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Role of CXCR3 Ligands in IL-7/IL-7R α -Fc mediated antitumor activity in Lung Cancer

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Abstract

Purpose—We evaluated the utility of chimeric γ c homeostatic cytokine, IL-7/IL-7R α -Fc, to restore host APC and T cell activities in lung cancer.

Experimental Design—Utilizing murine lung cancer models we determined the antitumor efficacy of IL-7/IL-7R α -Fc. APC, T cell, cytokine analyses, neutralization of CXCL9, CXCL10 and IFN γ were performed to evaluate the mechanistic differences in the antitumor activity of IL-7/IL-7R α -Fc in comparison to controls.

Results—IL-7/IL-7R α -Fc administration inhibited tumor growth and increased survival in lung cancer. Accompanying the tumor growth inhibition were increases in APC and T cell activities. In comparison to controls, IL-7/IL-7R α -Fc treatment of tumor bearing mice led to increased: i) levels of CXCL9, CXCL10, IFN γ , IL-12 but reduced IL-10 and TGF β , ii) tumor macrophage infiltrates characteristic of M1 phenotype with increased IL-12, iNOS but reduced IL-10 and arginase, iii) frequencies of T and NK cells, iv) T cell activation markers CXCR3, CD69 and CD127^{low} v) effector memory T cells and vi) T cell cytolytic activity against parental tumor cells. IL-7/IL-7R α -Fc treatment abrogated the tumor induced reduction in splenic functional APC activity to T responder cells. The CXCR3 ligands played an important role in IL-7/IL-7R α -Fc mediated antitumor activity. Neutralization of CXCL9, CXCL10 or IFN γ reduced CXCR3 expressing activated T cells infiltrating the tumor and abrogated IL-7/IL-7R α -Fc mediated tumor growth inhibition.

Conclusions—Our findings demonstrate that IL-7/IL-7R α -Fc promotes afferent and efferent antitumor responses in lung cancer.

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Keywords

Lung Cancer; IL-7 Chimeric Cytokine; CXC-Chemokines; APC and T Cell Activation

Introduction

Although tumor growth and invasion leads to inflammatory responses, the immune system generally develops tolerance to cancer. One way to induce potent immune responses against tumors is to activate key innate and immune effector mechanisms. Toward this end, we are evaluating the utility of chimeric γ c homeostatic cytokine, IL-7/IL-7R α -Fc, to restore host APC and T cell activities dysregulated in cancer patients (1, 2). It is evident from previous studies that intratumoral infiltration by relatively high numbers of activated T lymphocytes (3, 4) and APC (5) leads to better prognosis in lung cancer patients.

Interleukin 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 (6), T cell homeostasis (7–9) and maintenance (10, 11). IL-7 also promotes T cell cytolytic and innate responses (12, 13). 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 (14). Naïve T cells express high levels of the IL-7 receptor CD127 and respond rapidly to IL-7 stimulation (15) making this molecule an attractive agent for restoration of T cell activities in tumor bearing hosts. Our rationale for using the IL-7/IL-7R α -Fc chimera molecule is that it combines the T lymphocyte activation property of IL-7 with IgG Fc to augment innate effectors through enhanced APC activity. We anticipate that agents that activate both the innate and immune effector mechanisms will be more effective in controlling tumor growth.

Despite the expression of tumor antigens by lung cancer cells, the limited expression of MHC antigens, defective transporter associated with antigen processing (TAP), and lack of co-stimulatory molecules make them ineffective APC (16). The central importance of functional APC in the immune response against cancer was well defined by Huang *et al* (17). The study revealed that even highly immunogenic tumors require host APC for antigen presentation. Thus host APC, rather than tumor cells, present tumor antigen to CD8⁺ T cells. CD8⁺ T cell responses can be induced *in vivo* by professional APC that present exogenous antigens in a MHC I restricted manner (18) that is critical for effective antitumor responses (19). However, in tumor bearing hosts, there is a state of T cell unresponsiveness (20–22) through dysregulated APC activity (23).

Additionally, tumor cells produce immune inhibitory factors that promote escape from immune surveillance (24). The tumor microenvironment not only fails to provide the inflammatory signals needed for efficient APC activation but also inhibits APC differentiation and maturation through IL-10 (25). Immature APC produce little or no IL-12 which is required to support T cell proliferation. If APC fail to provide an appropriate costimulatory signal for T cells, tolerance or anergy can develop (25, 26).

Macrophages in the tumor microenvironment play an important modulatory role in the generation of anti tumor responses. The production of chemotactic factors such as CCL2, VEGF and M-CSF (27, 28) in the tumor microenvironment recruits macrophages. The type of macrophages infiltrating the tumor correlates with favorable or unfavorable prognoses (29). The M1 macrophages have potent antigen presentation function and stimulate Type1 immune responses that lead to tumor rejection, tissue destruction, and host defense. M1 macrophage density in the tumor islets is positively associated with extended survival of non

small cell lung cancer (NSCLC) patients (30). The M1 macrophages produce high levels of IL-12, CXCL10 and iNOS (31). In contrast, M2 macrophages are thought to promote tumor formation by enhancing wound healing and tissue remodeling via inhibition of Type1 immune responses by IL-10 and TGF β secretion. The M2 macrophages express high levels of IL-10 and arginase that suppress antitumor immune responses (31–34).

The numbers and types of leukocytes in the tumor infiltrate are related to the chemokines produced in the tumor microenvironment. Antitumor reactivity is due to the types of leukocytes infiltrating the tumors. The IFN γ inducible chemokines, CXCL9 and CXCL10 exert their biological effects by binding to the seven transmembrane domain G-protein coupled CXCR3 receptor (35). CXCL9 and CXCL10 expression in the tumor microenvironment recruit activated CXCR3 expressing effector T lymphocytes with antitumor reactivity. Thus mechanisms that increase the levels of CXCL9 and CXCL10 in the tumor microenvironment promote effective cell mediated antitumor activity through the CXCR3 expressing effector T lymphocytes.

Here we found that IL-7/IL-7R α -Fc treatment induced macrophages with M1 phenotype, inhibited tumor burden and extended survival in mice bearing lung cancer. Our findings demonstrate that IL-7/IL-7R α -Fc promotes the afferent M1 macrophage phenotype and the efferent (CXCR3/CXCR3 ligand biological axis) limbs of the immune response for sustained antitumor activity in lung cancer.

Materials and Methods

Reagents

The murine Lewis lung carcinoma (3LL, H-2^b, also known as LLC, ATCC CRL-1642) obtained from American Type Culture Collection (Manassas, VA) was used in these studies. The culture medium contained RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA), penicillin (100 units/ml), streptomycin (0.1mg/ml), and 2mmol/L glutamine (JRH Biosciences, Lenexa, KS). Fluorescein isothiocyanate-, phycoerythrin-, allophycocyanin-, PerCP- or PerCP-Cy7-conjugated anti-mouse mAbs to CD3 (145-2C11), CD4 (RM4-5), CD8a (53-6.7), CD69 (H1.2F3), CD127 (A7R34), subclass control antibody, unconjugated iNOS (6/iNOS/NOS Type II) and Arginase (19/Arginase I) were purchased from BD Biosciences (San Diego, CA). Anti-mouse mAbs to CD44 (IM7), CD49b (DX5), IL-10 (JES5-16E3), IFN γ (XMG1.2), CCR7 (4B12) and recombinant murine IL-7 (endotoxin level: less than 0.01 ng/ug cytokine as determined by the LAL assay) were purchased from eBioScience (San Diego, CA). Antibody to mouse F4/80 (BM8) was purchased from BioLegend. Anti-mouse mAbs to CXCR3 (220803), IL-7R α -Fc chimeric molecule (747-MR) (endotoxin level: less than 1.0EU per ug of protein by LAL method), ELISA antibody pairs for murine IFN γ , CXCL9, CXCL10 and IL-12 were purchased from R&D Systems (Minneapolis, MN), sensitivity of 3–5pg/ml. IL-2, IL-10, TGF β and TNF α were quantified with ELISA kits (eBioScience). Sensitivity: IL-2 (3pg/ml), IL-10 (30pg/ml), TGF β (60pg/ml) and TNF α (8pg/ml). Ovalbumin protein and Bradford protein quantification dye was obtained from Sigma (St. Louis, MO). Tissue digestion buffer consisted of [0.2 mg/ml of Collagenase A (Boehringer Mannheim/Roche, Indianapolis, IN), DNase 25U/ml (Sigma), and 0.3U/ml of Dispase (Invitrogen, Carlsbad, Ca)] in RPMI. Alamar blue was obtained from Cellular Technologies Ltd., (Camarillo, CA). Tissue homogenization reaction reagent1 buffer was obtained from Invitrogen. Monoclonal anti-mouse IFN γ (R4-462 from American Type Culture Collection) neutralizing Ab was purified from *SCID* mice ascites using antibody purification kits from Millipore (Billerica, MA). The ascites was generated 3–4 wk after *i.p.* injection of 10⁶ IFN γ hybridoma cells per mouse. Polyclonal goat anti-mouse CXCL9 and anti-mouse CXCL10 specific anti-serum were produced and characterized as previously described (36). F4/80

antibody for immunohistochemistry was from Abcam (Cambridge, MA). Brewers thioglycollate (Difco, BD Biosciences, San Jose, CA) was used to elicit peritoneal macrophages.

Mice

Pathogen-free C57BL/6 mice (6–8 weeks old) and UBC-GFPBL/6 (Jackson Laboratory, Bar Harbor, Maine) and FcR γ KO (Taconic, NY) were maintained in the West Los Angeles Veterans Affairs Animal Research vivarium. The institution's animal studies review board approved all studies.

Cell culture

3LL tumor cells were routinely cultured as monolayers in Corning T75 cm² tissue culture flask in humidified atmosphere containing 5% CO₂ in air in culture medium. The cell line was *Mycoplasma* and murine viral pathogen free and used up to the 10th passage before thawing frozen cells from liquid N₂.

Antigen processing and presentation assay

Day 21 purified splenic APC (5×10⁴/well) from naïve or 3LL tumor-bearing UBC-GFPBL/6 mice treated on days 7 and 14 with (1) Normal saline diluents (NS), (2) IL-7 (5 µg/dose), (3) IL-7/IL-7R α -Fc (5/5µg/dose) or (4) IL-7R α -Fc (5 µg/dose) were plated in triplicates in 96-well plates or in duplicates in 8-well chamber slides and co-cultured with OVA protein (2.5mg/ml) and the MHC Class I restricted CD8 T cell line B3Z (10⁵/well) for 24 hrs. IL-2 secreted by the activated CD8 T cells in the supernatant was quantified by ELISA. To determine the direct impact of IL-7/IL-7R α -Fc on APC activity, purified splenic APC were stimulated with Diluent, IL-7 (100ng/ml), IL-7/IL-7R α -Fc (100/100ng/ml), or IL-7R α -Fc (100ng/ml) for 6hr, washed, and then co-cultured with OVA and OVA specific CD8 T cells. IL-2 secreted by the activated CD8 T cells in the supernatant was quantified by ELISA. Peritoneal macrophages were elicited with 1 mL 4% thioglycollate intraperitoneally and the cells were recovered by lavage with 10 mL PBS after 5 days, RBC lysed and macrophage APC activity to process and present ovalbumin to CD8 T cells was evaluated as described for splenic APC. To determine the role of FcR γ expression on IL-7/IL-7R α -Fc mediated modulation in APC activity, splenic APC from WT and FcR γ KO mice was utilized.

Tumorigenesis model

1.5×10⁵ 3LL tumor cells were injected *s.c.* in the right supra scapular area of C57BL/6 mice. Mice bearing 7 day old palpable tumors were treated with NS or recombinant proteins via *i.p.* injections in 200µl. IL-7 (5µg/dose) was administered either daily or on the same schedule as IL-7/IL-7R α -Fc (5/5µg/dose) or IL-7R α -Fc (5µg/dose) that were administered once per week for 2 weeks. To form IL-7/IL-7R α -Fc complex, IL-7 (5µg) and IL-7R α -Fc (5µg) suspended in NS, were mixed and incubated for 30 min at 37 C. Tumor volumes were monitored by measuring two bisecting diameters of each tumor with calipers. Tumor volumes were calculated using the formula: $V=0.4ab^2$, with a as the larger diameter and b as the smaller diameter.

Orthotopic model

Implantation of the tumors in the lung was performed as previously described (37). Briefly, 10⁴ 3LL-GFP or unlabeled 3LL cells in 25µl sterile NS were injected by the transthoracic route of C57BL/6 or UBC-GFPBL/6 mice respectively utilizing a tuberculin syringe with a 30-gauge needle in the left lung under ketamine/xylazine anesthesia. UBC-GFPBL/6 mice express green fluorescent protein in all cells and were utilized to determine the APC activity in the tumor bearing mice. One week following tumor inoculation, a group of mice were

sacrificed to determine the baseline tumor burden before initiation of therapy. For tumor burden determination, the frequency of EpCam expressing 3LL cells were quantified in a single suspension of lung tumor digests by flow cytometry and H and E staining of tumor sections. One week following tumor inoculation, mice were treated with NS, IL-7 (5µg/dose) or IL-7Rα-Fc (5µg/dose) or IL-7/IL-7Rα-Fc (5µg/5µg/dose) *i.p.* once a week for two weeks. Three weeks after tumor implantation, lungs were harvested for evaluation of tumor burden and leukocytic infiltrates.

Cytokine neutralization

For *in vivo* neutralizations, mice bearing 5-day established subcutaneous tumors were treated with IL-7/IL7Rα-Fc on days 5 and 10. Twenty four hours prior to IL-7/IL7Rα-Fc treatment, and then three times per week, mice were injected *i.p.* individually with 100µg/dose of purified anti-IFNγ monoclonal Ab, or 1 ml/dose of anti-CXCL10, or 1 ml/dose of anti-CXCL9 or appropriate control antibodies (goat IgG, rat IgG, and anti mouse IgM) at equivalent doses for the duration of the experiment. Tumor volumes were assessed three times per week.

Flow Cytometry

Prior to therapy, day 7 orthotopic lung tumors were evaluated for the frequency of: i) macrophages (F4/80) and their intracytoplasmic expression of IL-12, IL-10, iNOS and Arginase, ii) T (CD4, CD8) cells and their intracytoplasmic expression of IL-10 and IFNγ and iii) NK cells. Following treatment (day 21 after tumor inoculation), flow cytometry was performed for the following T cell surface markers (CD3, CD4, CD8, CD127, CD44, CCR7, CD69, and CXCR3), NK cell surface marker (CD49b) and macrophage cell surface marker (F4/80) on single cell suspension of tumors or splenocytes. CD4 T, CD8 T and NK cells were evaluated for intracytoplasmic IL-10 and IFNγ expression. Macrophages were quantified for intracytoplasmic IL-10, IL-12, iNOS and arginase expression in the leukocytic cell population. For analyses of tumor leukocytic infiltrates, tumors were mechanically dissociated on a wire mesh by crushing with a 10ml syringe and incubated in tissue digestion buffer at 37°C for 25 min. The cells were filtered through 70µm nylon strainers (BD Biosciences, Bedford, MA) and stained with specific markers and analyzed by flow cytometry. The direct impact of IL-7/IL-7Rα-Fc [Diluent, IL-7 (100ng/ml), IL-7/IL-7Rα-Fc (100/100ng/ml), or IL-7Rα-Fc (100ng/ml)] on intracytoplasmic IL-12, IL-10, iNOS and Arg was evaluated on C57BL/6 peritoneal macrophages following a 48hr *in vitro* stimulation.

Samples were acquired on a FACSCanto (BD Biosciences/FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) in the University of California, Los Angeles, Jonsson Cancer Center Flow Cytometry Core Facility. A total of 10,000 to 25,000 gated events were analyzed using FCS Express 3 (De Novo Software, Canada). Cells incubated with irrelevant isotype-matched antibodies and unstained cells served as controls. The cutoffs were set according to control staining.

Cytotoxic T lymphocyte assay

T lymphocyte lytic responses were evaluated following therapy in 7-day old tumor bearing mice. One week following the last treatment (day 21), T cells were purified from spleens by negative selection using Miltenyi Biotec beads, and cytolytic activities were evaluated against autologous 3LL tumor cell line and the syngeneic control B16 melanoma tumor cell line. The T cell effectors were co-cultured with tumor cell targets (E:T of 20:1–60:1) in quadruplet wells in a 96-well plate, and 20 µl alamar blue was added to each well after 18 hours of incubation. Three hours after alamar blue addition, the plate was read with the Wallac 1420 fluorescence plate reader (Perkin-Elmer Life Science, Turku, Finland) with the excitation/emission set at 530/590 nm.

Cytokine ELISA

Splenocytes from naïve mice (2.5×10^6 /ml) were stimulated for 72 hours with IL-7, IL-7/IL-7R α -Fc or IL-7R α -Fc (IL-7 and IL-7R α -Fc range 12.5–100ng/ml) in triplicates and cytokines in the culture supernatants were quantified by ELISA. For *in vivo* experiments, one week following the last treatment (day 21) of tumor bearing mice the cytokines (IFN γ , TGF β , CXCL9, CXCL10, IL-10 and IL-12) in the spleen or tumor homogenates was quantified by ELISA. Tumors or spleens were homogenized with the polytron homogenizer (Kinematica, Switzerland) in tissue homogenization buffer in tubes on ice. Total protein concentration in tumor lysates was determined by the Bradford assay. TNF α was measured from supernatants of splenic APC (WT and FcR γ KO mice) following a 48hr stimulation with i) Diluent, ii) IL-7 (100ng), iii) IL-7R α -Fc (100ng) and iv) IL-7/IL-7R α -Fc (100ng/100ng). The ELISA plates were read at the specified wavelengths with a Microplate Reader (Amersham Biosciences, Sunnyvale, CA).

Tumor Tissue Sectioning and Immunohistochemistry

To determine the extent of tumor burden and macrophage infiltrate into the lung tumor sections, paraffin embedded lungs were serially sectioned to 5- μ m thickness. Sections were H&E and immune stained for F4/80 macrophages. Antigen retrieval was accomplished with sodium citrate 10 mmol/L (pH 6.0). Sections were blocked with 10% normal goat serum, and probed with an antibody against F4/80 using a working dilution of 1:200. Primary antibody was incubated overnight at 4°C. After incubation with secondary antibody (Vector Laboratories), staining was developed using DAB Substrate kit for Peroxidase (SK-4100, Vector Laboratories). Counter-stain was achieved with hematoxylin. The slides were observed under 1X71 Olympus Fluorescence microscope attached to a CCD camera. The images were acquired under 10X and 20X objectives using the Image Pro software.

Statistical analyses

All data are presented as mean $\pm$ SE. Statistical analysis was performed using Prism (GraphPad Software). We used analysis of variance for data with multiple groups, unpaired Student's *t*-test for dual comparison and log-rank test for comparison of survival in Kaplan-Meier survival plot. *P* values <0.05 were considered significant.

Results

IL-7/IL-7R α -Fc inhibits tumor growth and increases T cell activity in lung cancer

We and others have previously shown that IL-7 inhibits tumor growth in murine cancer models (37–39). Here we tested whether pre-association of IL-7 to IL-7R α -Fc could enhance the antitumor efficacy of IL-7 against subcutaneous 3LL tumor growth in C57BL/6 mice. IL-7/IL-7R α -Fc administered once a week for two weeks was more effective at inhibiting tumor growth compared to recombinant IL-7 administered on the same schedule or IL-7 administered daily for the duration of the experiment (Figure 1A). In comparison to IL-7 administered on the same schedule or control groups, IL-7/IL-7R α -Fc treated tumor bearing mice had increases in: tumor CD8 lymphocytic infiltrates expressing the activation markers CXCR3 and CD127^{low} (Figure 1B), tumor production of IFN γ , IFN γ inducible proteins CXCL9 and CXCL10, and IL-12 but reduced IL-10 and TGF β (Figure 1C), splenic T lymphocyte subsets (CD4 and CD8) and NK cells (Figure 1D), splenic CD8 T lymphocytes expressing the activated markers CD127^{low} and CD69 as well as CD8⁺CD44⁺CCR7⁻-effector memory T cells (Figure 1E–F) and T cytolytic activity against autologous 3LL tumors (Figure 1G) but not against the syngeneic B16 control (data not shown). The spleens of IL-7/IL-7R α -Fc treated mice had enhanced levels of IL-12, IFN γ ,

and the IFN γ inducible proteins CXCL9 and CXCL10 but reduced IL-10 and TGF β compared to IL-7 weekly or controls (Figure 1H).

IL-7/IL-7R α -Fc stimulates APC activity in 3LL tumor bearing mice

We evaluated the impact of IL-7/IL-7R α -Fc on splenic APC activity from 3LL tumor bearing mice. Photomicroscopy of the cells shows that splenic APC (green) from IL-7/IL-7R α -Fc treated group was more effective at attracting and sequestering OVA specific CD8 T cells (non green) than the IL-7 or diluent controls (Figure 2A). In comparison to IL-7 or controls, splenic APC activity from IL-7/IL-7R α -Fc treatment group was not as adversely impacted in ovalbumin antigen processing and presentation to activate OVA specific CD8 T cells to secrete IL-2 (Figure 2B). We found direct *in vitro* stimulation of naïve splenic APC by IL-7/IL-7R α -Fc augmented its capacity to process and present ovalbumin and activate CD8 T cells to secrete IL-2 as compared to IL-7 or diluent controls (Figure 2C). Similar results on APC activity were obtained from peritoneal macrophages following IL-7/IL-7R α -Fc stimulation *in vitro* (**data not shown**). To determine the impact of Fc portion of the IL-7/IL-7R α -Fc chimeric molecule on APC activity and TNF α production, antigen processing and presentation to CD8 T cells was evaluated following stimulation of splenocytes from FcR γ KO mice *in vitro*. The enhanced APC activity and TNF α production following IL-7/IL-7R α -Fc stimulation *in vitro* in the WT group was ablated in the FcR γ KO group (Figure 2D–E).

IL-7/IL-7R α -Fc induces splenocytes to secrete IFN γ , CXCL9 and CXCL10

Based on our findings of enhanced levels of IFN γ , CXCL9 and CXCL10 in the tumors and spleens of the IL-7/IL-7R α -Fc treated mice (Figure 1C), we determined if direct IL-7/IL-7R α -Fc stimulation of naïve splenocytes induce these cytokines *in vitro*. Compared to IL-7 and controls, IL-7/IL-7R α -Fc induced elevated levels of IFN γ , CXCL9 and CXCL10 from splenocytes (Figure 3A). Consistent with our murine data, human peripheral blood mononuclear cells (PBMCs) stimulated with IL-7/IL-7R α -Fc induced elevated levels of IFN γ compared to IL-7 and controls (Figure 3B).

Depletion of CXCR3 ligands CXCL9 or CXCL10 abrogates IL-7/IL-7R α -Fc mediated antitumor activity

To determine the importance of IFN γ , CXCL9 and CXCL10 in the IL-7/IL-7R α -Fc mediated tumor growth inhibition, these cytokines were depleted individually with neutralizing Abs to the respective cytokines in the IL-7/IL-7R α -Fc treated mice. Neutralization of IFN γ partially inhibited, whereas neutralization of CXCL9 or CXCL10 completely abrogated the antitumor benefit of IL-7/IL-7R α -Fc (Figure 4A–B). Following specific cytokine neutralization, there were decreases in the respective cytokines in the tumors (Figure 4C). Cytokine-specific ELISA of spleen lysates also showed that the neutralizing antibodies effectively neutralized the targets in the IL-7/IL-7R α -Fc treated mice (**data not shown**). Neutralization of CXCL9, CXCL10 or IFN γ reduced the frequency of CXCR3 expressing CD8 T lymphocytes in the tumors (Figure 4D).

IL-7/IL-7R α -Fc inhibits 3LL orthotopic lung tumor growth and extends survival

The antitumor efficacy of IL-7/IL-7R α -Fc was determined in the orthotopic 3LL lung cancer model (Figure 5).

Prior to therapy on day 7, a group of tumor bearing mice was evaluated for tumor burden and tumor leukocytic infiltrates. Tumor burden determination demonstrated a mean percentage of 8 ± 3 of total lung digest with multiple tumor satellites in the lungs by H&E (Figure 5A–B). Analyses of tumor leukocytic infiltrates demonstrated no significant changes

in the frequency of macrophages, CD4 T, CD8 T and NK cells between the naïve and tumor bearing mice (data not shown). However, compared to naïve non tumor bearing lungs, macrophages in the lung tumor leukocytic infiltrates had reduced IL-12 but increased IL-10 and arginase expression (Figure 5C). Compared to tumor burden on day 7, there was an 8-fold increase in the tumor burden in the diluent treated group on day 21 but only a 2-fold increase in the IL-7/IL-7R α -Fc treated group. IL-7/IL-7R α -Fc reduced tumor burden by 4-fold compared to diluent-treated mice (Figure 5D–E) and enhanced survival (Figure 5F). Whereas all diluent treated mice succumbed to their tumors by day 37, more than 50% of the mice in the IL-7/IL-7R α -Fc treatment group survived for over 90 days. As in the subcutaneous tumor model, IL-7 administered on the same schedule as IL-7/IL-7R α -Fc (once a week for two weeks) was synonymous to diluent control (data not shown). Compared to control (Figure 5D:5), immune staining of lung tumor sections showed increased F4/80 macrophages infiltrating the tumors of IL-7/IL-7R α -Fc treated mice (Figure 5D:6). Evaluation of intratumoral leukocytic populations (day 21) showed enhanced frequency of F4/80, CD4, CD8 and NK cells (Figure 5G). Accompanying the increased macrophage infiltrates in the IL-7/IL-7R α -Fc treated mice was the characteristic M1 phenotypic signature of increased IL-12 and iNOS but decreased IL-10 and arginase (Figure 5H). CD4 and CD8 T lymphocyte populations infiltrating the tumor had enhanced IFN γ but reduced IL-10 levels (Figure 5I–J). NK population infiltrating the tumors of IL-7/IL-7R α -Fc treated mice had elevated IFN γ levels (Figure 5K). IL-7/IL-7R α -Fc directly impacted macrophages by increasing the intracytoplasmic expression of IL-12 and decreasing IL-10 and arginase *in vitro* (Figure 5L).

Discussion

In a previous study we have shown that IL-7 treatment restores T cell activity in lung cancer bearing mice (37). In this study, we evaluated if IL-7/IL-7R α -Fc chimeric molecule can augment the antitumor activity of IL-7 against lung cancer. We hypothesized that IL-7/IL-7R α -Fc treatment of tumor bearing mice would combine the immune enhancing activities of IL-7 with the IgG Fc portion of the molecule to promote interaction between APC and T cells. The utilization of biological agents that promote both innate and adaptive immune cells for augmented antitumor activity will be a useful addition with other approaches for therapeutic intervention in lung cancer.

Many facets of host APC and T cell activity are suppressed thus allowing tumors to progress in immune-competent hosts. Our results demonstrate that IL-7/IL-7R α -Fc treatment of tumor bearing mice augments APC and T cell antitumor activity. IL-7/IL-7R α -Fc administered once a week for two weeks was more effective at inhibiting tumor burden compared to IL-7 administered daily or on the same schedule. This suggests that IL-7/IL-7R α -Fc administered less frequently is more effective than IL-7. Our rationale for selecting IL-7/IL-7R α -Fc dose used in the current study was based on IL-7 dose from a previous study (37). The frequency of IL-7/IL-7R α -Fc administration was based on pilot studies conducted for antitumor efficacy. For the initial experiments IL-7/IL-7R α -Fc was administered every other day for two weeks. As our studies progressed we noted that a less frequent weekly dosing had equivalent efficacy to the more frequent every other day dosing. For the data presented in the manuscript we have used the less frequent dosing of once per week for two weeks. Measurement of IL-7/IL-7R α -Fc from the serum or plasma samples one week following administration did not yield detectable amounts by ELISA that suggest that stability of the chimeric molecule cannot explain the differences in tumor burden between the IL-7 and IL-7/IL-7R α -Fc groups.

To explain the differences in tumor growth inhibition, we sought to determine the modulation in the frequency and activity of host APC, T lymphocyte and cytokine analyses

following treatment. Our rationale for quantifying APC activity is that the dominant mechanism underlying the development of antigen specific T cell unresponsiveness is thought to be through the downregulation of tumor antigen processing and presentation by APC (23). In tumor bearing mice, splenic APC function as determined by the processing and presentation of the OVA protein to CD8 T cells was reduced compared to naïve mice. IL-7/IL-7R α -Fc treatment of tumor bearing mice significantly restored the APC cross presentation of OVA protein to CD8 T cells. The enhancement in APC activity may be partially due to the direct effect of IL-7/IL-7R α -Fc on APCs. In comparison to IL-7 treatment alone, IL-7/IL-7R α -Fc stimulation *in vitro* enhanced function of APC from naïve mice. IL-7/IL-7R α -Fc treatment of tumor bearing mice led to qualitative changes in spleen APC by inducing cellular extensions and processes with a greater capacity to sequester and activate CD8 T cells. Based on these results we postulate that IL-7/IL-7R α -Fc induces more efficient T cell effectors than IL-7 alone by enhancing APC activities to CD8 T cells. This was further corroborated with the FcR γ KO mice where the effect of the Fc portion of the chimeric molecule was not evident in APC activity and TNF α release *in vitro* in comparison to WT. The purpose of utilizing the FcR γ KO mice in the APC activity assays was to determine the functional impact of the Fc portion of the IL-7/IL-7R α -Fc chimeric molecule. In comparison to IL-7 alone, IL-7/IL-7R α -Fc stimulation of splenocytes from WT spleens shows a 1.4 fold increase in IL-2 and TNF α that is abrogated in the FcR γ KO mice and levels return to IL-7 secreted amounts. This suggests that in addition to IL-7R mediated events, FcR γ is also required for IL-7/IL-7R α -Fc mediated increases in splenocyte IL-2 and TNF α . In the absence of the FcR γ , only the IL-7 portion of the molecule is active, which explains the similar levels of IL-2 and TNF α release between IL-7 and the IL-7/IL-7R α -Fc treatment groups. This data suggests that both the IL-7 and IL-7R α -Fc portions of the chimeric molecule are necessary for optimum APC activity for enhancing T cell responses. Our observations on APC activity using the FcR γ KO mice shows that the FcR γ is required for the full benefit of the IL-7/IL-7R α -Fc molecule. This data indicates that IL-7/IL-7R α -Fc acts as binary activation system with both the IL-7 and the IL-7R α -Fc parts required for full benefit of the chimeric molecule. However since there are several types of Fc γ R future work will delineate which Fc γ R is responsible for the antitumor activity of IL-7/IL-7R α -Fc utilizing the respective knockouts.

Compared to controls, IL-7/IL-7R α -Fc treated tumor bearing mice had increased T lymphocyte subsets, activated and memory T cell phenotype and NK cells systemically. IL-7/IL-7R α -Fc treatment enhanced tumor T cell infiltrates expressing the activation markers CXCR3 and CD127^{low}. We assessed the tumor lytic capacity of systemically mobilized T lymphocytes *in vitro*; IL-7/IL-7R α -Fc treatment led to the generation of T lymphocytes with enhanced specific lytic capacity against autologous tumors at lower effector to target ratios compared to the IL-7 treatment or control groups. These findings demonstrate that IL-7/IL-7R α -Fc treatment restores T cell responsiveness and generates a more effective functional repertoire of T lymphocyte effectors with augmented lytic activity against autologous tumor target in comparison to controls. Our data demonstrates that IL-7/IL-7R α -Fc treatment of tumor bearing mice leads to a higher frequency of activated CD8 T cells expressing CXCR3 in the tumors and that splenic T cells exhibit greater cytolytic activity compared to IL-7 treatment. This suggests that IL-7/IL-7R α -Fc administered less frequently generates a higher frequency of antitumor effector T cells. Future work will delineate the long term maintenance of antitumor activity of these T cell effectors and the role of the CXCR3 receptor in IL-7R α -Fc mediated antitumor activity in CXCR3 KO mice or through antibody mediated neutralization of CXCR3 effectors in WT mice.

IL-7/IL-7R α -Fc treatment of tumor bearing mice modulated the cytokine signature in the tumors and systemically in the spleens. The following cytokines were determined: IL-12, IL-10, TGF β , IFN γ , and the IFN γ inducible proteins CXCL9 and CXCL10. These cytokines

were evaluated because the tumor site has been documented to be abundant sources of IL-10 and TGF β that have been shown to suppress immune responses (24, 40) and to promote angiogenesis (41). Antibodies to TGF β and IL-10 suppress tumor growth *in vivo* in tumor model systems (42, 43). TGF β is known to suppress antigen presentation, and antagonize CTL generation and macrophage activation (40). IL-7/IL-7R α -Fc -treated tumor-bearing mice showed significant reductions in IL-10 and TGF β at the tumor sites. Thus, possible benefits of an IL-7/IL-7R α -Fc-mediated reduction in IL-10 and TGF β include the augmentation of antigen presentation, CTL generation and downregulation of immune suppression.

Apart from a decrease in TGF β and IL-10, the tumors of IL-7/IL-7R α -Fc-treated mice revealed significant increases in IFN γ , IL-12, CXCL9 and CXCL10. It is well documented that successful immunotherapy shifts tumor-specific T-cell responses to a Type 1 cytokine profile (44). Both IL-12 and IFN γ mediate a range of biological effects that facilitate antitumor immunity. IL-12, a cytokine produced by macrophages (45) and DCs (46), mediates potent antitumor effects that are the result of several actions involving the induction of CTL (47), Type1-mediated immune responses and NK activation (45), as well as the impairment of tumor vascularization (48). Following IL-7/IL-7R α -Fc treatment, the stimulatory cytokine (IFN- γ , IL-12) levels were increased compared to controls but the inhibitory cytokines (TGF β and IL-10), although reduced in response to IL-7/IL-7R α -Fc, remained relatively high. We postulate that TGF β and IL-10 may still be high because the tumors are not eradicated. Future experiments will investigate the impact of neutralizing the residual IL-10 or TGF β on the modulation of IL-7/IL-7R α -Fc anti tumor activity.

CXCL9 and CXCL10 are CXC chemokines that chemoattract activated T cells expressing the CXCR3 chemokine receptor (35), and are known to have potent antitumor and anti angiogenic properties (49–52). CXCL9 and CXCL10 are potent angiostatic factors that are induced by IFN γ (51, 53, 54). IL-7/IL-7R α -Fc chimeric molecule consists of two parts, the IL-7 and the IL-7R α -Fc portion. We have previously shown that IL-7 mediated CXCR3 ligand-dependent T cell antitumor activity in lung cancer (37). We evaluated CXCL9 and CXCL10 and the impact of these chemokines on IL-7/IL-7R α -Fc mediated antitumor reactivity to determine if the IL-7 portion of the chimeric molecule retains similar IL-7 mediated CXCR3 ligand-dependent T cell antitumor activity. The reductions in tumor growth observed in this study may be due to T cell-dependent lysis as well as participation by T cells secreting IFN γ that inhibit angiogenesis through induction of CXCL9 and CXCL10. Hence, an increase in IFN γ in the tumor in IL-7/IL-7R α -Fc -treated mice could explain the relative increases in CXCL9 and CXCL10. In addition both CXCL9 and CXCL10 are chemotactic for stimulated CXCR3-expressing T lymphocytes that could further amplify IFN γ in tumors. The tumor of IL-7/IL-7R α -Fc -treated mice revealed increased CXCR3 expressing CD8 T lymphocyte infiltrates in the tumor.

The CXCR3 biological axis has been shown to be important for cytokine mediated antitumor activity in several studies (37, 55, 56). To determine the importance of CXCL9, CXCL10, or IFN γ in IL-7/IL-7R α -Fc-mediated antitumor response, these cytokines were depleted in IL-7/IL-7R α -Fc -treated mice. For this study, an IL-7 group with IL-7 administered on the same schedule as IL-7/IL-7R α -Fc was not included because it did not have antitumor benefit compared to diluent control. Anti-CXCL9 or anti-CXCL10 or anti-IFN γ each significantly inhibited the antitumor response. In this model CXCL9 and CXCL10 appear to play redundant roles in the antitumor effect of IL-7/IL-7R α -Fc, because inhibition of the individual ligand significantly abrogated the IL-7/IL-7R α -Fc mediated antitumor activity. We have previously shown that individual neutralization of CXCL9, CXCL10 or IFN γ concomitantly decreases all three cytokines in the tumors (37, 55, 56). Thus one possible explanation for tumor growth inhibition following *in vivo* neutralization

of CXCL9 or CXCL10 is that these chemokines play interrelated roles in the recruitment of CXCR3-activated T cells in IL-7/IL-7R α -Fc-mediated antitumor responses. Depletion of any one of these cytokines led to a decrease in CXCR3-activated CD8 T cells in the tumors. *In vivo* depletion of IFN γ also inhibited the antitumor efficacy of IL-7/IL-7R α -Fc that could be due to a decrease in the IFN γ -dependent CXCR3 ligands CXCL9 or CXCL10, indicating that these chemokines are largely IFN γ dependent. These results indicate that modulation of the CXCR3/CXCR3 ligand biological axis may serve as potential therapeutic target for the treatment of lung cancer. Our findings demonstrate that in comparison to IL-7, IL-7/IL-7R α -Fc treatment of tumor bearing mice induces augmented levels of CXCL9, CXCL10 and IFN γ and reversal of these cytokines abrogates the antitumor activity. This data is consistent with IL-7 activity for the chimeric molecule. One interesting question is whether IFN γ or CXCL9 or CXCL10 are necessary in the afferent or efferent, or both phases of the immune response. This question will be addressed in future studies.

We evaluated the effect of IL-7/IL-7R α -Fc in the orthotopic lung cancer model. To compare the overall change in tumor growth we determined tumor burden on day 7 prior to therapy and following therapy on day 21 post tumor inoculation. The growth of the tumor was uninhibited in the control treatments compared to the IL-7/IL-7R α -Fc treatment group. H&E staining of tumor sections from the IL-7/IL-7R α -Fc group showed marked inhibition in tumor growth and mice had extended survival. Compared to tumor burden prior to therapy (day 7), control treatment had an 8-fold increase in tumor burden whereas the IL-7/IL-7R α -Fc group had a 2-fold increase at day 21. This demonstrates that IL-7/IL-7R α -Fc treatment was efficacious at inhibiting tumor growth. We anticipate that IL-7/IL-7R α -Fc would be most beneficial to patients who have their lung cancer detected early when the tumor burden is low and innate and immune effectors are more susceptible to modulation. In comparison to naïve mice, lung tumor digests from 7-day tumor bearing mice had no differences in the frequency of macrophages, T and NK cells. In comparison to naïve mice, macrophages in the lung tumors of day 7 tumor bearing mice had decreased IL-12 but increased IL-10 and arginase characteristic of M2 phenotype. The tumors of IL-7/IL-7R α -Fc treated mice (day 21) had increased frequency of macrophage, lymphocyte and NK infiltrates as compared to controls. Phenotypic evaluation of the macrophages showed that IL-7/IL-7R α -Fc caused a shift in the balance from tumor induced M2 to M1 phenotype (enhanced IL-12 and iNOS and decreased IL-10 and arginase). The T lymphocytes and NK cells from the IL-7/IL-7R α -Fc treatment group had increased IFN γ but reduced IL-10 expression. Our data suggests that IL-7/IL-7R α -Fc effectively promotes the afferent and efferent arms of the immune response for enhanced antitumor activity in lung cancer. Utilization of strategies that promote APC and T cell responsiveness will prove useful for therapeutic development against lung cancer.

Our combined data does not reflect that IL-7/IL-7R α -Fc is only a better IL-7 reagent. Had IL-7/IL-7R α -Fc simply been a better IL-7 reagent, the antitumor activity in comparison to IL-7 would not be so remarkable. We observed that IL-7/IL-7R α -Fc administered once a week for two weeks was more effective at inhibiting tumor growth compared to recombinant IL-7 administered on the same schedule or IL-7 administered daily for the duration of the experiment. We did not detect the chimeric molecule one week following IL-7/IL-7R α -Fc administration. IL-7R on T cells is rapidly downregulated in response to IL-7 or IL-7/IL-7R α -Fc *in vitro*. This suggests that even if the chimeric molecule was present for longer periods it would not improve signaling through the IL-7 receptor. We find that the antitumor activity of IL-7/IL-7R α -Fc molecule is more efficacious than IL-7 alone and IL-7/IL-7R α -Fc offers the advantage of combining the immune enhancing activities of IL-7 with the IgG Fc portion of the molecule for interaction with the Fc γ receptors present on APC cells. Our data indicates that IL-7/IL-7R α -Fc increases tumor macrophages infiltrates characteristic of the M1 phenotype with increased IL-12, iNOS but reduced IL-10 and arginase. Future work is

necessary to delineate the functional role of these macrophages in IL-7/IL-7R α -Fc mediated modulation in antitumor activity.

IL-7R is widely expressed on many leukocytic populations and priming these cell subsets will broadly impact immune responses that include both activating and suppressive activity. IL-7/IL-7R α -Fc treatment of tumor bearing mice did not alter the frequency and activity of Tregs compared to IL-7 administered daily treatment group (data not shown). Although we did not see any visible signs of autoimmunity, in future studies we will investigate if antibodies are generated in response to IL-7/IL-7R α -Fc therapy and their role on autoimmunity. In the current study, we do not know the impact of IL-7/IL-7R α -Fc on total B cell activity or on suppressor B cells. Future studies are needed to address this issue. It is plausible that in comparison to IL-7, IL-7/IL-7R α -Fc is more effective at reducing the cellular suppressor network that translates to a more effective antitumor benefit.

Our findings indicate that IL-7/IL-7R α -Fc provides the cues that address the deficits in the lung tumor microenvironment to achieve the requirements for the inhibition of tumor growth kinetics by: (i) generating sufficient numbers of T cells systemically (ii) increasing the activated T cell infiltrates in the tumor and (iii) activating the innate and immune cells in the tumor to manifest antitumor benefit. Although IL-7/IL-7R α -Fc is potent at reducing tumor growth kinetics, it does not lead to complete tumor eradication. However, the potent antitumor properties of IL-7/IL-7R α -Fc exhibited in the s.c. and orthotopic models await additional evaluation in a model in which tumors arise spontaneously in the lung for the full determination of cellular and molecular networks in the tumor microenvironment that eventually dampen the antitumor activity of IL-7/IL-7R α -Fc.

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References

1. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer*. 2005; 5:263–74. [PubMed: 15776005]
2. Almand B, Resser JR, Lindman B, et al. Clinical significance of defective dendritic cell differentiation in cancer. *Clin Cancer Res*. 2000; 6:1755–66. [PubMed: 10815894]
3. Johnson SK, Kerr KM, Chapman AD, et al. Immune cell infiltrates and prognosis in primary carcinoma of the lung. *Lung Cancer*. 2000; 27:27–35. [PubMed: 10672781]
4. Hiraoka K, Miyamoto M, Cho Y, et al. Concurrent infiltration by CD8+ T cells and CD4+ T cells is a favourable prognostic factor in non-small-cell lung carcinoma. *Br J Cancer*. 2006; 94:275–80. [PubMed: 16421594]
5. Dieu-Nosjean MC, Antoine M, Danel C, et al. Long-term survival for patients with non-small-cell lung cancer with intratumoral lymphoid structures. *J Clin Oncol*. 2008; 26:4410–7. [PubMed: 18802153]
6. Fry TJ, Mackall CL. The many faces of IL-7: from lymphopoiesis to peripheral T cell maintenance. *J Immunol*. 2005; 174:6571–6. [PubMed: 15905493]
7. Fry TJ, Connick E, Falloon J, et al. A potential role for interleukin-7 in T-cell homeostasis. *Blood*. 2001; 97:2983–90. [PubMed: 11342421]
8. Managlia EZ, Landay A, Al-Harthi L. Interleukin-7 signalling is sufficient to phenotypically and functionally prime human CD4 naive T cells. *Immunology*. 2005; 114:322–35. [PubMed: 15720434]

9. Aspinall R, Henson S, Pido-Lopez J, Ngom PT. Interleukin-7: an interleukin for rejuvenating the immune system. *Ann N Y Acad Sci.* 2004; 1019:116–22. [PubMed: 15247003]
10. Freitas AA, Rocha BB. Lymphocyte lifespans: homeostasis, selection and competition. *Immunol Today.* 1993; 14:25–9. [PubMed: 8442858]
11. Fry TJ, Mackall CL. Interleukin-7: from bench to clinic. *Blood.* 2002; 99:3892–904. [PubMed: 12010786]
12. Jicha DL, Mule JJ, Rosenberg SA. Interleukin 7 generates antitumor cytotoxic T lymphocytes against murine sarcomas with efficacy in cellular adoptive immunotherapy. *J Exp Med.* 1991; 174:1511–5. [PubMed: 1744582]
13. Naume B, Espevik T. Effects of IL-7 and IL-2 on highly enriched CD56+ natural killer cells. *J Immunol.* 1991; 147:2208–14. [PubMed: 1717551]
14. Mazzucchelli R, Durum SK. Interleukin-7 receptor expression: intelligent design. *Nat Rev Immunol.* 2007; 7:144–54. [PubMed: 17259970]
15. Bradley LM, Haynes L, Swain SL. IL-7: maintaining T-cell memory and achieving homeostasis. *Trends Immunol.* 2005; 26:172–6. [PubMed: 15745860]
16. Restifo NP, Esquivel F, Kawakami Y, et al. Identification of human cancers deficient in antigen processing. *J Exp Med.* 1993; 177:265–72. [PubMed: 8426105]
17. Huang AYC, Golumbek P, Ahmadzadeh M, Jaffee E, Pardoll D, Levitsky H. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science (New York, NY).* 1994; 264:961–5.
18. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I- restricted CTLs. *Nature.* 1998; 392:86–9. [PubMed: 9510252]
19. Bevan MJ. Antigen presentation to cytotoxic T lymphocytes in vivo. *J Exp Med.* 1995; 182:639–41. [PubMed: 7650475]
20. Staveley-O'Carroll K, Sotomayor E, Montgomery J, et al. Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Proceedings of the National Academy of Sciences of the United States of America.* 1998; 95:1178–83. [PubMed: 9448305]
21. Cuenca A, Cheng F, Wang H, et al. Extra-lymphatic solid tumor growth is not immunologically ignored and results in early induction of antigen-specific T-cell anergy: dominant role of cross-tolerance to tumor antigens. *Cancer Res.* 2003; 63:9007–15. [PubMed: 14695219]
22. Willimsky G, Blankenstein T. Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. *Nature.* 2005; 437:141–6. [PubMed: 16136144]
23. Sotomayor EM, Borrello I, Rattis FM, et al. Cross-presentation of tumor antigens by bone marrow-derived antigen-presenting cells is the dominant mechanism in the induction of T-cell tolerance during B-cell lymphoma progression. *Blood.* 2001; 98:1070–7. [PubMed: 11493453]
24. Huang M, Stolina M, Sharma S, et al. Non-small cell lung cancer cyclooxygenase-2-dependent regulation of cytokine balance in lymphocytes and macrophages: up-regulation of interleukin 10 and down-regulation of interleukin 12 production. *Cancer research.* 1998; 58:1208–16. [PubMed: 9515807]
25. Gerlini G, Tun-Kyi A, Dudli C, Burg G, Pimpinelli N, Nestle FO. Metastatic melanoma secreted IL-10 down-regulates CD1 molecules on dendritic cells in metastatic tumor lesions. *Am J Pathol.* 2004; 165:1853–63. [PubMed: 15579430]
26. Guermonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol.* 2002; 20:621–67. Epub 2001 Oct 4. [PubMed: 11861614]
27. Sica A, Larghi P, Mancino A, et al. Macrophage polarization in tumour progression. *Semin Cancer Biol.* 2008; 18:349–55. Epub 2008 Mar 26. [PubMed: 18467122]
28. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell.* 2006; 124:263–6. [PubMed: 16439202]
29. Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. *Cancer Res.* 2006; 66:605–12. [PubMed: 16423985]
30. Ma J, Liu L, Che G, Yu N, Dai F, You Z. The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time. *Bmc.* 10:112.

31. Mantovani A, Sica A, Locati M. New vistas on macrophage differentiation and activation. *Eur J Immunol.* 2007; 37:14–6. [PubMed: 17183610]
32. Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. *Immunity.* 2005; 23:344–6. [PubMed: 16226499]
33. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 2002; 23:549–55. [PubMed: 12401408]
34. Sinha P, Clements VK, Bunt SK, Albelda SM, Ostrand-Rosenberg S. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. *J Immunol.* 2007; 179:977–83. [PubMed: 17617589]
35. Loetscher M, Gerber B, Loetscher P, et al. Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. *J Exp Med.* 1996; 184:963–9. [PubMed: 9064356]
36. Belperio JA, Keane MP, Burdick MD, et al. Critical role for CXCR3 chemokine biology in the pathogenesis of bronchiolitis obliterans syndrome. *J Immunol.* 2002; 169:1037–49. [PubMed: 12097412]
37. Andersson A, Yang SC, Huang M, et al. IL-7 promotes CXCR3 ligand-dependent T cell antitumor reactivity in lung cancer. *J Immunol.* 2009; 182:6951–8. [PubMed: 19454692]
38. Sharma S, Batra RK, Yang SC, et al. Interleukin-7 Gene-Modified Dendritic Cells Reduce Pulmonary Tumor Burden in Spontaneous Murine Bronchoalveolar Cell Carcinoma. *Human Gene Therapy.* 2003:14.
39. Komschlies KL, Gregorio TA, Gruys ME, Back TC, Faltynek CR, Wiltout RH. Administration of recombinant human IL-7 to mice alters the composition of B-lineage cells and T cell subsets, enhances T cell function and induces regression of established metastases. *The Journal of Immunology.* 1994; 152:5776–84. [PubMed: 8207207]
40. Bellone G, Turletti A, Artusio E, et al. Tumor-associated transforming growth factor-beta and interleukin-10 contribute to a systemic Th2 immune phenotype in pancreatic carcinoma patients. *The American journal of pathology.* 1999; 155:537–47. [PubMed: 10433946]
41. Fajardo LF, Prionas SD, Kwan HH, Kowalski J, Allison AC. Transforming growth factor beta1 induces angiogenesis in vivo with a threshold pattern. *Lab Invest.* 1996; 74:600–8. [PubMed: 8600310]
42. Zhu LX, Sharma S, Stolina M, et al. Delta-9-tetrahydrocannabinol inhibits antitumor immunity by a CB2 receptor-mediated, cytokine-dependent pathway. *J Immunol.* 2000; 165:373–80. [PubMed: 10861074]
43. Hagenbaugh A, Sharma S, Dubinett S, et al. Altered immune responses in IL-10 transgenic mice. *Journal of Experimental Medicine.* 1997; 185:2101–10. [PubMed: 9182682]
44. Hu HM, Urba WJ, Fox BA. Gene-modified tumor vaccine with therapeutic potential shifts tumor-specific T cell response from a type 2 to a type 1 cytokine profile. *J Immunol.* 1998; 161:3033–41. [PubMed: 9743368]
45. Trinchieri G. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv Immunol.* 1998; 70:83. [PubMed: 9755338]
46. Johnson LL, Sayles P. Interleukin-12, dendritic cells and the initiation of of host -protective mechanisms against *Toxoplasma gondii*. *J Exp Med.* 1997; 186:1799. [PubMed: 9417472]
47. Ma X, Amezaga AM, Gri G, Gerosa F, Trinchieri G. Immunomodulatory functions and molecular regulations of IL-12. *Chem Immunol.* 1997; 68:1. [PubMed: 9329213]
48. Voest EE, Kenyon BM, O'Reilly MS, Truitt G, D'Amato RJ, Folkman J. Inhibition of angiogenesis in vivo by interleukin 12. *J Natl Cancer Inst.* 1995; 87:581–6. [PubMed: 7538593]
49. Brunda MJ, Luistro L, Warriar RR, et al. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J Exp Med.* 1993; 178:1223–30. [PubMed: 8104230]
50. Luster AD, Leder P. IP-10, a CXC chemokine, elicits a potent thymus-dependent anti-tumor response in vivo. *J Exp Med.* 1993; 178:1057–65. [PubMed: 8350046]
51. Arenberg DA, Kunkel SL, Polverini PJ, et al. Interferon-gamma-inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non-small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases. *J Exp Med.* 1996; 184:981–92. [PubMed: 9064358]

52. Sgadari C, Farber JM, Angiolillo AL, et al. Mig, the monokine induced by interferon-gamma, promotes tumor necrosis in vivo. *Blood*. 1997; 89:2635–43. [PubMed: 9108380]
53. Tannenbaum CS, Tubbs R, Armstrong D, Finke JH, Bukowski RM, Hamilton TA. The CXC chemokines IP-10 and Mig are necessary for IL-12-mediated regression of the mouse RENCA tumor. *J Immunol*. 1998; 161:927–32. [PubMed: 9670971]
54. Strieter RM, Kunkel SL, Arenberg DA, Burdick MD, Polverini PJ. Interferon gamma-inducible protein 10 (IP-10), a member of the C-X-C chemokine family, is an inhibitor of angiogenesis. *Biochemical and biophysical research communications*. 1995; 210:51–7. [PubMed: 7537965]
55. Sharma S, Yang SC, Hillinger S, et al. SLC/CCL21-mediated anti-tumor responses require IFNgamma, MIG/CXCL9 and IP-10/CXCL10. *Molecular cancer*. 2003; 2:22. [PubMed: 12740040]
56. Yang SC, Hillinger S, Riedl K, et al. Intratumoral administration of dendritic cells overexpressing CCL21 generates systemic antitumor responses and confers tumor immunity. *Clin Cancer Res*. 2004; 10:2891–901. [PubMed: 15102698]

Figure 1.

IL-7/IL-7Rα-Fc inhibits tumor growth, increases CD4, CD8 T cells subsets, NK cells and augments activated and memory T cells in lung cancer. **A**, C57BL/6 mice bearing 7-day established 3LL tumors were treated with Diluent, IL-7 (daily), IL-7 (weekly), IL-7/IL-7Rα-Fc and IL-7Rα-Fc via *i.p.* injections. In comparison to controls and IL-7 treatment, IL-7/IL-7Rα-Fc was the most effective at inhibiting tumor growth. **B–C**, Single cell suspension of the tumors were evaluated for CD8 T cell activation markers (**B**) and tumor lysates were evaluated for cytokine levels (**C**). IL-7/IL-7Rα-Fc treatment enhanced levels of IFNγ, CXCL9, CXCL10, IL-12, but reduced IL-10 and TGFβ in the tumors. **D–F**, Single cell suspensions of spleen cells were evaluated for the frequencies of CD4 T, CD8 T, NK cell (**D**), and CD8 T cell activation (**E**) and memory (**F**) phenotype. In comparison to IL-7 and controls, IL-7/IL-7Rα-Fc was the most effective in increasing the frequency of CD4, CD8 and NK cells and inducing CD8 T cells with the activation markers CD69 and CD127^{low} (**E**) and the effector memory (CD8/CD44/CCR7⁺) phenotype (**F**) in tumor bearing hosts. **G**, Enhanced cytotoxic activity was observed in purified splenic T cells from the IL-7/IL-7Rα-Fc treated mice against parental 3LL tumors *in vitro* (E:T of 20:1 and 60:1) but not against B16 control (data not shown). **H**, IL-7/IL-7Rα-Fc treatment generates enhanced levels of IFNγ, CXCL9, CXCL10 and IL-12 and reduced levels of IL-10 and TGFβ systemically. Data are representative of 2–3 independent experiments. Graphs, Mean ± SEM, p values: IL-7/IL-7Rα-Fc compared to the other treatment groups * p<0.01, n=8 mice/group.

Figure 2.

IL-7/IL-7R α -Fc treatment of tumor bearing mice increases the ability of splenic APC to process and present antigens to CD8T cell line. 10^4 3LL tumor cells were injected in the left lung by the transthoracic route in the UBC-GFPBL/6 mice. 3LL tumor bearing mice were treated with: Diluent, IL-7, IL-7/IL-7R α -Fc and IL-7R α -Fc on days 7 and 14 and splenic APC activity evaluated (day 21). **A**, Photomicroscopy of the cells (400X Mag.) show that APC (green) from IL-7/IL-7R α -Fc treated group was more effective at attracting, sequestering and activating OVA specific CD8 T cells (non green) than the control treated groups. **B**, APC from IL-7/IL-7R α -Fc treated tumor bearing mice were more effective at stimulating and activating the OVA specific CD8 T cells to secrete IL-2. **C–D**, *In vitro*, IL-7/IL-7R α -Fc stimulation enhanced splenic APC activity that was abrogated in the FcR γ KO mice. Purified splenic APC (naïve, WT and FcR γ KO) were stimulated with Diluent, IL-7, IL-7/IL-7R α -Fc, or IL-7R α -Fc for 6hr, washed, and then co-cultured with OVA and the OVA specific CD8 T cells. IL-2 secreted by CD8 T cells in the culture medium was quantified by ELISA. **E**, *In vitro*, IL-7/IL-7R α -Fc stimulation enhanced TNF α release from splenic APC that was abrogated in the FcR γ KO mice. TNF α in the APC culture supernatants was determined by ELISA. Data are representative of 2–3 independent experiments. Graphs, Mean $\pm$ SEM, p values: IL-7/IL-7R α -Fc compared to the other treatment groups, * $p < 0.005$, $n = 6–8$ mice per group.

Figure 3.

IL-7/IL-7Rα-Fc treatment augments IFN γ , CXCL9 and CXCL10 cytokines *in vitro*. **A**, 2.5×10^6 c/ml mouse spleen cells were incubated with: Diluent, IL-7, IL-7/IL-7Rα-Fc, and IL-7Rα-Fc for 72 hours and cytokines were measured in culture supernatants. IL-7/IL-7Rα-Fc was more effective than IL-7 in stimulating IFN γ , CXCL9 and CXCL10. Graphs, Mean $\pm$ SEM, p values: IL-7/IL-7Rα-Fc compared to the other treatment groups * $p < 0.005$ $n = 4$ mice/group. **B**, IL-7/IL-7Rα-Fc was more effective than IL-7 in stimulating IFN γ production from human PBMC. 2.5×10^6 /ml human PBMC were incubated with: Diluent, IL-7, IL-7/IL-7Rα-Fc, and IL-7Rα-Fc for 72 hours. IFN γ secreted in the culture medium was quantified. Data are representative of 2–3 independent experiments. Graphs, Mean $\pm$ SEM, p values: IL-7/IL-7Rα-Fc compared to controls, * $p < 0.001$

Figure 4.

CXCR3 pathway is required for IL-7/IL-7Rα-Fc mediated antitumor activity. For *in vivo* neutralizations, mice bearing 5-day established subcutaneous tumors were treated with IL-7/IL-7Rα-Fc on days 5 and 10. Twenty four hours prior to IL-7/IL-7Rα-Fc treatment, and then three times per week, mice were injected *i.p.* individually with the respective specific or appropriate control antibodies for the duration of the experiment. **A–B**, Neutralization of CXCL9 (**A**) or CXCL10 (**B**) or IFNγ (**B**) reversed the anti tumor benefit of IL-7/IL-7Rα-Fc [lower panels, photograph of tumors from diluents, IL-7/IL-7Rα-Fc and IL-7/IL-7Rα-Fc + anti-CXCL9, or anti-CXCL10 or anti-IFNγ (IL-7/IL-7Rα-Fc + control Ab was the same as IL-7/IL-7Rα-Fc treated group, data not shown)]. **C**, CXCL9, CXCL10 and IFNγ were reduced in tumors (day 15) following treatment of the tumor bearing mice with IL-7/IL-7Rα-Fc and the respective neutralizing antibodies: anti-CXCL9, or anti-CXCL10 or anti-IFNγ-antibody in comparison to control Abs. **D**, Neutralization of CXCL9, CXCL10 or IFNγ reduced the frequency of CXCR3 activated CD8 T cells in the tumor (day 15). Data, Mean ± SEM, * $p < 0.01$ for IL-7/IL-7Rα-Fc + control Ab compared to IL-7/IL-7Rα-Fc + anti-cytokine Ab or Diluent groups, (n=6/group).

Figure 5.

IL-7/IL-7R α -Fc inhibited orthotopic lung cancer tumor growth and prolonged survival. 10^4 3LL-GFP tumor cells were injected in the left lung. **A–C**, Prior to therapy mice bearing 7-day tumors were evaluated for lung tumor burden [H&E staining (**A**) and flow cytometry (**B**)] and tumor macrophage intracytoplasmic signature (**C**). Multiple tumor satellites were evident in the H&E stained sections from the 7-day old tumors and macrophages had reduced intracytoplasmic IL-12 and increased IL-10 and arginase expression compared to naïve. There were no changes in the frequency of CD4, CD8, NK and macrophages in the lung tumors between the day 7 and naïve (data not shown). **D–K**, Mice bearing one week established tumors were treated with IL-7/IL-7R α -Fc or controls (days 7 and 14) via *i.p.* injections. H&E staining of lung tumor sections from control-treated groups evidenced large tumor masses (**D:1**) without detectable leukocytic infiltration (**D:3**) as compared to IL-7/IL-7R α -Fc (**D:2 and D:4**). Compared to controls there was a marked decrease in tumor burden (solid arrow) in the lungs from IL-7/IL-7R α -Fc treated mice (**D:2**). **E**, Percentage of tumor cells was reduced in total lung digest from IL-7/IL-7R α -Fc treated groups compared to controls as analyzed by flow cytometry. **F**, IL-7/IL-7R α -Fc enhanced survival (67.5 days) as compared to control (26 days). **D:3–D:6 and G–H**, IL-7/IL-7R α -Fc treatment mediates increased: leukocytic infiltrates (**D:4, D:6 and G**), Macrophages with M1 phenotype (**H**) with increased IL-12, iNOS and decreased IL-10 and arginase expression as compared to control. **I–J**, CD4 and CD8 T lymphocytes had elevated IFN γ and reduced IL-10 and NK cells (**K**) have increased IFN γ expression after IL-7/IL-7R α -Fc treatment as compared to controls. **D:5–D:6**, Lung tumor section immune staining showed elevated levels of macrophages in the tumors of IL-7/IL-7R α -Fc (**D:6**) compared to control (**D:5**). For all data in this panel, results for IL-7 (same treatment schedule as IL-7/IL-7R α -Fc) and IL-7R α -Fc treatment groups were synonymous with diluent-treated group. **L**, IL-7/IL-7R α -Fc induces macrophages with M1 phenotype signature *in vitro* as compared to controls. Graphs, Mean $\pm$

SEM, (n=6 mice/group), *p<0.05 between the IL-7/IL-7R α -Fc and control group.
Percentage survival (n=10 mice/group), p<0.0001 between IL-7/IL-7R α -Fc and control.