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IL-2 REGULATES IL-24 PROTEIN EXPRESSION

LEADING TO GROWTH SUPPRESSION OF MELANOMA

by

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IL-2 REGULATES IL-24 PROTEIN EXPRESSION

LEADING TO GROWTH SUPPRESSION OF MELANOMA

A

DISSERTATION

Presented to the Faculty of

The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Emily Yea-Chian Jen, B.S.

Houston, Texas

May 2010

Dedication

For my mother

Everything I am and everything I will be is because of you.

Thank you for the years of support and encouragement. There is no way to repay you for all you have done for me.

Acknowledgments

I would like to thank Dr. Elizabeth Grimm for her continual support and assistance throughout graduate school. She is a wonderful role model both as a mentor and as a scientist. Thank you to Dr. Nancy Poindexter for her guidance, for teaching and encouraging me, and for getting me started in the right direction. I would also like to thank Dr. Russell Broaddus and Dr. Victoria Knutson for serving on all of my committees and for making time to chat about science and anything else that came to mind, and Dr. Alexander Lazar for all of his suggestions and for trying to get me to draw – I know it will come in handy. Thank you to Dr. Suhendan Ekmekcioglu for her friendship and for the advice, work-related or otherwise. Thanks to Dr. Julie Ellerhorst for all the help and scientific discussions. Thanks also to Deon Uffort, Dr. Vicky Greene, Dr. Elizabeth Farnsworth, Dr. Yong Qin, Dr. Chandrani Chattopadhyay, and Abdul Gabisi for all of your support, and for keeping me fed and caffeinated, making sure I made it to the end mostly in one piece. And, thanks to Sandra Kinney, Marilyn Johnson, and Carolyn Cooke for all the hugs. Many thanks to Alcena Doxley and Pearl Coleman for helping me with all the things that had to be done along the way. I am grateful for the National Institutes of Health Center for Clinical and Translational Science (CCTS) T32 Training Award which supported me through part of my work.

IL-2 REGULATES IL-24 PROTEIN EXPRESSION

LEADING TO GROWTH SUPPRESSION OF MELANOMA

Publication No._____

Emily Yea-Chian Jen

Supervisory Professor: Elizabeth A. Grimm, Ph.D.

(350 word limit)

Melanoma is known to be highly resistant to chemotherapy. Treatment with high dose IL-2 has shown significant clinical benefit in a minority of metastatic melanoma patients and has lead to long term survival in a few cases. However, this treatment is associated with excessive multiorgan toxicities, which severely limits its use. We hypothesize that one mechanism of effective IL-2 therapy is through the direct upregulation of IL-24 production in melanoma tumors and subsequent IL-24 mediated tumor growth suppression.

Five melanoma cell lines were treated with high dose recombinant hIL-2 at 1000U/ml. Three of the cell lines (A375, WM1341, WM793) showed statistically significant increases in their levels of IL-24 protein when measured by Western blotting, while the remaining two lines (WM35, MeWo) remained negative for IL-24 message and protein. This increase in IL-24 was abolished by either preincubating with an anti-IL-2 antibody or by blocking the IL-2 receptor directly with antibodies against the receptor chains. We also demonstrated by ELISA that these three cell lines secrete IL-24 protein

in higher amounts when stimulated with IL-2 than do untreated cells. These cells were found to contain IL-2R beta and gamma message by RT-PCR and also expressed higher levels of IL-24 when treated with IL-15, which shares the IL-2R beta chain. Thus we propose that IL-2 is signaling through IL-2R beta on some melanoma cells to upregulate IL-24 protein expression. To address the biological function of IL-2 in melanoma cells, five cell lines were treated with IL-2 and cell viability determined. Cell growth was found to be significantly decreased by day 4 in the IL-24 positive cell lines while no effect on growth was seen in WM35 or MeWo. Incubating the cells with anti-IL-24 antibody or transfecting with IL-24 siRNA effectively negated the growth suppression seen with IL-2. These data support our hypothesis that in addition to its immunotherapeutic effects, IL-2 also acts directly on some melanoma tumors and that the IL-24 and IL-2R beta status of a tumor may be useful in predicting patient response to high dose IL-2.

Table of Contents

i. Approval

ii. Title

iii. Dedication

iv. Acknowledgements

v. Abstract

x. List of Illustrations

xii. List of Tables

xiii. Abbreviations

Introduction

Skin Cancer and Melanoma ……...…… 1

Melanoma and Immunity ……...…… 5

Interleukin-2 and the IL-2 Receptor Complex ……. 5

High Dose Interleukin-2 (IL-2) as an Immunotherapy for

Metastatic Melanoma ……...… 8

Interleukin-24/melanoma differentiation associated gene-7 ……...…… 9

Preliminary data and rationale for hypothesis ……...……... 13

Materials and Methods

Cell lines and cell culture ……. 15

Charcoal stripped fetal bovine serum ……...……. 15

Reagents ……..16

Whole cell extracts and Western blotting analysis ……..…..….. 16

Detection of secreted IL-24 protein …….. 17

RNA isolation ……. 18

Real Time Quantitative – RT-PCR Probe based assay ……. 18

Growth suppression assay ……. 21

siRNA assay ……... 23

Statistical analysis ……..23

Results

IL-24 message is present in some melanoma cells ……... 24

Basal IL-24 levels in melanoma cells are affected by serum components ... 27

IL-2 upregulates IL-24 protein production and secretion in 3/5

melanoma cell lines ……... 29

Melanoma cells have IL-2R mRNA ……... 35

IL-15 upregulates IL-24 expression in melanoma cell lines ……... 39

IL-2 causes growth suppression in some melanoma cells ……. 41

IL-2 mediated growth suppression is dependent on IL-24 …….. 42

IL-15 causes growth suppression in A375 cells …….. 49

Serum levels of IL-24 in high dose IL-2 patients …….. 51

Discussion

IL-2 upregulates IL-24 protein expression leading to growth

suppression in melanoma cells …….. 53

Additional support for IL-24 as a predictive marker for response to IL-2 ... 61

IL-2Rβ or IL-15Rα coexistence with IL-24 as a predictive marker for response to IL-2 therapy……..... 63

Support for IL-15 as therapy for metastatic melanoma …….……. 64

Mechanism of IL-2 dependent IL-24 Upregulation in Melanoma Cell Lines ……..…… 65

Conclusions …….. 66

Reference List …… 68

Vita …….……. 100

List of Illustrations

1. Figure 1. Increased incidence of melanoma in individuals >45 years of age. 3
2. Figure 2. IL-2 receptor signaling 7
3. Figure 3. IL-24 associated pathways in cancer cell apoptosis 12
4. Figure 4. Some melanoma cell lines have message for IL-24 26
5. Figure 5. Charcoal stripped FBS media decreases background levels of IL-24 28
6. Figure 6. High dose IL-2 upregulates IL-24 expression in some melanoma cells 32
7. Figure 7. IL-24 is secreted by some melanoma cells 33
8. Figure 8. IL-2 upregulation of IL-24 can be neutralized with anti-IL-2 antibody 34
9. Figure 9. IL-2Rβ and IL-2Rγ chain mRNA is present in melanoma cell lines 37
10. Figure 10. IL-2 mediated IL-24 production is abolished by blocking the IL-2R 38
11. Figure 11. IL-15 upregulates IL-24 expression in some melanoma cells 40
12. Figure 12. Effects of IL-2 on melanoma cell growth by Trypan blue assay 44-47
13. Figure 13. IL-24 siRNA effectively knocks down IL-24 mRNA in melanoma cell lines 48

14. Figure 14. IL-15 causes growth suppression in A375 cells by day 4 50

15. Figure 15. Proposed novel mechanism for IL-2 direct effects on melanoma: IL-24 dependent IL-2 mediated growth suppression in melanoma cells 55

List of Tables

1. Table 1 – Simplified AJCC 2009 Melanoma Staging and Classification 4

2. Table 2 – IL-24 and IL-2R chain PCR primers and efficiency 22

3. Table 3 – Human melanoma cell line descriptions 25

Abbreviations

ADCC	Antibody dependent cell mediated cytotoxicity
BSA	Bovine Serum Albumin
CSFBS	Charcoal stripped fetal bovine serum
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluoresence activated cell sorting
FBS	Fetal Bovine Serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse Radish Peroxidase
Ig	Immunoglobulin
IL-x	Interleukin-x
IL-2R	Interleukin-2 receptor
LAK	Lymphokine activated killer
LPS	Lipopolysaccharide
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PHA	Phytohemagglutinin
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
siRNA	Short-interfering RNA

Introduction

Skin Cancer and Melanoma

Skin cancer is the most common type of all cancers, and melanoma is its deadliest form. Although melanoma accounts for less than 5 percent of total skin cancers, it caused about 75 percent of all skin cancer deaths last year. While the incidence of many other prevalent cancers has decreased in recent years, the incidence of malignant melanoma is steadily rising (Figure 1) [1, 2]. The number of new melanoma cases each year is increasing faster than many other cancers in the United States, and in Australia new cases of melanoma exceeded new cases of lung cancer for the first time in 2008. Although it receives little attention, melanoma is a very serious cancer problem. There were an estimated 68,720 new cases of melanoma (39,080 men and 29,640 women) and more than 8,650 melanoma deaths in the United States in 2009 [3] (NCI SEER Cancer Statistics Review, 2009). According to the American Cancer Society, the mortality rate for melanoma has increased by close to 50 percent since the early 1970s, and while surgical resection can be curative in the very early stages of disease, melanoma demonstrates extremely aggressive growth characteristics. The prognosis for patients with metastatic melanoma remains extremely poor. Median survival for stage III melanoma patients is around 24 months. Stage IV patient survival ranges from 6 to 8 months from the time of diagnosis, with 5-year survival rates of less than 4 percent [4, 5]. Because melanoma can strike early in life, it ranks among the top 5 malignancies in terms of years of potential life lost. Between 2002-2006, the median age at diagnosis for melanoma was 59 years of age, and greater than 40 percent of the cases diagnosed were in patients younger than 55. Melanoma is the most commonly occurring cancer in women aged

25-29 years, and it is second only to breast cancer in women aged 30-34 years [6]. Median age at death for melanoma between 2002-2006 was 68 years of age.

Cutaneous melanomas develop from melanocytes, the cells responsible for generating melanin in the skin. Melanocytes originate from multipotent neural crest cells, which give rise to numerous types of cells including neurons and glial cells, among others [7, 8]. During development, melanocyte precursors migrate from the neural crest to several locations within the body including the basal layer of the skin, hair, inner ear, uveal tract in the eye, and leptomeninges in the brain [9]. Any of these locations can become the site of a primary tumor. When melanoma metastasizes, the most common site is a lymph node closest to the primary tumor. Other common sites of metastasis include the skin, lungs, liver, brain, and bone. In patients with Stage I and II melanoma (local disease), significant beneficial prognostic factors include female sex, age >65 years, lesions on the extremities, tumor thickness of <0.1mm, and lack of ulceration. The most recent American Joint Committee on Cancer findings add mitotic rate (defined histologically as the number of mitoses/mm^2) as a prognostic factor (American Joint Committee on Cancer: AJCC Cancer Staging Manual, Seventh Edition;[10]). In Stage III disease (regional metastasis), smaller tumor burden and fewer involved lymph nodes were also significant beneficial factors.

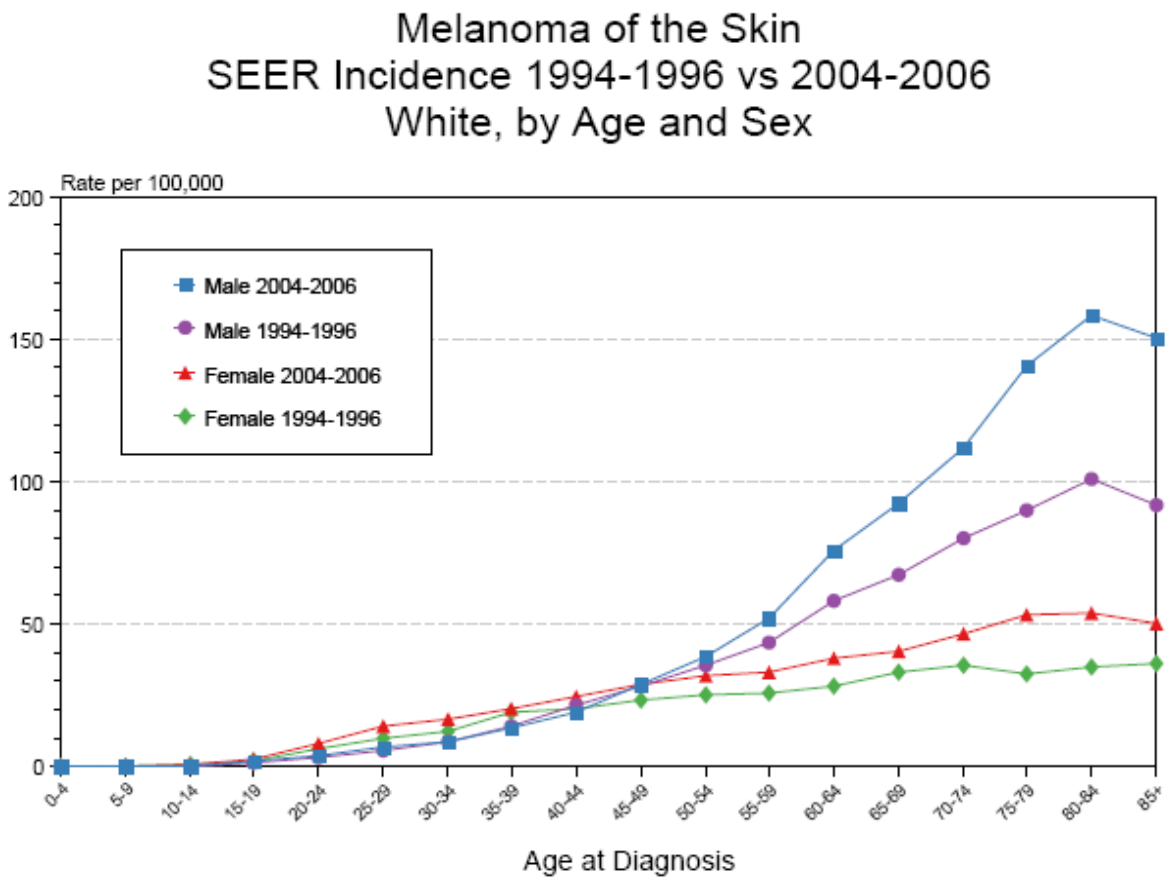

Figure 1. Increased incidence of melanoma in individuals >45 years of age. Comparison of three-year melanoma incidence between 1994 – 1996 and 2004 – 2006 grouped by gender. SEER Cancer Statistics Review 1975 – 2006, National Cancer Institute, 2008. Downloaded 04/2010 from
http://seer.cancer.gov/csr/1975_2007/browse_csr.php?section=16&page=sect_16_zfig.03.html

Table 1. Simplified AJCC 2009 Melanoma Staging and Classification

I	Primary 1.0 mm or less with or without ulceration and with or without mitoses $\geq 1/mm^2$, or primary 1.01 - 2.0 mm without ulceration, no lymph node or distant metastases
II	Primary 1.01 - 2.0 mm with ulceration, or primary > 2.01 mm with or without ulceration, no lymph node or distant metastases
III	Lymph node metastasis, or in-transit metastases/satellites, no distant metastases
IV	Distant metastases

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Melanoma and Immunity

Melanoma is notoriously resistant to both radiation therapy and cytotoxic chemotherapies. Treatment modalities for metastatic melanoma have included surgery, radiotherapy, chemotherapy, biologic therapy, hyperthermia limb perfusion, and intralesional immunotherapy. Treatment with chemotherapy, either alone or in combination with other chemotherapies, has resulted largely in only partial or nondurable responses in metastatic disease [11]. Throughout the course of treating patients, clinicians observed some interesting characteristics of the disease including spontaneous regression of primary lesions [12, 13] as well as disseminated disease, the presence of T cell infiltrates in early lesions, and the existence of a shared melanoma differentiation antigen in melanoma and vitiligo, an autoimmune skin disorder [14-16]. In fact, the presence of *de novo* vitiligo in patients with melanoma correlated with a better prognosis and response to immunotherapeutic tumor treatment; this was also true if patients had other coexisting autoimmune states such as autoimmune thyroid dysfunction [17-20]. This clinically evident relationship between the natural history of melanoma and immunity led to the suggestion that combining traditional chemotherapy with cytokine immunotherapy might yield cumulative immune and direct antitumor effects.

Interleukin-2 and the IL-2 receptor complex

Cytokines are protein molecules that are involved in regulating the immune response. Interleukin-2 was originally called T-cell growth factor based on its capacity to promote long-term proliferation of T cells grown in culture [21]. IL-2 is a member of a family of cytokines that includes IL-4, IL-7, IL-9, IL-15, and IL-21. It mediates its effects by signaling

through the IL-2 receptor complex (Figure 2). This complex consists of three chains – the IL-2Rα chain (CD25) which is specific for IL-2, the IL-2Rβ chain (CD122), and the common γ chain (CD132, γc), which is shared by all members of this family of cytokines. IL-2Rα serves to stabilize the interaction between IL-2 and its receptor but is not involved in signal transduction. All three chains combine to form a high affinity receptor complex, while IL-2Rβ and IL-2Rγ together form an intermediate affinity receptor. Binding of IL-2 to its receptor activates the Ras/MAPK, JAK/Stat, and PI 3-kinase/Akt signaling pathways. IL-2 is typically produced by the body during an immune response to foreign molecules or microbes [22, 23]. Foreign antigens bind to T cell receptors triggering the secretion of IL-2 and the expression of IL-2 receptor. IL-2 interaction with its receptor stimulates growth, differentiation and survival of antigen specific cytotoxic T cells by activating expression of specific genes [24, 25]. Therefore, IL-2 is essential for the development of T cell immunologic memory. IL-2 is also important during the development of T cells in the thymus; it is involved in the maturation of a subset of T cells called regulatory T cells or T-regs [26, 27]. After leaving the thymus, T-regs prevent other T cells from recognizing and responding to self antigens and leading to an autoimmune state. T-regs accomplish this by preventing other T cells from producing IL-2. Consequently, IL-2 is required to discriminate between self and non-self.

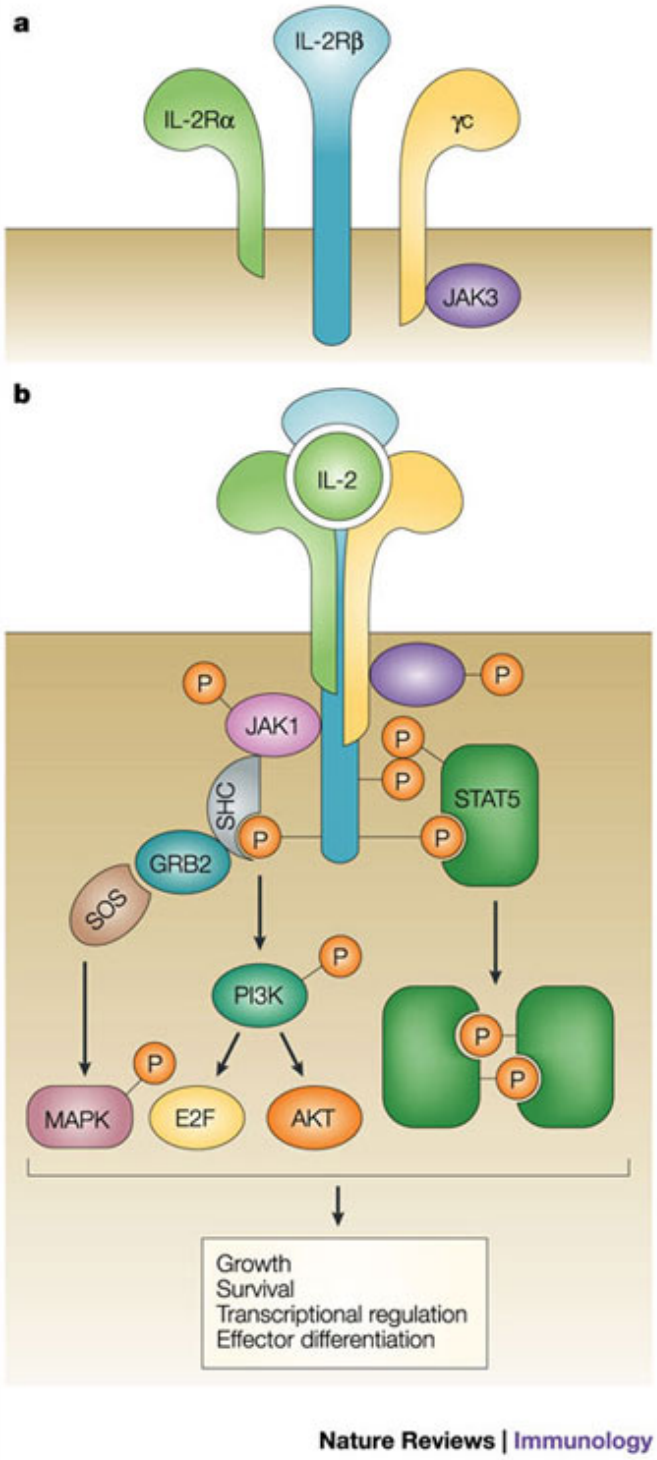


Figure 2. IL-2 receptor signaling. A. The high affinity IL-2 receptor is made up of three subunits - α, β, and γ. B. Binding of IL-2 to its receptor leads to signal transduction via Ras/MAPK, JAK/Stat and PI 3-kinase/Akt. Malek *et al*. Nature Reviews Immunology, 4, 665-674, Sept. 2004. Included with permission to reprint from Nature Publishing Group, License Number 2416781462303.

High Dose IL-2 as Immunotherapy for Metastatic Melanoma

The most promising adjuvant immunotherapy results in metastatic melanoma patients thus far have been observed with high dose bolus recombinant human IL-2 treatment, which has been shown to have significant clinical benefits and produce long term survival in a minority of patients [28-32]. IL-2 is a major regulator of the immune system, and is therefore a good contender to stimulate the immune system against cancer. It has also been shown to be effect in some patients with renal cell carcinoma [33-35]**.** Objective responses have been observed in 15% to 20% of melanoma patients, with 6% to 8% of patients achieving complete durable responses [36, 37]. Approximately 60% of patients who attain complete remission survive for more than 10 years. High dose IL-2 therapy is currently being utilized here at M. D. Anderson Cancer Center both alone and in combination with various chemotherapeutic regiments to treat patients with advanced metastatic disease. In initial trials, high dose IL-2 was given intravenously at 600,000 to 720,000 IU/kg every eight hours on days 1 to 5 and 15 to 19 with a maximum of 28 doses per course [38, 39]. IL-2 antitumor activity is thought to be mediated by activation of natural killer cells (NK) to lymphokine activated killer cells (LAK) [40-42], in essence enhancing the patient's immune system activity against melanoma.

However, high dose IL-2 therapy is associated with capillary leak syndrome (also commonly called vascular leak syndrome) resulting in severe vascular hypotension and extensive multiorgan toxicity, including renal, hepatic, and cardiac [43-47]. It also causes arrhythmias and fluid accumulation in the lungs. These highly toxic side effects severely limit its use to patients with healthier organ function and performance status, and thus it remains an obstacle to widespread treatment with IL-2. Only 6-8 % of patients benefit from

this treatment and we cannot predict who they are prior to applying a costly and toxic therapy. In addition, hospitalization is required for the duration of treatment, and because of the specialized monitoring and support services necessary to administer high dose IL-2 therapy this treatment is only offered at a limited number of centers. As of yet, there is no reliable marker to identify the melanoma patients who are most likely to respond positively to IL-2 therapy. Identification of such a marker is essential in order to effectively treat these patients.

Interleukin-24/melanoma differentiation associated gene-7

IL-24, initially called melanoma differentiation associated gene-7 (mda-7), is a unique multifunctional cytokine belonging to the IL-10 family [48, 49] The mda-7 gene was identified by subtraction hybridization from a melanoma cell line that was induced to differentiate with IFN-β and then stabilized by mezerin [50]. IL-24 was initially thought to be a transcription factor because it appeared to be localized in the nucleus [51]. However since sequence analysis showed a limited homology to IL-10, and IL-24 functions in an immune regulatory loop by stimulating monocyte production of a pro-Th-1 cytokine cascade (49), IL-24 was ultimately classified as a Th1 cytokine. IL-24 receptors were shown to consist of the class II heterodimeric receptors of IL-22R1/IL-20R2 and IL-20R1/IL-20R2 [52]. These receptors have been shown to be expressed primarily in the skin where they are present on keratinocytes [53, 54], but can also be found on endothelial cells, lung, testis, ovarian cells, as well as some cancer cells including melanoma [55]. In normal cells, endogenous IL-24 expression is limited to immune cells, including monocytes and stimulated T-cells, and skin cells including keratinocytes and melanocytes [48, 56-59]. Our laboratory

has shown that IL-24 protein is expressed by nevi and primary melanoma tumors. This protein expression is subsequently lost during melanoma tumor invasion and progression and is often undetectable in advanced metastatic disease [60-62]. IL-24 has also been shown to cause growth suppression and apoptosis specifically in tumor cells both *in vitro* and *in vivo*, including lung, pancreatic, and breast cancer cells, while demonstrating no apparent toxicity to normal cells [63-65] (Figure 3). Data from our laboratory further demonstrates the cytotoxicity of IL-24 to human melanoma cell lines. These data are all consistent with the tumor suppressor functions attributed to IL-24 [66]. A replication incompetent adenovirus expressing the mda-7 gene construct (Ad.*mda-7*; INGN 241) has been used in many studies to further elucidate the biological effects of IL-24. IL-24 has been observed *in vitro* and *in vivo* to possess certain anti-angiogenic properties [67, 68]. Human lung tumors treated with adenoviral *mda-7* had decreased tumor vascularization when compared to control tumors [64]. IL-24 protein has been shown to selectively inhibit endothelial cell differentiation, exhibiting direct anti-angiogenic actions [69]. In addition, IL-24 secreted by normal human prostate cells treated with Ad.*mda-7* also inhibited the invasion of cancer cells that express the IL-24 receptors [70]. IL-24 causes a G2/M block by increasing the accumulation of melanoma cell lines in S phase in-vitro, as well as having an anti-proliferative affect [71]. It also has been shown in glioma [72], prostate and ovarian carcinoma [73, 74], breast cancer [75], and non-small cell lung cancer [76, 77] that IL-24 generation of reactive oxygen species had a radiosensitization effect on those cells.

Accordingly, IL-24 appears to act as a tumor suppressor in melanoma and other cancers by a variety of mechanisms, and is currently being studied in clinical trials for its potential therapeutic usefulness. In addition to its direct tumor suppressive effects, several

studies confirm a bystander effect of the secreted protein from Ad.*mda-7* gene therapy (55) such that IL-24 secreted from Ad.*mda-7* transfected cells was shown to induce cell death in non-transfected tumor cells. The Ad.*mda-7* gene construct has been used in Phase I/II gene therapy clinical trials [56, 78-80] through intratumoral injection of the Ad.*mda-7* construct [78, 79]. In the phase I/II clinical trials IL-24 was well tolerated and showed considerable apoptotic activity in injected lesions [78, 81-83]. Clinically significant responses including tumor regression were found with repeat injections.

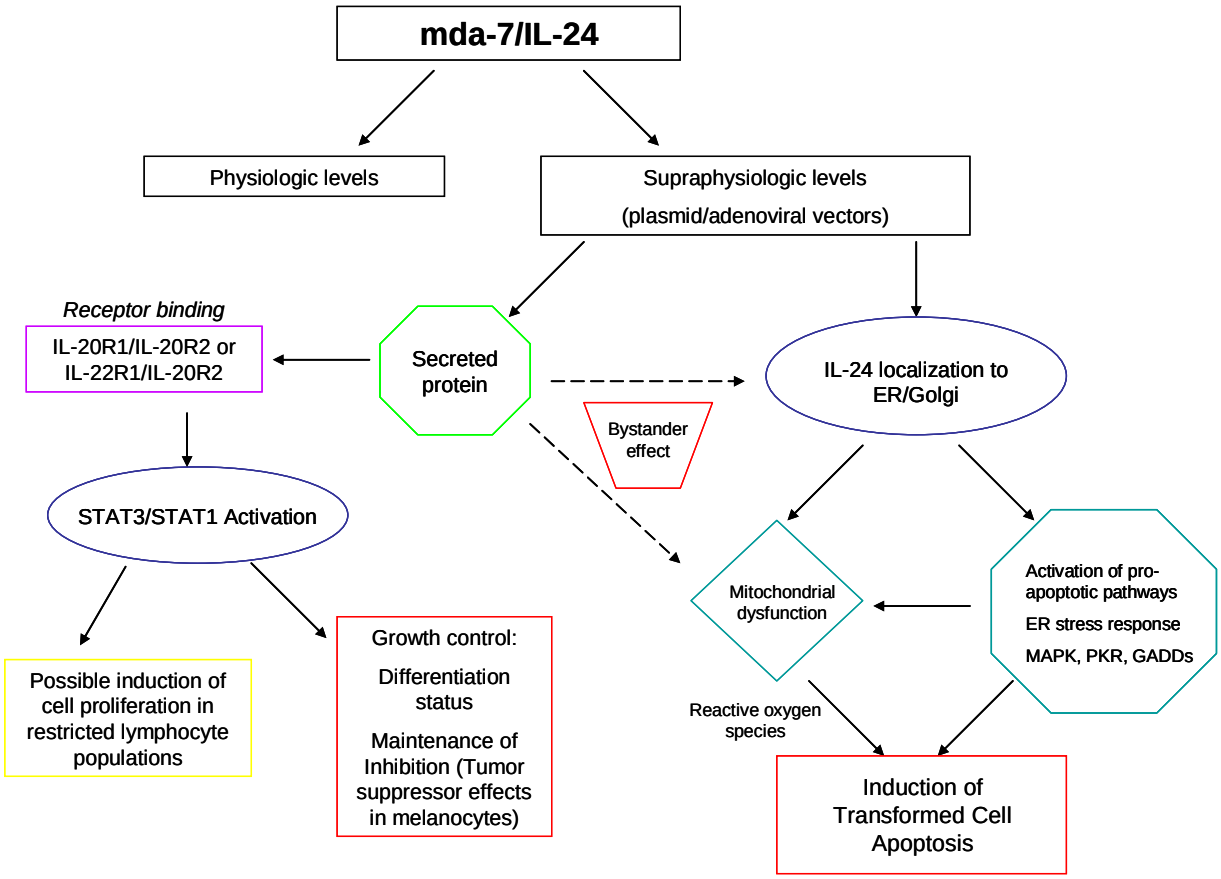


Figure 3. IL-24 associated pathways in cancer cell apoptosis. Summary of signaling pathways related to IL-24 activity.

Preliminary data and Rationale for Hypothesis

Preliminary data from our laboratory revealed by FACS analysis that stimulation of A375, a melanoma cell line, with high doses of IL-2 resulted in endogenous production of IL-24 in a dose dependent manner (data not shown). However, another melanoma cell line, MeWo, did not show any upregulation of IL-24 following IL-2 treatment. Subsequently, three out of five melanoma cell lines analyzed showed an increase in IL-24 protein production and secretion of IL-24 in response to treatment with high dose IL-2. Our laboratory has published *in vitro* data in normal peripheral blood mononuclear cells (PBMC) that show that PHA stimulation results in IL-2 secretion; this appears to stabilize IL-24 mRNA resulting in increased protein production [84]. It is possible that this mechanism also occurs in melanoma cells. These observations seemed to suggest that that there may exist a mechanism by which IL-2 acts directly on the melanoma cells in responsive patients instead of solely mediating its effects through the immune system.

Given these data, we propose that one mechanism by which high dose IL-2 is able to generate a durable response in these patients is through direct activation via the IL-2 receptor on melanoma cells of IL-24 mediated tumor growth suppression or apoptosis. Several articles in the literature report that IL-2 receptor chains are present on the surfaces of both human and murine cutaneous melanoma cells [85-90]. IL-2Rβ in particular has been identified in a number of cancer cell lines including small cell lung cancer and squamous cell carcinoma of the head and neck [91, 92], and melanoma. The mechanisms of IL-24 antitumor growth effects have been extensively studied by several groups. Based on this, we hypothesize that a subset of patients whose melanoma cells express IL-24 and IL-2 receptor will have a better response to IL-2 therapy due to IL-24 mediated growth control, and thus

expression of IL-24 and IL-2R may be useful as potential positive predictive markers to identify patients who are suitable candidates for IL-2 therapy. With this approach to treatment, therapy can then be targeted to the specific patients who will respond, while the patients who are unlikely to gain any benefits from IL-2 therapy will be spared its toxicity and can be directed to alternative treatments. Thus, we seek to improve the quality of life for melanoma patients. We hope that in identifying a potential predictive marker, this project will provide a prognostic test for the therapeutic efficacy of IL-2 therapy in melanoma patients.

The goal of this project was to mimic the conditions present in high dose IL-2 patients during therapy. Those patients receive approximately 600,000-720,000 IU/kg per dose every 8 hours infused intravenously over 15-20 minutes on days 1 to 5 and 15 to 19 with a maximum of 28 doses per course [93, 94]. A study was performed examining different cytokine levels in patients' serum at various time points during high dose IL-2 therapy. It showed that peak IL-2 levels in the serum of these patients averaged around 4000 U/ml and remained well above 1000 U/ml after the 4th dose [95, 96]. Based on these data, we feel confident that 1000U/ml is a reasonable conservative dose at which to test the effects of high dose IL-2 on melanoma cells.

Materials and Methods

Cell lines and cell culture

We obtained two human metastatic melanoma cell lines, A375 and MeWo, from the American Type Culture Collection (Rockville, MD). The WM1341, WM793, and WM35 human primary melanoma cell lines were developed by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA) [97] and were obtained from Dr. Robert Kerbel (University of Toronto, Toronto, Ontario, Canada) [98, 99]. All melanoma cell lines used were maintained in RPMI 1640 medium supplemented with 100 U/mL of penicillin, 100 mg/mL of streptomycin, 2 mM L-glutamine, and HEPES buffer with 4% fetal bovine serum (all purchased from Life Technologies, Inc., Grand Island, NY). Experiments were run in supplemented RPMI with 2% charcoal stripped FBS (description below) unless otherwise indicated.

Charcoal stripped fetal bovine serum

Fetal bovine serum (Life Technologies, Inc) was charcoal stripped to remove endogenous hormones and steroids and reduce any background levels of IL-24 in cell lines grown in serum caused by nonspecific activation from serum components. This was achieved by incubating the serum overnight at 4 degrees C with a mixture of charcoal, dextran, sucrose, magnesium chloride, and HEPES buffer from Sigma-Aldrich (St. Louis, MO) following the manufacture's protocol.

Reagents

IL-2 was obtained from Chiron (formerly Cetus) (Emeryville, CA). IL-2 was used at 1000 U/ml in all experiments described for this publication unless otherwise noted. IL-2 has an initial rapid redistribution phase half-life of approximately 13 minutes followed by a slower elimination phase half-life of 85 minutes in serum. As discussed in the introduction, the goal of this project was to mimic the conditions present in high dose IL-2 patients during therapy. Studies of high dose IL-2 patient serum showed that peak IL-2 levels in the serum averaged around 4000 U/ml and remained well above 1000 U/ml after the 4th dose [95, 96]. Based on these data, we feel confident that 1000U/ml is a reasonable conservative dose at which to test the effects of high dose IL-2 on melanoma cells. IL-15 was purchased from eBioscience (San Diego, CA). 7G11, an anti-IL-24 mouse monoclonal antibody (Introgen Therapeutics, Houston, TX), was used for Western blotting and for blocking IL-24 during growth assays. Mouse anti-human IL-2 monoclonal antibodies were purchased from BD BioSciences (San Jose, CA). Anti-IL-2 receptor (IL-2Rα, IL-2Rβ, and IL-2Rγ) antibodies were purchased from R&D Systems (Minneapolis, MN). Preimmune normal mouse IgG (Sigma-Aldrich, St. Louis, MO) was used as a negative control for receptor blocking studies. Anti-actin antibody from Santa Cruz Biotechnology (Santa Cruz, CA) was used as a standard loading control for all Western blot staining. Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO).

Whole cell extracts and Western blotting analysis

Cells were treated with brefeldin A (Sigma-Aldrich, St. Louis, MO) at 10ug/ml four hours prior to harvesting, washed with cold PBS, and subsequently harvested into PBS. Cell

pellets were then resuspended in protein lysis buffer (140 mM NaCl, 25 mM Tris HCl pH 7.4, and 1% NP-40) with fresh protease inhibitor cocktail (BD Biosciences, San Jose, CA). The supernatants were collected after agitation and protein concentration was measured by Bradford Assay (BioRad). Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane (Amersham-Hybond ECL – GE Healthcare, Piscataway, NJ), and blocked for 1 hour in 5% nonfat milk in PBS. Primary antibody was diluted in 5% nonfat dry milk/PBS/ 0.1% Tween and incubated overnight at 4°C. This was followed by a 45 minute incubation with an appropriate horseradish peroxidase labeled secondary antibody, also diluted in 5% nonfat dry milk/PBS/ 0.1% Tween. Membranes were then developed with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Western blot band protein quantification was performed using densitometry analysis with Scion Image software (Scion Corporation). Differences in protein levels between treatment conditions were determined by normalizing test proteins to beta-actin controls within each sample.

Detection of secreted IL-24 protein

Melanoma cells were plated at 1×10^6 cells/mL and cultured in supplemented RPMI-1640 medium (Invitrogen, Carlsbad, CA) + 2% charcoal stripped FBS with IL-2 added at 1000U/ml. Supernatants were harvested at 24 hours and secreted IL-24 levels were determined with an ELISA test kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's protocol. Reference standards for the ELISA were resuspended in cell culture medium. The amounts of IL-24 were quantified based on a standard curve determined by means of optical density readings measured at 450 nm with a reference of 550 nm on an

ELISA plate reader (Dynex Technologies, Chantilly, VA). The mean values at each time point were then used directly for the analysis reported.

RNA Isolation

RNA extraction from melanoma cells was accomplished using the Qiagen RNAeasy mini kit protocol. Total RNA was extracted from melanoma cells by first lysing and homogenizing the tissue by passing the cells back and forth through a syringe and needle. Ethanol was added to the lysates, and the samples were applied to the RNAeasy spin columns. Total RNA was isolated following the manufacturers protocol (Qiagen, Valencia, CA). DNase I treatment was accomplished by adding RNase-free DNase I (Sigma-Aldrich, St. Louis, MO) to the samples, incubating at 37°C for 30 minutes, and then heating at 75°C for 10 minutes to disable the DNAse I.

Real-Time Quantitative RT-PCR – Probe-based assay

Real-time qPCR assays were performed in the Quantitative Genomics Core Laboratory (QGCL) at The University of Texas Health Sciences Center in Houston, Texas. All real-time qPCR assays used in this publication were designed and validated by QGCL staff to ensure they pass the minimum requirements for efficiency, sensitivity and selectivity. Their protocol [100] follows:

"cDNA was synthesized in 5 µl (384-well plate) total volume by the addition of 3 µl/well RT master mix consisting of: 400 nM assay-specific reverse primer, 500 µM deoxynucleotides, Superscript II buffer and 1 U/µl Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), to a 96-well plate (ISC Bioexpress, Kaysville, UT) or 384-well

plate (Applied Biosystems, Foster City, CA) and followed by a 2 µl volume of sample (25 ng/µl). Each sample was assayed in triplicate plus a control without reverse transcriptase to access DNA contamination levels. Each plate also contained an assay-specific sDNA (synthetic amplicon oligo) standard spanning a 5-log template concentration range and a no template PCR control, also put into RT master mix with reverse transcriptase. Each plate was covered with Biofilm A (Bio-Rad, Hercules, CA) and incubated in a PTC-100 (96) or DYAD (384) thermocycler (Bio-Rad, Hercules, CA) for 30 minutes at 50°C followed by 72˚C for 10 min. PCR master mix, 15µl/well, was added directly to the 5 µl RT volume. Final concentrations for the PCR were 400 nM forward and reverse primers (IDT, Coralville, IA), 100 nM fluorogenic probe (Biosearch Technologies, Novato, CA), 5 mM $MgCl_2$, and 200 µM deoxynucleotides, PCR buffer, 150 nM SuperROX dye (Biosearch Technologies, Novato, CA) and 0.25 U JumpStart Taq polymerase per reaction (Invitrogen, Carlsbad, CA), final concentrations. RT master mixes and all RNA samples and DNA oligo standards were pipetted by a Tecan Genesis RSP 100 robotic workstation (Tecan US, Research Triangle Park, NC); PCR master mixes were pipetted utilizing a Biomek 2000 robotic workstation (Beckman, Fullerton, CA). Each assembled plate was then covered with optically clear film (Applied Biosystems, Foster City, CA) and run in a 7900 real-time instrument using the following cycling conditions: 95°C, 1 min; followed by 40 cycles of 95°C, 12 sec and 60°C, 30 sec. The resulting data were analyzed using SDS 2.3 (7900) software (Applied Biosystems, Foster City, CA) with FAM reporter and ROX as the reference dye.

Synthetic, PAGE purified DNA oligos used as standards (sDNA) encompassed at least the entire 5' – 3' amplicon for the assay (Sigma-Genosys, The Woodlands, TX). Each oligo standard was diluted in 100 ng/µl *E. coli* tRNA-H_2O (Roche Diagnostics, Indianapolis,

IN) and spanned a 5-log range in 10-fold decrements starting at 0.8 pg/reaction. It has been shown for several assays that *in vitro* transcribed RNA amplicon standards (sRNA) and sDNA standards have the same PCR efficiency when the reactions are performed as described above with PCR amplicons of less than 100 bases in length (G.L. Shipley, personal communication).

Due to the inherent inaccuracies in quantifying total RNA by absorbance, the amount of RNA added to an RT-PCR from each sample was more accurately determined by measuring the amount of at least one transcript that was invariant across all samples. The final data were normalized to b-actin. Due to the high abundance of the 3 ribosomal RNAs, a second set of samples diluted 1/500-fold was made in 100 ng/µl *E. coli* tRNA-H_2O for each sample. The final data are presented as the molecules of unknown transcript/molecules of normalizer transcript x 100 and expressed as the percent of the normalizer transcript. Unless otherwise stated, quantities for unknown samples were obtained from an internal standard curve run on each plate.

For Roche UPL probe-based or SYBR Green I dye-based assays, the dCt method was used for data analysis comparing test samples to an appropriate control 'calibrator' sample to determine a fold difference between the calibrator sample and the other unknown samples.

It is necessary to validate the hits using a second assay. Validation is based on Roche UPL hydrolysis probe assays. The determination of PCR efficiency and lowest limit of detection (LOD) was performed using template from a PCR diluted 100-fold into 100 ng/µl *E. coli* tRNA-H_2O. Seven $\log_{10}$ dilutions were made in diluent and a standard curve was produced using standard cycling conditions (see above for Taqman probes). From this curve, a PCR efficiency and LOD were determined."

Growth suppression assays

Cells were plated at 5 x 10^5 cells per well in 24 well plates. Cells were serum starved overnight and then treated with RPMI + 2% charcoal stripped FBS with or without IL-2 at 1000U/ml and other indicated conditions at day 0. Each condition was performed in triplicate. Cells were then trypsinized and collected, and viable cells were counted using a 0.2% Trypan blue dye exclusion assay at days 2 through 6 to assess cell growth. Mean values were determined for each condition and used for analysis.

Table 2. IL-24 and IL-2R chain PCR primers and efficiency

hIL-24 (IL-24) accession #: NM_006850	1484(+) AAGCAGATCCTCAATAAACATTTC 1556(-) ACCAAGGGAAAGGGATGATG 1511(+) FAM-TCCCACCCACACTCGCCAGC-BHQ1 PCR amplicon length =77 bases Lowest limit of detection = 190 copies PCR efficiency = 98%
hIL-2Ra (IL2RA) accession #: NM_000417	1061(+) CTAAATGGTCGCCCAGGAG 1128(-) TGTGATGTGACTTCAGAGCTT 1081(+) FAM-CATCCGTTGTGCTTGCCTGCGT-BHQ1 PCR amplicon length = 90 bases Lowest limit of detection = 160 copies (RT-PCR) PCR efficiency = 99%
hIL-2Rb (IL2RB) accession #: NM_000878	2037(+) CTCCCTCGTTAATCACAGGAT 2124(-) AGGACTGATATTGGTGAATAGCT 2068(+) FAM-TAGGCTCAGAAGCATCGCTCCTCT-BHQ1 PCR amplicon length = 90 bases Lowest limit of detection = 160 copies (RT-PCR) PCR efficiency = 99%
hIL-2Rg (IL2RG) accession #: NM_000206	380(+) CCACCTCTACCAAACATTTGTT 450(-) GCATCTGTGTGGCCTGTC 411(+) FAM-CAGGACCCACGGGAACCCAGG-BHQ1 PCR amplicon length = 80 bases Lowest limit of detection = 180 copies (RT-PCR) PCR efficiency = 99%

siRNA Assay

For these experiments, siGENOME SMARTpool for human IL-24 and siGENOME Non-Targeting siRNA Pool were purchased from Dharmacon, Inc. (Chicago, IL). Oligofectamine was obtained from Invitrogen (Carlsbad, CA). Melanoma cells were serum starved overnight and then transfected with 2 nM IL-24 siRNA or control siRNA in OptiMEM media. Fresh RPMI + 4% FBS was added 4 hours after the transfection. Cells were transfected a second time 24 hours after the initial transfection and harvested 24 hours later. RNA was extracted and analyzed for IL-24 mRNA levels by RT-PCR. For growth suppression assays, supplemented RPMI + 2% CSFBS media and IL-2 were added 24 hours after the second transfection. This time point was designated day 0, and viable cells were counted at day 6 using the Trypan blue dye exclusion assay.

Statistical Analysis

We determined means and standard errors for each of the variables and used the Student's *t* test to evaluate the statistical significance of the experimental results. All experiments were performed at least three times unless otherwise indicated. Statistical significance was set at $P < 0.05$.

Results

IL-24 message is present in some melanoma cells

Melanoma tumors are not homogeneous assemblages of cells. It has been observed that IL-24 protein is expressed by many nevi and primary melanoma tumors. One group looked at IL-24 expression by immunohistochemistry in various tumors and found that 100% of normal melanocytes and benign nevi and 71% of primary melanomas tested were positive for IL-24. This protein expression is often subsequently lost during melanoma tumor invasion and progression and can be undetectable in advanced metastatic disease [101-103]. Only 35% of metastatic melanomas examined had IL-24. Given this heterogeneity among melanoma tumors we hypothesize that only some melanoma tumors and cell lines will have IL-24 message.

Five melanoma cell lines representing various stages of disease and mutation status (Table 3) were examined by RT-PCR for the presence of IL-24 message. A375, WM1341, and WM793 were shown to possess IL-24 message while WM35 and MeWo had very low or undetectable levels of IL-24 mRNA (Figure 4). Data was normalized to beta-actin mRNA levels.

Table 3. Human melanoma cell line descriptions

Cell line	Source tissue	BRAF/NRAS Status
A375	Skin metastasis	Mutant / wild type
WM1341	1° nodular melanoma, early stage	Wild type / mutant
WM793	1° superficial spreading melanoma, vertical growth phase, sternum, stage 1	Mutant / wild type
WM35	1° superficial spreading melanoma, scalp/neck, stage 1	Mutant / wild type
MeWo	Lymph node metastasis – late stage	Wild type / wild type

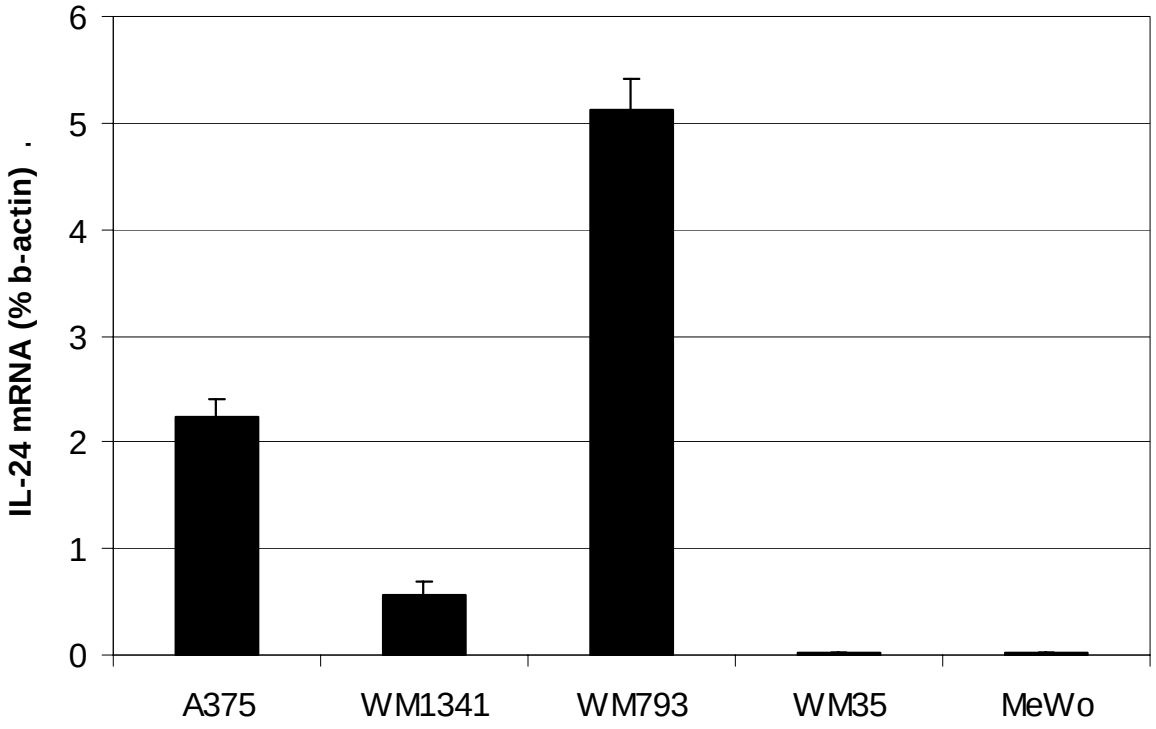


Figure 4. Some melanoma cells have message for IL-24. Cells were grown in FBS + 4% RPMI. Total RNA was extracted and analyzed for IL-24 mRNA by RT-PCR. Each sample was assayed in triplicate plus a control without reverse transcriptase to assess DNA contamination levels. Standard deviations are displayed above.

Basal IL-24 levels in melanoma cells are affected by serum components

To study the effects of cytokines *in vitro* it is often helpful to use charcoal stripping, an absorptive process, to minimize the presence of endogenous hormones, growth factors, and cytokines in serum. We hypothesized that any effects of treatment with IL-2 on IL-24 protein would be better exposed if background levels of IL-24 produced by cells, probably to some extent due to nonspecific activation from serum components, were decreased.

Melanoma cells grown in RPMI + 2% FBS were compared to those grown in RPMI + 2% charcoal stripped FBS. Those grown in charcoal stripped media exhibited significantly less background IL-24 than those grown in non-stripped media, and the effects of IL-2 treatment were more easily visualized (Figure 5). These melanoma cells are sensitive to components within the serum, and the decreased basal levels of IL-24 when grown in the charcoal stripped media allow for a better sense of the impact our treatments have on the cells.

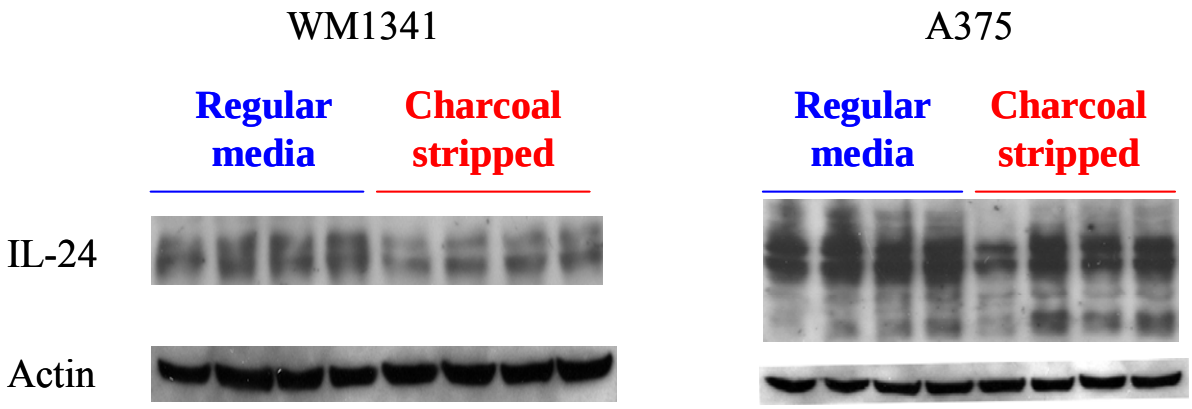


Figure 5. Charcoal stripped FBS media decreases background levels of IL-24. Cells were serum starved overnight and then grown for 24 hours in RPMI + 2% FBS or RPMI + 2% charcoal stripped FBS (CSFBS) under varying conditions. Brefeldin A was added 4 hours prior to harvesting the cells. Whole cell lysates were analyzed by Western blot for intracellular IL-24 and actin.

IL-2 upregulates IL-24 protein production and secretion in 3/5 melanoma cell lines

Our laboratory has published data that shows IL-2 stimulation triggers IL-24 production in PBMC [84]. Preliminary unpublished data showed by FACS analysis that IL-2 at high doses caused increased expression of IL-24 in A375 cells in a dose dependent manner, but no increase was seen in MeWo cells. We therefore hypothesized that in cell lines containing IL-24 mRNA, stimulation with IL-2 might also result in upregulation of IL-24 protein expression.

Melanoma cells were treated with high dose IL-2 at 1000U/ml in RPMI + 2% charcoal stripped FBS for 24 hours. It was discovered in early experiments that three of the cell lines actively secreted IL-24 protein, in some cases at levels high enough to be detected in the unconcentrated supernatant by Western blot analysis (data not shown). Therefore, in subsequent experiments all cells were treated with brefeldin A at 10ug/ml to prevent protein secretion for 4 hours prior to harvesting in order to better elucidate the levels of IL-24 being produced by our cells. LPS was used as a positive control since melanoma cells are known to respond via the Toll-Like Receptor-4 (TLR4); in monocytes and macrophages which express TLR4, IL-24 expression can also be induced by treatment with LPS [84]. Representative Western blots are shown here for the three responsive cell lines. A375, WM1341, and WM793 were all found to have basal levels of IL-24 protein. This result was anticipated from the previous IL-24 mRNA data and confirmed our hypothesis that IL-24 is constitutively present in some cells. These same cell lines also showed consistently increased levels of IL-24 protein when treated with high dose IL-2 (1000U/ml) as compared to cells cultured in media alone (Figure 6), and this increase was shown to be statistically significant. A375 exhibited an average increase in IL-24 levels of 43.0% ($p=0.01$) when

treated with IL-2. WM1341 showed an increase of 31.6% ($p<0.01$) and WM793 had an increase of 27.4% ($p<0.01$). WM35 and MeWo cells did not produce detectable levels of IL-24 regardless of treatment with IL-2 (data not shown). The actual percent increase in IL-24 levels varied between cell lines and experiments, but within the 3 responsive cell lines IL-2 stimulated cells always demonstrated increased levels of IL-24 protein. Each cell line was tested at least three times.

Based on cDNA analysis, soluble human IL-24 was predicted to be a monomeric 18 kDa protein [84]. However, data from several sources show that IL-24 protein derived from the HEK-293-IL-24 mammalian expression system exists as several different monomeric species with molecular weights ranging from approximately 18 to 32 kDa [104, 105]. These data point toward the possibility of post-translational modifications to the protein. Several groups, including our laboratory, have found that soluble IL-24 is N-glycosylated [106, 107], and secreted IL-24 is thought to contain three distinct N-glycosylation sites (49) which accounts for the immunoreactivity of multiple bands that our IL-24 antibody detects during Western blot analysis. IL-24 protein often appears or 2 or 3 separate bands in our blots depending on how far out the gels are run.

ELISA analysis of the cell lines showed that A375, WM1341, and WM793 cells secreted detectable levels of IL-24 into the supernatant constitutively, and that those cells treated with IL-2 for 24 hours secreted significantly more IL-24 protein than their untreated counterparts (Figure 7). IL-2 treatment caused an increase in IL-24 secretion in A375 by 56% ($p=0.02$), in WM1341 by 82% ($p<0.01$), and in WM793 by 40% ($p=0.01$). WM35 and MeWo cells do not secrete any detectable levels of IL-24. Charcoal stripped FBS media was included as a control.

In order to confirm that the increases we observed in IL-24 levels were specifically due to the actions of IL-2, the IL-2 was neutralized by preincubating with a mouse-anti-hIL-2 antibody for 1 hour at room temperature prior to stimulating the cells. A representative Western blot from the IL-24 positive cell line WM1341 is shown (Figure 8). Neutralization of the IL-2 resulted in a reversal of its effects on IL-24 and was enough to bring IL-24 levels down to below baseline. This indicates that the elevations of IL-24 levels in these cells are a direct result of treatment with high dose IL-2.

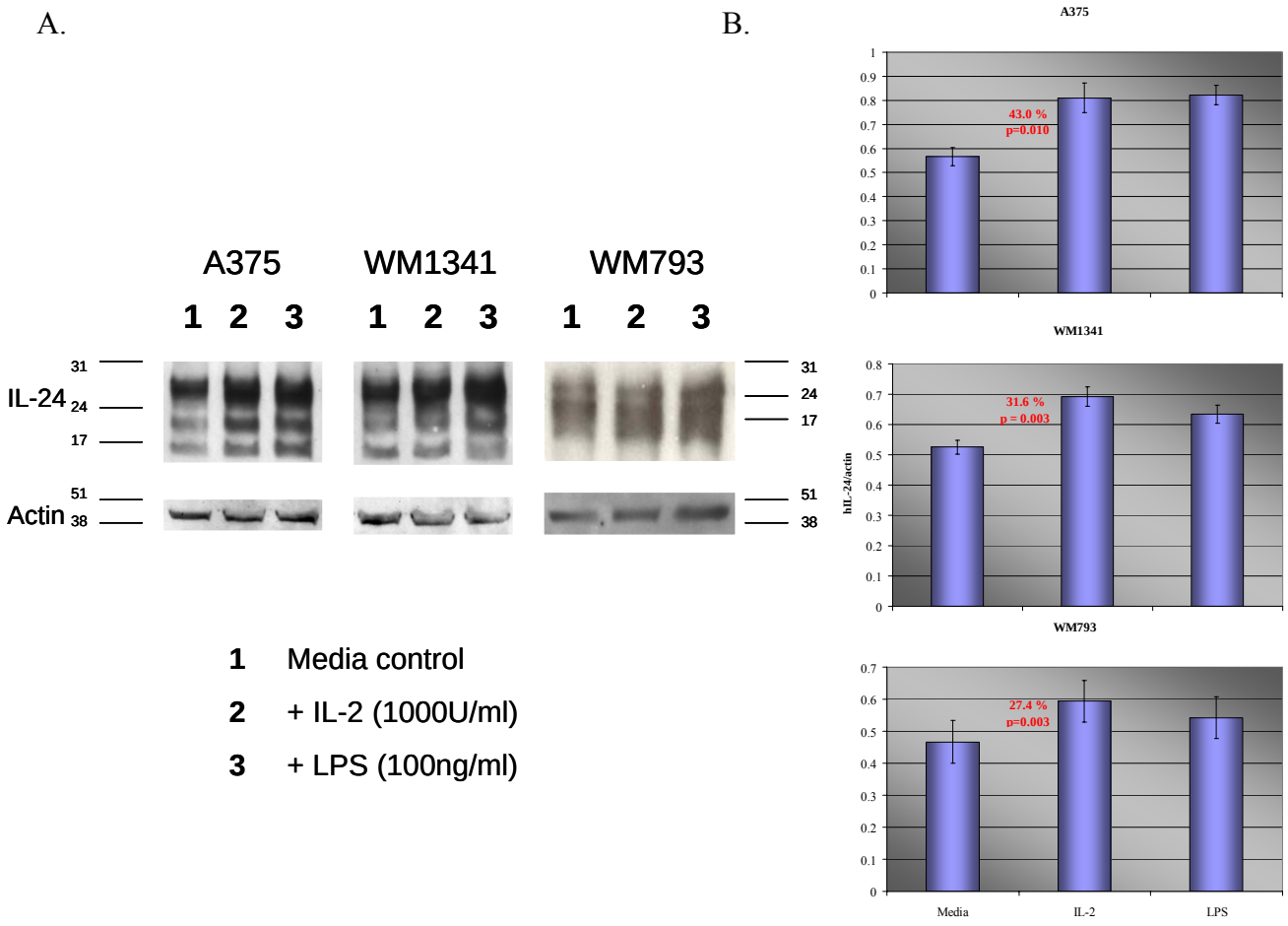


Figure 6. High dose IL-2 upregulates IL-24 expression in some melanoma cells – Cells were plated in 35mm dishes and then incubated with media alone, IL-2 (1000U/ml), or LPS (100ng/ml) for 24 hours. Brefeldin A was added 4 hours prior to harvesting the cells to halt protein secretion. a) Whole cell lysates were separated on a 12% SDS-PAGE gel and probed with 7G11 (mouse anti-human IL-24 antibody) and an anti-actin antibody as a protein loading control b) Western blot bands were quantified by normalizing to actin and then graphed as % of media control. Percent change and p-values were calculated by combining data from at least three experiments.

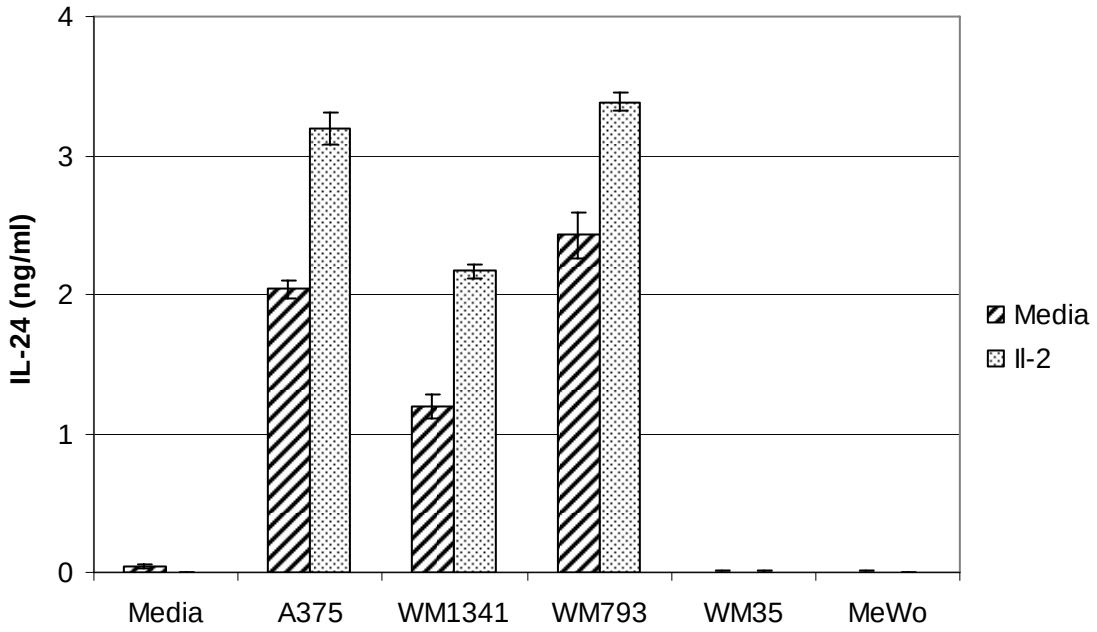
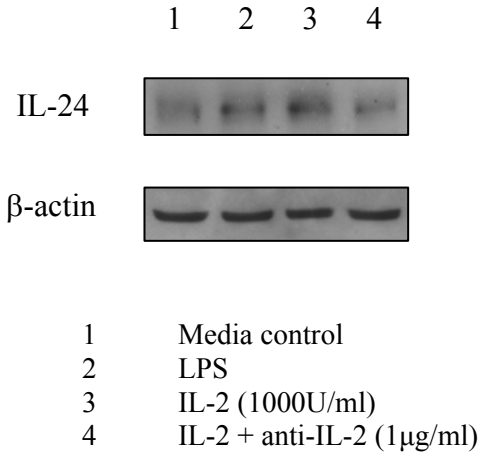


Figure 7. IL-24 is secreted by some melanoma cells. 1 x 10^6 cells were plated in 35mm dishes and serum starved overnight. Cells were then cultured in RPMI + 2% CSFBS with or without IL-2 (1000 U/ml). Supernatant was harvested at 24 hours and analyzed by ELISA in triplicate for secreted IL-24.

A.



B.

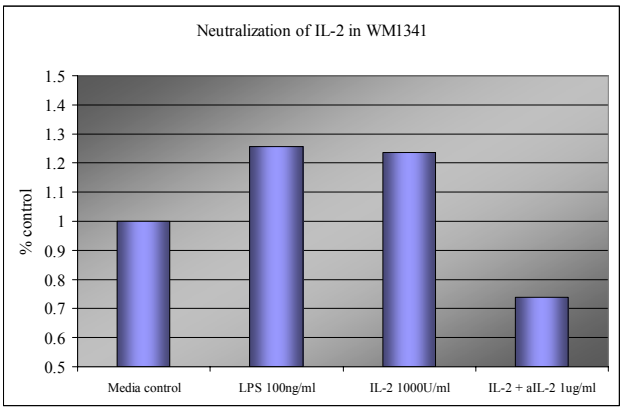


Figure 8. IL-2 upregulation of IL-24 can be neutralized with anti-IL-2 antibody. Shown here in a representative blot, IL-2 was preincubated with a neutralizing mouse-anti-hIL-2 antibody for 1 hour at room temperature before being added to WM1341 cells. A) Cells were harvested at 24 hours and analyzed by Western blot. B) Protein quantitation was performed by normalizing bands to actin.

Melanoma cells have IL-2R mRNA

Given our evidence that IL-2 has a direct biological effect on some melanoma cells, we hypothesized that the IL-2 receptor must therefore be present on these cells. IL-2 mediates its effects on T cells by binding and signaling through the IL-2 receptor [108]. The receptor complex on lymphocytes is composed of three chains - α, β, and γ which in different combinations form intermediate and high affinity receptors for IL-2 [109, 110]. Precedence exists for our hypothesis that some of our melanoma cells must contain one or more chains of the IL-2 receptor. Several articles in the literature report that IL-2 receptor chains are present on the surfaces of both human and murine cutaneous melanoma cells (85-90). IL-2Rβ in particular has been identified in a number of cancer cell lines including small cell lung cancer and squamous cell carcinoma of the head and neck (91;92), and melanoma.

Each of our five cell lines was found to have measurable IL-2Rβ and IL-2Rγ mRNA at varying amounts when analyzed by RT-PCR (Figure 9). IL-2Rα message was not detected in any of these cell lines. However, the IL-2Rα chain serves only to stabilize the interaction between IL-2 and its receptor; IL-2Rα is not required for IL-2R signal transduction [111]**.** IL-2Rβ and γ form an intermediate affinity receptor for IL-2. These findings support our hypothesis that IL-2 can have a direct effect on melanoma cells by signaling through its receptor.

To confirm that IL-2 signals specifically through its receptor to upregulate IL-24 in these cells, the IL-2 receptor was first blocked by preincubating WM1341 and A375 cells with antibodies specific for each of the three IL-2 receptor chains for 1 hour at room temperature and then stimulating with IL-2 (Figure 10). All antibodies were use at the concentrations suggested by the manufacturer, R&D Systems. As predicted, blocking with

antibodies to the IL-2 receptor chains prior to adding the IL-2 resulted in a decrease in IL-24 levels as compared to unblocked cells. This inhibition was seen most dramatically with the blocking of IL-2Rβ suggesting that the IL-2Rβ chain is essential for IL-2 upregulation of IL-24 expression. Mouse IgG was used at 60 ug/ml, a dose equal to the highest concentration of IL-2R antibody used, as a negative control antibody in order to rule out the effects of any nonspecific blocking. A representative Western blot from the IL-24 positive cell line WM1341 is shown.

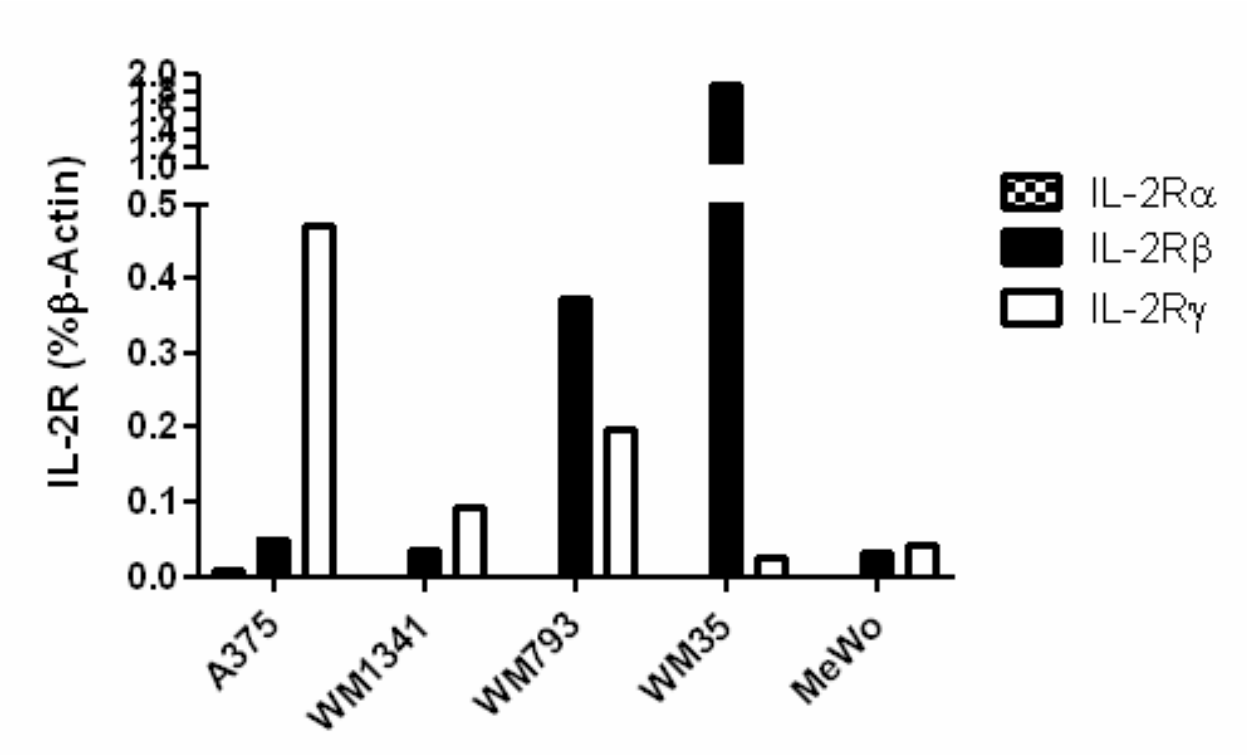


Figure 9. IL-2Rβ and IL-2Rγ chain mRNA is present in melanoma cell lines. Total RNA was extracted from 5 cell lines and analyzed by RT-PCR for IL-2 receptor chain message.

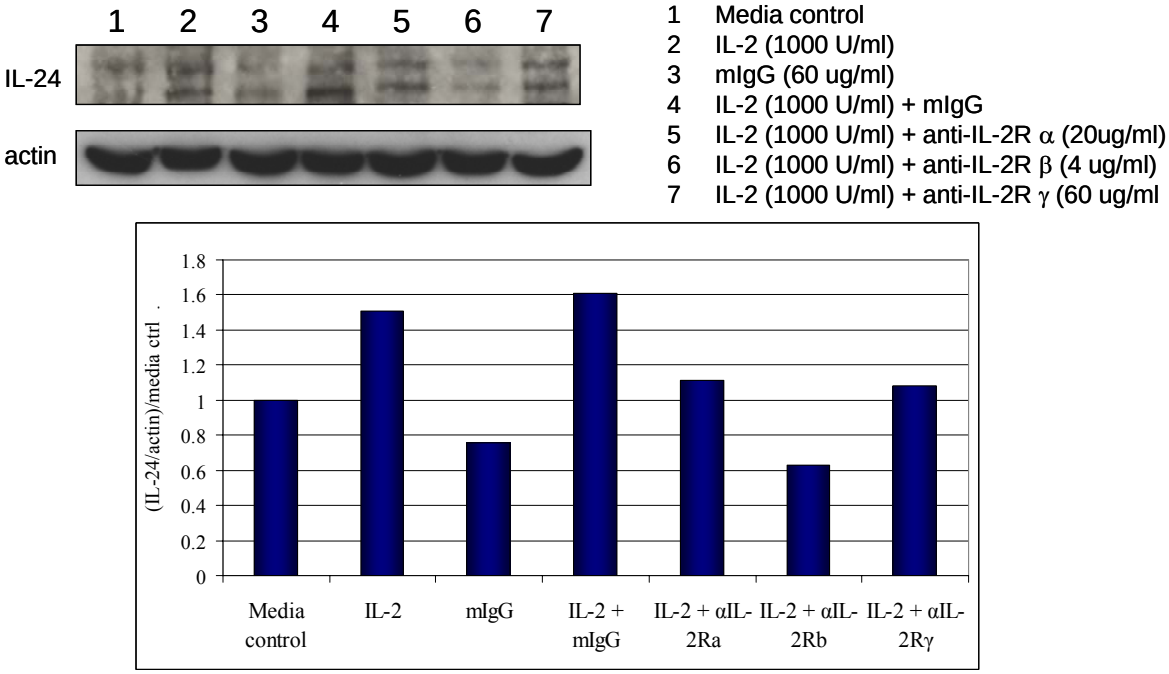


Figure 10. IL-2 mediated IL-24 production is abolished by blocking the IL-2R. Cells were preincubated with blocking antibodies against the three IL-2 receptor chains (α, β, and γ) at the concentrations noted per the manufacture's protocol for 1 hour at room temperature prior to the addition of IL-2 (1000U/ml). mIgG was used as an antibody negative control. Cells were harvested at 24 hours and analyzed by Western blot. A representative blot is shown here in WM1341 cells.

IL-15 upregulates IL-24 expression in melanoma cell lines

Once the presence and importance of IL-2Rβ on these melanoma cells were established, the next logical step was to determine if the upregulation of IL-24 is specific to stimulation with IL-2. Utilization of other cytokines which also signal through the IL-2R chains was considered. IL-15 was of particular interest because it is the only cytokine known to share both the IL-2Rβ chain and common gamma chain [112, 113]. Thus, we hypothesized that stimulating cells with IL-15 would also have an effect and result in increased levels of IL-24 expression.

Cells were stimulated with IL-15 (500 U/ml) for 24 hours and cell lysates were analyzed by Western blot analysis for IL-24 protein. IL-2 and LPS stimulated cells were included as controls. All three IL-24 positive cell lines showed upregulation of IL-24 when treated with IL-15 (Figure 11). In A375 and WM1341, this increase was even higher than that seen with high dose IL-2 stimulation. This data together with the IL-2 receptor blocking experiment confirms the significance of IL-2Rβ for IL-24 upregulation. It also points toward the potential therapeutic use of IL-15 as a treatment for melanoma.

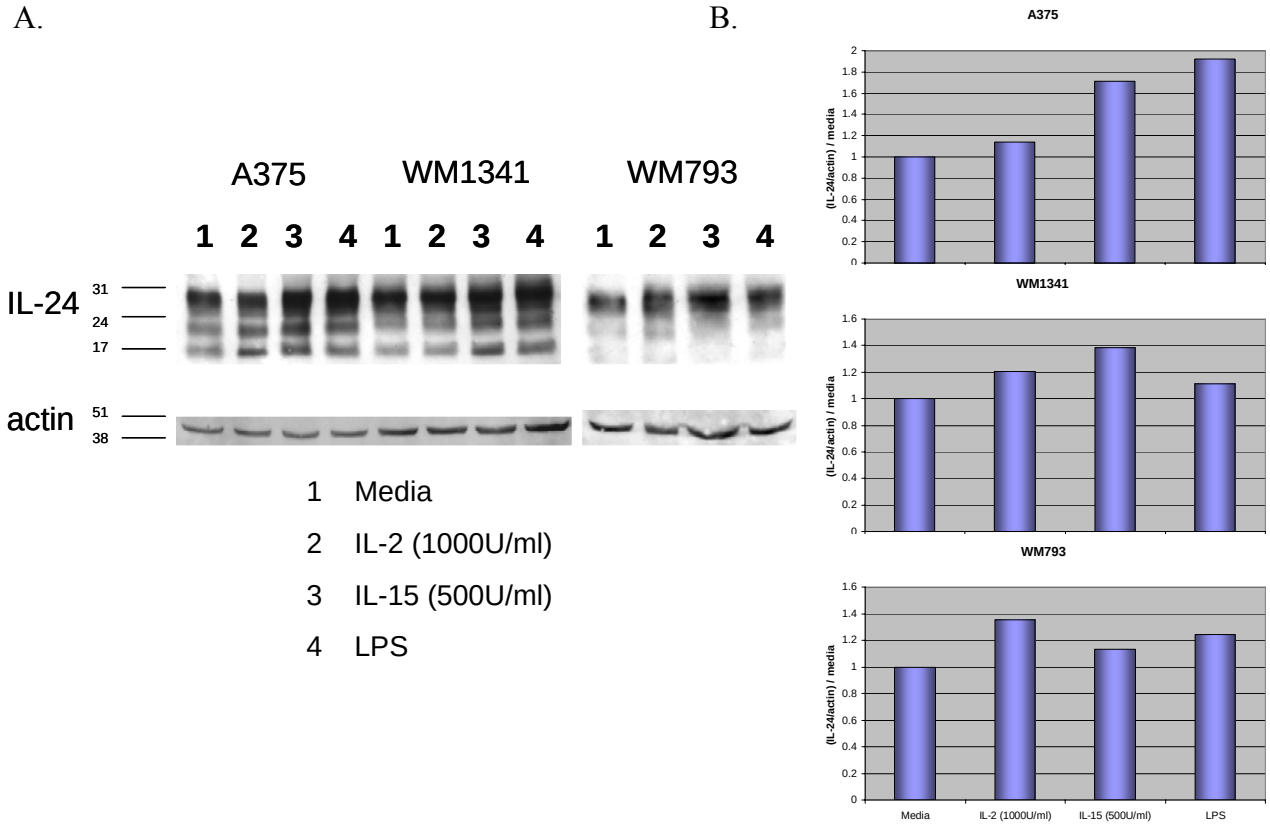


Figure 11. IL-15 upregulates IL-24 expression in some melanoma cells. Cells were incubated with media alone, IL-2 (1000U/ml), IL-15 (500U/ml), or LPS (100ng/ml) for 24 hours. Brefeldin A was added 4 hours prior to harvesting the cells to halt protein secretion. a) Cell lysates were separated on a 12% SDS-PAGE gel and probed with 7G11 and an anti-actin antibody as a protein loading control b) Western blot bands were quantified by normalizing to actin. Data is presented as % of IL-24 expression in media control.

IL-2 causes growth suppression in some melanoma cells

RT-PCR showed that IL-2Rβ is present on these melanoma cell lines. It has been reported that several other cancers including squamous cell carcinoma, renal cell carcinoma, and gastric carcinomas are growth inhibited by IL-2 signaling specifically through the IL-2Rβ chain [114, 115]. We hypothesized that this also might be the case in the subset of melanoma patients who respond to high dose IL-2 therapy and that these patients might be represented by some of our melanoma cell lines.

Cells for each condition were plated in triplicate in 24 well plates and grown in RPMI + 2% CSFBS with or without IL-2 (1000 U/ml) added at day 0. MTT assays were initially attempted but failed to show any differences in growth. Because the MTT assay relies on functional mitochondria to break down the tetrazolium bromide, it is not an ideal assay for melanoma cells which are known to have mitochondrial mutations [116-118]. Instead, cells were counted and cell viability assessed by Trypan blue dye exclusion assay on days 2 through 6 (Figure 11a). In WM1341 and WM793, cells treated with IL-2 had significantly fewer viable cells by day 4 ($p \leq 0.01$). A375 showed statistically significant growth suppression with IL-2 by day 5 ($p = 0.04$). By day 6, IL-2 treated cells exhibited 18-32% less growth than untreated cells. The growth of the IL-24 negative cell lines WM35 and MeWo was unaffected by treatment with IL-2. Although WM35 cells had the highest level of IL-2Rβ (Figure 9), IL-2 had no affect on its growth. This implies that IL-2 mediated growth suppression is dependent on the IL-24 status of the cells.

IL-2 mediated growth suppression is dependent on IL-24

As discussed in the introduction, the tumor suppressive characteristics of IL-24 have been well documented. IL-24 has been shown to cause tumor specific growth suppression and apoptosis both *in vitro* and *in vivo*, including lung, pancreatic, and breast cancer cells, while demonstrating no apparent toxicity to normal cells [56, 64, 65, 119-125]. Data from our laboratory further demonstrates IL-24's cytotoxicity to human melanoma cell lines. These data all support the tumor suppressor activity ascribed to IL-24 [126]. Based on our data which show that treatment with IL-2 causes increased IL-24 protein production and secretion in some melanoma cell lines, it is reasonable to conjecture that these amplified levels of IL-24 might subsequently cause growth suppression in those cell lines. According to the literature, melanoma cells transduced with Ad-mda7 (introduction of the IL-24 gene via adenoviral vector) can secrete IL-24 protein which then plays a tumor selective cytotoxic bystander role [70].

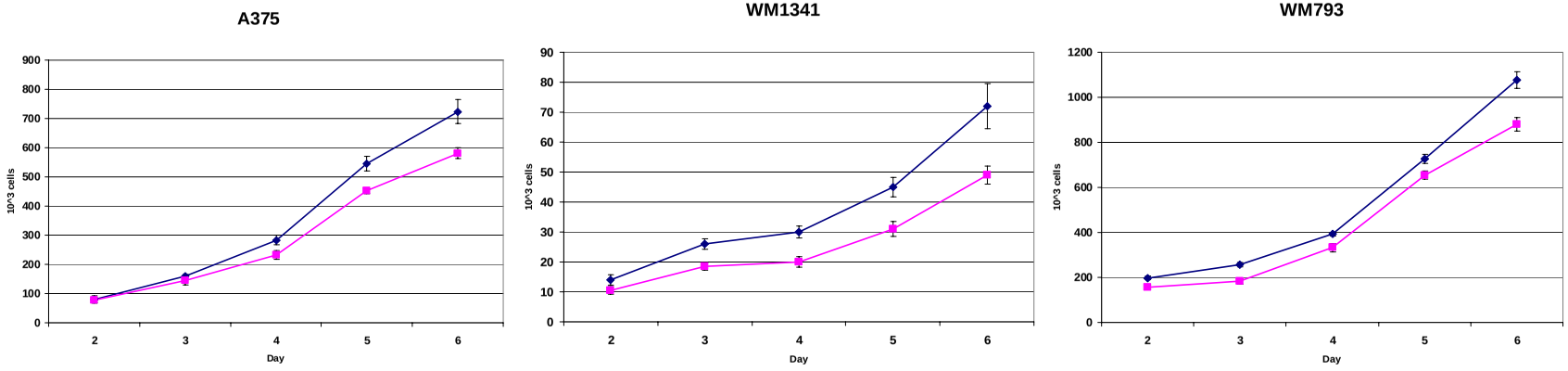
Therefore, we hypothesized that the growth suppression seen in response to IL-2 (Figure 12a) is IL-24 dependent. Consequently, blocking the effects of the secreted IL-24 with an anti-IL-24 monoclonal antibody should reduce the growth suppression observed in IL-2 responsive cells. In addition we hypothesized that blocking the production of IL-24 directly by silencing IL-24 mRNA should also inhibit the growth suppression seen with IL-2 stimulation.

Once again, cell viability was assessed by Trypan blue dye exclusion assay. A375 and WM1341 cells treated with 7G11 (an anti-IL-24 monoclonal antibody) to block the effects of secreted IL-24 displayed growth curve patterns similar to those cells grown in media alone without IL-2 (Figure 12b), i.e. no significant growth suppression was detected.

Cells were also treated with mIgG with or without IL-2 as controls; these cells behaved like their non-mIgG treated counterparts. The addition of 7G11 had no effect on the growth of WM35 and MeWo cells which do not express IL-24 protein. These data indicate that IL-2 mediated growth suppression in melanoma cell lines is directly due to IL-24.

In order to directly block IL-24 production, two IL-24 positive cell lines (A375 and WM1341) and one IL-24 negative cell line (WM35) were treated with IL-24 siRNA or a non-coding siRNA negative control for 48 hours prior to the addition of high dose IL-2 on day 0. Cell viability was assessed at day 6 as shown in Figure 12c. Although it is understood that the silencing effects of the siRNA transfection last only up to 72 hours, even this initial temporary inhibition of IL-24 production demonstrated a noticeable effect on cell growth. These growth suppression assays show that in IL-24 positive cell lines, silencing IL-24 at the mRNA level counteracts the IL-2 mediated growth suppressive effects previously observed (Figure 12c). In these cell lines, there was no significant difference seen between the IL-24 silenced cells that were cultured with IL-2 and those without IL-2. There was a loss of total cell number due to toxicity in both the control oligofectamine treated cells and non-coding siRNA treated cells, but IL-2 maintained its growth suppressive effects in both of these groups ($p \leq 0.05$). In WM35, an IL-24 negative cell line, treating with IL-24 siRNA had no effect on growth between IL-2 treated and non-treated cells. IL-24 knock-down was verified by RT-PCR for IL-24 message in cells transfected with either non-coding control siRNA or IL-24 siRNA (Figure 13). These data also confirm that IL-2 mediated growth suppression is directly dependent on IL-24.

A. IL-24 positive cells lines

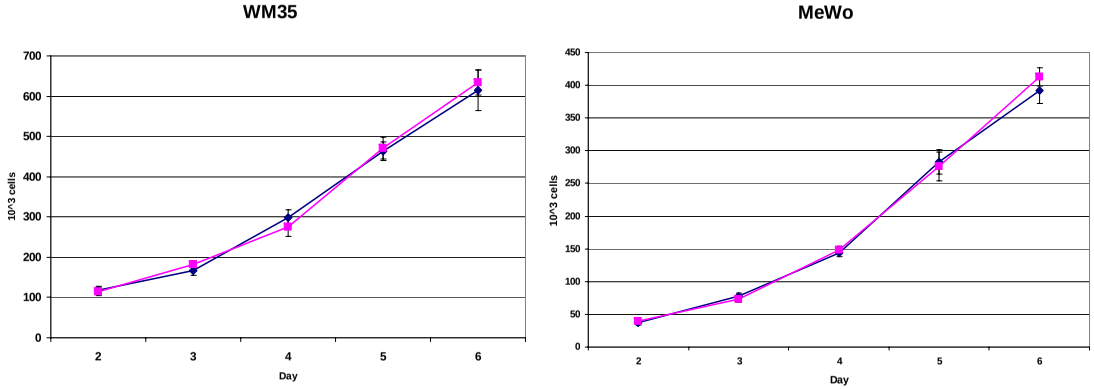


Media

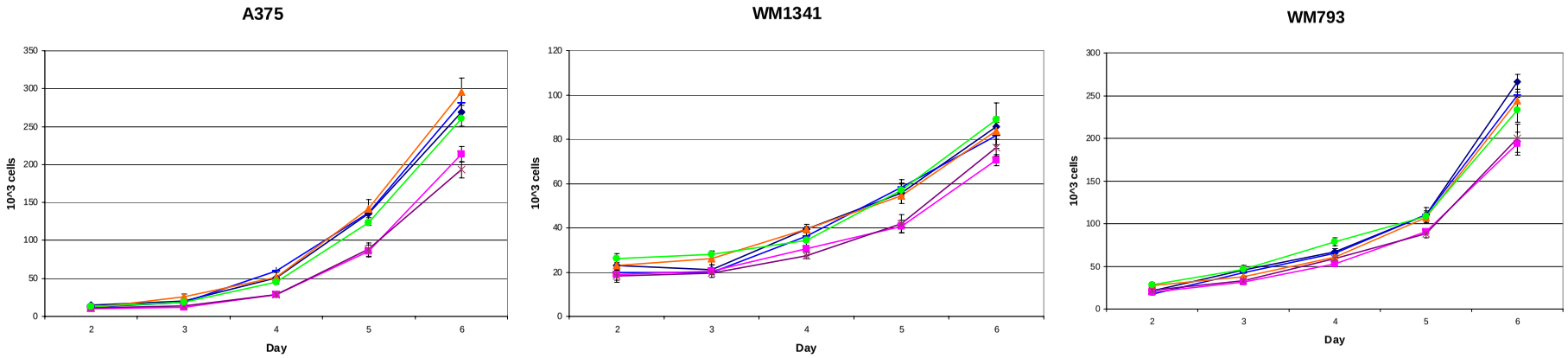
Media + Il-2 (1000U/ml)

	Day 4		Day 5		Day 6	
	% decrease	p	% decrease	p	% decrease	p
A375	18	0.14	17	0.04	20	0.04
WM1341	33	0.01	31	0.04	32	0.02
WM793	15	<0.01	10	<0.01	18	<0.01

IL-24 negative cells lines



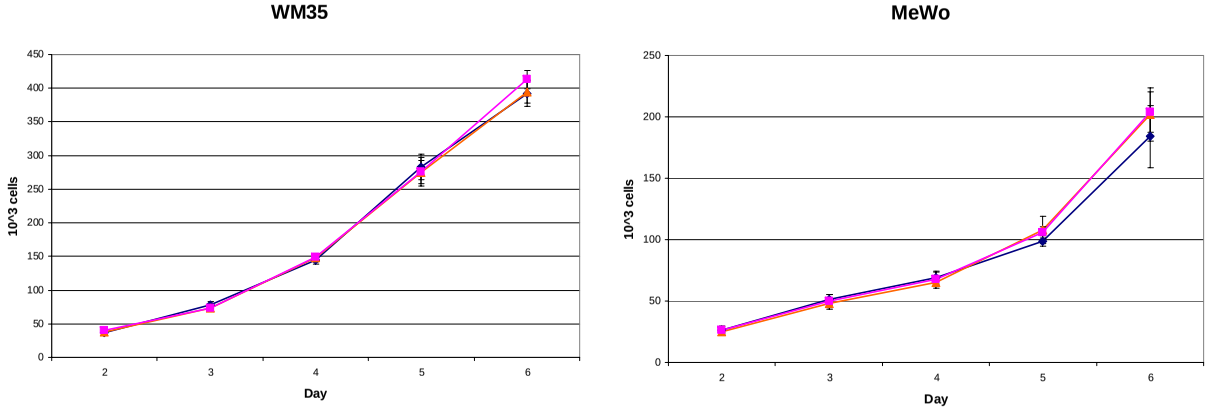
B. IL-24 positive cells lines



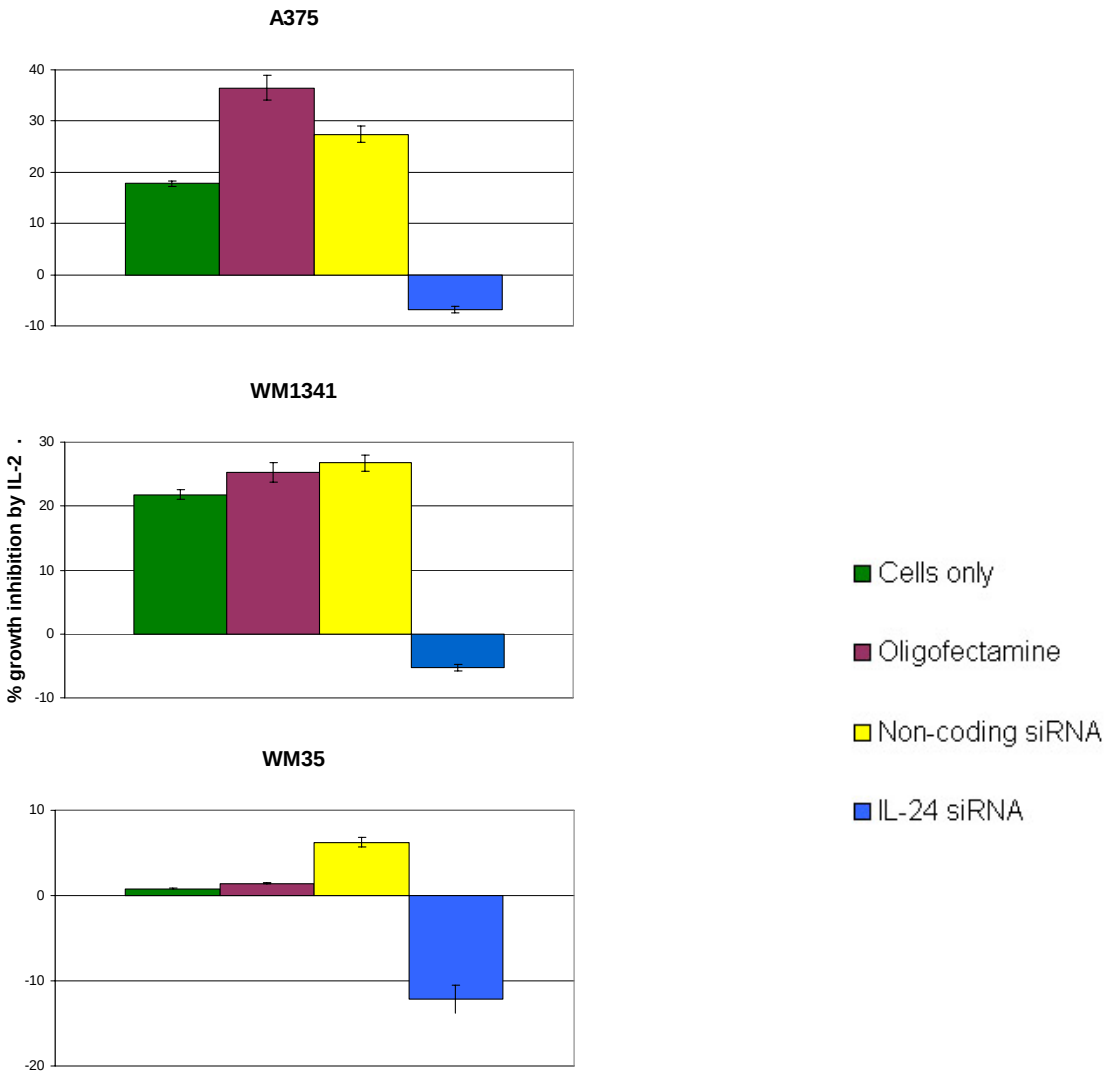
Cell growth with IL-2 vs. media alone.	Day 4		Day 5		Day 6	
	% decrease	p	% decrease	p	% decrease	p
A375	43	0.01	36	0.02	20	<0.01
WM1341	22	0.01	27	0.04	18	0.01
WM793	21	<0.01	18	0.02	27	0.03

- Media
- mIgG (5ug/ml)
- 7G11 (5ug/ml)
- IL-2 (1000U/ml)
- IL-2 (1000U/ml) + mIgG (5ug/ml)
- IL-2 (1000U/ml) + 7G11 (5ug/ml)

IL-24 negative cells lines



C.



	Media % inhibition		Oligofectamine % inhibition		Non-coding siRNA % inhibition		IL-24 siRNA % inhibition	
A375	18	p=0.03	36	p=0.01	27	p<0.01	-7	p=0.65
WM1341	22	p=0.01	25	p=0.05	27	p=0.05	-5	p=0.74
WM35	0	p=0.94	1	p=0.88	6	p=0.58	-12	p=0.62

Figure 12. Effects of IL-2 on Melanoma Cell Growth by Trypan Blue Assay. Cells were plated in 24 well plates with each condition in triplicate. Cells were serum starved overnight and then stimulated on day 0. The table indicates the % decrease in cell growth observed when treated with IL-2 as compared to cells grown in media alone. A. IL-2 effects on melanoma cell growth. B. Blocking the effects of secreted IL-24 with 7G11 (mouse-anti-IL-24) antibody. C. siRNA transfected melanoma cell growth at day 6. All data is presented as % growth inhibition by IL-2 compared to cells grown in media alone (1-(IL-2 treated cells/media)).

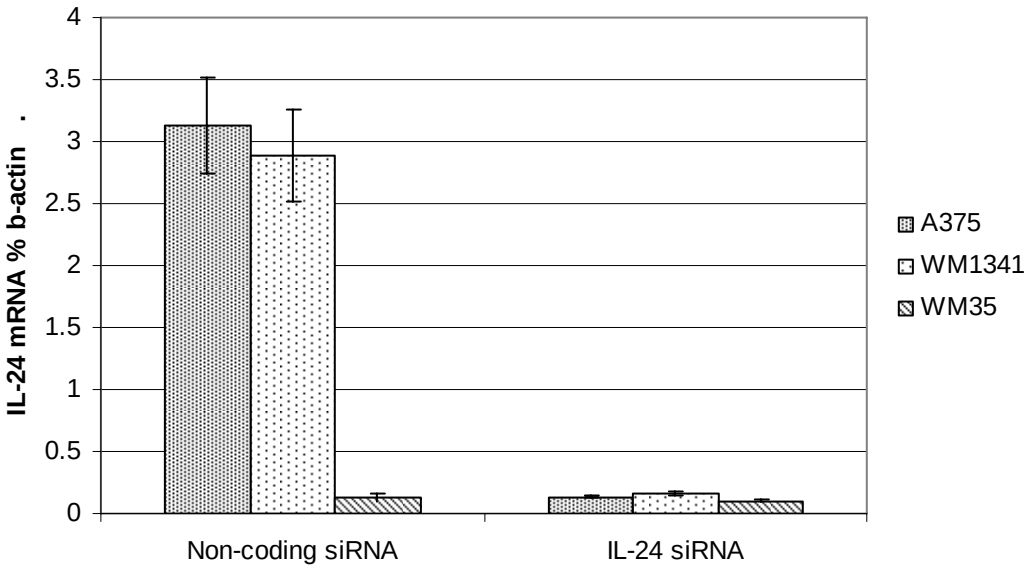


Figure 13. IL-24 siRNA effectively knocks down IL-24 mRNA in melanoma cell lines. Cells were harvested 24 hours after treatment with siRNA and analyzed for IL-24 and actin mRNA by RT-PCR. IL-24 mRNA data is shown as % b-actin.

IL-15 causes growth suppression in A375 cells

Our data has established that in A375, WM1341, and WM793 cells IL-2 causes growth suppression in an IL-24 dependent manner. Stimulating those cell lines with IL-15 also results in upregulation of IL-24 protein expression. It is known that IL-15 signals through the same IL-2Rβ and IL-2Rγ chains as IL-2. Thus, we hypothesized that IL-15 should also cause growth suppression in IL-24 positive cell lines. This hypothesis was investigated in the A375 melanoma cell line. The experimental design was identical to the growth suppression assays previously described and were done in parallel with IL-2. Cells were grown in media alone, IL-15 (500 U/ml), or IL-2 (1000 U/ml). Results showed that cells treated with IL-15 demonstrated significant growth suppression of approximately 19-25% over days 4 through 6 ($p=0.02$ by day 4). These results are comparable to those cells treated with IL-2 (Figure 14).

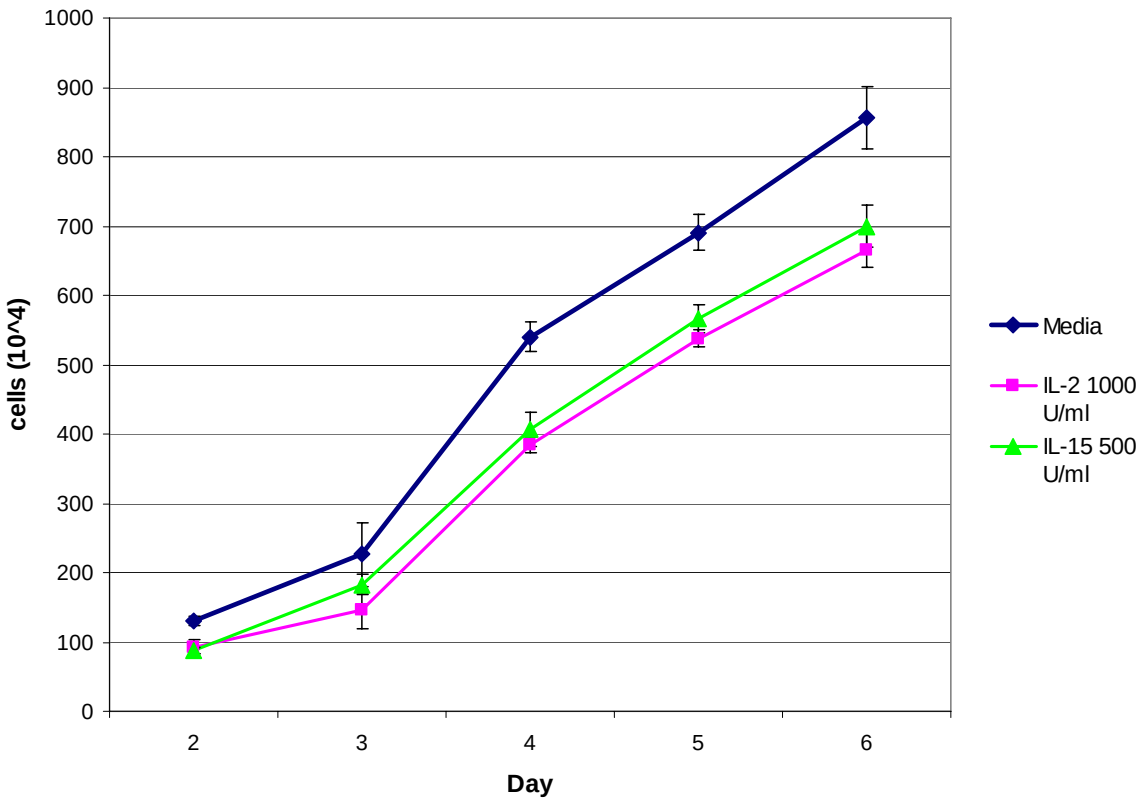


Figure 14. IL-15 causes growth suppression in A375 cells by day 4. Cells were plated in 24 well plates with each condition in triplicate. Cells were serum starved overnight and then stimulated on day 0.

Serum levels of IL-24 in high dose IL-2 patients

We hypothesized that because IL-2 upregulates IL-24 protein expression in some cells, it might be possible to measure any subsequent systemic changes in the serum of patients. Dr. Laslo Radvanyi (M. D. Anderson Cancer Center, Houston, TX) generously donated serum from 14 high dose IL-2 patients receiving treatment at M. D. Anderson Cancer Center (Protocol LAB06-0762, P.I. Dr. Laslo Radvanyi). Rich Joseph in Dr. Radvanyi's laboratory compiled a clinical database of the responses in those patients.

On this protocol, each cycle of high dose IL-2 therapy consists of 720,000 IU/kg of Aldesleukin (human rIL-2) via intravenous infusion over 15 min given up to 12 times per cycle, with each dose/infusion spaced 8 h apart. Cycle 2 is given 21 days later; patients can receive up to 12 cycles of therapy depending on tolerance and toxicity. Patients are assessed after cycle 2 by CT and if they progress, they are taken off IL-2 therapy. Patients with disease stabilization or a partial response continue with therapy cycles until no more tumor shrinkage occurs, or until they progress or undergo a complete response. Most people withdraw after 2 or more cycles due to toxicity (vascular leak syndrome, fever, fatigue, mental confusion, liver dysfunction). During each cycle, these toxicities are closely monitored (especially liver) and if liver dysfunction begins, the infusions are stopped. Most patients can take up to 6-8 infusions per cycle of therapy; some can take all 12.

Serum was obtained from 10 nonresponders and 4 responders at three separate time points – prior to the first dose, 5 hours after the fourth dose, and 2 days after the last dose of cycle 1. Data from our laboratory examining normal serum from 15 donors shows that IL-24 binds to other serum proteins; markedly higher amounts of IL-24 were detectable by ELISA after serum samples were heat released by heating at 56 degrees C for 30 minutes. Based on

this data, patient serum samples were heat released before being analyzed by ELISA for IL-24 and IL-2 levels.

Six of 10 nonresponder patients had measurable levels of IL-24 that peaked or continued to increase >20% over pretreatment levels after the fourth high dose IL-2 treatment time point. IL-24 levels measured with this assay varied over a wide range from 130 pg/ml to 13,700 pg/ml. The remaining 4 nonresponders as well as all 4 responders had serum levels of IL-24 below the limits of our ELISA kit standard curve (<62 picograms/ml) at any of the time points tested. We observed no obvious correlations between quantifiable serum levels of IL-24 and any specific responses recorded in the clinical database for this small group of patients. 75% of the 4 nonresponders with undetectable IL-24 levels died of their disease while the only one third of the 6 nonresponders with measurable IL-24 had died at the time these experiments were run. However, the 4 patients who responded to therapy also did not have measurable IL-24 by our assay. It is possible that any changes in IL-24 level might be better detected at an earlier time point. Furthermore we believe that it is more likely that any clinical relationship between measurable differences in IL-24 before and after high dose IL-2 treatment and patient response to treatment would be better detected in the tumor microenvironment and not in the serum.

Discussion

IL-2 upregulates IL-24 protein expression leading to growth suppression in melanoma cells

In this dissertation, we have shown a novel mechanism of IL-2 action in melanoma (Figure 15). We have established that A375, WM1341, and WM793 melanoma cells have IL-24 mRNA while WM35 and MeWo cells lack it. We have confirmed that high dose IL-2 has a direct effect on some melanoma cells, causing upregulation of IL-24 protein expression and secretion in cell lines that possess IL-24 mRNA. IL-2 mediates this activity by signaling through the IL-2 receptor on these cells; the IL-2Rβ chain in particular appears to be essential for this action. These IL-2 produced increases in IL-24 protein levels subsequently result in the suppression of tumor cell growth, and this growth suppression has been confirmed to occur in an IL-24 dependent manner. IL-15, which shares the IL-2Rβ chain with IL-2, has also been discovered to be capable of upregulating IL-24 protein expression, consequently causing growth suppression in IL-24 positive cells.

While we do not disagree with the valuable role of IL-2 as an established immunotherapy, these data together give us novel confirmatory evidence that high dose IL-2 directly affects melanoma tumor cells via its receptor, causing IL-24 upregulation and secretion and subsequent tumor growth suppression. It is possible that the group of patients whose melanoma cells respond to IL-2 by producing increased IL-24 are the subset of patients with durable responses to high dose IL-2. We therefore envision exciting implications for possible predictive biomarkers of IL-2 response for targeted therapy and potential alternative therapeutics for melanoma. This is promising given our evidence that the IL-2Rβ chain plays a significant role in our novel pathway and that only the IL-24

positive melanoma cells lines showed growth suppression with high dose IL-2 treatment. Additional support for the use of these two molecules as biomarkers of IL-2 response is discussed in further detail later in this section. Of course, we cannot discount the effects of high dose IL-2 actions on the immune system. Although we have shown the direct effects of IL-2 on melanoma cells *in vitro* in the absence of any immune cells and believe that it may contribute to patient response to treatment, this mechanism must also be researched *in vivo*. It may be that in the *in vivo* setting, the effects of IL-2 as an immunotherapy on the immune system far outweigh the direct effects seen *in vitro*. Therefore, more work must be done to determine the direct effects of high dose IL-2 on melanoma cells *in vivo.*

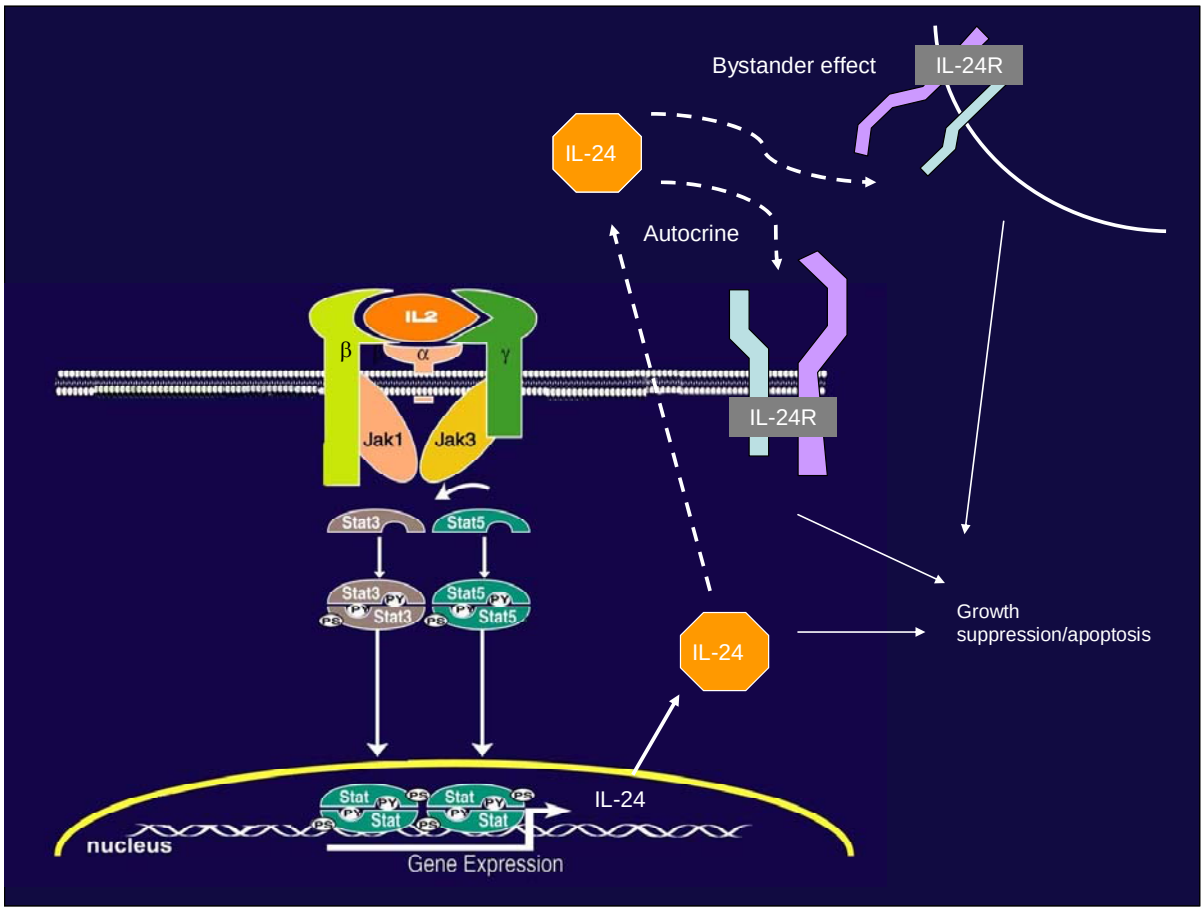


Figure 15. Proposed novel mechanism for IL-2 direct effects on melanoma: IL-24 dependent IL-2 mediated growth suppression in melanoma cells. IL-2 binds its receptor (i.e. IL-2Rβ) on melanoma cells causing upregulation of IL-24 protein expression (possibly via message stabilization). These increased levels of IL-24 subsequently cause melanoma tumor cell growth suppression both in an autocrine and paracrine (bystander) manner in tumor cells with IL-24 receptor.

Upregulation of IL-24 expression has also been reported in the inflammatory dermatologic process psoriasis, which involves overactive immune stimulation and excessive skin production (psoriatic plaque formation) [127, 128]. Conflicting data has been presented in the literature regarding the role of IL-24 in psoriasis. The pattern of expression of IL-24 and its receptor *in vivo* indicate that a major physiological function of IL-24 is its involvement in wound healing [59, 129]. Keratinocytes from the epidermis are one of the major targets of IL-24 [53, 107]. It is hypothesized that irregularities in IL-24 function may contribute to pathological skin conditions such as psoriasis [130]. In psoriasis, IL-24 is produced by infiltrating mononuclear cells migrating to the dermal layer directly below the psoriatic epidermis [131]. IL-24 protein and receptor overexpression have also been observed in the epidermal keratinocytes of psoriatic skin [130, 131], suggesting a potential link between the over-activation of IL-24 signaling pathways and overgrowth of keratinocytes. As with other pro-inflammatory cytokines, excess IL-24 can exacerbate immunological conditions. Therefore, constitutive production of IL-24 by the monocytes in the dermis in addition to the overexpression of IL-24 and its receptor on keratinocytes could lead to continual receptor activation and result in abnormal cell proliferation of the keratinocytes in the epidermis; this process would subsequently lead to the development of psoriasis.

However, it has also been reported by other groups that the expression of three specific IL-20/IL-24 combined haplotypes demonstrated a significant protective effect against plaque-type psoriasis [132]. One of the IL20/IL24 haplotypes was also associated with a reduced risk for palmoplantar pustulosis (pustular psoriasis) [133]. Our laboratory has published that IL-24 protein actually inhibits growth factor stimulated proliferation and

migration of keratinocytes during wound healing, causing the contraction of the wound and return of keratinocytes to their normal differentiating processes [134]. Therefore, the loss of IL-24 expression, not upregulation, could lead to uncontrolled keratinocyte proliferation and abnormal healing.

Elevated levels of IL-24 are also seen in patients with latent tuberculosis (TB) [135]. IL-24 has been determined to modulate IFN-γ expression in patients with tuberculosis infection [136]. A correlation exists between IFN-γ expression and the development of TB. T cells and NK cells secrete IFN-γ in response to *Mycobacterium tuberculosis* (*Mtb)* [137]; a defect in IFN-γ would leave the host susceptible to *Mtb* infection [138]. Therefore, an inability to mount a Th1 response may contribute to progression to active tuberculosis. IL-24 increases the expression of IFN-γ stimulating cytokines including IL-12, IL-23, and IL-27 in PBMC. Increased IL-24 expression has been reported during viral and bacterial infections [139], and IL-24 expression is elevated in newborns that have been BCG vaccinated [140]. Latent tuberculosis is defined as infection with *Mycobacterium tuberculosis*, but not active tuberculosis disease. Studies found that patients with latent *Mtb* infection produce high levels of IL-24 and IFN-γ while patients who progress to TB disease have low levels of IL-24 and IFN-γ [141]. Addition of exogenous IL-24 boosts expression of IFN-γ while neutralization of IL-24 results in low expression of IFN-γ. If the patient progresses to TB disease, treatment with exogenous IL-24 may be useful.

There is significant evidence in the literature substantiating the role of IL-24 as a tumor suppressor. IL-24 has been shown to cause growth suppression and apoptosis in numerous different cancer cells including melanoma [124, 125, 142-144]. The Ad.*mda-7* gene construct has been used in Phase I/II gene therapy clinical trials [56, 78, 79, 145]

through intratumoral injection of the Ad.*mda-7* construct [78, 79]. In the phase I/II clinical trials IL-24 was well tolerated and showed considerable apoptotic activity in injected lesions [78, 83, 146, 147]. Therefore, overexpression of IL-24 in cancers has a well documented anti-tumor effect, and this supports our data that upregulated IL-24 causes melanoma growth suppression.

The idea that IL-2 could have a direct effect on melanoma cells is not unprecedented. Other cytokines have also been shown to impose direct effects on a variety of cancer cells. The actions of transforming growth factor – beta (TGF-β) in particular have been extensively studied in many cancers. In normal cells TGF-β acts as a tumor suppression regulating a wide spectrum of cellular functions including the restriction of cell growth, differentiation, and apoptosis [148-150]. Many cancers have been shown to possess the TGF-β receptor. In pancreatic cancer, vascular smooth muscle cancer, and cervical cancers, TGF-β has been discovered to induce apoptosis by signaling through its receptor to activate Smad3 signaling and repress telomerase reverse transcriptase (TERT) [151]. However, during the development of some carcinomas, including colon, breast, and prostate cancer, various elements to the TGF-β signaling pathway become mutated. Research shows that one third of colon cancer tumors have mutated TGF-β receptors, and that other tumors have mutations in the signaling pathway activated by TGF-β [152-154]. Both of these pro-oncogenic conditions lead to unrestricted cell proliferation. Further studies must been done to determine the mechanism and IL-2 signaling pathway elements involved in the direct tumor suppressive effects of IL-2 that we have shown in melanoma cells.

IL-2 mediates its actions by binding to its receptor [109, 155, 156]. This ligand-receptor interaction results in activation of Ras/MAPK, JAK/Stat, and PI3-kinase/Akt signaling pathways leading to downstream gene transcriptional regulation and the proliferation and activation of lymphocytes as shown previously in Figure 2 (151).

As an immunotherapeutic agent, IL-2 directs its anti-tumor functions through cell-mediated immunity. IL-2, formerly called T cell growth factor, drives T cell differentiation and proliferation. It activates T cells and NK cells transforming them into more efficient cytotoxic cells called lymphokine activated killer (LAK) cells [157-159]. These LAK cells then destroy tumor cells by antibody dependent cell mediated cytotoxicity (ADCC). They bind to the tumor cells directly and secrete cytolysins (perforin and granulysin) which kill the target cell. Early studies of IL-2 as an immunotherapy demonstrated that injection of LAK cells and rhIL-2 inhibited the growth of pulmonary metastases and subcutaneous melanoma in mice [160].

High dose IL-2 received FDA approval for patients with advanced melanoma in 1998 based on its ability to produce durable responses. Clinical tumor regression in response to high dose IL-2 is well documented in a minority of patients. Data collected from numerous Phase II studies showed a response rate of 16%, with 6% of patients achieving a complete response and 10% a partial response [161-164]. One group studied the anti-tumor activity of systemic treatment with *Salmonella typhimurium* expressing IL-2 in a melanoma mouse model. They reported that mice treated with the bacteria showed decreased angiogenesis as well as increased tumor necrosis and increased survival times [165]. In humans, a phase II trial found that in melanoma patients treated with intralesional IL-2 injections histologic studies of patient biopsies showed evidence of tumor necrosis. Immunofluorescence analysis

by confocal laser scanning microscopy revealed caspase 3 staining of tumor cells undergoing apoptosis [78, 166, 167].

Although there have been a few reports of IL-2 receptor and direct IL-2 effects on cancer cells, the mechanism of this action is still unclear. Some groups have reported that IL-2 can increase proliferation in some murine and human melanoma cell lines (85-90). One group reported that IL-2 at low doses in the absence of serum appeared to stimulate proliferation of some melanoma cell lines but not others during the first 24 hours, while cell growth was somewhat suppressed or not seen at higher doses around 500U/ml over the same time period [168].

IL-2 has been shown to directly inhibit the growth of human squamous cell carcinoma of the head and neck (SCCHN) both *in vitro* and in nude mice bearing SCCHN xenografts [169-172]. Growth inhibition in SCCHN cells was completely reversed by blocking with an anti-IL-2Rβ antibody and partially reversed by blocking with an anti-IL-2Rα antibody; this indicates the importance of the intermediate affinity IL-2R for the growth suppressive effect of IL-2 in cancer cells (158). This strongly supports our data that IL-2 signals through the IL-2Rβ chain and has a direct growth suppressive effect on melanoma cells.

Historically, the general assumption has been that the therapeutic effects of IL-2 are a result of its immunomodulatory actions. However, it is highly likely that the anti-tumor effects of high dose IL-2 on cancer cells in responsive patients can be attributed to the direct actions of IL-2 on tumors which express the IL-2R in addition to its enhanced immune functions. There is evidence in the literature for the presence of IL-2R on cancer cells and IL-2 inhibition of cancer cell growth at high doses. The literature also confirms the positive

effects on disease processes of elevated IL-24, both in its role as a cytokine and as a tumor suppressor. Together with the data we have presented in this dissertation, this evidence supports our hypothesis that high dose IL-2 signals through IL-2Rβ directly causing growth suppression in some melanoma cells in an IL-24 dependent manner (Figure 15), and that the IL-24 and IL-2Rβ status of a tumor may therefore be useful in predicting patient response to high dose IL-2 therapy. Further studies are needed to determine the molecular mechanism for IL-2 regulation of IL-24 expression in these cells.

Additional support for IL-24 as a potential predictive marker for response to IL-2 therapy

Although we are aware that IL-24 secreted from melanoma cells may also act as a pro-Th1 type cytokine [173] by inducing secondary cytokine production in PBMCs including the proinflammatory cytokines IFNγ, IL-1β, IL-12 and TNFα (49), consequently enhancing T cell mediated immunity by recruiting tumor infiltrating leukocytes to the site of the tumor, this project has focused specifically on the tumor growth suppressive characteristics of IL-24.

IL-24 has been shown to cause growth suppression and apoptosis in numerous different tumor cells including melanoma [144]. Melanoma cells transduced with Ad-*mda7* secrete IL-24 protein which then plays a tumor specific cytotoxic bystander role [70]. Our data have now established that high dose IL-2 causes increased IL-24 protein production in melanoma cells with IL-24 mRNA; in addition, those same melanoma cells also secrete IL-24 constitutively and do so at even higher levels following treatment with high dose IL-2. It is possible that the melanoma cells are well acclimated to survival at the concentrations of IL-24 they generate endogenously, but that the elevated levels of IL-24 produced in response to IL-2 pushes their equilibrium towards growth suppression. Therefore it is to be expected

that this additional secreted IL-24 could also have a paracrine effect by impacting neighboring cells within the tumor microenvironment. It has been reported that the IL-24 receptor chains are present on melanoma cell lines [174]. Because melanomas are so heterogeneous, it may be unlikely that all cells within a tumor will respond directly to IL-2. However, even if only a fraction of the cells are capable of responding to IL-2, the remaining unresponsive cells which likely possess the IL-24 receptor could be indirectly affected by the secreted IL-24 from responding cells. We propose that given the potential autocrine and paracrine bystander tumor growth suppressive effects of elevated IL-24 in high dose IL-2 patients, IL-24 is an excellent candidate to be a positive predictive marker for response to high dose IL-2 therapy.

Ideally, pretreatment and posttreatment biopsy or fine needle aspirate (FNA) samples from high dose IL-2 patients could be examined using immunohistochemistry techniques for IL-24 and then follow their clinical response to treatment. Alternatively, archived tissues could be obtained from metastatic melanoma patients through the MD Anderson Melanoma Core and perform a retrospective study by examining tissue from responders and nonresponders to IL-2 therapy and then correlating IL-24 staining with survival data. These data would substantiate the importance of IL-24 as a predictive marker for responsiveness to IL-2 therapy in melanoma patients. We would expect that patients who respond to high dose IL-2 treatment are more likely to express IL-24 in their melanoma tumor cells.

If showing IL-24 on patient biopsy samples by immunohistochemistry is unsuccessful, some alternative approaches for detection of protein on tissue samples include qPCR from paraffin embedded tissue, in situ hybridization in tissue, and direct tissue proteomics [175].

IL-2Rβ or IL-15Rα coexistence with IL-24 as a predictive marker for response to IL-2 therapy

Our data demonstrated that stimulating some melanoma cells with IL-2 increases IL-24 expression via signaling through the IL-2 receptor. Blocking the IL-2Rβ chain in particular had the greatest effect on IL-2 upregulation of IL-24 protein expression. IL-15, which also signals through the IL-2Rβ chain, was likewise able to upregulate IL-24 protein expression. All of these data emphasize the essential role that IL-2Rβ plays in controlling IL-24 expression and subsequent tumor growth suppression in these cells. This observation is encouraging because it has been shown that several other cancers, including squamous cell carcinoma, renal cell carcinoma, and gastric carcinomas, are growth inhibited by IL-2 signaling specifically through the IL-2Rβ chain [114, 115]. It happens that the five melanoma cell lines tested all contained IL-2Rβ mRNA. However, this does not imply that all melanoma cells will have IL-2Rβ present. Therefore we propose that the coexistence of IL-2Rβ and IL-24 in melanoma cells could potentially be a stronger predictive marker of response to IL-2 than IL-24 alone.

Other groups have also shown that IL-15Rα is expressed in the melanoma cell lines MELP and MELREO [176] as well as in 12 uveal melanoma cell lines [177, 178]. Secondary cytokines released by IL-2 stimulated lymphocytes include TNFα and IFNγ which are known to induce the production of IL-15. Therefore, IL-15 levels are very likely to be elevated in patients receiving high dose IL-2 therapy. Thus, patients whose melanoma tumor cells express IL-15Rα might also respond better to IL-2 therapy by upregulation of IL-24.

Support for IL-15 as therapy for metastatic melanoma

IL-15 is known to be similar to IL-2 in terms of function [179, 180]. Both cytokines are capable of facilitating the manufacturing of immunoglobulins produced by B lymphocytes and induce the differentiation and proliferation of natural killer (NK) cells [180]. The principal differences between IL-2 and IL-15 are associated with the adaptive immune response. IL-2 is involved in maintaining T-Regs and decreases self-reactive T cells resulting in a more self tolerant state. IL-15 is required for maintaining highly specific T cell responses by supporting the survival of CD8+ memory T cells [181]. In a recent publication, regression of subcutaneous melanoma tumors was seen after intratumoral delivery of an IL-15 expressing plasmid [182]. Treatment with IL-15 also lacks the secondary cytokine storm response seen with IL-2 which causes its capillary leak syndrome side effects [183]. Thus dose limiting toxicity of IL-15 is considerably less than that of high dose IL-2. IL-15 may therefore be potentially promising as therapy for melanoma either alone or in combination with IL-2 or chemotherapeutics. In fact, the National Cancer Institute is currently sponsoring a Phase I trial exploring the use of recombinant hIL-15 as a treatment for metastatic malignant melanoma and metastatic renal cell carcinoma (ClinicalTrials.gov ID: NCT01021059, http://clinicaltrials.gov/ct2/show/NCT01021059). While this study focuses on the immunotherapuetic aspect of IL-15, once again, our data show that IL-15 has a direct effect on melanoma cells by upregulating expression of IL-24 and causing tumor cell growth suppression. By this reasoning, IL-24 also has the potential to be a promising predictive marker for response to IL-15 therapy.

Mechanism of IL-2 dependent IL-24 Upregulation in Melanoma Cell Lines

We have established that in those tumor cells which do respond to IL-2 treatment, IL-2 mediates this activity by signaling through the IL-2 receptor. It is important to elucidate the mechanism by which this upregulation of IL-24 expression occurs. We have published *in vitro* data in normal peripheral blood mononuclear cells (PBMC) that show that PHA stimulation causes IL-2 secretion which stabilizes IL-24 mRNA resulting in increased protein production [84]. It is recognized that the IL-2/IL-2 receptor interaction results in the activation of downstream Janus Kinase (Jak)-signal transducer and activator of transcription (STAT) pathways which in turn regulate gene transcription. In T cells, IL-2Rβ signals through Jak1 and IL-2Rγ signal through Jak3. These in turn phosphorylate and activate STAT5 and STAT3.

Since STATs control gene transcription, it is possible that activation by IL-2 causes stabilization of IL-24 mRNA message via STAT activation in melanoma cell lines as has previously been reported occurs in PBMC [184]; this can be determined by real time RT-PCR. To determine whether message stabilization is occurring cells can be treated with IL-2 followed by Actinomycin D to stop any new RNA transcription. If there is message stabilization, levels of IL-24 mRNA will remain steady when measured over time by real time RT-PCR.

IL-2 induces and regulates the expression of many genes. As previously discussed, IL-2 signals via its receptor through the activation of several different pathways, including the JAK/STAT pathway and the p38 MAPK pathway [185-187]. In IL-2 activated Th1 cells, IL-2 induces TNFβ production by activating the p38 MAPK pathway (in addition to the JAK/STAT pathway) [188]. Another study demonstrated that IL-1β induces p38 MAPK

dependent expression of IL-24 in normal human keratinocytes. The study shows that p38 MAPK regulates the gene expression of IL-24 in keratinocytes by interfering with IL-24 mRNA degradation caused by 3' UTR destabilization [189]. Given these data, another possible mechanism for IL-2 regulation of IL-24 expression is by interfering with IL-24 mRNA degradation via signal transduction through the p38 MAPK pathway.

Although the three IL-24 positive cell lines we investigated all responded to high dose IL-2, it is completely possible that there exist melanoma cells which contain IL-24 mRNA but are unresponsive to IL-2. Once we understand the mechanism of IL-2 induction of IL-24 in cell lines that do respond we can investigate why the process might not work in other cells. IL-2 unresponsive IL-24 positive cells may lack functional IL-2 receptor chains, or could contain mutations or splice variants of downstream elements [178, 190]. To probe these possibilities, further downstream events along the IL-2 signaling pathways must be examined to determine if differences exist that correlate with melanoma cells' responsiveness to IL-2.

Conclusion

Based on the data contained in this dissertation and the available literature reviewed, we have presented a novel model for the direct effects of high dose IL-2 on melanoma cells. In this model, treatment with high dose IL-2 upregulates IL-24 protein expression which leads to melanoma tumor growth suppression. Currently no reliable marker exists to identify and predict the melanoma patients who are most likely to respond positively to IL-2 therapy. The results from this study offer insights into several potential predictive biomarkers for targeted high dose IL-2 therapy. Identification of such a marker is essential in order to

effectively treat these patients. IL-2 upregulation of IL-24 may be important for predicting clinical response to high dose IL-2 therapy. Potential markers include the presence of IL-24, or coexpression of IL-24 and the IL-2Rβ chain in melanoma cells. This study also offers evidence to support the exploration of IL-15 either alone or in combination with other treatments as a potential therapy for melanoma. We have shown it to be equally capable of causing IL-24 dependent melanoma cell growth suppression in comparison with IL-2, and the existing literature observes that IL-15 has fewer side effects and toxicity than high dose IL-2 therapy.

Cancers are incredibly complex diseases, and increasing our understanding of the molecular mechanisms of both the diseases and the treatments is essential. Selection of an appropriate therapy should be tailored to the individual patient based on his specific tumor and immunologic profile. In fact, the prevailing trend in cancer medicine is toward genetic profiling and customized courses of therapy. It is our hope that this work helps provide a predictive marker for IL-2 responsiveness and an identifiable targeted therapy for melanoma.

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Vita

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