometry. For in vivo effects of IL-7 in tumor-bearing mice, the above cell surface markers as well as the markers CD4, CXCCL7, and CD69 were analyzed on single-cell suspensions of spleen or LN cells. For T cell marker analyses in the tumor tissue, tumors were mechanically dissociated on a wire mesh by crushing with a 10-ml syringe and then digested at 37°C for 25 min with 0.2 mg/ml collagenase A (Boehringer Mannheim/Roche), 5 U/ml DNase (Sigma-Aldrich), and 0.3 U/ml dispase (Invitrogen) in RPMI 1640. The digested cells were filtered through a 70-μm nylon strainer (BD Biosciences). CD3, CD4, and CD8 as well as CXCCL3 and CXCCL7 expression on the T cell subsets were quantified in the single-cell digests of tumors, LN, and spleens by gating within the lymphocyte population. T cell subsets in single-cell suspensions of lung tumors, LN, and spleens were stained for intracytoplasmic IFN-γ with PE-labeled Abs and reagents (BD Pharmingen) according to the manufacturer’s instructions.

To determine the effect of IL-7 on Treg cells, 5-day-old tumor-bearing mice were treated with IL-7 or NS for 7 days. One day following the last IL-7 administration, CD4+CD25+ Foxp3+ Treg cells were quantified in the murine splenocytes by staining with fluorochrome (PE, FITC, and PerCP)-labeled Abs and the cells were detected by flow cytometry.

Samples were acquired on a FACSCanto (BD Biosciences)/FACSCalibur flow cytometer (BD Biosciences) in the University of California, Los Angeles, Jonsson Cancer Center Flow Cytometry Core Facility. Between 10,000 and 25,000 gated events were collected and analyzed using FCS Express 3 (De Novo Software). Cells incubated with irrelevant isotype-matched Abs (BD Biosciences) and unstained cells were used as controls. The cutoffs were set according to the findings in controls.

**T cell cytolytic activity**

Lymphocytic antitumor responses were evaluated following IL-7 (5 μg/dose/day for 7 days) therapy in 5-day-old tumor-bearing mice. One day following the last IL-7 treatment, T cells were purified from spleens by negative selection using Miltenyi Biotec beads, and cytolytic activities were evaluated against the autologous 3LL tumor cell line and the syngeneic control B16 melanoma tumor cells. The T cell effectors were incubated with tumor cell targets (E:T of 32:1 and 64:1) in quadruplet wells in a 96-well plate, and 32P-labeled B16 melanoma tumor cells. The T cell effectors were incubated with tumor cell targets (E:T of 32:1 and 64:1) in quadruplet wells in a 96-well plate, and 32P-labeled B16 melanoma tumor cells. The T cell effectors were incubated with tumor cell targets (E:T of 32:1 and 64:1) in quadruplet wells in a 96-well plate, and 32P-labeled B16 melanoma tumor cells.
buffer from Invitrogen in tubes on ice according to the manufacturer’s instructions with the polytron homogenizer (Kinematica). Total protein concentration in tumor and LN lysates was determined by the Bradford assay. The plates were read at 490 nm with a Micro Plate (Molecular Devices). The sensitivity of the IFN-γ/H9253, CXCL9, CXCL10, and IL-12 ELISA was 2 pg/ml.

**Apopopsis**

To determine the effect of IL-7 on the T cell apoptosis rate, 5-day-old tumor-bearing mice were treated with IL-7 (5 μg/dose/day for 7 days) or diluent. One day following the last IL-7 injection, T cells were purified from spleens by negative selection using Miltenyi Biotec beads. Apoposis was determined by the effect of IL-7 on the T cell apoptosis rate, 5-day-old tumor-bearing mice were treated with IL-7 (5 μg/dose/day for 7 days) or diluent. One day following the last IL-7 injection, T cells were purified from spleens by negative selection using Miltenyi Biotec beads. Apoposis

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**FIGURE 1.** CD4 and CD8 T lymphocytes are required for IL-7-mediated tumor reduction. A, C57BL/6 mice bearing 5-day established 3LL tumors were treated with PBS, IL-7, IL-7 + control (Crl) Ab, IL-7 + anti-CD8 Ab, or IL-7 + anti-CD4 Ab via i.p. injections. IL-7 was administered daily. Neutralization of CD4 or CD8 reversed the antitumor activities of IL-7. Data, mean ± SEM, p values: IL-7, IL-7 + anti-CD8 Ab, or IL-7 + anti-CD4 Ab compared with control groups: ***, p < 0.005 and ****, p < 0.001 (n = 6/group). Enhanced T lymphocyte tumor infiltration (B) and activation (C–E) following IL-7 treatment. B, UBC-GFP/BL6 mice bearing 5-day established tumors were treated with PBS or IL-7 (5 μg/day for 7 days) via i.p. injections. OCT frozen tissue was sectioned, fixated onto slides, and counterstained with the nuclear dye DAPI (blue). Control tumors (left panel) demonstrate very limited green fluorescence (infiltrating cells) under a low-power view (original magnification, ×100, upper panel). Under a high-power view (original magnification, ×400, lower left panel), most green fluorescent cells are large polygonal stromal cells (thick arrow) while tumor cells are counterstained blue by DAPI. In contrast, prominent tumor-infiltrating lymphocytes, small round green fluorescent cells (thin arrow), are evident in tumors from the IL-7-treated mice (right panel). C–E, Tumor digest (C), LN cells (D), and spleen cells (E) were prepared and evaluated for CD3, CD4, and CD8 T cell subsets, and activation by cell surface staining/flow cytometry. IL-7 treatment (E) mediates increased frequency of CD3, CD4, and CD8 cells in tumors (C), LN (D), and spleens (E), and the T cell activation markers: CXCR3 and CD127low in tumors (C), CXCR3, CD69, and CD127low in LN (D), and CD127low as well as increased effector memory cells (CD8+CD44+CCR7+) in the spleen (E) (n = 6/group). F, Enhanced cytolytic activity was observed in purified splenic T cells from the IL-7-treated mice against parental 3LL tumors in vitro (ET of 32:1 and 64:1; n = 6/group). G, IL-7 stimulation leads to a marked increase in activation in vitro. Purified spleen T cells (2.5 × 10⁶ cells/ml) were incubated with IL-7 (100 ng/ml) for 48 h and evaluated for CD4, CD8, and CD127low by cell surface staining/flow cytometry (n = 3/group). Data, mean ± SEM, p values: IL-7 compared with control: *, p < 0.05, ***, p < 0.01, and ****, p < 0.001.
in the T cell population was quantified immediately after isolation and at 24 h after culture in vitro using an Annexin V FITC Kit according to the manufacturer’s protocol. For flow cytometry, 10,000 events were collected and analyzed with CellQuest software.

Total RNA preparation, cDNA synthesis, and real-time PCR for Foxp3 and SMAD7

Five-day-old tumor-bearing mice were treated with IL-7 (5 μg/dose/day for 7 days) or diluent. One day following the last IL-7 treatment, tumor tissue or T cells from spleens were isolated using Miltenyi Biotec beads for the quantification of Foxp3 and SMAD7 gene expression by quantitative PCR (QPCR). To determine whether IL-7 could reverse PGE2 and the TGFβ-mediated increase in Treg Foxp3 gene expression, splenocytes (10 million cells per condition) were stimulated with PGE2 (26 μM) or TGFβ (10 ng/ml) for 24 h and then IL-7 (100 ng/ml) was added for an additional 48 h. For QPCR analyses, RNA was isolated using a Qiagen kit. The cDNA was prepared with a kit (Invitrogen) according to the manufacturer’s instructions. Foxp3 gene expression was quantified using a SYBR Green Quantitative PCR Kit in the iCycler (Bio-Rad) and corrected with the β-actin housekeeping control gene. Amplifications were done in a total volume of 20 μl for 40 cycles of 15 s at 95°C, 20 s at 64°C, and 30 s at 72°C. For β-actin the annealing temperature was 68°C. Samples were run in triplicate and their relative expression was determined by normalizing expression of each target to β-actin and then comparing this normalized value with the normalized expression in a reference control sample to calculate a fold change value. The primers for the amplicons spanned intron/exon boundaries to minimize amplification of genomic DNA. Primer sequences were as follows: β-actin forward, 5'-CCACAGCTTGAGGAGGAATC-3' and reverse, 5'-TCTCCAGGGAGGAAAGGAT-3'; Foxp3 forward, 5'-CCC AGGAAAAAGACAGACCAT-3' and reverse, 5'-TTCTCACAACAGGC CACTTG-3'; and SMAD7 forward, 5'-GCAGCCCCATCTTCTAC TAGTCCG-3' and reverse, 5'-GTAGGGAGAACCAGGAAAC-3'.

SMAD7, Foxp3, and Bim Western blot analyses

Five-day-old tumor-bearing mice were treated with IL-7 (5 μg/dose/day for 7 days) or diluent. One day after the last IL-7 treatment, spleen T cells were isolated for the detection of Foxp3 and SMAD7 by Western blot analyses as described previously (30). For Foxp3 detection, we used anti-foreskin anti-mouse IgG1 Ab (eBioscience), for SMAD7 detection we used rabbit anti-SMAD7 IgG (Santa Cruz Biotechnology), and for Bim detection we used rabbit anti-Bim (Cell Signaling Technology) at dilutions of 1:1000. The bands were visualized by the Amersham Life Sciences Kodak Image Station 440.

T cell proliferation assay

Five-day-old tumor-bearing mice were treated with IL-7 or diluent for 1 wk. One day after the last IL-7 treatment, splenocytes, murine spleen CD4+ CD25+, and CD4+ CD25- T cells were purified using Miltenyi Biotec beads according to the manufacturer’s instructions. Flow cytometric evaluation of Miltenyi Biotec bead-purified CD4+ CD25+ T cells showed >98% of the T cells staining positive for CD4+ CD25+. The purified CD4+ CD25+ cells revealed <1% of the cells staining positive for CD4+ CD25-. CD4+ CD25- T or CD4+ CD25+ (2 × 10^6 cells) were added to plate-bound, anti-CD3-coated plates (1 μg/ml) and soluble anti-CD28 (1 μg/ml) containing 2 × 10^6 spleen T cells in quadriplate wells per condition in 24-well plates for 72 h. The ratio of Treg/effectector T cells was 1:1. Proliferation was measured by adding 20 μl of Alamar blue for the last 3 h of the 72 h of incubation and plates were read with a Wallac fluorescence plate reader with the excitation/emission set at 530/590 nm.

Statistics

Statistical analysis was performed using Prism (GraphPad Software). Dual comparisons were made using the unpaired Student t test or two-way ANOVA with Bonferroni post test to compare tumor volume in the treatment groups.

Results

IL-7-mediated reduction in tumor burden requires CD4 and CD8 T lymphocytes

We tested the effect of IL-7 on 3LL tumor burden and T cell activities in C57BL/6 mice (Fig. 1, A–E). Compared with diluent-treated controls, systemic IL-7 treatment of tumor-bearing mice significantly reduced tumor burden. To determine the T cell requirements for antitumor reactivity, CD4 and CD8 T cells were neutralized in IL-7-treated tumor-bearing mice. Individual neutralization of CD4 or CD8 T cells reversed the antitumor benefit of IL-7 (Fig. 1A). The tumors of IL-7-treated mice had increased lymphocytic infiltrates (Fig. 1B) that expressed the activation markers CXCR3 and CD127low (Fig. 1C). Systemically, IL-7 treatment increased CD4 (12%) and CD8 (22%) T cell subsets, augmented the frequency of CD8+ CD127low-activated (2-fold) as well as CD8+ CD44+ CCR7+ effector memory T cells (2-fold; Fig. 1, D and E). Compared with diluent-treated controls, IL-7 enhanced splenic T cell cytolytic activity (1.9- to 3-fold) against parental 3LL tumor cells (Fig. 1F) but not against the syngeneic B16 control (data not shown). IL-7-treated splenocytes had a marked increase in CD127low-activated CD4+ (6-fold) and CD8+ (17-fold) T lymphocyte populations compared with controls (Fig. 1G).

**FIGURE 2.** IL-7 reduces T cell apoptosis rates (A) and the proapoptotic protein Bim (B). Purified spleen T cells, from diluent (○) and IL-7- (□) treated tumor-bearing mice, were evaluated immediately after isolation and at 24 h after in vitro culture for apoptosis by annexin V staining/flow cytometry (A) (n = 6/group). T cell total Bim and GAPDH levels were quantified by Western blot (uneven numbers = diluent, even numbers = IL-7; B). Graph depicts densitometric analyses of Bim normalized by GAPDH (B, lower right) (n = 5/group). Data, mean ± SEM, p values: IL-7 compared with control: ***p < 0.001.

IL-7 reduced T cell apoptosis rates and the proapoptotic protein Bim

IL-7 is known to increase the longevity of T cells (31). We determined the effect of IL-7 on the apoptosis rates in T cells from diluent and IL-7-treated tumor-bearing mice. Compared with diluent-treated tumor-bearing mice, IL-7 treatment significantly reduced the apoptosis rates (2.4-fold) in the T cell population (p < 0.001; Fig. 2A). The outcome for rates of apoptosis may be related to changes in the balance of pro- and antiapoptotic proteins. To determine whether the decrease in T cell apoptotic rates was associated with a decrease in the T cell proapoptotic protein Bim, we quantified Bim levels in T cells from IL-7- and diluent-treated tumor-bearing mice. Compared with controls, the proapoptotic marker Bim protein was reduced by 40% in T cells from the IL-7 treatment group (p < 0.001; Fig. 2B).

IL-7 induces IFN-γ, CXCL9, CXCL10, and IL-12 in vivo

Following IL-7 treatment of 3LL tumor-bearing C57BL/6 mice, we determined the levels of IFN-γ, CXCL9, CXCL10, and IL-12 in vivo (Fig. 3). Compared with diluent-treated controls, IL-7 treatment led to high levels of IFN-γ (4-fold), CXCL9 (2.5-fold), CXCL10 (2-fold), and IL-12 (2-fold) from the tumors (Fig. 3A), LN (Fig. 3B), and spleens (Fig. 3C).
We determined the impact of IL-7 on splenocyte IFN-γ, CXCL9, and CXCL10 production. IL-7 induced splenocyte production of IFN-γ (>20-fold), CXCL9 (>2.5-fold), and CXCL10 (>30-fold) compared with controls (Fig. 3D).

To determine the importance of IFN-γ, CXCL9, and CXCL10 in the IL-7-mediated tumor reduction, these cytokines were depleted individually with neutralizing Abs to the respective cytokines in the IL-7-treated mice. Neutralization of IFN-γ partially inhibited, whereas neutralization of CXCL9 or CXCL10 completely reversed the antitumor benefit of IL-7 (Fig. 3E, p < 0.001 compared with the control Ab group). Cytokine-specific ELISA of spleen or LN lysates showed that the neutralizing Abs effectively neutralized the targets in the IL-7-treated mice (data not shown).

**IL-7 treatment reduces Treg activities and Foxp3 expression in vivo**

The impact of IL-7 on SMAD7 and Treg Foxp3 expression was assessed by QPCR and Western blot analyses in tumor-bearing mice (Fig. 4, A–D). There was a reciprocal relationship between Treg Foxp3 and SMAD7 expression in vivo.

**FIGURE 3.** IL-7 generates high levels of IFN-γ, CXCL9, CXCL10, and IL-12 in vivo (A–C). Lysates from tumors (A), LN (B), and spleens (C) were prepared from diluent (□)- and IL-7 (▲)-treated tumor-bearing mice and analyzed for cytokine production by ELISA (n = 6/group). D, Supernatants from spleen cells (2.5 × 10⁶ cells/ml) stimulated with IL-7 (100 ng/ml) for 24 h were analyzed for IFN-γ, CXCL9, and CXCL10 secretion (n = 3/group). Data, mean ± SEM, p values: IL-7 compared with control: ***, p < 0.001 and **, p < 0.01. E, Neutralization of CXCL9 and CXCL10 completely reverse, whereas IFN-γ neutralization partially reverses the antitumor effect of IL-7. C57BL/6 mice bearing 5-day established 3LL tumors were treated with PBS, IL-7, IL-7 + control (Ctrl) Ab, IL-7 + anti-IFN-γ Ab, IL-7 + anti-CXCL9 Ab, or IL-7 + anti-CXCL10 Ab via i.p. injections. IL-7 (5 μg/dose) was administered daily. The Abs (anti-IFN-γ (100 μg/dose), anti-CXCL9, and anti-CXCL10 (1 ml/dose) were administered i.p. 24 h before IL-7 treatment and then three times a week for the duration of the experiment. Tumor burden was assessed three times a week (n = 6/group). Data, mean ± SEM, p values: IL-7 and IL-7 + anti-cytokine Ab compared with control groups: **, p < 0.01 and ***, p < 0.001.

**FIGURE 4.** Treg activities and Foxp3 expression are reduced in IL-7-treated mice. A and B, IL-7 decreases Foxp3 and increases SMAD7 expression. mRNA isolated from tumors and purified spleen T cells of diluent- and IL-7-treated tumor-bearing mice was analyzed for Foxp3 and SMAD7 expression by real-time QPCR (n = 6/group). A, Foxp3 and SMAD7 were quantified from purified spleen T cells, diluent- or IL-7-treated tumor-bearing mice, by Western blot (B). C, IL-7 treatment decreases tumor-induced increases in splenic Tregs. Flow cytometry analyses were performed on T cells from the spleen following staining with CD4, CD25, and Foxp3. Compared with naive mice, diluent-treated mice had increased Treg cells that were reduced by IL-7 treatment (n = 6/group). D, IL-7 significantly decreased the tumor-induced increase in Treg activity. CD4⁺CD25⁺ T cells were sorted and cultured with IL-7 (10 ng/ml) for an additional 48 h, and then analyzed for CD25 expression by real-time QPCR (n = 6/group). Data, mean ± SEM, p values: IL-7 compared with control: *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
IL-7 reduces tumor burden in an orthotopic murine lung cancer model

The antitumor efficacy of IL-7 was determined in the orthotopic 3LL lung cancer model (Fig. 5). IL-7 reduced tumor burden by 4.5-fold compared with diluent-treated mice (Fig. 5A). Following therapy, T cell infiltrates increased in a single-cell suspension of lung tumor digests, with significant increases in the CXCR3-expressing CD4 and CD8 T cells producing intracellular IFN-γ (Fig. 5B). Similar T cell phenotypic increases were observed in the LN and the spleens of tumor-bearing mice in response to IL-7 (Fig. 5, C and D).

Discussion

In this study, we evaluated the capacity of IL-7 to restore host antitumor reactivity in a murine lung cancer model. The ability of IL-7 to enhance immune response against malignancies in several tumor models and its therapeutic potential in cancer supports the rationale for mechanistic investigations on its antitumor capacity. An understanding of the underlying antitumor mechanisms will enable the design of strategies for optimum tumor control.

Many facets of T lymphocyte activities are suppressed, thus allowing tumors to progress in immune-competent hosts. Our results indicate that IL-7 treatment of tumor-bearing mice restores T cell activities. IL-7 treatment caused a significant reduction in tumor burden that correlated with increases in the CD4 and CD8 T cell populations in the tumors and systemically in the LN and spleens. Within the CD8 T cell population, there were significant increases in both the activated and effector memory phenotypes. Purified splenic T cells from IL-7-treated tumor-bearing mice had enhanced specific T cell cytolysis against parental tumor but not against the syngeneic control B16. The enhanced T cell cytosis of the parental tumor in response to IL-7 signifies activation of a wide repertoire of T cells. Compared with controls, the tumors of IL-7-treated mice had a higher frequency of T cell infiltrates in the tumors and secondary lymphoid organs that expressed CXCR3 and CD127high.

Our rationale for selecting the 5-μg dose of rIL-7 was based on a study by Komshlies et al. (32), who showed that this dose effectively induced the proliferation in CD4 and CD8 T cell subsets in vivo. We found that the 5 μg/dose of rIL-7 reduced tumor burden when therapy was initiated on day 10 after tumor inoculation in the s.c. tumor model (data not shown) or on day 7 in the orthotopic tumor model. Our findings are consistent with the results of Komshlies et al. (32) who showed that recombinant human IL-7 administered systemically reduced tumor burden in mice.
bearing early renca adenocarcinoma pulmonary metastasis. The antitumor reactivity was also evident at lower doses of rIL-7 (0.2–0.8 µg/dose) in the 5- to 6-day-old palpable s.c. tumor model (data not shown).

T cells played a crucial role in the generation of an efficient IL-7-mediated antitumor immune response which is supported by the evidence from neutralization experiments. Neutralization of CD4 or CD8 T cells led to the complete reversal of the antitumor immune responses, indicating cooperation between the CD4 and CD8 T cell effectors for the full antitumor benefit of IL-7. In addition to the cellular infiltrates, the tumors of IL-7-treated animals had an enhanced elaboration of IFN-γ, CXCL9, CXCL10, and IL-12. CXCL9 and CXCL10 are chemotactic for CXCVR3-expressing cells and are important for antitumor immune responses (33). The high level expression of these chemokines in the tumors can explain the high frequency of CXCVR3-expressing lymphocytes infiltrating the tumors and the subsequent reduction in tumor burden. We found an enhancement in CXCVR3-expressing CD4 and CD8 T cells that stained for intracytoplasmic IFN-γ in the tumors as well as in the secondary lymphoid organs in response to IL-7. Based on this data, we postulate that the IFN-γ-expressing CXCVR3 T cells are the effectors that lyse the tumors. Based on our data, we anticipate that a relationship exists between the dose and/or kinetics of IL-7 with the recruitment of CXCVR3-expressing T cells into the tumors.

The importance of IFN-γ, CXCL9, and CXCL10 were assessed in neutralization experiments. Neutralization of IFN-γ partially reversed, whereas neutralization of the CXCVR3 ligands CXCL9 or CXCL10 completely ablated the antitumor benefit of IL-7. These results indicate a pivotal role for CXCL9 and CXCL10 in IL-7-mediated lung cancer antitumor reactivity.

The immune response to tumor Ags is altered in patients with cancer (34, 35). These alterations concern many elements of the immune response that prevent effective proliferation of tumor Ag-specific T cells and their subsequent recognition of tumor cells (35, 36). A balance between proliferation and apoptotic cell death controls immunological activity. We determined the impact of IL-7 on T cell apoptotic rates in the tumor-bearing mice. IL-7 significantly reduced the T cell apoptotic rates with an associated decrease in the proapoptotic protein Bim. It is possible that other proteins in the apoptotic pathway may also be altered in response to IL-7. Thus, IL-7 is crucial for the survival and/or proliferation of a much broader range of T lymphocytes and for the maintenance of a polyclonal naive T cell pool that can respond to the tumor.

Based on our recent studies showing that IL-7 can reverse the immune suppressive action of human Treg cells in vitro (37), in this study, we determined the role of IL-7 on Treg cell activities in vivo. In addition to tumor-associated macrophages contributing to the immunosuppressive milieu, a subset of CD4+ lymphocytes, commonly expressing cell surface CD25 and expressing high levels of the transcription factor Foxp3, has been shown to be a potent suppressor of immune function (38). These Treg cells can suppress the ability of antitumor T cells that accumulate in lung cancer tissues (17, 18) of patients as well as suppress the activity of T cells to effectively treat established murine cancers (22–24).

Foxp3 is a specific marker for Treg cells (39) and forced expression of the Foxp3 gene can convert murine naive T cells to Treg cells that phenotypically and functionally resemble naturally occurring CD4+CD25+ Treg cells (39–41). Furthermore, inoculations of CD4+CD25+ T cells prepared from normal mice can prevent autoimmune disease in Foxp3-deficient mice (41). Collectively, these findings indicate that Foxp3 is a critical control gene for the development and function of natural CD4+CD25+ Treg cells. Consistent with this concept, Rudensky and colleagues (41) have shown that CD4+CD25+ T cells from Foxp3-deficient mice lack regulatory activity. Because Foxp3-expressing Treg cells can generate immune suppression in the tumor-bearing host, we determined the impact of IL-7 on Treg Foxp3 and activities in vivo. Based on our previous study that IL-7 inhibition of TGFβ signaling in fibroblasts was associated with an increase in Smad7 (42), we evaluated the impact of IL-7 on Smad expression in the tumors. SMAD7 is an inhibitory Smad that acts as a negative regulator of signaling by TGFβ superfamily proteins. SMAD7 is induced by TGFβ, stably interacts with activated TGFβ type I receptor, and interferes with the phosphorylation of receptor-regulated Smads (43). TGFβ is known to induce Foxp3 in CD4+CD25+ T cells and Foxp3 down-regulates SMAD7 directly by binding to the promoter and decreasing its expression, thus rendering these T cells with regulatory characteristics (44). IL-7 decreased Foxp3 in the tumors with a reciprocal increase in SMAD7. The down-regulation of Foxp3 by IL-7 could explain the increase in SMAD7. Based on these results on SMAD 7 levels, it is probable that other SMAD proteins in the TGFβ family may also be altered in response to IL-7. In addition, Treg cells from IL-7-treated tumor-bearing mice had reduced activity. The results of our study are consistent with the findings of Rosenberg et al. (45) who found that patients with metastatic melanoma showed decreases in CD4+ Treg cells following IL-7 administration.

TGFβ that has been shown to induce the differentiation of CD4+CD25+ T cells into Foxp3-expressing CD4+CD25 Treg cells (44). TGFβ also regulates the in vivo expansion of Foxp3-expressing CD4+CD25+ Treg cells (46). We have previously shown that tumor cyclooxygenase-2/PGE2 promotes Foxp3 expression and CD4+CD25 Treg cell activities in lung cancer (47). Based on this evidence, we determined the effect of IL-7 on TGFβ- and PGE2-mediated enhancement in Treg Foxp3 gene expression in vitro. IL-7 treatment markedly reduced PGE2- and TGFβ-mediated Foxp3 gene expression in vitro.

Taken together, the enhancement in CXCVR3 ligand-dependent anti-tumor immune responses requiring both CD4 and CD8 T cells and the reduction of Treg activities by IL-7 show the potential of this cytokine as an immune-enhancing adjuvant. The fact that IL-7 significantly reduced tumor burden without completely rejecting the tumors implies that suppressor mechanisms are operative or that the T cells generated in response to IL-7 are eventually exhausted. Additional studies are necessary to define the suppressor mechanisms and the signaling pathways operative in T cells to explain the incomplete tumor resolution in response to IL-7.

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**Disclosures**

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**References**


