A Cytokine Odyssey: From Interleukin-2 Signaling to Cytokine Therapy for Cancer

by

Eric Tran
B.Sc., University of Victoria, 2004,

A Dissertation Submitted in Partial Fulfillment
of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biochemistry and Microbiology

© Eric Tran, 2010
University of Victoria

All rights reserved. This thesis may not be reproduced in whole or in part, by photocopy or other means, without the permission of the author.

Supervisory Committee

A Cytokine Odyssey: From Interleukin-2 Signaling to Cytokine Therapy for Cancer

by

Eric Tran
B.Sc., University of Victoria, 2004

Supervisory Committee

Dr. Brad H. Nelson, (Department of Biochemistry and Microbiology)
Supervisor

Dr. Terry W. Pearson (Department of Biochemistry and Microbiology)
Co-Supervisor

Dr. Perry L. Howard (Department of Biochemistry and Microbiology)
Departmental Member

Dr. Juan Ausio (Department of Biochemistry and Microbiology)
Departmental Member

Dr. Ben F. Koop (Department of Biology)
Outside Member

Abstract

Supervisory Committee
Dr. Brad H. Nelson, (Department of Biochemistry and Microbiology)
Supervisor

Dr. Terry W. Pearson (Department of Biochemistry and Microbiology)
Co-Supervisor

Dr. Perry L. Howard (Department of Biochemistry and Microbiology)
Departmental Member

Dr. Juan Ausio (Department of Biochemistry and Microbiology)
Departmental Member

Dr. Ben F. Koop (Department of Biology)
Outside Member

T cells are a crucial component of the immune system and play an important role in responses to pathogens, tumours, and transplanted tissues. In many human cancers, elevated numbers of tumour-infiltrating CD8+ killer T cells are associated with favourable outcomes, suggesting that enhancing T-cell responses could provide major therapeutic benefit for cancer patients. Thus, identifying factors that can promote protective T-cell responses is of great clinical importance. The cytokine interleukin-2 (IL-2) is a major inducer of T-cell proliferation and differentiation, and is used clinically to treat melanoma and renal cell carcinoma. The first two chapters of this thesis focus on the biochemical mechanisms by which IL-2 induces T-cell proliferation. By using mutant and chimeric cytokine receptors expressed in lymphocyte cell lines, the interplay between Shc and STAT5, two major mitogenic signaling pathways activated by the IL-2 receptor, are investigated, revealing an essential synergy between the two pathways for optimal lymphocyte proliferation.

The third chapter of this thesis describes work done to identify cytokines that promote T-cell responses within the ovarian cancer microenvironment. In human diseases such as HIV/AIDS and cancer, high numbers of "polyfunctional" T cells (i.e., T cells capable of multiple effector functions) are associated with favourable outcomes. Using clinical ovarian cancer samples in a novel *ex vivo* assay, it was found that the ovarian tumour environment inhibits polyfunctional T-cell responses to varying extents among patients. After surveying a large panel of cytokines, the cytokine combination of IL-2, IL-12, and IL-18 was found to overcome the immunosuppressive environment to potently enhance CD8+ T-cell proliferation and polyfunctionality in all patient samples. The polyfunctional profiles induced by these cytokines are associated with protective immunity in various human conditions. Thus, these findings suggest that given the right signals, T cells can become highly polyfunctional effectors in the ovarian cancer microenvironment, which offers promise for the development of effective T-cell based therapies for this clinically challenging disease.

Table of Contents

List of Tables

List of Figures

Acknowledgments

I would first like to thank my family. Thank you mom, dad, and brother for taking care of me, being so supportive, and allowing me to pursue my passion. I know it must have been difficult at times not being able to spend much time with me, and I apologize for that, and thank you for your patience and understanding. Research can so easily pull one away from some of the simple, but important, things in life, but you helped me keep a balance. Thank you.

Next, I would like to thank my second family, the DRC, past and present. From the administrative staff to the high school interns, undergraduate students, graduate students, post-docs, and research staff, I feel extremely fortunate to have worked alongside such a fine group of individuals. You have all contributed to a wonderfully rich research environment. I will miss the positive, collaborative atmosphere in the lab, the scientific (and dare I say, non-scientific?) chats, and journal club in the foyer. I really felt at home with all of you, and that's why you truly are my second family. There are a few DRC'ers that I would like to specifically thank (in somewhat chronological order): Heather Lockyer, for being my first (and fantastic!) teacher in the lab, you taught me about MoBi and Westerns, the steak and potatoes of molecular immunology; Erika "Elika" Wall, for many helpful discussions and teaching me how to use multi-colour flow cytometry, the steak and potatoes of cellular immunology; Taimei "the Surgeon" Yang, for all the discussions about cytokines and ascites; Melanie Mawer, for all your behind the scenes work, and for endowing me with the "Junior VP of Lab Tasks" position; Rob "Bobby S" Sahota, for being so helpful in the lab and being an outstanding lab citizen,

and for the scientific chats; Michele “Miggy Bunz” Martin, for always looking out for your “Erkel-pie” and being my big sis’ in the lab; Darin “D-Wick” Wick, for your technical excellence and help with experiments, and for the many thoughtful immunology discussions; Julie “Jupes” Nielsen, for all your help with experiments, various chats about immunotherapy, enthusiasm for science, and for rivalling me on “hours spent at the DRC on weekends” (I think you won by the way); and Nathan “Eggman, Chief Soaring Egg” West, for being my partner in “DRC crimes”, and for all the times we’ve talked about cytokine signaling and T cells. Due to space constraints, I apologize for not being able to specifically thank each and every one of you who I have met at the DRC. I cherish each friendship and the times we had together. You have made my second home a happy one. Thank you.

I would also like to thank my committee, Drs. Terry Pearson, Perry Howard, Juan Ausio, and Ben Koop, for your helpful comments and discussions as I progressed throughout my graduate studies. It has been an honour to have you as my committee. Thank you.

And of course, my biggest thank you goes to my mentor, Brad Nelson. I could easily write an entire chapter (or even maybe thesis!) on all the things that you have done for me. I look back to the first time I met you, and I feel extremely lucky that you accepted me as a graduate student. Here I was, scientifically green as grass and not knowing what real immunology research was, and yet you still took me in. From there, you have devoted countless hours of your time and energy to teach me how to think, write, and present like a scientist. I remember times when I was disappointed when experiments didn’t work out, but you were always there with understanding,

encouragement, and guidance. Even as the DRC grew larger and you got busier, you always found time for me. The myriad discussions we had about cytokines, T cells, and immunotherapy were always insightful. You challenged me to think a little bigger and to ask the right questions. You have a relentless passion for research that is inspiring. In essence, you have taught me how to be a scientist; you are my scientific father. You are one of the most outstanding scientists, and people, I have ever met, and I look up to you as the measure of scientific excellence that I hope to one day achieve. I truly appreciate all the times we've had, both in and out of the lab, and I am and forever grateful to have you as my mentor and friend. Thank you.

Dedication

To my family, the DRC, and the women on Vancouver Island who have fought, and are fighting, ovarian cancer.

Chapter 1: An Introduction to Cytokines

1.1 Prologue

Cytokines are soluble, low molecular weight proteins that play critical roles in orchestrating both protective and pathological immune responses by regulating processes such as cell proliferation, death, migration, inflammation, and angiogenesis (1-3). Given that cytokines are involved in protective immunity, and dysregulation of cytokine networks contributes to human diseases, there has been much interest in unraveling the mechanisms by which these molecules mediate their effects at the cytokine, cytokine receptor, and cellular signaling levels. Directly relevant to the clinic is the therapeutic use of cytokines or cytokine antagonists to treat a variety of infectious and autoimmune diseases, and cancers (3-5). This thesis explores both the molecular mechanisms of cytokine signaling, namely how the classic T-cell growth factor IL-2 promotes T-cell proliferation, and the potential use of cytokine combinations to enhance T-cell responses against ovarian cancer.

1.2 Cytokines, a brief historical perspective

1.2.1 The 1950s, in the beginning, there was a fever

The cytokine field began in the early 1950s, when Bennett and Beeson described a heat labile substance from leukocyte extracts and supernatants that could induce fever in rabbits (6). It is now known that the fever-inducing effects of this pyrogenic substance were likely due to the pro-inflammatory cytokine IL-1 (3). Close behind was the report of

nerve growth factor (NGF) by Levi-Montalcini and Hamburger in 1953 (7), and the landmark description of interferons (IFNs) by Isaacs and Lindenmann in 1957 (8). Their observation that an "interfering substance" was present in the fluid of cells infected with influenza virus, and that this fluid protected cells from virus infection, provided hope that IFNs could one day be used therapeutically to protect against and/or treat viral infections (3).

1.2.2 The 1960s, lymphocytes "blast" onto the scene

Lymphocytes are a class of white blood cells comprised of T cells, B cells, and natural killer (NK) cells. One major characteristic of lymphocytes is their extraordinary ability to rapidly proliferate upon antigen encounter, which allows for the formation of a lymphocyte "army" to protect the host from disease. Although it is now well established that lymphocytes are essential mediators of protective immunity, prior to the 1960s, no one really understood what lymphocytes did (9). Text books of those days described lymphocytes as uninteresting, terminally differentiated cells that lacked the ability to proliferate (9). This all changed with a single observation by the young scientist Peter Nowell: he noticed that lymphocytes could in fact proliferate (9). Nowell was using the kidney bean extract phytohemagglutinin (PHA) to separate (agglutinate) red blood cells from white blood cells, and unintentionally left the non-agglutinated fraction which contained white blood cells and PHA, in the incubator for several days (9, 10). Nowell came back to these cultures and observed that the cells were much larger and undergoing mitosis, resembling leukemic blast cells (9, 10).

Adding to the excitement of Nowell's findings were discoveries from multiple groups demonstrating that lymphocyte proliferation could also be induced by other mitogens (in addition to the plant lectin PHA used by Nowell) and mixing lymphocytes from different donors together (i.e., mixed leukocyte reactions) (11-14). The cytokine field was given another boost by several groups in the mid 1960s. Two groups in particular, Kasakura and Lowenstein, and Gordon and Maclean, simultaneously detected mitogenic activity, a "blastogenic factor" as it was coined, in the culture supernatants of PHA-stimulated cells and peripheral white blood cells stimulated with soluble protein antigen (15, 16). Moreover, this "blastogenic factor" was not an antibody and was synthesized by lymphocytes, and so was also called a "lymphokine", to denote that the factor was derived from lymphocytes (17). Factors involved in processes other than proliferation were also described in the supernatants of stimulated lymphocytes. For example, macrophage migration inhibitory factors (MIFs) were described by David (18), and Bloom and Bennett (19), and a cell cytotoxicity factor ("lymphotoxin") was described by Ruddle and Waksman (20), and Granger and Williams (21).

Thus, the 1960s saw a newfound excitement for studying lymphocytes. Lymphocytes could indeed proliferate and they could secrete factors that controlled important biological and immunological processes. Lymphocytes were no longer "boring"; the age of cellular immunology had arrived.

1.2.3 The 1970s, T-cell growth factor (TCGF) takes the spotlight

Not surprisingly, the activities of more cytokines were described in the 1970s, such as a macrophage activating factor (MAF) which promoted macrophages to kill intracellular bacteria (22); tumor necrosis factor (TNF) which induced tumor cell death (23); and lymphocyte activating factor (LAF) which was produced by macrophages and stimulated lymphocyte proliferation (24). However, it was the use of the originally described lymphocyte-conditioned media containing a "blastogenic factor" that made major head-way in T-cell biology. Prior to this point, the study of T cells was limited by the inability to easily grow and sustain normal T cells in culture. Robert Gallo's group was the first to show that conditioned media from PHA-stimulated lymphocytes could be used to support long-term growth of normal T cells (25). Lymphocyte-conditioned media were also used by Kendall Smith's group to generate the first monoclonal, tumor-specific T-cell cultures (26, 27). These were landmark discoveries, as it meant, from a therapeutic standpoint, that a T-cell growth factor (TCGF) could be used to generate a large number of tumor (antigen)-reactive T cells *in vitro*, which could potentially be infused back into patients for therapeutic benefit (i.e., adoptive immunotherapy).

It is important to note that due to the limited molecular tools of the day, all of the different observed activities ascribed to the various lymphocyte-conditioned media were based on non-purified supernatants. The purification and identification of the factors (i.e., cytokines) responsible for mediating the observed effects would have to wait until the 1980s.

1.2.4 The 1980s, the dawn of molecular immunology and cytokine therapy

The advent of newer biochemical technologies ushered in the molecular cytokine era (2). The combination of high performance liquid chromatography (HPLC), microsequencing and cytokine-specific antibodies allowed the purification and amino acid sequencing of the small amounts of cytokines present in conditioned media (2). The production of T-cell growth factor (TCGF)-specific monoclonal antibodies allowed the purification of large (milligram) quantities of TCGF, which when radio-labeled, further resulted in the identification of the first cytokine receptor (28). These techniques were also used to show that TCGF was different than lymphocyte activating factor (LAF), despite the fact that both were observed to be mitogenic for lymphocytes. Specifically, experiments by Kendall Smith and colleagues revealed that LAF promoted lymphocyte proliferation by inducing the production of TCGF by T cells, and thus TCGF was the true mitogenic cytokine for T cells (29, 30). Since LAF acted upstream of TCGF, it was renamed IL-1, while TCGF was designated IL-2 (9).

Advances in molecular biology techniques revolutionized the cytokine field. In 1980, Tada Taniguchi cloned the first cytokine gene, IFN-β1 (31), which was followed by the cloning of a rash of other cytokines such as IFN-α1 by Nagata and colleagues in 1980 (32), and IFN-γ by Gray et al. in 1982 (33). In 1983, Taniguchi also cloned the first interleukin, IL-2, which allowed researchers to definitively demonstrate that IL-2 was a major mitogenic factor for T cells (i.e., TCGF was IL-2) (34). Many other cytokines were cloned during the 1980s, including TNF-α (35), TNF-β (36), transforming growth factor beta (TGF-β) (37), IL-1 (38), and IL-6 (39). Moreover, the age of "the cytokine

receptor" arrived in 1984 when Warren Leonard and colleagues cloned the first cytokine receptor, the IL-2 receptor alpha chain (40).

Importantly, these molecular advancements meant large quantities of pure cytokines could be produced, which was required if cytokines were to be used therapeutically. At the forefront of cytokine therapy for human cancer were Steven Rosenberg and colleagues, with their studies using the T-cell growth factor IL-2 (41, 42). Using IL-2 to treat cancer was based on two main ideas: 1) T cells were capable of killing tumors; and 2) IL-2 potently expanded T cells. Thus, giving IL-2 to cancer patients may "boost" the number of tumor-reactive T cells in the patient. After decades of clinical testing, IL-2 therapy appears to be effective only in a small subset of cancers. Nevertheless, it still remains the only approved curative treatment for metastatic melanoma and renal cell carcinoma, with objective clinical responses of ~20% and an overall cure rate of ~6-8% for these otherwise incurable diseases (43, 44). The proof-of-principle was in: cytokines could be used to treat human disease.

Thus, the 1980s saw the advent of new molecular techniques that allowed tremendous growth in the cytokine field. Researchers could finally begin to tease apart the cytokines found in "lymphocyte-conditioned media". The cloning of cytokines and their receptors brought the field one giant step closer to understanding what cytokines did and how they did it, and catalyzed the use of cytokines for treating human disease.

1.2.5 The 1990s, the arrival of cytokine signaling

The torrid pace of cytokine research from the 1980s continued into the 1990s. More cytokines and cytokine receptors were cloned, allowing researchers to begin

deciphering the mechanisms underlying how these molecules worked to mediate a biologic effect. Up to this point, researchers knew that cytokines bound to receptors, which caused the receptors to "do something" within the cell; however that "something" was a big "black box". Adding to this, a major peculiarity of the cytokine receptors was the lack of any protein tyrosine kinase (PTK) domain or any other enzymatic domain that resembled something that could initiate an intracellular signaling cascade (9). This was unlike some of the known receptors of the time, such as the epidermal growth factor receptor (EGFR), which has a kinase domain and exhibits autophosphorylation of tyrosine residues upon ligand binding (9).

Thus, it was timely that a new class of kinases called the **Ja**nus **k**inases (JAKs) was discovered around this time (45-48). These kinases were named after the two-faced god Janus since sequence analysis predicted the presence of two PTK domains arranged in opposite orientation (9). In 1992, Sandra Pellegrini and colleagues were the first to demonstrate that a JAK (Tyk2) was linked to cytokine (IFN-α/β) receptor signalling (49). This was closely followed by a wave of discoveries linking more JAKs to other cytokine receptors such as the Erythropoietin (Epo) (50), Growth Hormone (GH) (51), IFN-γ (52, 53), IL-3 (54), and IL-2 (48) receptors, among others. Collectively, these findings importantly provided a mechanistic link behind how a cytokine receptor, which lacks intrinsic enzymatic activity, initiates an intracellular signaling cascade. Concurrently, James Darnell's group was investigating how IFNs mediated their effects at the molecular level. They initially found two proteins that were involved with the interferon response and called them **S**ignal **T**ransducers and **A**ctivators of **T**ranscription (STAT) 1

and 2 (STAT1 and STAT2), to denote the fact that these proteins transduced the cytokine signal and also modulated gene transcription (55-58). Subsequently, Darnell's group also discovered STAT3 and STAT4 (59, 60), while STAT5 was cloned by Groner's group (61), and STAT6 was discovered in Steven McKnight's lab (62). Further work in the 1990s by many groups revealed that specific cytokines activated distinct combinations of JAKs and STATs, thereby providing a mechanism by which cytokines induced a specific cellular response. To date, four JAKs and seven STATs have been identified and are further discussed below.

1.2.6 The 2000s, cytokine antagonists go primetime, and TCGF (IL-2) takes the spotlight (again)

While the therapeutic use of cytokines such as the IFNs, IL-2, and IL-12 were extensively evaluated in the '80s and '90s, the 2000s brought promising new cytokines such as IL-7, IL-18, and IL-21 into oncology clinical trials. Details of these cytokines and their use in cancer patients are described in a later section. However, in parallel with cytokine immunotherapy was the development of anti-cytokine therapy. Given that cytokines contribute to the pathology of many chronic diseases such as rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and cancer, great effort has been, and continues to be made in identifying and blocking the cytokines that mediate disease progression. Strong pre-clinical data in the '90s led to the development of commercialized cytokine antagonists in the 2000s, ushering in a revolution for the treatment of a number of diseases (63). Indeed, over 40 different cytokine antagonists targeting over 30 cytokines implicated in the pathogenesis of over 40 different chronic

diseases are currently in clinical trials (63). Perhaps the most successful example of a disease greatly affected by cytokine antagonist treatment is rheumatoid arthritis (RA), a disease characterized by chronic joint inflammation and destruction (64). The pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) is a major contributor to the pathogenesis of RA and thus this cytokine has become the target of 3 commercially available TNF-α antagonists, Infliximab, Adalimumab, and Etanercept (63-66). Infliximab (Remicade®) and Adalimumab (Humira®) are monoclonal antibodies specific for TNF-α, while Etanercept (Enbrel®) is a TNF-α decoy receptor, and together, these cytokine antagonists have been used to treat over 1 million patients (64). IL-1 is another pro-inflammatory cytokine that is implicated in the pathogenesis of RA and has been targeted with the IL-1 receptor antagonist Anakinra (Kineret ®), although Anakinra appears to be less clinically effective than the TNF-α antagonists (63, 65, 66). In the cancer setting, vascular endothelial growth factor (VEGF) is a key cytokine for driving angiogenesis, tumor development and metastasis, which prompted the development of VEGF antagonists (63). Bevacizumab (Avastin ®) is a monoclonal antibody that has been approved for the treatment of colorectal and non-small cell lung carcinoma, and is currently in late phase clinical trials for a number of other cancer types (63).

The 2000s also brought the discovery of a new, critical role for the "old" cytokine IL-2. The potent ability of IL-2 to induce T-cell proliferation led to the notion that the main role of this cytokine was to amplify and promote T-cell immunity. Paradoxically, however, mice deficient in IL-2 were not immunodeficient, but rather, suffered from lymphoproliferative disease and fatal autoimmunity, suggesting that the main role of IL-2

in vivo was to promote T-cell tolerance (67, 68). This paradox was resolved with the discovery that IL-2 is critical for the development and function of regulatory T cells (Tregs) (69), which prevent autoimmunity by suppressing self-reactive T cells. Tregs are also widely implicated in inhibiting anti-tumor immune responses (70), which has major implications for the immunotherapy of cancer. For example, IL-2 therapy of cancer could have the undesired effect of promoting Treg expansion and function. Indeed, IL-2 administration to metastatic melanoma and renal cell carcinoma patients was found to elevate and sustain the number and frequency of Tregs in clinical non-responders compared to responders (71), suggesting that IL-2 may not be the optimal cytokine for treating cancer patients. Similar to anti-cytokine therapies, a number of "anti-Treg" depletion strategies (e.g., the anti-CD25 monoclonal antibody, daclizumab, and the IL-2-diptheria toxin fusion protein, Ontak) are currently being investigated in cancer patients as a means to enhance anti-tumor immune responses (70).

Thus, the 2000s saw a great explosion in the clinical evaluation of cytokines and cytokine antagonists for treating human diseases. In addition, IL-2 made major headlines by demonstrating that it was essential for Treg development and consequently, peripheral T-cell tolerance, which questions the use of IL-2 for treating cancer. Although there are successful examples of cytokine and anti-cytokine therapy in humans, it should be noted that these successes represent the minority. However, the efficacy of cytokine and anti-cytokine therapy will inevitably improve as immunologists are continually developing a better understanding of how cytokines function at the molecular, structural, cellular, and whole organism level.

1.3 General mechanisms of cytokine signaling

1.3.1 The JAK and STAT paradigm

Fig.1 illustrates the general paradigm of JAK-STAT5 signaling. Cytokines bind to their cognate receptors, which induces receptor oligomerization and subsequent activation of membrane-proximal, receptor associated JAKs (72-75). The activated JAKs phosphorylate tyrosine residues on the receptor, thereby creating docking sites for proteins containing a Src homology 2 (SH2) domain (72-75). STATs are recruited to the receptor via their SH2 domain, where they become substrates of the JAKs. After phosphorylation and release from the receptor, phosphorylated STATs dimerize through reciprocal phospho-tyrosine-SH2 domain interactions, and translocate to the nucleus where they modulate transcription by binding to specific DNA sequences called γ-activated sequences (GAS) (72-75).

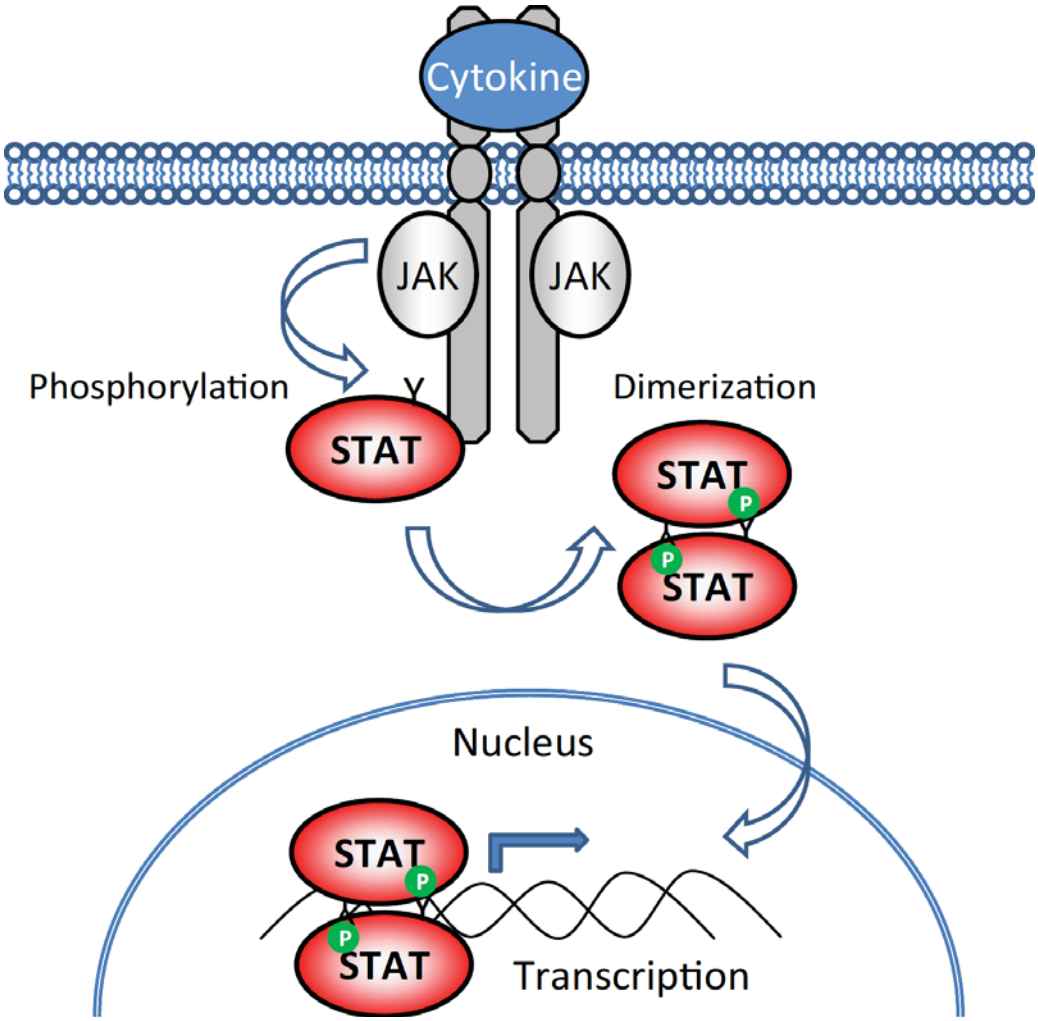


Figure 1. JAK-STAT signaling.

Cytokines bind to their receptors and induce activation of receptor-associated JAKs, which in turn phosphorylate receptor tyrosine residues thereby creating docking sites for STATs. Recruited STATs are then phosphorylated on key tyrosines by JAKs, causing STATs to dimerize and translocate to the nucleus where they bind to specific DNA recognition motifs (γ-activated sequences, GAS) and regulate transcription. Adapted from (72-75).

1.3.2 The JAKs

The mammalian JAK family is comprised of four members: JAK1, JAK2, JAK3, and TYK2 (tyrosine kinase 2) (72-75). JAKs are ubiquitously expressed, except for JAK3, which is restricted to leukocytes (72). JAKs range from 120-140 kDa in size and contain seven conserved JAK homology (JH) domains, known as JH1-JH7 (see Fig. 2A for domain structure) (72, 73). The carboxy terminal JH1 and JH2 domains represent the "two-faced Janus" aspect of the JAKs, since JH1 is the catalytically active tyrosine kinase domain, and JH2 is the catalytically inactive pseudo-kinase domain (72-74). Although lacking kinase activity, the pseudo-kinase domain is thought to regulate JAK tyrosine kinase activity (73, 74). The amino-terminal JH4-JH7 domains make up a FERM domain, (named after the proteins from which it was first described: **f**our point-one, **e**zrin, **r**adixin, and **m**oesin) which mediates binding to the proline rich, membrane-proximal box1/box2 domains found on cytokine receptors (72). JAKs also contain an "SH2-like" domain of unknown function (72). The importance of JAKs in the immune system is illustrated by patients who have defects in JAK3 signaling: these patients are severely immunocompromised due mainly to the impairment of T- and NK-cell development (76).

1.3.3 The STATs

There are seven members of the mammalian STAT family: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (72-75). STATs range from 750 to 900 amino acids in size and contain seven conserved domains, the most highly conserved of which is the SH2 domain, due to its paramount role in receptor recruitment and STAT dimerization (see Fig. 2B for STAT domain structure) (72). The amino-terminal (NH2)

domain appears to serve several important functions, including nuclear import and export of STATs, and directing dimerization of inactive STATs (72). In addition, the NH2 domain, like the DNA-binding domain, can also facilitate STAT binding to the GAS family of enhancers (72). The coiled-coil domain is a four-α-helix bundle that can bind proteins involved in regulating gene transcription and nuclear export (72). The C-terminus of STATs contain a tyrosine activation domain directly adjacent to the SH2 domain (at approximate amino acid residue 700), and a highly variable transactivation domain (TAD), which allows a specific STAT to associate with different transcriptional regulators (72). Tyrosine phosphorylation is critical for STAT function, as it induces a conformational change that promotes both the formation of STAT-STAT complexes and binding to DNA (75).

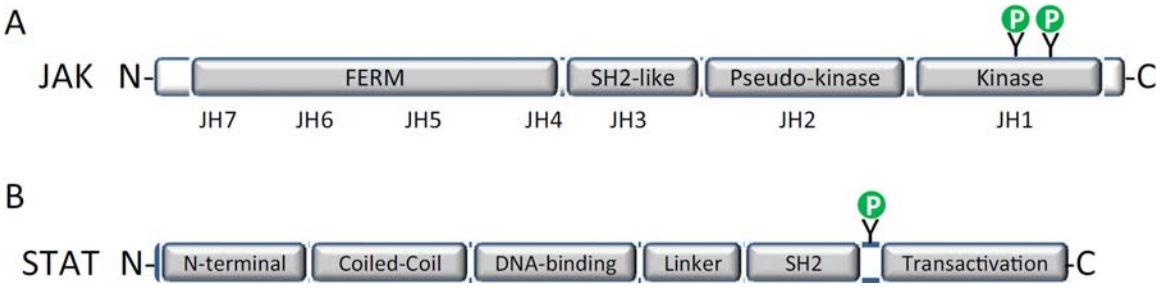


Figure 2. Domain structure of JAKs and STATs.

(A) JAKs share 7 regions of high homology, designated JH1-7, where JH1 is the kinase domain and contains two tyrosine residues (Y) that can be phosphorylated upon cytokine stimulation. The FERM domain mediates binding to cytokine receptors. **(B)** STATs contain several domains, the most highly conserved of which is the SH2 domain which mediates both the binding of STATs to tyrosine phosphorylated residues on the receptor and STAT dimerization via reciprocal phospho-tyrosine interactions. Adapted from (72-75) .

1.3.4 Other pathways activated by cytokines

In addition to the JAK/STAT pathway, cytokines activate a multitude of other signaling pathways. It is outside the scope of this thesis to cover all these signaling pathways, but directly relevant to this thesis are the pathways activated by the IL-2 receptor, which are described in more detail in Chapter 2. Briefly, the IL-2 receptor activates the JAK/STAT pathway as well as two other major pathways, PI3K/AKT and the RAS/ERK, both of which are mediated by the adapter protein Shc. Other common signaling pathways activated by cytokines include the other mitogen activated protein kinase (MAPK) pathways p38 and c-Jun N-terminal kinase (JNK), Myeloid differentiation primary response gene 88 (MyD88), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), SMADs, and caspase pathways. Thus, cytokines induce the coordinate activation of the JAK/STAT and other signaling pathways, and cells integrate these signals to produce a signature biological effect.

1.4 Cytokines for cancer therapy, past and present

As mentioned, given a pivotal role of cytokines for mediating protective immunity, many cytokines have been, and are being, evaluated for the treatment of various cancers in pre-clinical models. However, the following section will focus on the therapeutic efficacy of some of the most extensively evaluated cytokines, and some that are early in clinical development but hold great promise for treating human cancers.

1.4.1 The common gamma chain cytokines

Background and Rationale: The common gamma chain (γc) cytokines include IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, and are so named because they all bind to the γc receptor and require it for functional cytokine signaling. Patients with defects in γc signaling exhibit severe combined immunodeficiency disease (SCID), which highlights the importance of these cytokines in protective immunity. Although each of the γc cytokines possess unique, non-redundant roles in immunity, the general basis for their use in cancer therapy lies primarily in their common ability to promote T and NK-cell proliferation and/or effector function.

1.4.1.1 IL-2

IL-2 can potently enhance tumor-specific T-cell proliferation, and promote tumor-reactive NK-cell expansion and effector function (77). Thus, IL-2 infusion into cancer patients may activate and expand endogenous, tumor-reactive lymphocytes, which may consequently mediate tumor regression.

The FDA approved the use of IL-2 (Proleukin®, Prometheus) for the treatment of metastatic renal cell carcinoma (RCC) in 1992, and malignant melanoma in 1998. As mentioned earlier, high dose IL-2 treatment of patients with these cancers induces objective clinical response rates of ~20% and can cure approximately 6-8% of these "incurable" patients (43, 44); however, this is usually accompanied with toxic, but treatable, side effects such as vascular leak syndrome. In other cancer types such as leukemia and lymphoma, IL-2 does not appear to improve survival (4). Intraperitoneal

administration of IL-2 to ovarian cancer patients appears to show modest benefit, but prolongation of survival has not been validated in randomized phase III trials (78, 79). IL-2 has also been combined with a large number of other modalities, such as chemotherapies, antibodies, and various vaccines, with varying success (80).

Given the limited therapeutic efficacy of IL-2, and toxic side effects, efforts have been made to identify patients that are likely respond to treatment. In line with this, it has been demonstrated that high levels of vascular endothelial growth factor (VEGF) and fibronectin predict a poor response to IL-2 (81). In contrast, renal cell cancer patients with a clear cell histology and high levels of carbonic anhydrase-IX are likely to respond favourably to high dose IL-2 therapy (82). Focus has also been on modifying IL-2 to increase half-life and/or specificity through: 1) Pegylation (covalently attaching polyethylene glycol) to increase half-life *in vivo* (83); 2) altering the amino acid sequence to preferentially bind the high affinity IL-2 receptor found on activated T cells (and not on NK cells) (84); 3) generation of immunocytokines, i.e., a molecule made up of an antibody fragment specific to a tumor antigen conjugated to IL-2, which theoretically increases the IL-2 concentration at the tumor site (85); and 4) mixing IL-2 specific antibodies with IL-2 to create "IL-2 antibody-cytokine complexes", which depending on the antibody used, can preferentially present IL-2 to effector CD8+ T cells or regulatory T cells (Tregs) (86). Since Tregs have been shown to blunt effector anti-tumor T-cell responses and are expanded by IL-2, use of the antibody-IL-2 complex that preferentially expands effector CD8+ T cells may prove beneficial in the cancer setting.

1.4.1.2 IL-7

IL-7 appears to have essential, non-redundant roles for T-cell development and function. IL-7 can dramatically increase peripheral T cell numbers, and in pre-clinical models, when used as an adjuvant to anti-tumor vaccines, can enhance anti-tumor CD4+ and CD8+ T-cell responses and significantly prolong survival of tumor bearing hosts (87, 88). IL-7 also appears to be less toxic that IL-2, and does not promote expansion of immunosuppressive Tregs (87).

The use of IL-7 for the treatment of human cancer is in its infancy. Three small phase I dose escalation studies from the National Cancer Institute (NCI) in the USA, have demonstrated that IL-7 enhances both CD4+ and CD8+ T-cell numbers (and repertoire) in metastatic cancer patients, with a preference for naïve T cells, although central memory T cells are also expanded (89-91). Notably, there is little effect of IL-7 on Treg expansion and the cytokine is well tolerated with no grade 4 toxicities seen (89-91). No anti-tumor effects were observed in these studies, but the maximum tolerated dose (MTD) was not reached, and being phase I trials, many factors such as dose number and kinetics may also not have been optimal (89-91). As IL-7 preferentially expands naïve T cells, its main utility may lie as an "immune-restorative" for patients who have experienced lymphocyte depletion due to disease or therapy. IL-7 also holds promise in the cancer clinic as an adjuvant for tumor vaccines or other modalities (87).

1.4.1.3 IL-15

IL-15 has similar properties to IL-2 in that IL-15 is also a potent T-cell mitogen and inducer of CTL function (92). In contrast to IL-2, IL-15 does not induce activation

induced cell death (AICD), has a propensity for expanding and maintaining effector memory T cells, and does not appear to expand the immunosuppressive Treg population, characteristics of which are desirable for the immunotherapy of cancer (92). Moreover, IL-15 therapy has promising anti-tumor activity, and in some cases is superior to IL-2, in a wide variety of pre-clinical tumor models including lung adenocarcinoma, colon cancer, melanoma and breast cancer (80).

Despite being cloned in 1994 and having numerous positive pre-clinical studies, IL-15 has not yet made it into clinical trials. However, it is actively being developed at the NCI and will soon be evaluated in cancer patients. It should be noted that caution is warranted when using IL-15, since some tumors such as renal cell carcinoma, and various leukemias and lymphomas, can use IL-15 as a mitogenic factor (80).

1.4.1.4 IL-21

IL-21 enhances the proliferation, cytotoxicity, and IFN-γ production of CD8+ T cells (4, 80, 93). In addition, IL-21 promotes NK-cell cytotoxicity and effector cytokine production (4, 93). In various pre-clinical mouse models, IL-21 could induce regression of established tumors (80, 93).

It took well less than a decade from the time IL-21 was first reported in literature to its appearance in clinical trials for the treatment of cancer (94-96). In two phase I trials involving metastatic melanoma and renal cell carcinoma patients, the maximum tolerated dose for single agent IL-21 was found to be 30 μg/kg, and encouragingly, some antitumor effects were observed (objective clinical responses of ~4% for melanoma and ~21% for RCC) (95, 96). In a phase II study with 24 metastatic melanoma patients, an overall

response rate of 8.3% was reported (1 complete response and 1 partial response), although none of these responses were durable (97). Based on strong pre-clinical data, trials with IL-21 in combination with other treatments such as tyrosine kinase inhibitors and therapeutic antibodies are on-going and await outcomes data (97).

1.4.2 The Interferons (IFNs)

Background and Rationale: Interferons are a large family of proteins that induce a wide range of biological effects. In addition to their originally described function of promoting resistance to viral infections, IFNs have demonstrated promising anti-tumor activity, which is mainly attributable to: 1) enhancing anti-tumor immunity by augmenting NK and T-cell function and upregulating tumor antigen presentation; and 2) inhibiting tumor cell proliferation and angiogenesis (98, 99). Moreover, endogenous IFN-γ protects against tumor development, and in a number of tumor models, is critical for anti-tumor immunity (100). These properties and promising pre-clinical activities provided the major impetus for using IFNs for the treatment of human cancers. The most widely evaluated IFNs for the treatment of human cancers are IFN-α (Intron®A, Schering Corporation, and Roferon-A, Roche), and IFN-γ (ActImmune®, Intermune).

1.4.2.1 IFN-α

Recombinant human IFN-α is one of the most widely evaluated cytokines for treating human cancer, and has been used to treat hematological malignancies such as hairy cell leukemia, chronic myelogenous leukemia (CML), B- and T-cell lymphomas, and multiple myeloma (98, 99). Treatment of hairy cell leukemia and CML patients with

IFN-α results in an approximately 75-85% hematological response rate (both partial and complete), which is associated with decreased morbidity (98, 99). However, more effective therapies have since superceded IFN-α for the treatment of these diseases (98, 99). The response rate of IFN-α is much lower for multiple myeloma (~10-20%) (98), but even when combined with chemotherapies, IFN-α does not appear to improve overall survival (101). In contrast, IFN-α therapy in combination with various chemotherapy regimens can increase disease-free and overall survival in non-Hodgkin's lymphoma.

IFN-α has also been evaluated in several solid tumor types, most notably for metastatic melanoma and renal cell carcinoma (98). For melanoma, although response rates of 2-29% were observed, little impact was seen on progression-free and overall survival (98). However, in the adjuvant setting (i.e., after primary surgery), IFN-α may increase the time to disease recurrence and overall survival (98). Similar response rates are observed in renal cell carcinoma patients treated with IFN-α, but unlike melanoma, large phase III trials have demonstrated a significant survival benefit of 2 ½ months for single agent IFN-α therapy, and 7 ½ months for combination IFN-α and chemotherapy (98). Other solid tumors, such as pancreatic, midgut carcinoid, ovarian, bladder, and basal cell tumors, have shown variable responses to IFN-α, but for the most part, major increases in survival (over the current standard of care) were not seen. It should be noted that IFN-α therapy is often associated with substantial toxicities including fatigue (> 70% of patients), neurological toxicities, mood disorders, endocrine dysfunction, and 8-20% of patients develop autoimmune-mediated thyroid dysfunction (102).

1.4.2.2 IFN-γ

IFN-γ has been tested in several cancer types including ovarian and superficial bladder cancer, and adult T-cell leukemia (103). Results from two large phase III trials for ovarian cancer using IFN-γ in combination with platinum-based chemotherapy are conflicting, with one trial demonstrating that the inclusion of IFN-γ increased complete response rates (104), while the other trial had to be stopped prematurely since the cohort that received IFN-γ had a significantly shorter survival rate and more adverse events (105). The beneficial effect of IFN-γ appears to be more clear for bladder cancer and adult T-cell leukemia, as targeted delivery of IFN-γ to the tumor site can protect against cancer recurrence (106).

1.4.3 The "IFN-γ inducing" cytokines

Background and Rationale: As mentioned above, IFN-γ has been shown to be important in protecting against tumor development (100). In some tumor models, the efficacy of an immunotherapy is critically dependent on IFN-γ (100). Moreover, there is a high correlation between IFN-γ production and tumor regression mediated by cancer immunotherapies (103). IL-12 and IL-18 are both strong inducers of IFN-γ, which made them promising cytokines for treating human cancers.

1.4.3.1 IL-12

In addition to stimulating IFN-γ production, IL-12 also enhances NK and T-cell cytotoxicity (4, 107, 108). IL-12 also plays critical roles in polarizing CD4+ T cells into the T-helper 1 cells (Th1), thereby stimulating CD8+ and NK cells, and in upregulating

tumor antigen presentation (4, 107, 108). IL-12 is also an anti-angiogenic factor (4, 107, 108). In a plethora of pre-clinical mouse tumor models, IL-12 potently inhibited tumor growth, and in some cases, completely eradicated tumors (107-109).

IL-12 therapy has been evaluated for a wide variety of human cancers, including AIDS-related Kaposi's sarcoma, melanoma, multiple myeloma, various lymphomas, head and neck, renal cell, abdominal, bladder and cervical carcinomas (108, 110). However, with the exception of AIDS-related Kaposi's sarcoma, non-Hodgkin's and cutaneous T-cell lymphomas, the objective response rates for IL-12 as a monotherapy have been low, ranging from 0-8.3% (108, 110). Given the low response rates and associated toxicities, single agent IL-12 therapy has largely been abandoned, and instead, combinational therapies with IL-12 are being investigated. In combination with other cytokines, vaccines, or anti-tumor antibodies, IL-12 can mediate slightly improved response rates (6-11%) in melanoma, renal cell carcinoma, and breast cancer patients (108). To minimize systemic toxicities, targeting IL-12 to the tumor site using gene therapy has been attempted using various techniques (108). In perhaps one of the more clinically successful IL-12 gene therapy studies, *in vivo* electroporation was used to introduce plasmid encoding IL-12 directly into melanoma lesions (111), which resulted in 10/19 (53%) of patients experiencing disease stabilization and/or objective regressions, and 2/19 patients achieving complete regression of primary and distant lesions (111).

1.4.3.2 IL-18

IL-18 is a member of the IL-1 family of cytokines and can synergize with IL-12 to stimulate IFN-γ production in NK and CD8+ T cells and promote Th1 T-cell responses

(4). IL-18 has also shown anti-tumor activity in pre-clinical tumor models as a single agent and in combination with other cytokines such as IL-2 or IL-12 (4).

Two phase I trials with IL-18 as a monotherapy demonstrated evidence of biological activity and potential clinical responses in patients with advanced cancers (112, 113). However, a phase II trial involving 64 metastatic melanoma patients was ended prematurely due to a lack of clinical efficacy of IL-18 treatment (114). Given the synergistic nature of IL-18, there is still hope for using IL-18 in combination with other immunomodulators (114).

1.4.4 Cytokines for cancer therapy, the future

It is clear that the major successes seen with cytokine therapy in pre-clinical tumor models have not translated to human cancer patients. In some pre-clinical mouse models, cytokines, even as a monotherapy, could regress large established tumors, and in some cases, cure mice. However, in humans, these same cytokines at best modestly enhance survival and often at the expense of significant toxicities. An exception is IL-2, where a small percentage of patients of specific cancer types can be cured, but also at the expense of toxicities. Perhaps the lack of clinical efficacy is not entirely surprising, given the extreme biological complexity of humans. Within just the cytokine field, there are several hundred (pleiotropic) cytokines, their receptors, all with distinct expression patterns on different cell types, with complex regulation, and interactions and synergies. With this in mind, perhaps it is even more surprising that a simple regimen of high dose IL-2 can “trump” all immunological and tumor cell networks to produce a cure for some patients.

An important lesson learned from both mouse and human studies is that cytokines often work better, and sometimes synergistically, when used in combination with other treatment modalities, such as other cytokines, tumor vaccines, adoptive T-cell therapy, and chemotherapy. Thus, combining cytokines with other therapies holds promise for the effective treatment of cancer.

1.5 Thesis hypotheses

This thesis encompasses both basic and translational aspects of cytokine research, and thus there are two major hypotheses. Chapters 2 and 3 focus on better defining the biochemical mechanisms by which the potent T-cell growth factor IL-2 induces lymphocyte proliferation. Although it is known that the IL-2 receptor activates the Shc and STAT5 pathways, the relative contribution of these pathways to lymphocyte proliferation is controversial. Thus, the major hypothesis is that STAT5 is required for sustaining activation of the PI3K/AKT pathway (downstream of Shc) and together these pathways cooperatively regulate genes that are essential for lymphocyte proliferation. The exploration of this hypothesis will contribute to our understanding of IL-2 receptor signaling, cell cycle regulation, and may provide biochemical insights into various hematological malignancies where STAT5 and PI3K/AKT activity are aberrant, potentially allowing for the development of more effective cancer therapies.

Chapter 4 investigates the potential use of cytokines for ovarian cancer therapy. The presence of tumor-infiltrating killer T cells is associated with favourable outcomes in ovarian cancer, suggesting that these T cells are actively opposing tumorigenesis. We

therefore wanted to determine whether cytokines could enhance T-cell function within the ovarian cancer environment. Thus, the major hypothesis is that a defined set of cytokines can override the varied immunosuppressive ovarian cancer environment to promote T-cell proliferation and function. The identification of cytokines that can improve T-cell responses within the tumor environment holds great promise for improving future cancer immunotherapies.

Chapter 2: STAT5 is essential for Akt/p70S6 kinase activity during IL-2-induced lymphocyte proliferation

Adapted from: Heather M. Lockyer,[*] Eric Tran[*†] and Brad H. Nelson[*†]. *The Journal of Immunology,* 2007, 179(8), 5301 -5308. *These authors contributed equally to this work.

[*]British Columbia Cancer Agency: Trev and Joyce Deeley Research Centre, 2410 Lee Ave, Victoria BC, V8R 6V5, Canada;

[†]Department of Biochemistry and Microbiology, University of Victoria, PO Box 3055 STN CSC, Victoria BC, V8W 3P6, Canada

ET designed research, performed research, collected data, analyzed and interpreted data, and wrote the manuscript

HML designed research, performed research, collected data, analyzed and interpreted data, and wrote the manuscript

BHN designed research, analyzed and interpreted data, and wrote the manuscript

Copyright 2007. The American Association of Immunologists, Inc.

2.1 Abstract

The IL-2 receptor (IL-2R) activates two distinct signaling pathways mediated by the adaptor protein Shc and the transcription factor STAT5. Prior mutagenesis studies of the IL-2R have indicated that the Shc and STAT5 pathways are redundant in the ability to induce lymphocyte proliferation. Yet paradoxically, T cells from STAT5-deficient mice fail to proliferate in response to IL-2, suggesting that the Shc pathway is unable to promote mitogenesis in the genetic absence of STAT5. Here we show in the murine lymphocyte cell line Ba/F3 that low levels of STAT5 activity are essential for Shc signaling. In the absence of STAT5 activity, Shc was unable to sustain activation of the Akt/p70S6 kinase (p70S6K) pathway or promote lymphocyte proliferation and viability. Restoring STAT5 activity via a heterologous receptor rescued Shc-induced Akt/p70S6K activity and cell proliferation with kinetics consistent with a transcriptional mechanism. Thus, STAT5 appears to regulate the expression of one or more unidentified components of the Akt pathway. Our results not only explain the severe proliferative defect in STAT5-deficient T cells, but also provide mechanistic insight into the oncogenic properties of STAT5 in various leukemias and lymphomas.

2.2 Introduction

Interleukin-2 (IL-2) is a potent cytokine used for the *in vitro* expansion of T-cells and to treat diseases such as melanoma, renal cell carcinoma and HIV/AIDS (115-117). IL-2 initiates a program of lymphocyte proliferation by binding the IL-2 receptor (IL-2R), which consists of three transmembrane proteins, IL-2Rα, IL-2Rβ and γc (77).

Intracellular signaling is mediated by IL-2Rβ and γc, which undergo IL-2-induced heterodimerization followed by activation of the associated tyrosine kinases Jak1 and Jak3 (77, 118). Downstream signals arise from Jak1/Jak3-mediated phosphorylation of tyrosine residues on IL-2Rβ, which creates docking sites for the adaptor protein Shc and the transcription factor STAT5 (signal transducer and activator of transcription 5) (119-124).

Once recruited to IL-2Rβ, Shc activates at least two downstream pathways, the Ras/Erk (extracellular signal-related kinase) pathway and the PI3K (phosphatidylinositol 3- kinase) pathway (Figure 3) (125-128). Shc activates the PI3K pathway by recruiting the adaptor protein Grb2, which in turn recruits the adaptor protein Gab2 followed by the p85 PI3K regulatory subunit (127-129). Formation of the Shc/Grb2/Gab2/p85 complex ultimately leads to catalytic activation of p110α PI3K, which converts phosphatidylinositol 4,5-bisphosphate (PIP_2) into the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3) in the cell membrane (128, 130-133). PIP_3 recruits to the cell membrane proteins containing pleckstrin homology (PH) domains, such as 3-phosphoinositide-dependent kinase 1 (PDK1) and Akt. Akt is a key mediator of PI3K-mediated cell survival, growth and proliferation (130-134). In parallel, the Shc/Grb2 complex also recruits the guanine nucleotide exchange factor Sos. Sos activates the GTPase Ras, which ultimately leads to the phosphorylation and activation of Erk (135).

We and others have studied the mechanism by which Shc promotes lymphocyte proliferation in the context of IL-2 signaling. Although IL-2 activates the Ras/Erk

pathway, this is not essential for proliferative signaling (136, 137). By contrast, several groups have shown that the PI3K/Akt pathway is essential for T-cell proliferation (137-139). The PI3K/Akt pathway was found to activate the E2F transcription factor, which is pivotal for G1 to S phase progression (138). Additionally, the PI3K/Akt pathway was shown to be necessary, although not sufficient, for maximal induction of the mitogenic genes *c-myc*, *cyclin D2*, *cyclin D3*, *cyclin E*, and *bcl-x_L* (137, 140). Finally, PI3K pathway-specific inhibitors have been used to show that a late phase of PI3K activity is required for IL-2-induced lymphocyte proliferation (139). Although essential, the PI3K pathway is not sufficient for proliferation, indicating the involvement of other pathways downstream of IL-2Rβ (137, 138).

The transcription factor STAT5, which refers to two highly homologous proteins STAT5a and STAT5b, also promotes mitogenesis and anti-apoptosis in lymphocytes (141). Indeed, dysregulated STAT5 activity is found in various leukemias and lymphomas (142-144). Upon tyrosine phosphorylation, STAT5 dimerizes via its SH2 domain and translocates to the nucleus where it directly transactivates target genes such as *c-myc, cyclin D2, cyclin D1, bcl-x_L, bcl-2, p21^{waf1}, pim-1, CIS,* and *IL-2Rα (CD25)* through a C-terminal transactivation domain (TAD) (142, 145-151). Though best characterized as a transcription factor, STAT5 can also act as an adaptor protein in the Gab2/p85 signaling complex (152, 153). Specifically, both phosphorylated wild-type STAT5 and a constitutively active mutant of STAT5 (caSTAT5) were found to co-precipitate with the scaffolding protein Gab2 and the PI3K regulatory subunit p85 (152-154). Moreover, the ability of caSTAT5 to induce cell proliferation was dependent on

Gab2, as expression of a functionally inactive Gab2 mutant prevented the ability of caSTAT5 to activate the PI3K/Akt and Ras/Erk pathways and induce lymphocyte proliferation (152). Altogether, these results indicate that STAT5 is capable of at least two mechanistically distinct modes of signaling.

In addition to having a major role in survival and proliferative signaling, activated STAT5 can also promote apoptosis under some conditions (151, 155). IL-2 plays a major role in sensitizing T cells to activation induced cell death (AICD), and this was found to depend on STAT5 signaling (155). Furthermore, in a lymphocyte cell line, a constitutively active mutant of STAT5 was shown to promote apoptosis by inducing expression of the growth inhibitory protein JAB (JAK-binding) (151). Finally, naturally occurring isoforms of STAT5 can be produced by alternative splicing or proteolytic cleavage by enzymes such as cathepsin G or calpain (156-158). Although the exact physiological significance of these isoforms remains to be determined (158, 159), overexpression of isoforms lacking the C-terminal transactivation domain can exert a dominant-negative effect on STAT5 signaling and induce apoptosis in certain cell types (156, 160-163).

Prior mutagenesis studies, in which the Shc or STAT5 docking sites on IL-2Rβ were selectively removed, indicated that Shc and STAT5 are redundant in the ability to induce lymphocyte proliferation (119, 120, 126, 137, 148, 164, 165). However, T cells rendered genetically deficient in STAT5 are completely non-proliferative upon T-cell receptor and IL-2 stimulation, suggesting that STAT5 is absolutely required for mitogenesis irrespective of the Shc pathway (149). This could mean that STAT5

contributes to proliferative signaling even when not activated by the IL-2R, as has been shown for STAT1 (166). Alternatively, IL-2Rβ mutants reported to activate Shc alone might also activate STAT5 to low but functionally significant levels. To distinguish these possibilities, we expressed mutant cytokine receptors that selectively activate Shc or STAT5, either alone or in combination, in subclones of the lymphoid cell line Ba/F3. We find that, unexpectedly, a low level of STAT5 activity is essential for sustained activation of the Akt/p70S6K pathway by Shc. Our results demonstrate a novel, essential connection between the Shc and STAT5 pathways, explain the severe proliferative defect in STAT5-deficient lymphocytes, and provide insight into the oncogenic role of STAT5 in various leukemias and lymphomas.

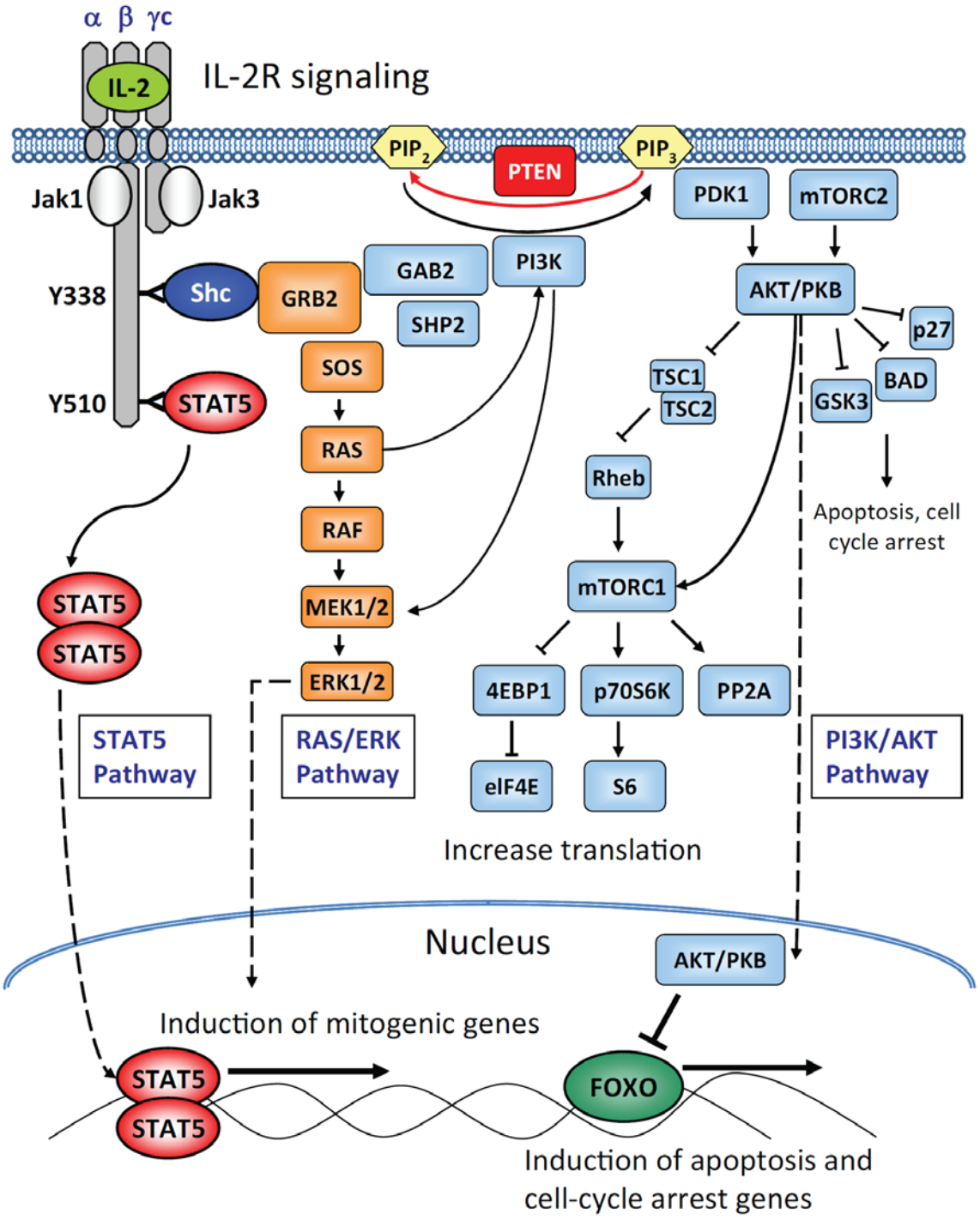


Figure 3. IL-2 receptor signaling.

Shown are the major pathways activated by IL-2R. See Chapter 2 Introduction and Discussion for more details.

2.3 Materials and Methods

Plasmid construction. β-wt, β-Y338, β-Y510 and wtSTAT5A have been previously described (137, 148). All receptor mutants were generated by standard PCR-based techniques. β-Y338GG was created from β-Y338 by modification of the C-terminus to the following sequence Y^{338}GFG[stop]. G-Y510 was created by joining the human G-CSFR extracellular domain to human gp130 at EcoRI to incorporate the transmembrane and Jak binding domains (Box1&2) of gp130. The Shp2 and STAT3 binding sites of gp130 were then replaced with a single STAT5 docking site corresponding to Y510 and flanking residues from human IL-2Rβ (YLSLQELQ[stop]). All receptor mutants were sequenced and cloned into a human β-actin promoter-driven expression vector containing a neomycin resistance gene (167). The caSTAT5A1*6 expression plasmid has been described elsewhere (168).

Cell culture. Murine pro-B Ba/F3 cells stably transfected with human GM-CSFRα, designated BAF.GM, were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin and 10% WEHI-conditioned media (source of murine IL-3). Upon human GM-CSF stimulation, the GM-CSFRα chain dimerizes with the murine common β-chain and induces strong lymphocyte proliferation mediated by STAT5, Shc, Gab2, ERK, and PI3K, which are signaling intermediaries also utilized by the IL-2R (169). GM-CSF can be purchased at low cost through the hospital pharmacy and therefore represents an inexpensive yet high quality cytokine to serve as a positive control. The murine IL-2-dependent T-cell lines CTLL-2 (CD8+) and HT-2 (CD4+) were maintained in RPMI 1640 supplemented with 10% FBS,

2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 25 mM HEPES, 1 mM sodium pyruvate, and 25 μM 2-mercaptoethanol. For the generation of stable transfectants using Ba/F3 lymphocytes, linearized plasmids were electroporated into cells and stably transfected subclones were selected at limiting dilution for G418 resistance (0.8 μg/mL, Sigma). Receptor expression was assessed by flow cytometry with antibodies to human IL-2Rβ or human G-CSFR (BD Biosciences, San Diego, CA). For all experiments, we used subclones with receptor expression levels between 0.5-1.5 log fluorescence units (Fig. 4B).

Western blots. Cytoplasmic and nuclear extracts of BAF.GM cells expressing either β-wt, β-Y338, β-Y338GG or a combination of β-Y338GG and G-Y510 were prepared and immunoblotted as described (170) with the following modifications: cells were washed 3 times with 1X PBS, and following 4 h incubation in medium without added cytokine, 20 x 10^6 cells were stimulated with recombinant human GM-CSF (100 ng/mL), IL-2 (100 U/mL), G-CSF (100 ng/mL) or a combination of IL-2 and G-CSF at 37 ^oC for the indicated time points. Extracts from 2 x 10^6 cells were run on 3-8% tris-acetate gels (Criterion XT, BioRad Laboratories, Hercules, CA) and transferred to nitrocellulose. Western blotting was performed by blocking membranes in pH 7.5 TBS-T (0.1 M Tris, 0.9% NaCl, 0.05% Tween) containing 1% (wt/vol) bovine serum albumin (BSA). Membranes were incubated for 3 h in blocking buffer containing antibodies to phospho-STAT5 (Tyr694), phospho-Shc (Tyr317 or Tyr239/240), phospho-Gab2 (Tyr452), phospho-Shp2 (Tyr542), phospho-(Tyr) p85 PI3K, phospho-Akt (Ser473), phospho-p70S6K (Thr421/Ser424), phospho-S6 (Ser235/236), or phospho-ERK-p44/42

MAPK (Thr202/Tyr204) (all from Cell Signaling Technology). Membranes were washed with TBS-T and incubated with horseradish peroxidase-conjugated goat-anti-rabbit antibodies (Jackson Laboratories). Bound antibodies were detected by enhanced chemiluminescence (Amersham). Following detection, membranes were stripped for 1 h at 60 ^{o}C with 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.1 M 2-Mecaptoethanol before washing with TBS-T, blocking in TBS-T + 1%BSA and re-probing with control rabbit antibodies specific for: Gab2 (Upstate Biotechnology), p70S6K (Santa Cruz), STAT5, Shc, Shp2, p85 PI3K, Akt, S6 or ERK-p44/42 MAPK (Cell Signaling Technology).

Proliferative assays. 5-bromo-2'-deoxyuridine (BrdU) incorporation was assessed using the Cell Proliferation Biotrak ELISA system (Amersham). Assays were conducted in triplicate with 10^4 transfected BAF.GM cells cultured in 200 μL medium plus the appropriate stimulus. After 48 h, cells were fixed, permeabilized and incubated with peroxidase-labeled anti-BrdU (1:100 in antibody dilution solution) for 90 min. Bound antibodies were detected by TMB substrate and read at 450 nm on Molecular Devices plate reader.

Transient Transfections. BAF.GM lymphocytes stably expressing β-Y338GG were resuspended at 12.5 x 10^6 cells/ml in 10 mM $MgCl_2$ PBS solution with 100 μg total of plasmid DNA (wtSTAT5A or caSTAT5A in combination with a GFP vector) and electroporated (350 V, 975 μF) with a GenePulser Xcell (BioRad). Cells were then rested overnight in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin, 50 μ/mL streptomycin and 10% WEHI-conditioned media (a source of murine IL-3). Following recovery, cells were washed 3 times with 1X PBS, starved of cytokines

for 4 h and then stimulated with 100 U/ml IL-2 or media alone. At t=1 and 10 h, cells were harvested for intracellular flow cytometry as described below. Successful transfectants, as demarcated by GFP expression, were analyzed for phospho-S6 content (see below). Transfection efficiency typically ranged between 2-5%.

Intracellular flow cytometry. Cells were stimulated for the indicated time-points with the appropriate cytokine(s), and fixed with formaldehyde (2% v/v final concentration) for 10 minutes at 37 °C. Fixed cells were then spun at 500x g and cell pellets were permeabilized with 100% ice cold methanol and incubated on ice for 20 minutes to achieve complete permeabilization. Cells were rehydrated by washing twice with >10 volumes of 1X PBS + 0.5% BSA. Cells were then stained with antibodies to phospho-STAT5 or phospho-S6 (1:200; Cell Signaling Technology) for 30-60 min at RT, washed twice with 1X PBS + 0.5% BSA and then stained with anti-rabbit IgG (conjugated to phycoerythrin (PE) at 1:100) for 30-60 min at RT in the dark (CalTag Laboratories). Events were collected with a BD FACSCalibur flow cytometer and CellQuest Pro software. Data analysis was performed using FlowJo software (Tree Star, Inc).

Quantitative PCR (QPCR) analysis. Cells were snap frozen in an ethanol/dry ice bath and stored at -80 ºC. Total RNA was isolated using the RNeasy Mini kit (Qiagen) following the manufacturer's protocol and quantified using a NanoDrop® ND-1000 spectrophotometer. RNA was reverse transcribed to cDNA using the iScript cDNA synthesis kit (BioRad). *CIS* expression was measured by QPCR using the intron-spanning primer set: *CIS* Forward 5'-CGT TGT CTC TGG GAC ATG GTC-3'; *CIS* Reverse 5'-

CAA TTT GCT CCA CAG CCA GC-3'. *c-myc* expression was determined with the intron-spanning primer set: *c-myc* Forward TTT GTC TAT TTG GGG ACA GTG TT; *c-myc* Reverse CAT CGT CGT GGC TGT CTG. GAPDH was used as a reference gene and transcript levels were assessed using the primers: *GAPDH* Forward 5'-AAC TTT GGC ATT GTG GAA GG-3'; *GAPDH* Reverse 5'-ACA CAT TGG GGG TAG GAA CA-3'. QPCR was performed using the iCycler MyiQ Real-Time PCR detection system (BioRad) with the following 2-step protocol: initial denaturation at 95 °C for 1:30, followed by 40 cycles of denaturation at 95 °C for 10 sec and 30 sec extension at 55 °C. After final denaturation at 95 °C for 1 min, a melt curve analysis was performed starting at 55 °C and increasing by increments of 1 °C up to 95 °C. Relative gene expression was calculated using Bio-Rad's Gene Expression Macro™ Version 1.1 software. Expected product sizes were verified by standard agarose gel electrophoresis.

2.4 Results

An IL-2Rβ mutant reported to exclusively activate Shc induces low levels of STAT5 activity.

To assess the possibility that IL-2R mutants thought to exclusively activate Shc might also activate low levels of STAT5, we first re-evaluated a previously described IL-2Rβ truncation mutant designated β-Y338 (also known as ββΔ355) which contains the Shc docking site at Y338 but lacks all other cytoplasmic tyrosine residues, including all previously defined STAT5 activation sites (Fig. 4A and 4B) (121, 126). In accord with prior reports, β-Y338 induced robust activation of the Shc pathway, as evidenced by IL-

2-induced phosphorylation of Shc, p70S6K, S6, and ERK (Fig. 5A), and this was associated with strong proliferation in the lymphocyte cell line BAF.GM, a derivative of Ba/F3 cells (Fig. 5B and Materials and Methods). Sensitive immunoblotting with phospho-specific antibodies revealed that the β-Y338 mutant also induced low-level tyrosine phosphorylation of the full-length isoform of STAT5, despite lacking all known STAT5 docking sites (Fig. 5A). Furthermore, quantitative PCR (QPCR) analysis revealed low but reproducible induction of the STAT5-specific target gene *CIS* by β-Y338 (Fig. 5C). Thus, previous studies concluding that the Shc pathway alone could induce lymphocyte proliferation may be confounded by low-level STAT5 activation by receptor mutants such as β-Y338 (119, 126, 137, 155).

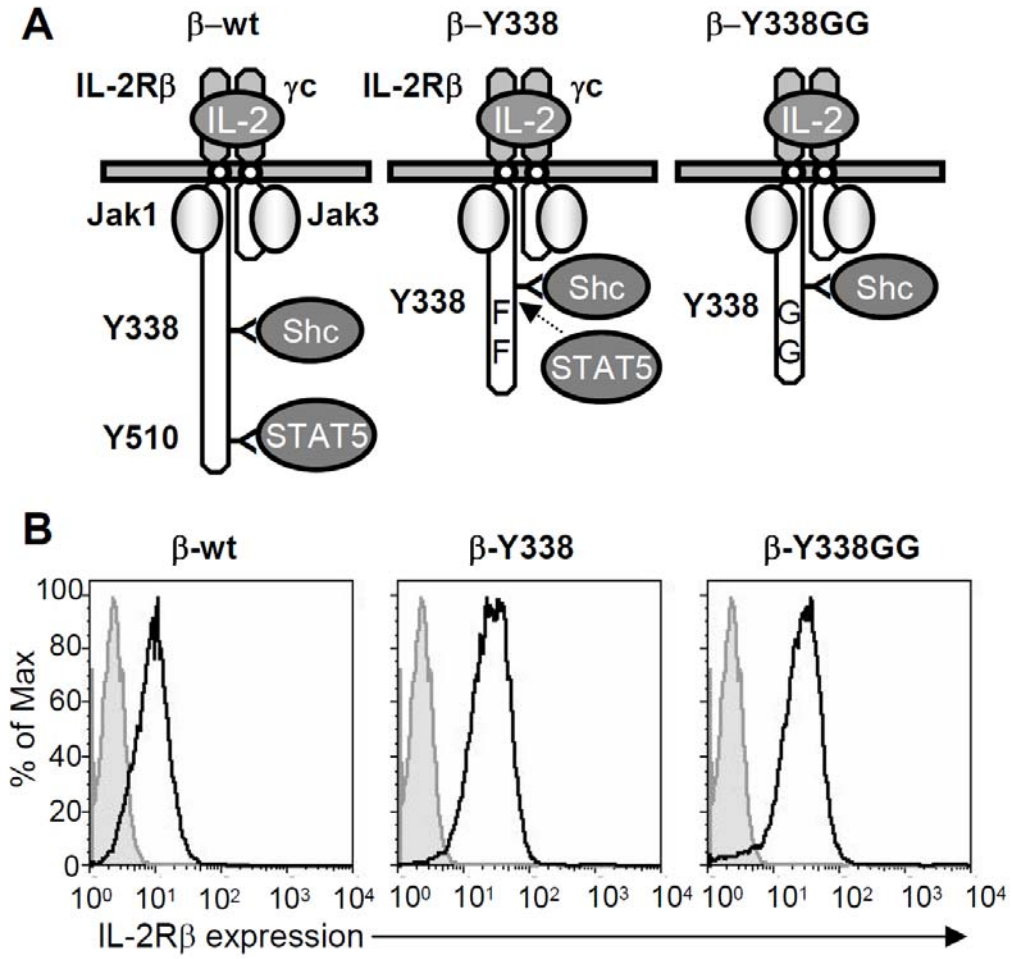


Figure 4. Experimental IL-2 receptor systems used.

(A) Schematic diagram of wild-type (β-wt) and mutant IL-2 receptors with key tyrosine (Y) residues highlighted. Upon IL-2 stimulation, β-wt delivers a wild-type IL-2 signal (Shc + STAT5); β-Y338 activates Shc and modest levels of STAT5; and β-Y338GG exclusively activates Shc. **(B)** Flow cytometric analysis of IL-2Rβ expression on BAF.GM cells expressing β-wt, β-Y338 or β-Y338GG. Subclones displaying IL-2Rβ expression within 0.5 - 1.5 log fluorescent units were selected for use in subsequent experiments. A representative clone for each receptor construct is shown.

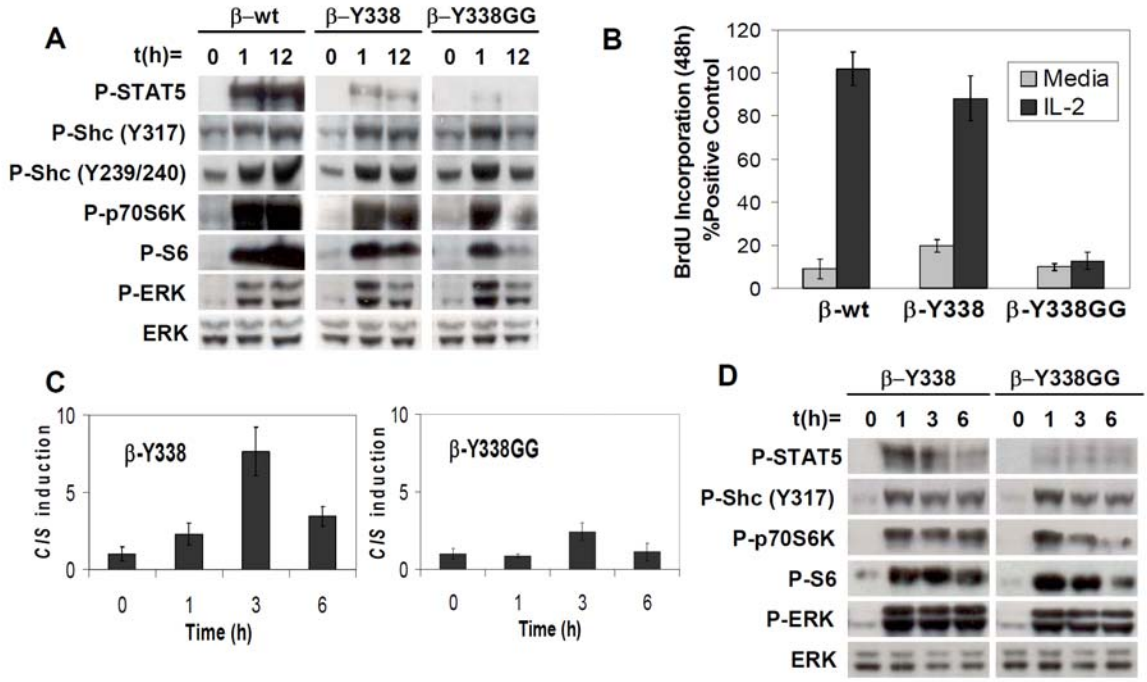


Figure 5. Shc is unable to sustain activation of the Akt/p70S6K pathway and promote lymphocyte proliferation in the absence of STAT5 activity.

(A) Phospho (P)-specific Western Blot analysis of Shc (P-Shc, P-p70S6K, P-S6 and P-ERK) and STAT5 (P-STAT5) pathway activation by β-wt, β-Y338, and β-Y338GG. BAF.GM cells stably expressing the indicated receptors were deprived of cytokine for 4 h, followed by a 1 or 12 h stimulation with 100 U/ml IL-2 before cell extracts were prepared and analyzed by SDS-PAGE and immunoblotting. IL-2 stimulation of βY338GG does not activate STAT5 or sustain long-term (t=12 h) activation of the Akt/p70S6K pathway. Total ERK serves as a loading control. **(B)** β-Y338GG fails to promote lymphocyte proliferation. BAF.GM cells stably expressing the indicated receptors were stimulated with 100 ng/ml GM-CSF (positive control), 100 U/ml IL-2, or medium alone (negative control), and bromodeoxyuridine (BrdU) incorporation was measured 48 h later. Data are plotted as a percent of the proliferation observed with the positive control cytokine, GM-CSF. **(C)** Differential induction of the STAT5-specific target gene *CIS* by β-Y338 and β-Y338GG, assessed by quantitative PCR. **(D)** Time-

course of Shc pathway activation by β-Y338 and β-Y338GG reveals a selective loss of p70S6K activity between 3-6 h. BAF.GM cells were treated and analyzed as per (B). All data are representative of 3 or more independent experiments.

Activation of the Shc pathway alone does not sustain long-term Akt/p70S6K activity or lymphocyte proliferation and viability.

To further reduce STAT5 activation while sparing the Shc pathway, a second IL-2R mutant was constructed, designated β-Y338GG, in which two phenylalanine residues immediately C-terminal to the Shc docking site at Y338 were mutated to glycine and the C-terminus was further truncated to residue 341 to eliminate any potential binding to Y338 by the SH2 domain of STAT5 (Fig. 4A and 4B). We expected the phospho-tyrosine binding (PTB) domain of Shc to still bind to β-Y338GG, since the PTB domain recognizes residues N-terminal to Y338 (171). As intended, the β-Y338GG mutant showed reduced STAT5 tyrosine phosphorylation and *CIS* induction in response to IL-2 while tyrosine phosphorylation of Shc still occurred (Fig. 5A and 5C). Remarkably, this was associated with a major reduction in cell proliferation and viability, suggesting that the Shc signal, when isolated from STAT5, is not sufficient for mitogenesis and cell survival (Fig. 5B and data not shown). To understand the biochemical basis of this proliferative impairment, we evaluated key signaling events associated with the Shc pathway. At 1 h post-stimulation, the β-Y338GG mutant showed normal phosphorylation of Shc, ERK, p70S6K and its substrate S6, suggesting that early Shc signaling was intact (Fig. 5A). However, by 12 h post-stimulation, the β-Y338GG mutant showed greatly impaired phosphorylation of p70S6K and S6, whereas Shc and ERK phosphorylation were only modestly diminished (Fig. 5A). A more refined time course revealed that p70S6K and S6 phosphorylation began to diminish 3-6 h after stimulation of β-Y338GG, despite normal phosphorylation of Shc and ERK at these time-points (Fig. 5D).

STAT5 and Shc cooperate to sustain Akt/p70S6K pathway activation and lymphocyte proliferation.

The failure of β-Y338GG to sustain p70S6K and S6 phosphorylation could result from reduced STAT5 activation by this mutant. Alternatively, the β-Y338GG mutation could disrupt the interaction of Y338 with other unidentified signaling proteins that regulate p70S6K and S6. To distinguish these possibilities, we attempted to rescue p70S6K and S6 phosphorylation by restoring STAT5 activation through a second receptor. We chose a receptor that could be stimulated independent of β-Y338GG and was structurally distinct from the IL-2R, such that STAT5 represented one of the few shared signaling elements. Specifically, we made a chimeric receptor that placed STAT5 under the control of a second cytokine, G-CSF. Several groups have described a chimeric G-CSF/gp130 receptor that generates an IL-6-like signal in response to G-CSF that is mediated by Jak1, Jak2, Tyk2, Shp2 and STAT3 (172, 173). We replaced the Shp2 and STAT3 activation sites of G-CSF/gp130 with the STAT5 activation site from IL-2Rβ (Y510) to generate a chimeric receptor designated G-Y510 (Fig. 6A). As expected, G-Y510 induced STAT5 phosphorylation and *CIS* expression in response to G-CSF, without inducing phosphorylation of STAT3 and Shp2 (components of the IL-6 signal), or Jak3 and Shc (components of the IL-2 signal) (Fig. 6B, Fig. 8B and data not shown). The level of STAT5 phosphorylation was modest compared to the wild type IL-2R, and consequently G-CSF-induced cell proliferation was weak (Fig. 6C). Nevertheless, when G-Y510 was co-expressed with β-Y338GG, the combination of IL-2 + G-CSF induced a

proliferative response equivalent to that of the wild-type IL-2R (Fig. 6C). Thus, the Shc and STAT5 pathways exhibit strong cooperativity even when triggered by heterologous receptors.

We next evaluated G-Y510 and β-Y338GG for synergistic effects on p70S6K and S6. As before, β-Y338GG alone induced strong phosphorylation of p70S6K and S6 at 1 h, and this was greatly reduced by 12 h (Fig. 6B). By contrast, G-Y510 alone induced little or no p70S6K/S6 phosphorylation at 1 or 12 h (Fig. 6B). Importantly, when cells were co-stimulated with IL-2 + G-CSF, strong phosphorylation of p70S6K and S6 was observed at both 1 h and 12 h (Fig. 6B). To quantify these results, we measured S6 phosphorylation by flow cytometry using the same cell cultures. Consistent with the immunoblotting results, the combination of IL-2 + G-CSF induced at least a 4-fold increase in the mean fluorescence intensity (MFI) at 12 h relative to IL-2 alone (Fig. 6D). Thus, Shc and STAT5 cooperatively regulate p70S6K and S6, even when activated through heterologous receptors. Notably, the quantitative data obtained by flow cytometry demonstrates that this was a synergistic rather than additive effect of the two cytokines.

To further demonstrate that STAT5 was the factor synergizing with the Shc pathway, we generated BAF.GM cells stably co-expressing β-Y338GG and the IL-2 receptor mutant β-Y510 (formerly known as ββΔ325+Y510). This mutant has undergone extensive investigation to show that it signals exclusively through the activation of STAT5 by Y510 (140, 148, 174). Similar to the results observed with β-Y338GG + G-Y510, IL-2-induced co-activation of β-Y338GG and β-Y510 also led to sustained

phosphorylation of S6 at late time-points, as assessed by intracellular flow cytometry (Fig. 6E). Together, these results imply an essential role for STAT5 in sustaining the Akt/p70S6K pathway.

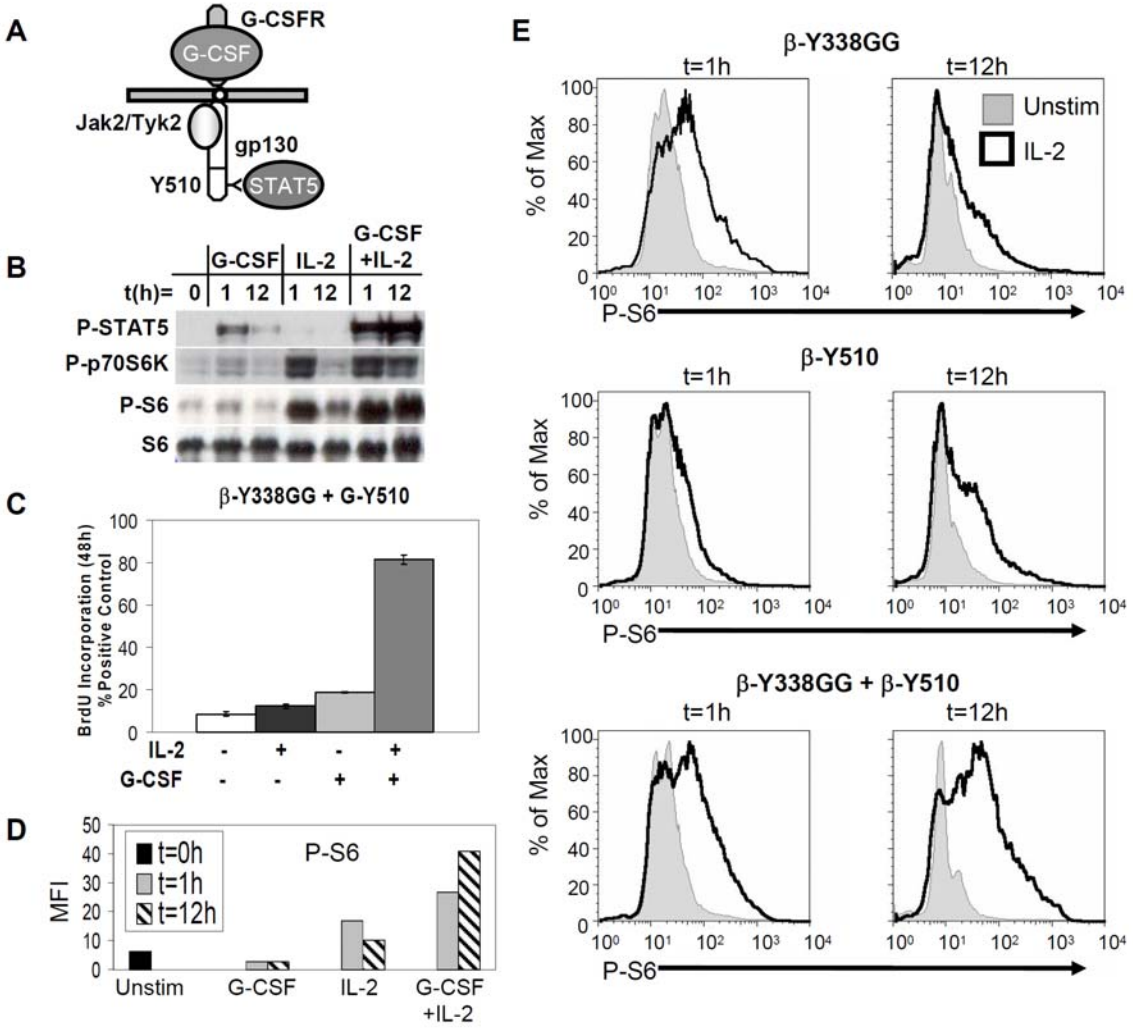


Figure 6. Shc and STAT5 pathways exhibit strong functional synergy even when triggered by heterologous receptors.

(A) Schematic diagram of the chimeric cytokine receptor G-Y510. G-Y510 is characterized by the G-CSFR extracellular domain fused to the gp130 intracellular domain modified to contain the STAT5 activation site (Y510) from IL-2Rβ. **(B)** Western Blot analysis showing synergistic activation of the STAT5 (P-STAT5) and Shc (P-p70S6K and P-S6) pathways upon co-activation of β-Y338GG and G-Y510. BAF.GM cells stably co-expressing the indicated receptors were deprived of cytokine for 4 h, followed by a 1 or 12 h stimulation with either 100 ng/ml G-CSF alone to activate

STAT5, 100 U/ml IL-2 alone to activate Shc, or both cytokines to activate both Shc and STAT5. **(C)** Synergistic effects of Shc and STAT5 on lymphocyte proliferation. BAF.GM cells stably co-expressing β-Y338 + G-Y510 were stimulated for 48 h with medium alone (negative control), 100 ng/ml GM-CSF (positive control), 100 U/ml IL-2, 100 ng/ml G-CSF, or a combination of IL-2 and G-CSF, and assayed for BrdU incorporation. Data are plotted as percent proliferation relative to the positive control, GM-CSF. **(D)** Intracellular flow cytometry analysis showing the synergistic enhancement of phospho-S6 (P-S6) by STAT5 activation at t=1 and 12 h after cytokine stimulation. MFI, mean fluorescence intensity. All data are representative of 3 or more independent experiments. **(E)** Intracellular flow cytometric analysis of phospho-S6 (P-S6) to assess activation of the Akt/p70S6K pathway in BAF.GM lymphocytes stably expressing β-Y338GG (activates Shc), β-Y510 (activates STAT5), or both β-Y338GG and β-Y510 (which together activate Shc and STAT5). Cells were washed, starved, and then stimulated with 100 U/ml of IL-2 for 1-12 h. Unstimulated cells served as a negative control at each time point (shaded region).

Constitutively active STAT5 can independently induce S6 phosphorylation.

In theory, the G-Y510 chimeric receptor, and even the well-characterized β-Y510 IL-2 receptor, should exclusively activate STAT5. However, the possibility remained that these receptors might activate cellular proteins in addition to STAT5 which may in turn have effects on the Akt/p70S6K pathway. To ensure that STAT5 was indeed the factor responsible for sustaining the Akt/p70S6K pathway, we took advantage of a well-characterized constitutively active form of STAT5, caSTAT5A1*6 (168). caSTAT5A1*6 harbours two amino acid substitutions that confer constitutive tyrosine phosphorylation, nuclear localization and transcriptional activity (168). As expected, when transiently expressed in BAF.GM lymphocytes, caSTAT5A1*6 demonstrated strong, constitutive tyrosine phosphorylation (data not shown). This was associated with potent phosphorylation of S6 to levels exceeding that induced by β-Y338GG or β-wt (Fig. 7 and data not shown). Expression of wild-type STAT5A (wtSTAT5A) did not affect S6 phosphorylation (Fig. 7), therefore activated STAT5 is required for this process. Transient expression of caSTAT5A1*6 in the murine T-cell lines CTLL-2 and HT-2 also induced phosphorylation of S6 (data not shown), therefore the link between the STAT5 and Akt/p70S6K pathways is operant in multiple cell types.

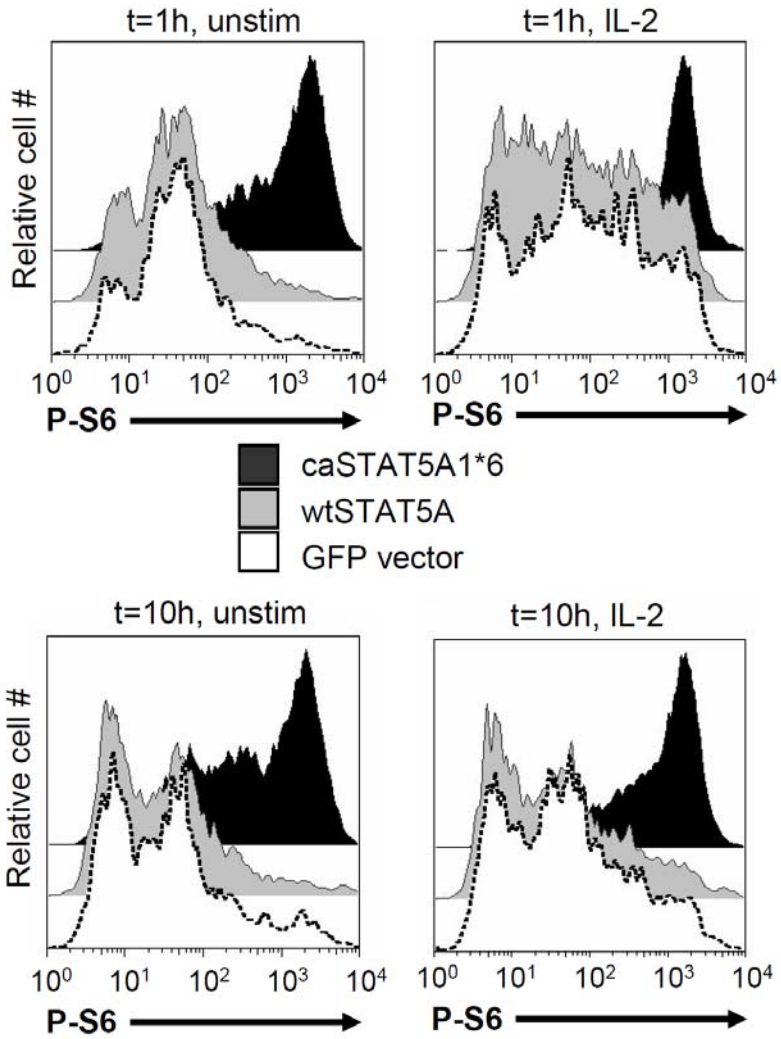


Figure 7. Constitutive activation of STAT5 results in strong S6 phosphorylation. Intracellular flow cytometry for phospho-S6 to assess activation of the Akt/p70S6K pathway. BAF.GM lymphocytes stably expressing β-Y338GG were transiently co-transfected with plasmids encoding constitutively active STAT5 (caSTAT5A1*6) and GFP. Cells were rested overnight, starved for 4 h, and stimulated for 1-10 h with IL-2 to activate Shc, or left unstimulated. Plasmids encoding GFP alone, or wtSTAT5A served as negative controls. Data are gated on the GFP positive population and is representative of 3 independent experiments.

STAT5 cooperates with the Shc pathway at the level of Akt.

We examined other signaling events downstream of Shc to determine the point at which STAT5 synergizes with the Shc pathway. In cells co-expressing β-Y338GG and G-Y510, IL-2 induced the phosphorylation of Shc, Gab2, Shp2, p85 and ERK, and these events were not enhanced by G-CSF at any time-point (Fig. 8A). By contrast, G-CSF enhanced the phosphorylation of Akt and p70S6K at 6 and 12 h, and an even greater effect was seen on the downstream effector protein S6 (Fig. 8A). These results indicate that STAT5 acts at or near the level of Akt. Intriguingly, in addition to STAT5 facilitating Shc signaling, activation of the Shc pathway by β-Y338GG enhanced the tyrosine phosphorylation of STAT5 as well as induction of the STAT5 target genes *CIS* and *c-myc* (Fig. 8A and 8B). Thus, there appears to be bi-directional cooperative signaling between the Shc and STAT5 pathways.

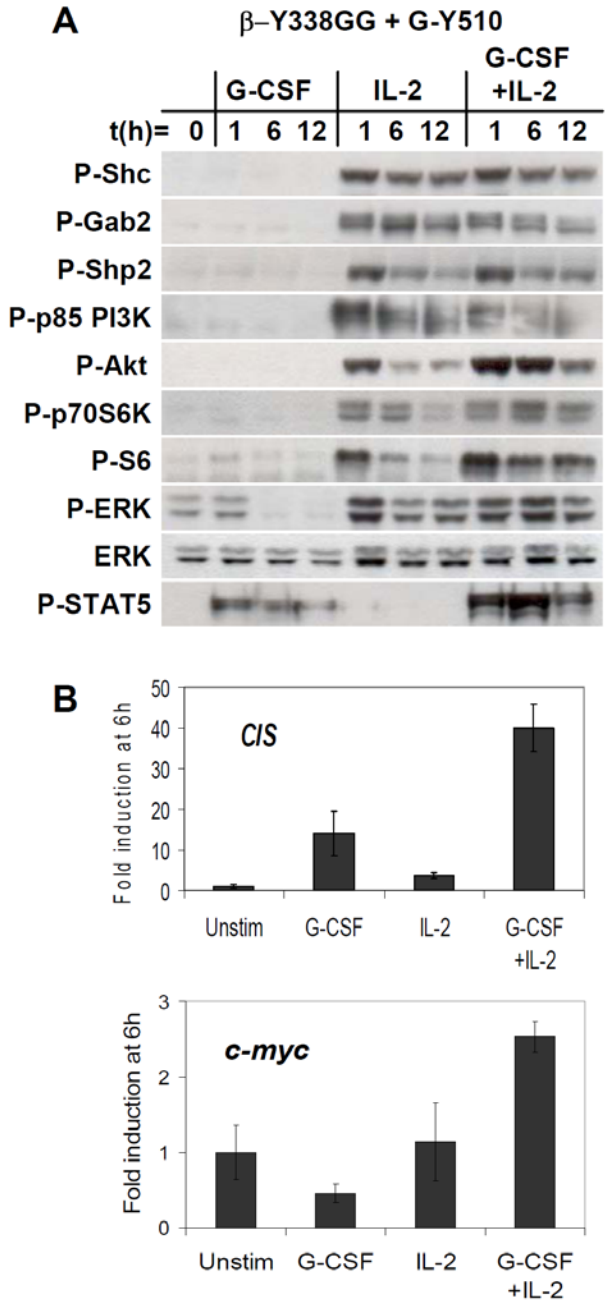


Figure 8. Co-stimulation of the Shc and STAT5 pathways results in enhanced phosphorylation of Akt, p70S6K, S6 and STAT5.

(A) Western Blot analysis of Shc and STAT5 signaling events. BAF.GM cells stably co-expressing β-Y338GG and G-Y510 were deprived of cytokine for 4 h, followed by a 1, 6

or 12 h stimulation with either 100 ng/ml G-CSF alone to activate STAT5, 100 U/ml IL-2 alone to activate Shc, or both cytokines to activate Shc and STAT5. Total ERK is used as a loading control. **(B)** QPCR analysis showing synergistic upregulation of the STAT5 target genes *CIS* and *c-myc* upon activation of both Shc and STAT5. BAF.GM lymphocytes co-expressing β-Y338GG and G-Y510 were unstimulated (t=0 h) or stimulated with 100 ng/ml G-CSF, 100 U/ml IL-2, or both cytokines for 6 h prior to RNA isolation, cDNA synthesis and QPCR. GAPDH was used as the reference gene. All data are representative of 3 or more independent experiments.

Temporal dissociation of Shc and STAT5 signaling impairs S6 phosphorylation and cell proliferation.

STAT5 has been reported to weakly activate the PI3K pathway through its conventional role as a transcription factor (139, 140). In addition, STAT5 can serve as an adaptor protein, forming a complex with Gab2 and the p85 regulatory subunit of PI3K (152). To investigate which of these mechanisms underlies the synergy between STAT5 and Shc, we determined how rapidly STAT5 could rescue the Shc signal, reasoning this should occur immediately if STAT5 served as an adaptor protein or more slowly if STAT5 served as a transcription factor. Cells co-expressing β-Y338GG and G-Y510 were stimulated with IL-2 alone for 9 h to allow the Shc signal to initiate and then decay. When G-CSF was added, STAT5 phosphorylation increased within 15 minutes whereas S6 phosphorylation increased over a 1-6 h period (Fig. 9A and 9B). These results are consistent with STAT5 sustaining S6 phosphorylation by serving as a transcription factor rather than an adaptor protein. Importantly, if cells were washed prior to the addition of G-CSF, such that the IL-2-induced Shc signal was extinguished prior to the initiation of the STAT5 signal, there was only negligible rescue of S6 phosphorylation (data not shown). This demonstrates that S6 phosphorylation is not regulated through a simple bi-phasic mechanism involving an initial Shc phase followed by a STAT5 phase. Rather, both pathways need to be simultaneously active to achieve maximal S6 phosphorylation. Similar to the results for S6 phosphorylation, cell proliferation was maximal only when the Shc and STAT5 pathways were both activated throughout the entire stimulation cycle (data not shown). Thus, continuous, simultaneous Shc and STAT5 signaling is required

to achieve maximal S6 phosphorylation and cell proliferation, demonstrating an essential synergy between these pathways.

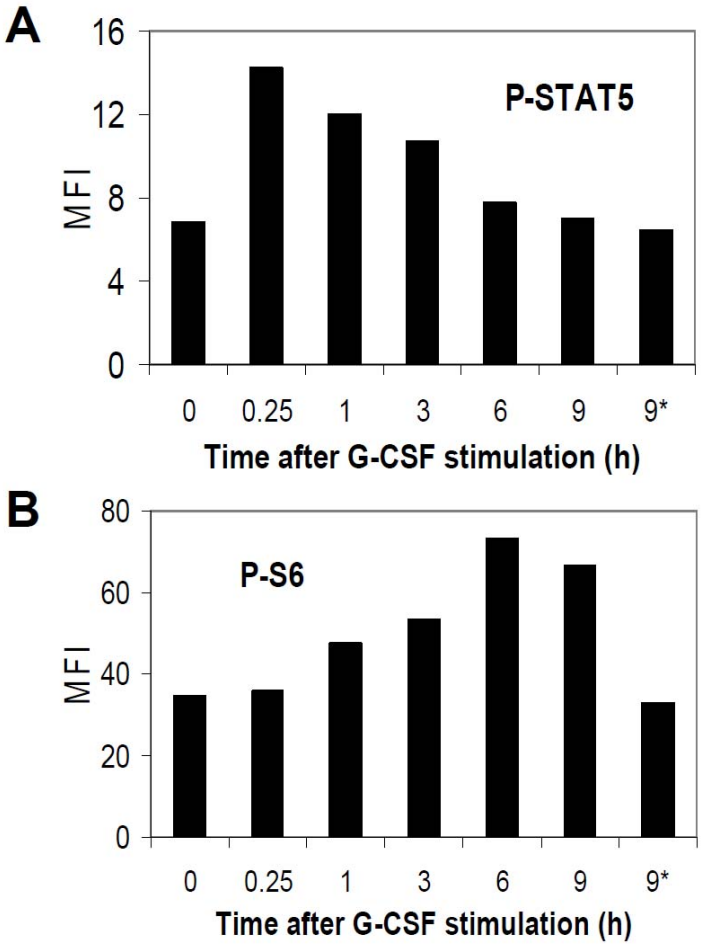


Figure 9. Rescue of S6 phosphorylation by G-Y510 is delayed relative to STAT5 phosphorylation.

Intracellular flow cytometry analysis of P-STAT5 **(A)** and P-S6 **(B)** in BAF.GM cells harbouring both β-Y338GG and G-Y510 receptors. Cells were stimulated with 100 U/ml IL-2 for 9 h followed by 100 ng/ml G-CSF for the indicated times. 9* denotes no G-CSF stimulation. MFI, mean fluorescence intensity. Data are representative of 3 independent experiments.

2.5 Discussion

We show here that STAT5 is essential for sustained activation of the Akt/p70S6K pathway, which was previously thought to be an exclusive function of Shc. At early time points after IL-2 stimulation, our results are consistent with the conventional model in which a complex containing Shc, Grb2, Gab2, Shp2 and p85 mediates activation of the Akt/p70S6K pathway (127-129). However, when concurrent STAT5 activity was absent, the Akt/p70S6K signal waned after 3-6 hours of IL-2 stimulation, and this was associated with drastically impaired lymphocyte proliferation and viability. Restoration of STAT5 activity through a heterologous receptor rescued maximal Akt/p70S6K activity and lymphocyte proliferation. The Shc pathway further promoted these events by enhancing the transcriptional activity of STAT5. This bi-directional, cooperative signaling by Shc and STAT5 appears to operate throughout the proliferative cycle, as temporal dissociation of the Shc and STAT5 signals lead to sub-optimal S6 phosphorylation and lymphocyte proliferation. Thus, the Shc and STAT5 pathways do not operate as independent signaling modules, as prior studies have suggested, but instead are intimately linked at the level of the Akt/p70S6K pathway and possibly other regulatory nodes as well. These findings likely explain the severe mitogenic defect seen in STAT5-deficient T cells (130, 131, 133, 142, 149), as lack of STAT5 signaling would also impair the ability of Shc to activate the Akt/p70S6K pathway.

It has been recently demonstrated that STAT5 can serve as an adaptor protein in the Gab2/Shp2/p85 complex, which could potentially explain the observations reported here (152-154). However, the physiological relevance of this mode of signaling by

STAT5 has yet to be fully established. In fact, constitutively active STAT5 mutants that are defective in transactivating STAT5 target genes, but are theoretically capable of acting as adaptor proteins, were found incapable of inducing leukemia in mice (175, 176). By contrast, constitutively active STAT5 mutants capable of tetramerisation and strong DNA binding efficiently induced leukemogenesis, which implicates an important role for STAT5 transcriptional activity, and not adaptor function, in oncogenesis (175, 176).

In the present study, we provide four lines of evidence that STAT5 regulates the Akt pathway through a transcriptional mechanism: 1) time-course experiments showed a delayed (1-3 h) rescue of the Akt pathway by STAT5 (Fig. 9); 2) the cooperation between the Shc and STAT5 pathways occurred even in the context of heterologous receptors, suggesting it involved signaling intermediaries rather than direct complex formation between Shc and STAT5; 3) short term (1 h) activation of the Akt pathway by Shc occurred in the absence of STAT5 activity, indicating that any adaptor function of STAT5 is not essential for formation of the Shc/Grb2/Gab2/p85 complex; and 4) expression of caSTAT5A1*6 alone (i.e., without co-activation of the Shc/Grb2/Gab2/p85 complex) strongly activated the Akt/p70S6K pathway (Fig. 7) and indeed promotes factor-independent proliferation of Ba/F3 cells (151, 168). Since caSTAT5A1*6 predominately localizes to the nucleus, possesses strong transcriptional activity, and can bind DNA in the absence of cytokines (168), this is also consistent with a transcriptional mechanism. Unfortunately, attempts to directly confirm the transcriptional activity of STAT5 using the protein translation inhibitor cycloheximide were confounded by direct effects of this agent on p70S6K (data not shown), consistent with a prior report (177).

Nevertheless, even though STAT5 can form a complex with Gab2, Shp2 and p85, the collective evidence strongly suggests that it regulates the Akt/p70S6K pathway predominantly by a transcriptional mechanism.

We found that STAT5 does not affect the IL-2-induced phosphorylation of Shc, Grb2, Gab2, Shp2, or p85. Rather, the effect of STAT5 is seen at the level of Akt and the downstream effectors p70S6K and S6 (Fig. 8A). Although Akt plays a central role in cell survival, growth, and proliferation, its mechanism of regulation remains unresolved (134). Maximal activation of Akt requires both translocation to the plasma membrane and phosphorylation of Thr308 and Ser473 (131, 134, 178). Akt is recruited to the plasma membrane by the PI3K product PIP_3, which is bound by the PH domains of Akt and PDK1. Subsequently, PDK1 phosphorylates Thr308 and PDK2 phosphorylates Ser473. The precise identity of PDK2 remains controversial, although recent evidence suggests it may consist of the Rictor/mTOR complex (179). Therefore, STAT5 could potentially promote Akt activation by upregulating the activity of p110α (the catalytic subunit of PI3K), PDK1, PDK2, or other components of the mTOR pathway. Alternatively, STAT5 could activate Akt by transcriptionally repressing a negative regulator(s). To date, no Akt-specific phosphatase has been found (134). However, the carboxy-terminal modulator protein (CTMP) negatively regulates Akt by preventing its phosphorylation (180). Finally, because Akt is downstream of PI3K, negative regulators of PI3K, such as the tumour suppressors PTEN and p53, could also oppose Akt activity (133). With these candidates in mind, we are currently attempting to identify the STAT5 target gene(s) that regulate the PI3K/Akt pathway.

In addition to showing that STAT5 is essential for sustaining the Akt/p70S6K pathway, we also found that optimal STAT5 transcriptional activity depends on concurrent Shc signaling. This suggests that the Shc pathway is regulating a kinase or phosphatase that controls the phosphorylation of STAT5. Time-course experiments revealed that the Shc pathway enhances STAT5 phosphorylation with delayed kinetics, which suggests a transcriptional mechanism (data not shown), however, the molecular basis of this finding remains to be elucidated.

Prior studies with the IL-2R mutant ββΔ325+Y510 and the wild type IL-7R have shown that STAT5 can activate Akt to a low level in the absence of Shc signaling (139, 140). Together with our current results, this might suggest a bi-phasic mode of Akt regulation, wherein initial activation of the PI3K/Akt pathway is mediated by the well-characterized Shc/Grb2/Gab2/p85 complex followed by a second phase of Akt activity mediated by STAT5. Indeed, the PDGF receptor activates the PI3K pathway through a bi-phasic mechanism (181). However, we do not believe the data supports such a model for the IL-2R, simply because STAT5 alone is a very weak activator of Akt (139, 140). It is only when STAT5 is combined with Shc that strong phosphorylation of Akt, p70S6K, and S6 occurs. Instead, we propose a model in which Akt is predominantly regulated by the Shc/Grb2/Gab2/p85 complex, as indicated by prior studies, but that one or more unidentified factors downstream of this complex depends on STAT5 transcriptional activity for continued expression. Thus, further study of STAT5 target genes will shed new light on the mechanism of Akt regulation by cytokine receptors.

Activated STAT5 has been observed in a number of human cancers, including various leukemias and lymphomas as well as prostate, uterine, ovarian, breast, and head and neck cancers (142-144). STAT5 is traditionally thought to contribute to oncogenesis by transactivating genes involved in cell cycle progression and survival, such as *c-myc, pim-1*, *bcl-*x_L, *mcl-1* and *D-type cyclins* (142, 143, 168). Our findings, together with others (152-154), indicate that the oncogenic properties of STAT5 may also be attributable to its ability to promote activation of the PI3K/Akt pathway, which is also widely implicated in oncogenesis (130, 131, 133). If so, then strategies aimed at inhibiting STAT5 activity may have the additional benefit of disrupting the PI3K/Akt pathway, thereby further promoting tumor cell apoptosis. Likewise, inhibitors of the PI3K/Akt pathway might prove efficacious against tumors with dysregulated STAT5 activity.

2.6 Acknowledgements

We thank Dr. Toshio Kitamura for kindly providing the caSTAT5A1*6 plasmid and James Lord, James Moon and Bryan Carson for valuable materials and advice.

Chapter 3: Identification of genes that are cooperatively regulated by Shc and STAT5 and are associated with IL-2-induced lymphocyte proliferation

Eric Tran, Heather M. Lockyer, and Brad H. Nelson.

ET designed research, performed research, collected data, analyzed and interpreted data, and wrote the chapter

HML performed research

BHN designed research and analyzed and interpreted data

Introduction

As demonstrated in Chapter 2, the interleukin-2 receptor (IL-2R) promotes the viability and proliferation of lymphocytes through activation of the Shc and STAT5 pathways. Importantly, there appears to be an essential synergy between the Shc and STAT5 pathways, as maximal proliferation occurs only with concurrent activation of Shc and STAT5 (182). Although we showed that STAT5 was critical for sustaining long-term activation of the Akt/p70S6K pathway downstream of Shc, the underlying mechanisms by which these pathways integrate to mediate lymphocyte proliferation remain poorly defined (182).

The activation of intracellular signaling pathways by cytokine receptors induces changes in gene expression that are essential for cellular process such as proliferation (i.e., some genes have to be up-regulated, while others repressed). The gene expression profile induced by IL-2 has been relatively well studied using microarray technologies, and these studies have identified upwards of ~ 2800 putative IL-2 regulated genes (183-188). Given the large number of genes, it is not surprising that the importance of the vast majority of these genes in the biological response mediated by IL-2 remains unknown. However, many IL-2 regulated genes that play a role in viability and proliferation and/or effector function have been identified, such as *il-2rα* (189), *pim-1* (190), *pim-2* (191), *bcl-2* (192), *bcl-xL* (192), the *d-type cyclins* (193, 194), *c-myc* (192), *c-myb* (195), *perforin* and *granzyme B* (196), *cis* (197), *socs1* (198), and *dusp5* (184), among others. Interestingly, all of these genes are up-regulated by IL-2 stimulation, demonstrating the importance of up-regulated genes in T-cell responses promoted by IL-2. However, IL-2 is

also known to repress genes (183-188), and genes that are actively repressed by IL-2 could theoretically be just as important as up-regulated genes for augmenting physiological processes such as proliferation (i.e., IL-2 could down-regulate genes that inhibit proliferation).

Unlike other cytokines such as TGF-β, where the importance of gene repression in the cell cycle process is relatively well studied (199), the significance of repressed genes in the proliferative response mediated by IL-2 is unknown. In fact, there have only been a handful of studies directly addressing the physiological significance of down-regulated IL-2 target genes in T cells, and these are not in the context of proliferative signaling. A study from Xue et al. showed that IL-2 represses IL-7 receptor expression in T cells (200), while another study by Lindemann and colleagues showed that IL-2 negatively regulated IL-17 receptor expression (201). IL-2 was also demonstrated to down-regulate Cytochrome p-450 gene expression in rat hepatocytes (202).

The goal of research presented in Chapter 3 was to better define the mechanisms by which the Shc and STAT5 pathways converged to induce lymphocyte proliferation, with the ultimate goal of identifying IL-2 regulated genes that play an important role in T-cell proliferation. Since maximal proliferation only occurs upon concurrent activation of the Shc and STAT5 pathways, we used our unique dual receptor system to identify genes that were dually regulated upon concurrent Shc and STAT5 activation, reasoning that genes important for proliferation would be differentially expressed upon co-activation of Shc and STAT5, compared to activation of either pathway alone. Moreover, since little is known about the genes that are actively repressed by IL-2, we also focused

on identifying genes that were down-regulated upon IL-2 stimulation. During the writing of this thesis, a subset of these down-regulated genes was being evaluated for their role in proliferative signaling by the IL-2 receptor.

Methods and Materials

Plasmid constructs. β-wt, β-Y338GG, and G-Y510 receptors were expressed under a human β-actin promoter-driven vector as previously described in Chapter 2 (182).

Cell culture. Murine proB Ba/F3 cells stably expressing human GM-CSFRα and above plasmid constructs, and murine cytotoxic CTLL-2 cells were maintained as described in Chapter 2 (182). Although not shown for all experiments, GM-CSF (100 ng/ml) was used as an internal positive control. Receptor expression was assessed by flow cytometry with antibodies to human IL-2Rβ or human G-CSFR (BD Biosciences). Unless otherwise stated, for all cytokine stimulation experiments, cells were washed 3X with 1X PBS and then starved of cytokine for 4-6 h prior to stimulation with the indicated cytokine for the indicated time. IL-2 was used at 100 U/ml and G-CSF was used at 100 ng/ml.

Gene expression analysis and cooperation response gene (CRG) identification: Affymetrix and Quantitative PCR (QPCR). Cells were snap frozen in an ethanol-dry ice bath and stored at -80 °C. Total RNA was isolated using the RNeasy Mini kit (Qiagen) following the manufacturer's protocol and quantified using a NanoDrop ND-1000 spectrophotometer. Total RNA from 3 independent clones was pooled and for

preliminary gene expression analysis, microarray experiments were conducted using Mouse Genome 430 2.0 arrays (Affymetrix).

For QPCR experiments, RNA from 3 independent clones were pooled and then reverse transcribed to cDNA using the iScript cDNA synthesis kit (BioRad). See Table 1 for primer details for all genes assessed by QPCR. QPCR was performed using Quanta PerfeCTa Sybr Green Supermix (Quanta Biosciences) and the iCycler MyiQ real-time PCR detection system (Bio-Rad) with the following two-step protocol: initial denaturation at 95 °C for 90s, followed by 40 cycles of denaturation at 95 °C for 10s and 30 s extension at 60 °C. After final denaturation at 95 °C for 1 min, a melt curve analysis was performed starting at 55 °C and increasing by increments of 1 °C up to 95 °C. All reactions were run in triplicate and relative gene expression was calculated using the Bio-Rad Gene Expression Macro version 1.1 software. GAPDH was used as a reference gene (forward 5'-AACTTTGGCATTGTGGA AGG-3'; reverse 5'-ACACATTGGGGGTAGGAACA-3'). Expected product sizes were verified by standard agarose gel electrophoresis.

Genes that responded synergistically upon activation of both Shc and STAT5 (i.e., fold change of a gene upon concurrent Shc and STAT5 activation was greater than the sum of the fold changes upon individual activation of Shc and STAT5) were named cooperation response genes (CRG) and were selected based on a similar procedure as described in (203), with the following modifications. Let *a* represent gene expression magnitude upon Shc activation, *b* corresponds to gene expression magnitude upon STAT5 activation, and *c* represent the gene expression upon concurrent activation of Shc

and STAT5. Then, an upregulated CRG is defined as: $c / (a + b) > 1$, and a downregulated CRG is defined as $(c/a) + (c/b) < 1$. For consistency, the reciprocal value was taken for the down-regulated CRG, such that a value greater than 1 is considered a down-regulated CRG.

Proliferation assay. BrdU incorporation was assessed using the Cell Proliferation Biotrak ELISA system (GE Healthcare). Assays were conducted in triplicate with 2 - 4 x 10^4 cells cultured in 200 μl of medium plus the appropriate stimulus. After the indicated time (24 or 48 h), cells were fixed, permeabilized, and incubated with peroxidase-labeled anti-BrdU (1/100 in Ab dilution solution) for 90 min. Bound Abs were detected by TMB substrate and read at 450 nm on a Molecular Devices plate reader.

Intracellular flow cytometry. Cells were stimulated for the indicated time points with the appropriate cytokine(s), and fixed with formaldehyde (2% v/v final concentration) for 10 min at 37 °C. Fixed cells were then spun at 500 g, and cell pellets were permeabilized with 100% ice-cold methanol and incubated on ice for 20 min to achieve complete permeabilization. Cells were rehydrated by washing twice with 10 volumes of 1X PBS + 0.5% BSA (FACS buffer). Cells were then stained with Abs to phospho-STAT5 or phospho-S6 (1/200; Cell Signaling Technology) for 30–60 min at room temperature, washed twice with FACS buffer and then stained with anti-rabbit IgG-PE (1/100; CalTag Laboratories) for 30–60 min at room temperature in the dark. Events were collected with a BD FACSCalibur flow cytometer and CellQuest Pro software. Data analysis was performed using FlowJo software (Tree Star).

Table 1. Sequences of all intron-spanning primers (5'- 3') used in QPCR experiments.

Gene	Forward primer	Reverse primer	Amplicon size (bp)
CIS	CGTTGTCTCTGGGACATGGTC	CAATTTGCTCCACAGCCAGC	69
C-MYC	TTTGTCTATTTGGGGACAGTGTT	CATCGTCGTGGCTGTCTG	128
HBEGF	CGTACTCCCTCTTGCAAATGC	GAGTCAGCCCATGACACCTGT	61
PIM-2	CTGCTTCACGATGAGCCG	CCCCACAGACCATGTCATAG	142
PIK3IP1	CTGGCATTATCGTGGGCTAC	GTCTCACAGGTGGGGTTTG	133
ADORA3	GCT TCC ATC ATG TCC TTG CTG	GTC AGC CCC ACC AGA AAG	146
ID-1	GCGAGATCAGTGCCTTGG	CTCCTGAAGGGCTGGAGTC	111
CCNB1IP1	CCTGGAACCAGGTATGATCC	TGGTCCAAAGAAAACTTTGAGTTA	78
TNFRSF12A	ATTCGGCTTGGTGTTGATG	CCATGCACTTGTCGAGGTC	104
DHRS3	AGATGTTCCAGGGCATGAGA	GGGCTACTGTCTCTGGCTTC	69
IL-17RA	TGGGATCTGTCATCGTGCT	ATCACCATGTTTCTCTTGATCG	74
BBC3	TTCTCCGGAGTGTTCATGC	TACAGCGGAGGGCATCAG	146
RRP1B	ATGTGGGTGCAGGACGAG	CGTGGATGAGTTGGGAAATAA	67
GAS5	TCTCACAGCCAGTTCTGTGG	CTGTTATCCAGCTTCCTGACG	69
DUSP5	GATCGAAGGCGAGAGAAGC	GGAAGGGAAGGATTTCAACC	107
LRP12	CAGGCTGGCGTATTTTTCA	ATTTCCACAGCGGAACTGAT	70
IFRD1	TTTGGCACTTCTGTTTGAATTG	GGGTCAAAGAATCCATATCTTCA	74
LSM7	TTGACCCACTGCTCAACCT	CCGTCAGCTTGTACTGGTCAT	78
PLEKHA3	CTGGTCTCTCCCGTGTCG	AGCTGCTCCTCTCGGAACT	88
SLC25A33	TGCAGCTAGAACGCAAGGT	GGACGCCTTCTGTCTGGTAG	84
GPD2	GGCAAAGAGCGAGTGAGC	GGCTCTCACGTCAAATTCCT	69
HIVEP3	GCAAAAAGTGCCAAGAGACG	GCAGGTCATCACTGGTTCCT	69
CCDC86	CACCTGGAGGAGGAGAAACA	TGGACAATCTCAGCCTTGC	104
RRP9	AGCGATTCTGAGAGCGAGAG	CTTTTCCTGTGCGGTCTCTT	84
YPEI3	CACTTTGGGCTGGAAATATGA	CAATGATGTATTTCCCCTCTTTG	71
SPNA1	AGGCTCTAAGAGATCAGGCAGA	GACCAGCCTCGTCCACTG	66
KLHL24	GCAGTGACAAGCTGTATAGGAAAA	TCTGGGTCATAAGATTGGACCT	92
NBEAL2	AGCGGCTCTACGAGTTGTG	TTCTCGAAGACACCCACAAAG	94
PRKACB	TCTGAGGAAATGGGAGAACC	CGAGGGTTTTCTTCCTCTCAA	71
HIP1R	GAAGCTCATGTTTAAGCTGCAC	GTGGAACCGATCCCTGTG	70
PARP16	TCCAAGATCCTGACAATCCAC	TCAGCTGCTGGATCTTTTCA	61
MXD4	TCTGAAGCGTGCTAAGATGC	TGTTCCTTGATGCTCAGTGC	72
ASCC2	TTTTGCAGCTGAACATCTCG	AGAGCTGGCATTGTGCTGT	65
ATP6V1E1	CAAAAAGGATGTTGATGTCCAG	ATCTCAACGCCACCAGCTAT	72

Results

Temporal requirements for maximal lymphocyte proliferation

We and others have shown that the Shc and STAT5 pathways are both required for maximal proliferation and viability of lymphocytes (139, 182, 204, 205). However, the mechanisms behind how Shc and STAT5 integrate signals to induce lymphocyte proliferation are not well defined. In Chapter 2, we found that simultaneous activation of Shc and STAT5 was required for maximal S6 phosphorylation and lymphocyte proliferation, and that temporal dissociation of the Shc and STAT5 pathways by 9 h resulted in markedly impaired S6 phosphorylation and lymphocyte proliferation (data not shown). However, this strategy did not address whether temporal plasticity exists for lymphocyte proliferation if the Shc and STAT5 pathways are temporally dissociated from each other by less than 9 h. Therefore, we first set out to better define the temporal requirements required for maximal lymphocyte proliferation. We utilized the previously described dual receptor system expressed in Ba/F3 lymphocytes, whereby the Shc and STAT5 pathways can be selectively activated within a single cell by the addition of IL-2 or G-CSF, respectively (Fig. 10A-C and (182)). As expected, proliferation was markedly impaired in cells stimulated with IL-2 (Shc) alone or G-CSF (STAT5) alone compared to cells simultaneously stimulated with G-CSF and IL-2 (Fig. 11A), confirming that activation of both pathways is required for optimal lymphocyte proliferation.

To define the temporal requirements for Shc and STAT5 activation for inducing maximal proliferation, we temporally dissociated the Shc and STAT5 signals in Ba/F3 cells for 1, 3, 6, or 9 h and assessed the effects on lymphocyte proliferation (see Fig. 11B

for schematic). When cells were stimulated with IL-2 alone for the first 6 or 9 h, addition of G-CSF did not significantly enhance proliferation over that of IL-2. Likewise, in cells stimulated with G-CSF alone for the first 6 or 9 h, addition of IL-2 had little effect on proliferation over that of G-CSF (Fig. 11C). However, when the Shc and STAT5 pathways were temporally dissociated for 3 hours or less, the magnitude of proliferation was similar to cells that were given both signals simultaneously (Fig. 11C). Thus, although there is some temporal plasticity, maximal lymphocyte proliferation only occurs when both the Shc and STAT5 pathways are activated within ~3 h of each other.

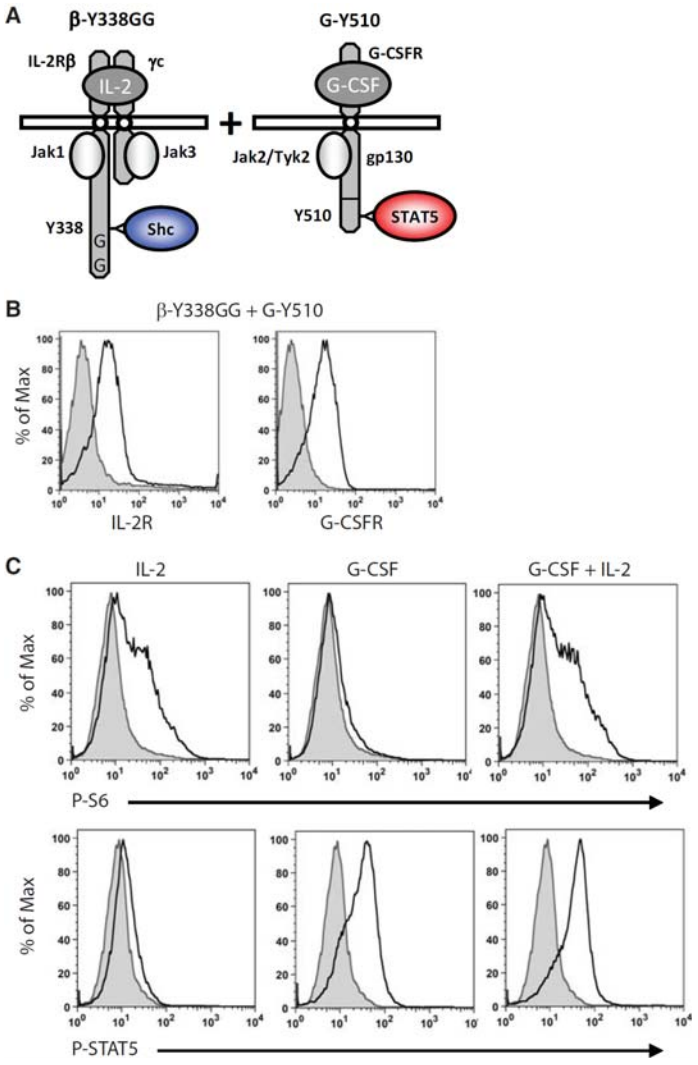


Figure 10. Experimental system used to study the relative contribution of the Shc and STAT5 pathways to proliferative signaling by the IL-2R.

(A) Diagram of the receptor constructs used. Ba/F3 lymphocytes were stably co-transfected with the truncated IL-2R β-Y338GG and the chimeric receptor G-Y510. **(B)** Representative expression levels of β-Y338GG and G-Y510 as determined by flow cytometry. **(C)** Intracellular flow cytometry analysis to ensure receptor function. Cells were stimulated with the indicated cytokine(s) for 1h and then processed for intracellular flow cytometry as described in Materials and Methods. β-Y338GG activates the Shc

pathway as assessed by phosphorylation of S6 (P-S6) upon IL-2, but not G-CSF, stimulation. G-Y510 activates STAT5 as assessed by phosphorylation of STAT5 (P-STAT5), but not Shc, upon G-CSF stimulation. Data are representative of 3 independent clones.

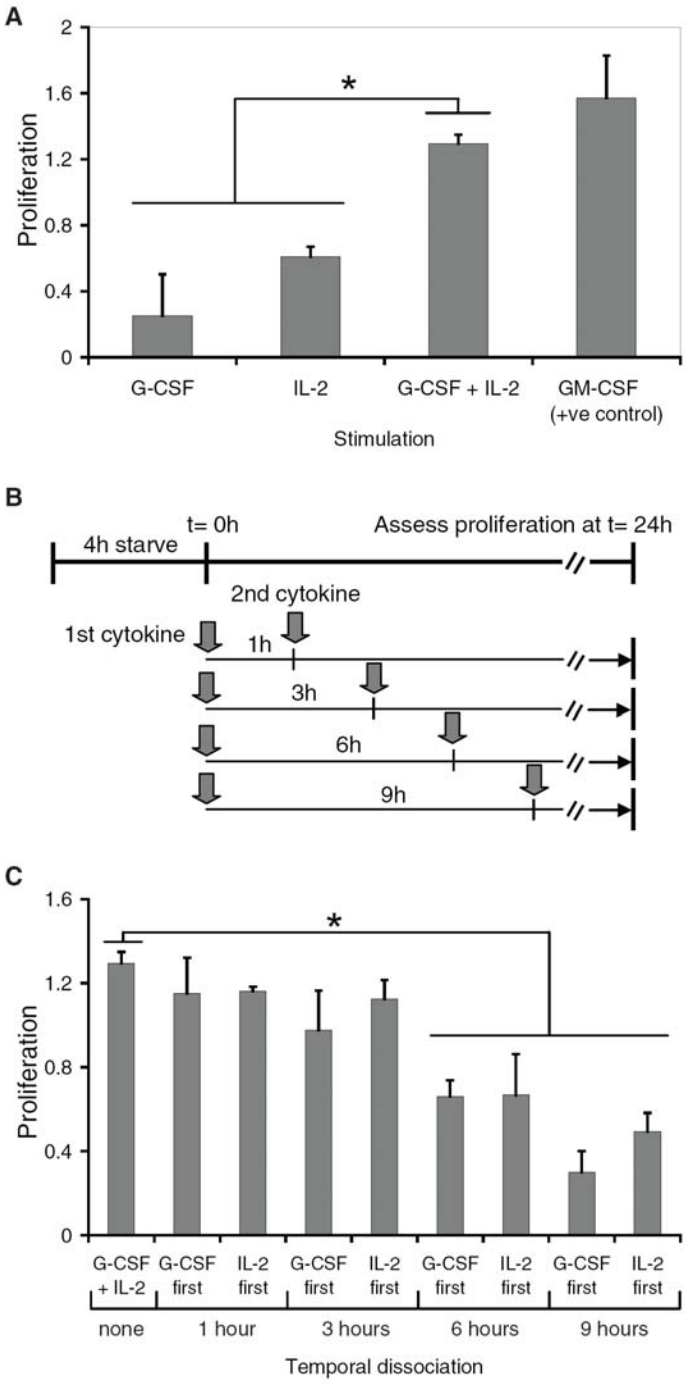


Figure 11. Temporal requirements for optimal lymphocyte proliferation mediated by the Shc and STAT5 pathways.

(A) Optimal proliferation requires co-activation of the Shc and STAT5 pathways. Ba/F3 lymphocytes stably expressing both β-Y338GG and G-Y510 were starved of cytokine for

4 h and then stimulated with medium (negative control), G-CSF (100 ng/ml) to activate STAT5, IL-2 (100 U/ml) to activate Shc, or a combination of G-CSF and IL-2, and proliferation (BrdU incorporation) was assessed 24 h later. GM-CSF stimulation serves as a positive control for proliferation (see Materials and Methods). The effect of combined G-CSF and IL-2 stimulation was significantly greater than either one alone (* $p < 0.05$, 1 way ANOVA, Tukey's multiple comparisons test). **(B)** Experimental design used to define the temporal requirements for Shc and STAT5-mediated lymphocyte proliferation. **(C)** Proliferative impairment upon temporal dissociation of the Shc and STAT pathways. Ba/F3 lymphocytes stably expressing both β-Y338GG and G-Y510 were stimulated with G-CSF (or IL-2) first, followed by IL-2 (or G-CSF) 1, 3, 6, or 9 h later as illustrated in (B), and proliferation was measured at 24 h. Temporally dissociating the Shc and STAT5 pathways by 6 or 9 h (but not 1 or 3 h) significantly impairs proliferation compared to no temporal dissociation (* $p < 0.05$, 1 way ANOVA, Tukey's multiple comparisons test). Proliferation (Y-axis) indicates OD 450 nm readings. Background (medium) control is subtracted from all values. Data are averaged from 3 independent clones and error bars represent standard error means (SEM).

Cooperatively regulated genes of the Shc and STAT5 pathways

Given that maximal proliferation occurs upon concurrent activation of Shc and STAT5 (Fig. 10A-C and (182)), we sought to further define the biochemical mechanism behind how the Shc and STAT5 pathways integrate signals to induce lymphocyte proliferation. As an initial survey, we used Affymetrix arrays to profile the gene expression signature of cells stimulated through Shc, STAT5, or both pathways, reasoning that genes critical for proliferation would exclusively be found differentially expressed when both the Shc and STAT5 pathways were activated. To this end, we first stimulated cells for 6 h with G-CSF alone, IL-2 alone, or the combination of G-CSF and IL-2.

We first searched for genes from the Affymetrix data that were synergistically regulated upon activation of both Shc and STAT5. These genes were termed "cooperation response genes" (CRGs) as described in Materials and Methods. We identified 81 putative upregulated CRGs from the Affymetrix data (Table 2), and used QPCR to validate a subset of these genes (Table 2, highlighted in yellow). The majority of the evaluated upregulated CRGs, 16/20, showed similar expression trends to the Affymetrix data (i.e., gene expression was strongest with concurrent Shc and STAT5 activation, Table 3 for raw QPCR data) but only nine (*pim-2*, *cis*, *c-myc*, *ccnb1ip1*, *adora3*, *id-1*, *lrp12*, *rrp9*, and *hbegf*) were validated by QPCR to be a true CRG by our strict definition (Fig. 12A). Interestingly, with the exception of *cis*, *c-myc*, and *pim-2*, little or nothing is known about the role these genes play in proliferative signaling induced by the IL-2 receptor.

Table 2. List of putative up-regulated cooperation response genes (CRGs) identified by Affymetrix analysis.

Data are sorted on descending signal strength upon co-stimulation of with G-CSF and IL-2 (co-activation of STAT5 and Shc). Highlighted genes were further evaluated with QPCR. Gene names were annotated using the Gene ID Conversion tool from The Database for Annotation, Visualization and Integrated Discovery 2008 (DAVID), NIAID, NIH.

Gene Name	Gene Symbol	Affymetrix ID	G-CSF Stimulation	IL-2 Stimulation	G-CSF + IL-2 Stimulation	CRG Score
SPLICING FACTOR, ARGININE/SERINE-RICH 3 (SRP20)	SFRS3	1434512_x_at	2897.1	3670.3	7759.8	1.18
INHIBITOR OF DNA BINDING 1	ID1	1425895_a_at	978.9	577.5	5308.3	3.41
MYELOCYTOMATOSIS ONCOGENE	MYC	1424942_a_at	1540.9	2810.7	4375.5	1.01
RIKEN CDNA 2600005C20 GENE	RRP1B	1456117_at	1474.9	1663.1	3315.1	1.06
GENE MODEL 288, (NCBI)	CCNB1IP1	1435998_at	886.7	1180.3	3006.7	1.45
NUCLEOLAR AND COILED-BODY PHOSPHOPROTEIN 1	NOLC1	1450087_a_at	1220.1	1701.2	2958.2	1.01
GROWTH ARREST SPECIFIC 5	GAS5	1455904_at	835.3	1284.6	2530.3	1.19
RIBOSOMAL PROTEIN L37A	RPL37A	1416218_x_at	657.8	1303.9	2197.6	1.12
GROWTH ARREST SPECIFIC 5	GAS5	1436222_at	669.7	1096.1	2043.6	1.16
DUAL SPECIFICITY PHOSPHATASE 5	DUSP5	1437199_at	744.9	1120.8	1954.8	1.05
RNA, U22 SMALL NUCLEOLAR	SNHG1	1437658_a_at	709.9	1044.3	1953.3	1.11
LOW DENSITY LIPOPROTEIN-RELATED PROTEIN 12	LRP12	1433864_at	1013	728.4	1859.4	1.07
RIKEN CDNA 5033413D16 GENE	5033413D16RIK	1428694_at	516.1	994	1782.7	1.18
INTERFERON-RELATED DEVELOPMENTAL REGULATOR 1	IFRD1	1416067_at	677.3	1021.7	1748.8	1.03
PROVIRAL INTEGRATION SITE 2	PIM2	1417216_at	577.4	249	1723.2	2.09
GLYCEROL PHOSPHATE DEHYDROGENASE 2, MITOCHONDRIAL	GPD2	1452741_s_at	831.4	667.4	1575.6	1.05
METEORIN, GLIAL CELL DIFFERENTIATION REGULATOR-LIKE	METRNL	1424356_a_at	647.2	411.7	1508.4	1.42
LSM7 HOMOLOG, U6 SMALL NUCLEAR RNA ASSOCIATED (S. CEREVISIAE)	LSM7	1417313_at	522.5	544.9	1267.3	1.19
PLECKSTRIN HOMOLOGY DOMAIN-CONTAINING, FAMILY A (PHOSPHOINOSITIDE BINDING SPECIFIC) MEMBER 3	PLEKHA3	1420840_at	513.8	530.9	1233.1	1.18
CYTOKINE INDUCIBLE SH2-CONTAINING PROTEIN	CISH	1448724_at	459	225.4	1044.6	1.53

RIKEN CDNA 5730438N18 GENE	SLC25A33	1424211_at	406.9	531.1	1021	1.09
HUMAN IMMUNODEFICIENCY VIRUS TYPE I ENHANCER BINDING PROTEIN 3	HIVEP3	1458802_at	156.1	580.2	1017.7	1.38
RIKEN CDNA 4933411H20 GENE	CCDC86	1454197_a_at	338.1	651.6	999.7	1.01
RNA, U3 SMALL NUCLEOLAR INTERACTING PROTEIN 2	RRP9	1451293_at	378.7	494	963.4	1.10
SPERMATOGENESIS ASSOCIATED 5	SPATA5	1417254_at	446.4	447.6	954.2	1.07
RIKEN CDNA 2210411K19 GENE	2210411K19RIK	1454639_x_at	260.1	593.9	945.4	1.11
TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY, MEMBER 12A	TNFRSF12A	1418571_at	165.5	422.9	882.1	1.50
RIKEN CDNA 2210411K19 GENE	2210411K19RIK	1433530_at	257.4	462.4	866.4	1.20
TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY, MEMBER 12A	TNFRSF12A	1418572_x_at	171.3	462.4	814.6	1.29
CELLULAR NUCLEIC ACID BINDING PROTEIN 1	CNBP	1438739_at	410	298.1	760.7	1.07
BRUNO-LIKE 4, RNA BINDING PROTEIN (DROSOPHILA)	BRUNOL4	1426929_at	159.2	566.5	755.4	1.04
SPLICING FACTOR PROLINE/GLUTAMINE RICH (POLYPYRIMIDINE TRACT BINDING PROTEIN ASSOCIATED)	SFPQ	1436898_at	399.6	299	709.8	1.02
ADENOSINE A3 RECEPTOR	ADORA3	1430482_at	146.7	115.3	625.5	2.39
PRP31 PRE-MRNA PROCESSING FACTOR 31 HOMOLOG (YEAST)	PRPF31	1453005_at	325.1	296.1	624.5	1.01
PC4 AND SFRS1 INTERACTING PROTEIN 1	PSIP1	1442148_at	297	181.1	575.2	1.20
RIBOSOMAL PROTEIN L39	RPL39	1423032_at	249.4	242.3	572.9	1.17
METHIONINE ADENOSYLTRANSFERASE II, ALPHA	MAT2A	1439386_x_at	120.1	315.6	562.6	1.29
RIKEN CDNA 5730408K05 GENE	5730408K05RIK	1429207_at	161.9	311.8	527.9	1.11
PODOCALYXIN-LIKE	PODXL	1417396_at	312.5	183.8	525.6	1.06
BRUNO-LIKE 4, RNA BINDING PROTEIN (DROSOPHILA)	BRUNOL4	1426930_at	156.8	351.5	524.9	1.03
GROWTH ARREST AND DNA-DAMAGE-INDUCIBLE 45 BETA	GADD45B	1449773_s_at	205.1	281.5	493.8	1.01
SPLICING FACTOR 3B, SUBUNIT 3	SF3B3	1430075_at	165.7	230.9	488.3	1.23
HARVEY RAT SARCOMA VIRUS ONCOGENE 1	HRAS1	1424132_at	94.1	336.6	454.1	1.05
COAGULATION FACTOR II (THROMBIN) RECEPTOR-LIKE 2	F2RL2	1421407_at	147.3	191.5	453.4	1.34
NUCLEAR RECEPTOR COACTIVATOR 6 INTERACTING PROTEIN	TGS1	1421905_at	307.2	132.3	441.9	1.01
SHORT COILED-COIL PROTEIN	SCOC	1430999_a_at	197	243.2	441	1.00
CAPPING PROTEIN (ACTIN FILAMENT), GELSOLIN-LIKE	CAPG	1447803_x_at	128.6	149.3	412	1.48
PUTATIVE HOMEODOMAIN TRANSCRIPTION FACTOR 2	PHTF2	1437637_at	138.3	215.1	392.6	1.11
MATRIN 3	MATR3	1441272_at	189.2	106.5	364	1.23
RIKEN CDNA 1810032O08 GENE	1810032O08RIK	1436677_at	179.2	163.3	352.9	1.03

AXIN1 UP-REGULATED 1	AXUD1	1434350_at	62	242.1	320.8	1.05
SIMILAR TO HEAT SHOCK COGNATE 71 KDA PROTEIN	LOC434047	1431182_at	81.9	163.9	316.1	1.29
RIBOSOMAL PROTEIN S13	EG625298	1415913_at	75.8	161.9	307.6	1.29
RAN BINDING PROTEIN 3	RANBP3	1457711_at	171.3	97	286.9	1.07
NEURTURIN	NRTN	1449281_at	42.5	208.4	286.7	1.14
EXPRESSED SEQUENCE C81203	C81203	1439988_at	93.9	110.7	284.2	1.39
PHOSPHORIBOSYLGLYCINAMIDE FORMYLTRANSFERASE	GART	1441911_x_at	102.7	109.4	283.4	1.34
HEPARIN-BINDING EGF-LIKE GROWTH FACTOR	HBEGF	1418350_at	64.3	87.5	278.9	1.84
RIKEN CDNA 3110003A22 GENE	3110003A22RIK	1439932_at	120.6	155.8	278	1.01
CHAPERONIN SUBUNIT 4 (DELTA)	CCT4	1456572_x_at	98.7	159.4	269.7	1.04
RIKEN CDNA 4930515K21 GENE	ZFP451	1456350_at	99	104.7	262.2	1.29
NUCLEAR RECEPTOR SUBFAMILY 0, GROUP B, MEMBER 2	NR0B2	1449854_at	80.1	151.8	256.2	1.10
TRANSMEMBRANE PROTEIN 69	TMEM69	1447966_a_at	89	114.5	250.7	1.23
NUCLEOREDOXIN	NXN	1446804_at	78.9	11.4	243.9	2.70
SLAM FAMILY MEMBER 8	SLAMF8	1425294_at	18.3	97.9	243.4	2.09
CEREBELLAR DEGENERATION-RELATED 2	CDR2	1417430_at	67.3	116.7	242.3	1.32
INSULIN-LIKE GROWTH FACTOR I RECEPTOR	IGF1R	1445600_at	78.7	148.4	242.2	1.07
HYPOTHETICAL LOC433022	PLCXD2	1455324_at	131.7	72.6	240.9	1.18
RIKEN CDNA 5230400J09 GENE	TYW3	1435728_at	63.9	175.2	240.1	1.00
SPROUTY-RELATED, EVH1 DOMAIN CONTAINING 2	SPRED2	1436892_at	100	118.4	239.4	1.10
POLIOVIRUS RECEPTOR	PVR	1451160_s_at	35.3	163.8	223.5	1.12
NA	NA	1445935_at	119.5	64.7	218.7	1.19
RIKEN CDNA 1810006J02 GENE	1810006J02RIK	1431552_at	61.8	136.4	217.9	1.10
ATP-BINDING CASSETTE, SUB-FAMILY B (MDR/TAP), MEMBER 4	ABCB4	1449818_at	14.4	93.2	217.2	2.02
RIKEN CDNA 2310014L17 GENE	2310014L17RIK	1435572_at	72.5	71.1	212.8	1.48
SUPPRESSOR OF CYTOKINE SIGNALING 3	SOCS3	1455899_x_at	99.3	102.8	212	1.05
UBIQUITIN SPECIFIC PEPTIDASE 2	USP2	1417169_at	133.9	71.8	207.9	1.01
NA	NA	1456303_at	124.2	67	202.9	1.06
NA	NA	1457342_at	123.3	68.8	201.5	1.05
LEUCINE RICH REPEAT AND FIBRONECTIN TYPE III DOMAIN CONTAINING 1	LRFN1	1421364_at	100.4	50.9	200.5	1.33
NA	NA	1455275_at	152.9	43.9	200.4	1.02

Table 3. QPCR expression data for up-regulated CRG determination

	QPCR expression					Standard Deviations			
	Medium	**G-CSF**	**IL-2**	**G+2**	**CRG score**	**Medium**	**G-CSF**	**IL-2**	**G+2**
GAS5	1.00	1.71	2.57	1.45	0.34	0.35	0.38	0.55	0.46
IFRD1	1.00	0.79	1.79	1.20	0.47	0.08	0.15	0.53	0.19
LSM7	1.00	1.22	1.54	1.36	0.49	0.35	0.19	0.54	0.29
HIVEP3	1.00	0.68	3.05	3.00	0.80	0.21	0.15	0.66	0.54
PLEKHA3	1.00	1.04	2.38	2.78	0.81	0.11	0.23	0.43	0.38
CCDC86	1.00	0.79	3.39	3.64	0.87	0.30	0.41	0.48	0.24
GPD2	1.00	0.85	2.15	2.64	0.88	0.11	0.31	0.31	0.51
DUSP5	1.00	1.05	3.00	3.62	0.89	0.13	0.12	0.38	0.63
RRP1B	1.00	1.12	2.76	3.54	0.91	0.09	0.15	0.41	0.61
SLC25A33	1.00	0.99	3.23	4.07	0.96	0.10	0.24	1.11	0.63
TNFRSF12A	1.00	1.69	4.81	6.33	0.97	0.19	0.28	1.73	1.49
HBEGF	1.00	1.24	3.70	5.00	1.01	0.26	0.07	0.66	0.69
RRP9	1.00	1.72	3.23	5.23	1.06	0.40	0.43	0.89	0.91
LRP12	1.00	2.00	2.82	5.54	1.15	0.07	0.28	0.62	1.13
ID1	1.00	4.45	2.76	9.45	1.31	0.34	0.62	1.17	2.46
ADORA3	1.00	1.82	2.91	6.85	1.45	0.19	0.31	0.33	1.37
CCNB1IP1	1.00	3.27	5.13	12.41	1.48	0.19	0.71	1.11	2.30
MYC	1.00	1.60	3.88	11.34	2.07	0.18	0.68	2.07	3.29
CISH	1.00	2.10	1.61	8.03	2.16	0.14	1.40	1.00	2.17
PIM2	1.00	2.06	1.02	10.58	3.44	0.18	1.49	0.31	4.76

Highlighted genes indicate that the gene expression trend seen with QPCR did not match the trend seen with Affymetrix.

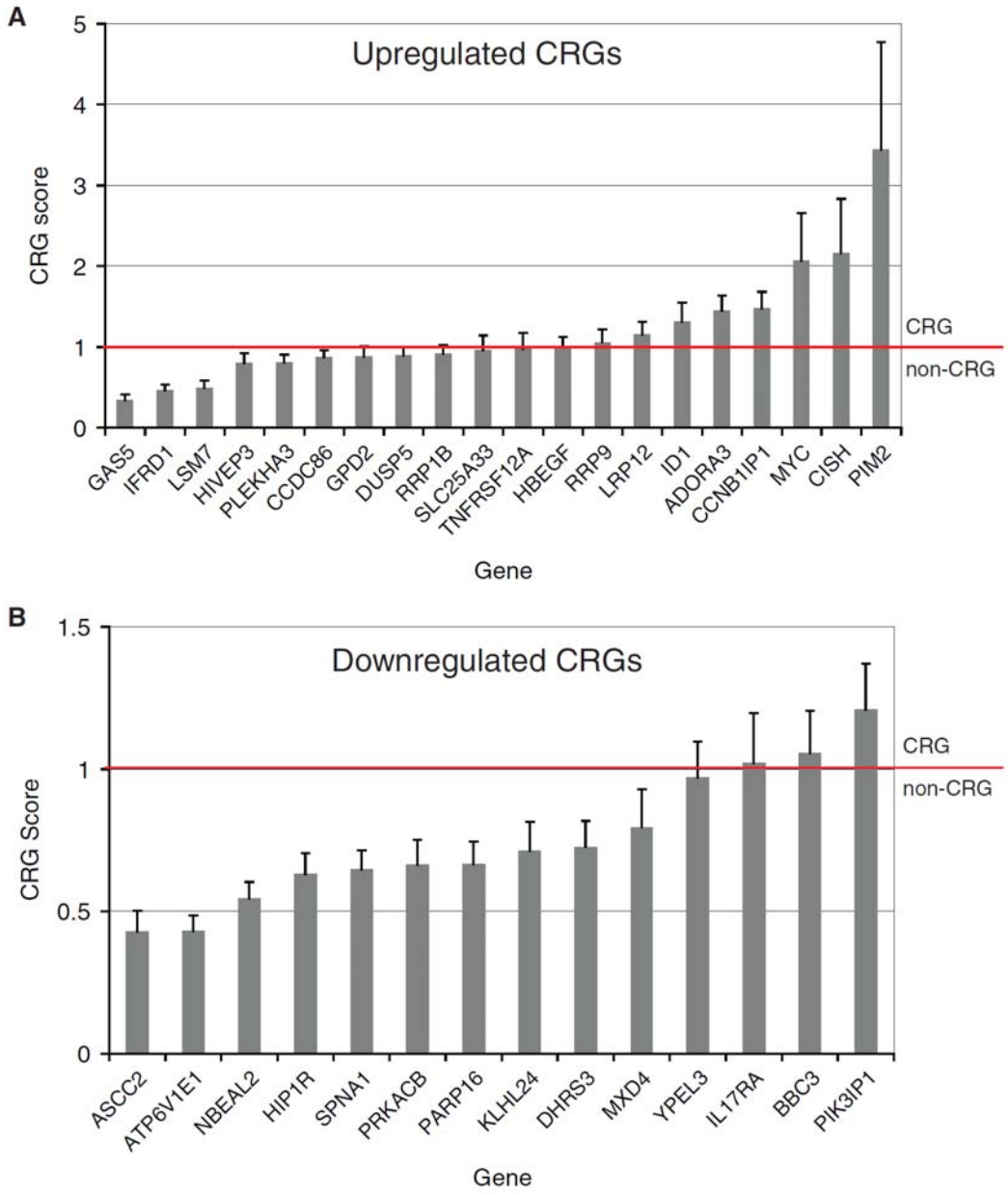


Figure 12. QPCR validation of Shc and STAT5 cooperation response genes (CRGs) identified from Affymetrix.

Identification of **(A)** up-regulated or **(B)** down-regulated CRGs. Ba/F3 lymphocytes stably expressing both β-Y338GG and G-Y510 were starved of cytokine for 4 h and then stimulated for 6 h with medium (negative control), G-CSF (100 ng/ml) to activate

STAT5, IL-2 (100 U/ml) to activate Shc, or a combination of G-CSF and IL-2. Total RNA was isolated, converted to cDNA, and QPCR was performed on the specified genes and normalized with GAPDH. CRGs are defined in the Materials and Methods. Genes with a score greater than 1 are considered synergistically regulated by both Shc and STAT5 (i.e., a CRG). Data are an average of 3 independent clones and error bars represent SEM.

The Shc and STAT5 pathways could also promote lymphocyte proliferation by cooperatively down-regulating genes that negatively regulate proliferation. Thus, as with the upregulated CRGs, we searched the Affymetrix data for down-regulated CRGs (as described in Materials and Methods). We found 59 putative down-regulated CRGs (Table 4), and used QPCR to validate a subset of these genes (Table 4, highlighted in yellow). Although 11/14 genes showed the same expression trends to the Affymetrix data (i.e., gene expression was lowest with concurrent Shc and STAT5 activation, Table 5 for raw QPCR data), only three (*pik3ip1*, *bbc3*, and *IL-17Ra*) met the strict definition of a CRG (Fig. 12B). Of these three CRGs, *pik3ip1* has not been associated with cytokine signaling and thus represents an entirely novel IL-2 regulated gene.

Since our experiments thusfar have been in the pro-B lymphocyte cell line Ba/F3, we wanted to ensure that the identified CRGs were also regulated by IL-2 in T cells. To this end, the murine cytotoxic T-cell line CTLL-2 was stimulated with IL-2 or left unstimulated for 6 h, followed by RNA isolation and QPCR analysis of the initial 34 candidate CRGs. Although two genes (*ccnb1ip1* and *spna1*) were undetectable transcripts in CTLL-2 cells, we found that the majority of initial putative CRGs found in Ba/F3 cells were also regulated by IL-2 in the cytotoxic T-cell line CTLL-2 (Fig. 13A and B), demonstrating that our results are relevant in multiple cell types. Thus, concurrent activation of Shc and STAT5 results in the synergistic induction and repression of a subset of genes, some of which may be involved in IL-2 mediated T-cell proliferation.

Table 4. List of putative down-regulated cooperation response genes (CRGs) identified by Affymetrix analysis.

Data are sorted on descending signal strength upon co-stimulation of with G-CSF and IL-2 (co-activation of STAT5 and Shc). Highlighted genes were further evaluated with QPCR. Gene names were annotated using the Gene ID conversion tool from The Database for Annotation, Visualization and Integrated Discovery 2008 (DAVID), NIAID, NIH.

Gene Name	Gene Symbol	Affymetrix ID	G-CSF Stimulation	IL-2 Stimulation	G-CSF + IL-2 Stimulation	CRG Score
DEHYDROGENASE/REDUCTASE (SDR FAMILY) MEMBER 3	DHRS3	1448390_a_at	2137.2	1830	799.2	1.23
SYNAPTOTAGMIN 11	SYT11	1456464_x_at	2445.2	1088	571.3	1.32
INTERLEUKIN 17 RECEPTOR	IL17RA	1420905_at	1355.7	1189	557.3	1.14
YIPPEE-LIKE 3 (DROSOPHILA)	YPEL3	1426624_a_at	1788.6	1088	530.9	1.27
SPECTRIN ALPHA 1	SPNA1	1421277_at	1323.7	1069	527.9	1.12
BCL-2 BINDING COMPONENT 3	BBC3	1423315_at	1122.9	899	401.9	1.24
RIKEN CDNA 1500004A08 GENE	PIK3IP1	1439087_a_at	1489.3	683	390	1.20
RIKEN CDNA 1500004A08 GENE	PIK3IP1	1428332_at	1497.9	680	369.9	1.26
KELCH-LIKE 24 (DROSOPHILA)	KLHL24	1428651_at	1313.4	609	335.3	1.24
RIKEN CDNA 1110014F23 GENE	NBEAL2	1456881_at	723.8	824	298.5	1.29
PROTEIN KINASE, CAMP DEPENDENT, CATALYTIC, BETA	PRKACB	1420610_at	717.6	682	291.2	1.20
HUNTINGTIN INTERACTING PROTEIN 1 RELATED	HIP1R	1425553_s_at	527.1	696	261.3	1.15
POLY (ADP-RIBOSE) POLYMERASE FAMILY, MEMBER 16	PARP16	1426950_at	512.8	458	208.8	1.16
SIMILAR TO HISTONE H2B-616	HIST2H2BE	1455095_at	499.4	415	190.8	1.19
ACTIVATING SIGNAL COINTEGRATOR 1 COMPLEX SUBUNIT 2	ASCC2	1418348_a_at	399.6	425	183.1	1.12
MAX DIMERIZATION PROTEIN 4	MXD4	1434379_at	694.3	536	181.9	1.66
BETA-SITE APP CLEAVING ENZYME 1	BACE1	1456399_at	558.8	288	169.7	1.12
VATPASE, H+ TRANSPORTING, LYSOSOMAL V1 SUBUNIT E1	ATP6V1E1	1449711_at	632	591	156.7	1.95
CYCLIC NUCLEOTIDE PHOSPHODIESTERASE 1	CNP	1418980_a_at	321.4	439	152.6	1.22
CALPAIN 1	CAPN1	1417229_at	355.6	390	134.7	1.38
NUCLEAR RECEPTOR COACTIVATOR 2	NCOA2	1450458_at	308.4	298	131.6	1.15
NON-POU-DOMAIN-CONTAINING, OCTAMER BINDING PROTEIN	NONO	1447160_at	596.6	386	130	1.80
PROCOLLAGEN, TYPE V, ALPHA 1	COL5A1	1416740_at	483.3	407	124.9	1.77

RIKEN CDNA 4632417N05 GENE	4632417N05RIK	1427082_at	292.5	407	123.9	1.37
TETRASPANIN 7	TSPAN7	1417502_at	346.8	263	123.5	1.21
WD REPEAT DOMAIN 22	WDR22	1436438_s_at	302.5	289	114.5	1.29
SORBITOL DEHYDROGENASE	SORD	1438183_x_at	385.4	245	112.6	1.33
TRANSMEMBRANE PROTEIN 110	TMEM110	1438693_at	306	236	110.4	1.21
RIKEN CDNA 1110014K08 GENE	1110014K08RIK	1443835_x_at	258.5	288	103.8	1.31
PYROGLUTAMYL-PEPTIDASE I	PGPEP1	1460001_at	250.5	250	101.8	1.23
HLA-B-ASSOCIATED TRANSCRIPT 1A	BAT1A	1447779_x_at	208.3	295	99.9	1.22
RIKEN CDNA 4733401I18 GENE	CD200R3	1430645_at	223.9	236	99.2	1.16
E74-LIKE FACTOR 4 (ETS DOMAIN TRANSCRIPTION FACTOR)	ELF4	1421338_at	268.3	289	98.4	1.41
KELCH-LIKE 24 (DROSOPHILA)	KLHL24	1436968_x_at	341.7	184	97.8	1.22
RIKEN CDNA 0610030I10 GENE	CYP4F13	1418767_at	269.2	215	97.4	1.23
TRANSLOCASE OF INNER MITOCHONDRIAL MEMBRANE 22 HOMOLOG (YEAST)	TIMM22	1448518_at	283.5	207	95.5	1.25
F-BOX AND WD-40 DOMAIN PROTEIN 9	FBXW9	1425857_at	309.3	215	93.6	1.35
PAIRED-IG-LIKE RECEPTOR A2	PIRA2	1420464_s_at	265.1	205	87.5	1.32
NA	NA	1441367_a_at	205.3	205	87.2	1.18
GA REPEAT BINDING PROTEIN, BETA 2	GABPB2	1453682_at	236.1	247	82.5	1.46
PANTOTHENATE KINASE 1	PANK1	1429813_at	228.9	201	82.1	1.30
CITRON	CIT	1426028_a_at	246	226	80	1.47
RHO GTPASE ACTIVATING PROTEIN 18	ARHGAP18	1431133_at	226.9	189	79.9	1.29
TRYPSIN DOMAIN CONTAINING 1	TYSND1	1441856_x_at	288.5	565	78.5	2.43
RIKEN CDNA 1110020G09 GENE	1110020G09RIK	1437286_x_at	240.3	195	75	1.44
RIKEN CDNA 5930418K15 GENE	TMEM181	1440862_at	214.8	157	74.3	1.22
RIKEN CDNA F830020C16 GENE	HMBOX1	1452449_at	235.2	173	72.1	1.38
RAS-LIKE WITHOUT CAAX 1	RIT1	1428710_at	221.5	124	70.2	1.13
NUCLEAR FACTOR OF ACTIVATED T-CELLS, CYTOPLASMIC, CALCINEURIN-DEPENDENT 1	NFATC1	1447084_at	234.8	238	60.9	1.94
ASPARAGINE-LINKED GLYCOSYLATION 12 HOMOLOG (YEAST, ALPHA-1,6-MANNOSYLTRANSFERASE)	ALG12	1427557_at	162.7	266	60.7	1.66
DNA SEGMENT, CHR 15, WAYNE STATE UNIVERSITY 169, EXPRESSED	D15WSU169E	1447521_x_at	254.9	189	60.4	1.80
RIKEN CDNA 3021401N23 GENE	3021401N23RIK	1453277_at	213.5	145	55.6	1.55
RIKEN CDNA 4631402N15 GENE	EG70793	1453694_at	235.7	129	52.6	1.58
HEAT SHOCK PROTEIN 1	HSPB1	1427853_a_at	252.3	157	51.2	1.89
WD REPEAT DOMAIN 62	WDR62	1424849_at	161.4	202	50.1	1.79
NEOGENIN	NEO1	1434931_at	209.5	237	49.4	2.25
TRIPARTITE MOTIF PROTEIN 12	TRIM12	1446939_at	204.2	77.6	45.3	1.24
NUCLEOPORIN 153	NUP153	1441689_at	224.6	132	42.2	1.97

RIKEN CDNA 9130404D08 GENE	9130404D08RIK	1431070_a_at	275.8	166	39.3	2.64
RAN, MEMBER RAS ONCOGENE FAMILY	RAN	1436490_x_at	246	115	35.6	2.21
RIKEN CDNA 1700010I14 GENE	1700010I14RIK	1419624_a_at	211.2	205	19.7	5.28

Table 5. QPCR expression data for down-regulated CRG determination

	QPCR expression					Standard Deviations			
	Medium	G-CSF	IL-2	G+2	CRG score	Medium	G-CSF	IL-2	G+2
ASCC2	1.00	1.09	1.49	1.46	0.43	0.10	0.34	0.47	0.26
ATP6V1E1	1.00	1.26	1.78	1.71	0.43	0.07	0.29	0.47	0.18
NBEAL2	1.00	1.52	2.02	1.59	0.55	0.13	0.26	0.53	0.13
HIP1R	1.00	1.13	1.55	1.03	0.63	0.08	0.25	0.20	0.16
SPNA1	1.00	1.93	1.54	1.32	0.65	0.22	0.38	0.34	0.14
PRKACB	1.00	1.32	1.71	1.12	0.66	0.11	0.23	0.49	0.16
PARP16	1.00	0.68	0.59	0.47	0.67	0.22	0.12	0.19	0.05
KLHL24	1.00	1.67	1.20	0.98	0.71	0.09	0.45	0.26	0.17
DHRS3	1.00	1.79	1.26	1.02	0.73	0.25	0.21	0.55	0.10
MXD4	1.00	1.71	1.82	1.11	0.80	0.10	0.49	0.40	0.25
YPEL3	1.00	1.44	1.04	0.62	0.97	0.12	0.38	0.16	0.10
IL-17R	1.00	1.46	2.36	0.88	1.02	0.23	0.37	0.52	0.22
BBC3	1.00	1.85	1.54	0.79	1.06	0.07	0.47	0.59	0.07
PIK3IP1	1.00	2.09	1.20	0.63	1.21	0.22	0.25	0.26	0.13

Highlighted genes indicate that the gene expression trend seen with QPCR did not match the trend seen with Affymetrix.

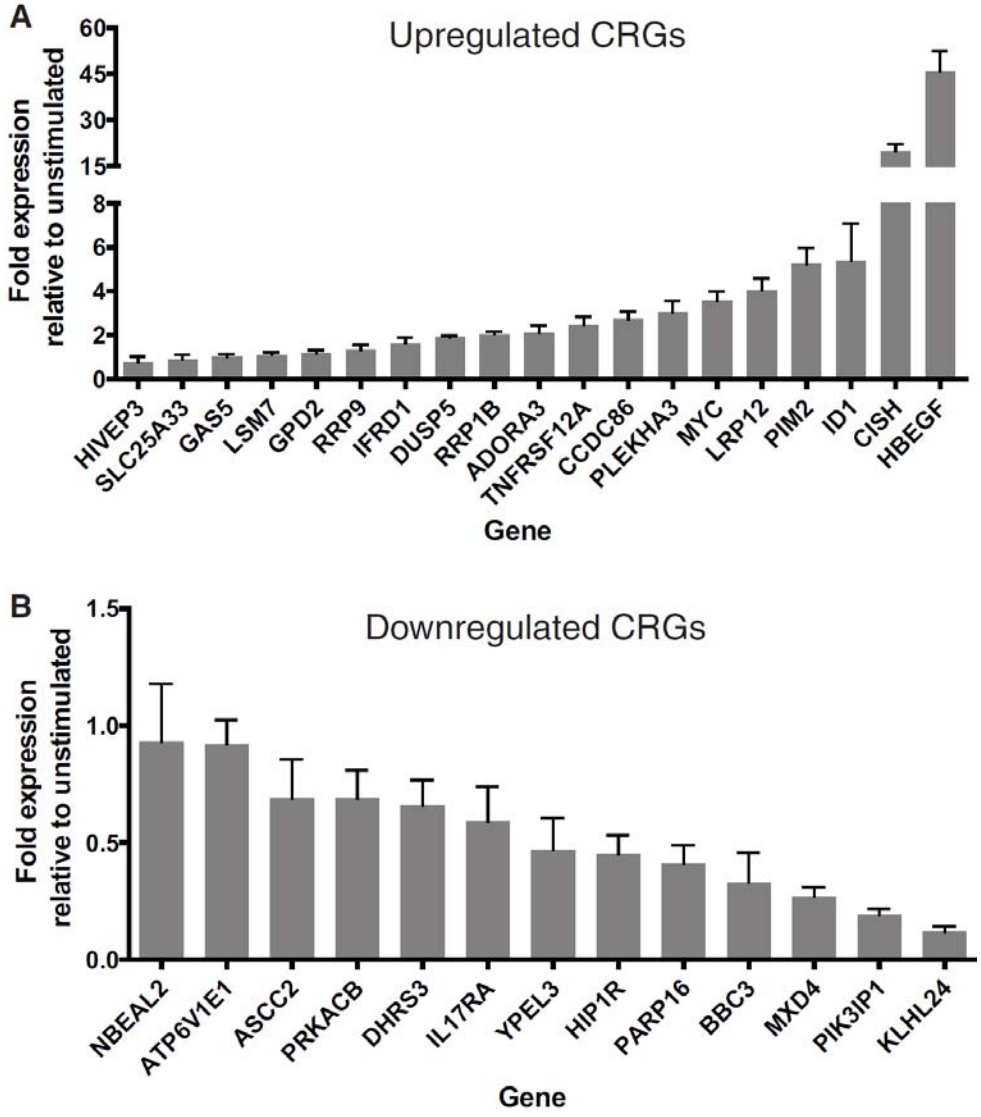


Figure 13. Validation of putative CRGs in the cytotoxic T-cell line CTLL-2. CTLL-2 cells were starved of cytokine for 4 h and then stimulated with medium (negative control) or IL-2 (100 U/ml) for 6 h. Total RNA was isolated, converted to cDNA and QPCR was performed on the specified genes. Expression of putative **(A)** up-regulated and **(B)** down-regulated CRGs upon stimulation through the wild-type IL-2 receptor, which activates both Shc and STAT5, in CTLL-2 T cells. Data shown is relative to unstimulated cells (harvested after 4 h cytokine starve) and normalized to GAPDH.

Temporal requirements for maximal gene induction

To narrow the number of candidate genes that may play an important role in proliferation, we assessed gene expression patterns upon temporal dissociation of the Shc and STAT5 pathways. We included all 34 of the candidate CRGs from the initial Affymetrix analysis. Given that temporal dissociation of Shc and STAT5 for 3 h or less does not significantly affect proliferation, we analyzed gene expression of the 34 genes upon temporal dissociation of 1 and 3 h, reasoning that genes important for proliferation would not be significantly affected by temporal dissociation of 1 or 3 h. On the other hand, if a gene was markedly perturbed by temporal dissociation for 1 or 3 h, then that gene is probably not important for optimal lymphocyte proliferation. We first assessed the effects of temporal dissociation on *c-myc*, since expression of *c-myc* has always tracked positively with cell proliferation. Thus, the Shc and STAT5 pathways were simultaneously activated, or temporally dissociated for 1 or 3 h, and gene expression was assessed at 6 h post stimulation. As expected, *c-myc* levels significantly correlated with cell proliferation as assessed by Pearson's correlation (Fig. 14A).

Using the same approach, we found that 18 of the remaining 33 genes significantly correlated with proliferation, the majority (15/18) of which were up-regulated genes. Of the nine up-regulated CRGs validated by QPCR (Fig. 12A), eight significantly correlated with proliferation (Table 6), with *adora3* demonstrating the strongest correlation (Fig. 14B). Of the down-regulated genes, only 3/14 genes (*parp16*, *ypel3*, and *pik3ip1*) were significantly (negatively) associated with proliferation (Table 7), with *parp16* showing the strongest negative correlation with proliferation (Fig. 14C).

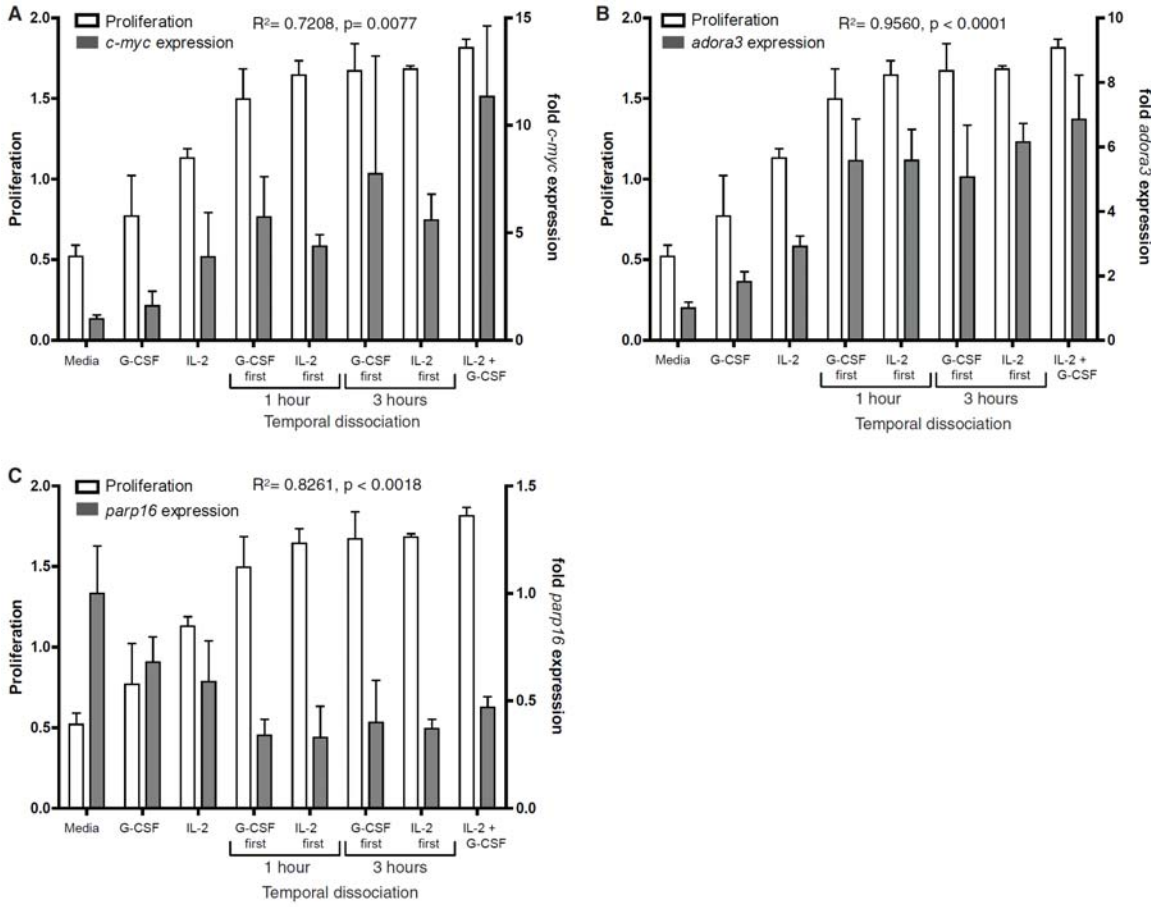


Figure 14. Temporal dissociation of the Shc and STAT5 pathways identifies genes that are correlated with lymphocyte proliferation.

Ba/F3 lymphocytes stably expressing both β-Y338GG and G-Y510 were starved of cytokine for 4 h and then stimulated for 6 h with medium (negative control), G-CSF (100 ng/ml) to activate STAT5, IL-2 (100 U/ml) to activate Shc, or a combination of G-CSF and IL-2. Additionally, G-CSF and IL-2 stimulation were temporally dissociated from one another by 1 or 3 h. Total RNA was isolated at t=6 h post stimulation, converted to cDNA, and analyzed by QPCR. **(A)** Correlation of *c-myc* expression with proliferation upon temporal dissociation. Correlation of the up-regulated CRG **(B)** *adora3*, and down-regulted CRG **(C)** *parp16*, with proliferation upon temporal dissociation. Pearson correlation was used for statistical analysis and significance was set at p=0.05. See Tables 6 and 7 for results for all putative CRGs.

Table 6. Temporal dissociation of the Shc and STAT5 pathways identifies upregulated genes that are correlated with lymphocyte proliferation.

See Fig. 14 legend for details.

Gene	Pearson r	R^2	P-value (two-tailed)
ADORA3	0.9777	0.956	**< 0.0001**
RRP9	0.9484	0.8995	**0.0003**
CIS	0.9444	0.8918	**0.0004**
DUSP5	0.925	0.8557	**0.001**
GPD2	0.9235	0.8529	**0.0011**
LRP12	0.9189	0.8444	**0.0013**
PLEKHA3	0.9154	0.838	**0.0014**
HBEGF	0.9095	0.8271	**0.0017**
SLC25A33	0.9107	0.8293	**0.0017**
RRPB1	0.9021	0.8138	**0.0022**
TNFSFR12A	0.887	0.7869	**0.0033**
ID1	0.8732	0.7624	**0.0046**
C-MYC	0.849	0.7208	**0.0077**
HIVEP3	0.7601	0.5777	**0.0286**
CCDC86	0.7594	0.5766	**0.0289**
CCNB1IP1	0.7437	0.5531	**0.0344**
PIM-2	0.6899	0.476	0.0583
ATP6V1E1	0.629	0.3956	0.0948

Table 7. Temporal dissociation of the Shc and STAT5 pathways identifies down-regulated genes that are correlated with lymphocyte proliferation.

See Fig. 14 legend for details.

Gene	Pearson r	R^2	P-value (two-tailed)
PARP16	-0.9089	0.8261	**0.0018**
YPEL3	-0.7753	0.6011	**0.0238**
PIK3IP1	-0.7403	0.548	**0.0357**
KLHL24	-0.586	0.3434	0.1269
LSM7	0.5858	0.3432	0.127
BBC3	-0.5085	0.2586	0.1981
DHRS3	-0.4858	0.236	0.2223
IFRD1	0.4798	0.2302	0.2289
MXD4	-0.4548	0.2068	0.2576
GAS5	0.4217	0.1779	0.298
HIP1R	-0.3742	0.14	0.3611
SPNA1	-0.3064	0.09389	0.4604
NBEAL2	0.2459	0.06045	0.5572
IL-17R	-0.2147	0.04609	0.6097
ASCC2	0.1564	0.02448	0.7114
PRKACB	-0.1413	0.01997	0.7386

Expression kinetics of down-regulated genes

Given the relative paucity of information on genes that are repressed by IL-2, we further characterized some of these genes by defining their expression kinetics upon IL-2 stimulation. CTLL-2 cells were washed, starved of cytokine for 4 h and then stimulated with IL-2 for 1, 3, and 6 h, followed by gene expression analysis by QPCR. As seen in Fig. 15, different genes exhibited different kinetics of repression by IL-2. Some genes appeared to be maximally repressed by ~ 3 h (Fig. 15D and G for *bbc3* and *parp16*, respectively), while the remaining genes required greater than 3 h for maximal repression (Fig. 15, A-C, E, F). Notably, some of these genes, such as *klhl24* and *pik3ip1*, were potently repressed (~10-25 fold) by IL-2 within 6 h (Fig. 15A and B, respectively).

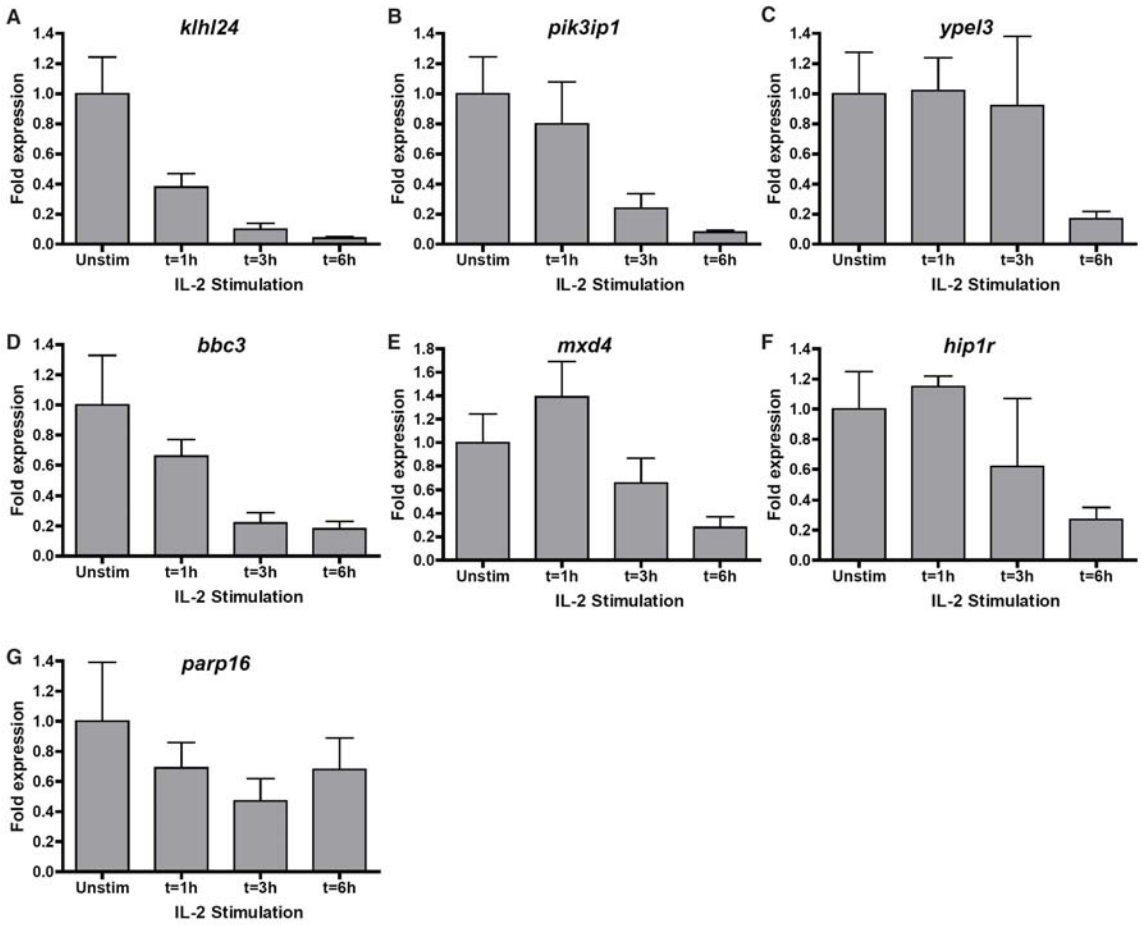


Figure 15. Kinetics of down-regulated genes.

CTLL-2 cells were washed 3X and then starved of cytokine for 4 h followed by stimulation with or without IL-2. Total RNA was isolated and converted to cDNA for QPCR analysis. **(A-G)** Gene expression kinetics of the indicated down-regulated gene.

Discussion

Maximal proliferation mediated by the IL-2 receptor requires activation of both the Shc and STAT5 pathways; however, the mechanisms by which these two pathways cooperate to induce proliferation remain poorly defined. Here we show that the Shc and STAT5 pathways display some temporal plasticity for inducing proliferation, i.e., proliferation does not appear to be significantly affected provided that both signals are delivered within ~3 h of each other. We also identified a subset of genes that are cooperatively regulated upon activation of both the Shc and STAT5 pathways and that are correlated with lymphocyte proliferation. We found that IL-2 signaling also potently repressed the expression of some genes. Thus, the Shc and STAT5 pathways appear to promote lymphocyte proliferation by both co-inducing and co-repressing a variety of genes.

Given that cell cycle progression is a highly regulated process, it is intriguing to find that a moderate degree of temporal plasticity exists between the Shc and STAT5 pathways for inducing proliferation. That is, with respect to proliferation, the cell does not appear to notice if the Shc and STAT5 pathways have been temporally dissociated for up to ~3 h. However, when the two pathways are temporally dissociated by ~6 h or more, the cell "appears to notice" and cellular proliferation is impaired. The reasons behind why both signals are required within ~3 h of each other are unknown, but may have to do with a temporal requirement of unique signals delivered by each pathway, or alternatively, may reflect the requirement of both signals for optimal regulation of proliferation genes, as discussed below.

The Shc pathway activates the PI3K/AKT and ERK pathways, which both have important roles in cell cycle progression (206, 207). However, we and others have shown that Shc signaling alone is not sufficient to promote maximal proliferation, and that STAT5 is critical for lymphocyte proliferation mediated by the IL-2R (182, 208). This could mean that the Shc and STAT5 pathways each regulate a unique set of genes that cooperate to induce cell cycle progression, or alternatively, it could mean that both pathways cooperate to enhance the induction or repression of common target genes that are important for proliferation. In support of the latter, previous studies demonstrated that both Shc and STAT5 were required for maximal induction of key cell cycle genes such as *cyclin D2*, *cyclin D3*, and *c-myc*, and this enhanced gene induction was correlated with increased proliferation (140, 204). Therefore our current study focused on identifying other IL-2R target genes that are similarly dependent on both Shc and STAT5 for optimal regulation. Using Affymetrix gene chip analysis, we identified a large number of genes that were putatively co-regulated by Shc and STAT5, and further validated a number of these by QPCR. Of the QPCR validated up-regulated CRGs, several genes, such as *c-myc* (192), *cis* (197), *adora3* (209), and *pim-2* (191), have been shown to play a role in T-cell proliferation, while others, such as low density lipoprotein receptor-related protein 12 (*lrp12*), Heparin binding EGF-like growth factor (*hbegf*), and Inhibitor of Differentiation/DNA-binding-1 (*id1*) have not. In fact, *lrp12* and *id1* have not yet been reported to be regulated by IL-2. Little is known about *lrp12*, but it is interesting to note that *lrp12* has been documented to be upregulated in oral cancers (210), suggesting that *lrp12* may have a role in cell proliferation.

HBEGF is a potent mitogen for fibroblasts, smooth muscle, and a wide variety of tumors (211). HBEGF is initially synthesized as a precursor membrane anchored protein known as pro-HBEGF (212), which when proteolytically cleaved, allows HBEGF to signal through two distinct mechanisms. First, soluble HBEGF can directly bind to and activate the growth factor receptors Erbb1 (EGFR) and Erbb4 (212), and signaling through Erbb1 can activate the RAS/ERK pathway to promote cell cycle progression (213). Second, the cytoplasmic C-terminal fragment (CTF) of HBEGF can translocate to the nucleus and enhance cell cycle progression by inhibiting transcriptional repressors such as promyelocytic leukemia zinc finger (PLZF) (214), and Bcl6 (215). Specifically, the CTF of HBEGF can induce nuclear export of PLZF, which leads to expression of cyclin A2 and promotion of S-phase cell cycle entry (214). The CTF of HBEGF can also interact with Bcl6, and this association can enhance cyclin D2 expression and cell cycle progression (215). To our knowledge, since T cells do not express Erbb receptors, it is unlikely that soluble HBEGF would contribute to T-cell proliferation. However, since human T cells have been documented to synthesize HBEGF (216, 217), it is possible that the CTF of HBEGF could contribute to T-cell proliferation through the mechanisms described above. Thus, IL-2 stimulation of T cells induces expression of HBEGF, which could theoretically contribute to proliferative signaling through its CTF.

Id-1 is a basic helix-loop-helix (bHLH) protein that is expressed in proliferating cells, and was originally described as a negative regulator of cellular differentiation (218). More recently, Id-1 has been found to promote cancer cell growth and survival, and is over-expressed in over 20 different tumor types (218). Ectopic expression of Id-1

can promote cell survival and proliferation through enhancement of the RAS/ERK, NFκB, and Akt pathways (219, 220) and inactivation of tumor suppressor pathways such as the p53 and retinoblastoma pathways (219). Id-1 also appears to have key roles in T-cell biology. Thymocytes from Id-1 transgenic mice undergo massive apoptosis; however, despite this massive apoptosis, Id-1 transgenic mice often develop T-cell lymphoma, suggesting that Id-1 plays critical roles in both T-cell development and proliferation (221). Id-1 can also promote T-cell proliferation and cytokine production by enhancing TCR-mediated NFκb pathway activation (222, 223). Thus, IL-2R signaling may promote proliferation by upregulating Id-1, which in turn could further augment T-cell proliferation by impinging on multiple signaling pathways. Overall, the novel genes identified to be cooperatively up-regulated by Shc and STAT5 may play a role in proliferative signaling mediated by the IL-2R, an idea that awaits validation with functional studies.

We also identified a subset of genes that were repressed upon concurrent Shc and STAT5 signaling. Although the QPCR CRG validation rate for the negatively regulated genes was low, it should be noted that our definition of a CRG was arbitrary and was used merely to help narrow down the number of genes for further study. What potentially is more biologically relevant is the degree of gene repression upon stimulation through the wild-type IL-2 receptor. In this scenario (i.e., with CTLL-2 cells), stimulation with IL-2 resulted in the repression of some genes, such as *ypel3*, *hip1R*, *parp16*, *bbc3*, *mxd4*, *pik3ip1*, and *klhl24*. Interestingly, several of these genes have documented roles in proliferation and/or apoptosis.

Although there is no documented role of Ypel3 (also known as small unstable apoptotic protein, SUAP) in T cells, Ypel3 has been shown to promote apoptosis in myeloid cells (224). More recently, Ypel3 was found to be regulated by the tumor suppressor p53, and induction of Ypel3 induced tumor cell senescence and decreased tumor cell viability (225). Moreover, gene expression analysis revealed that Ypel3 was reduced in ovarian, lung and colorectal tumours compared to normal controls, suggesting that tumors may down-regulate *ypel3* as a mechanism to promote tumor cell survival and decrease tumor cell senescence (225). The Ypel family members have also been shown to localize with centrosome or mitotic spindles, which may suggest a role for Ypel proteins in cell division (226). Thus, down-regulating *ypel3* may be one of the mechanisms used by IL-2 to promote lymphocyte proliferation.

Hip1R is a clathrin-binding protein that can induce apoptosis via a BAK-dependent mechanism (227). However, Hip1R was also found to enhance cell survival by augmenting receptor tyrosine kinase signaling through a mechanism that involved inhibition of receptor degradation (228). Moreover, overexpression of *hip1R* has been reported in chronic lymphocytic leukeumia (CLL) and is correlated with trisomy 12 (an extra chromosome 12), which suggests that Hip1R may play a role in the pathogenesis of CLL (229). Thus, Hip1R appears to have dual roles in cell survival, but its relevance in T-cell biology remains to be determined.

Bbc3, also known as PUMA, is a proapoptotic Bcl-2 family member that interacts with mitochondria to induce both p53-dependent and -independent cell death (230). Myeloid cells deficient in Bbc3 are resistant to death upon growth factor withdrawal

(231), and similarly, Bbc3-deficient T cells exhibit abnormally prolonged *in vivo* persistence after virus infection and enhanced survival *in vitro* (232). Bbc3 also regulates T-cell responses by inducing apoptosis of antigen-specific T cells after pathogen clearance (232). T cells deprived of IL-2 upregulate Bbc3, and this was found to be mediated by FOXO3a, which is a transcription factor downstream of the PI3K/AKT pathway (233). Collectively, these results implicate a critical role for Bbc3 in cell death induction. Thus, IL-2 may repress Bbc3 in order to enhance T-cell survival.

Mxd4 (formerly known as Mad4) is a member of the Mxd-family of proteins whose main role is to antagonize Myc activity (234, 235). Given that Myc has a central role in promoting cell proliferation (234, 235), it is possible that IL-2 may repress a negative regulator of Myc as a means to enhance proliferation. Although nothing is known about the role of Mxd4 in T cells, expression of the related protein Mxd1 (Mad1) has been documented to impair T-cell growth and proliferation (236, 237). The redundant nature of Mxd proteins (234) suggests that like Mxd1, Mxd4 may also be capable of inhibiting T-cell proliferation.

The genes most potently repressed by IL-2 were *klhl24* and *pik3ip1*. Notably, nothing is known about Klhl24 (also known as Krip6) with respect to the immune system; however, Klhl24 appears to play a role in the nervous system by modulating currents induced by glutamate receptors (238). Specifically, Klhl24 was found to bind the ionotropic glutamate receptor GluR6 and reduce peak currents in neurons (238). Given that ion flux is critical for T-cell proliferation (239), and the fact that T cells express glutamate receptors (240-243), it is tempting to speculate that IL-2 may down-regulate

klhl24 as a means to enhance ion flux and thus T-cell proliferation. Indeed, a number of studies have shown that glutamate can positively regulate T-cell proliferation and function through various classes of glutamate receptors (240, 241).

The role of PIK3IP1 in the immune system is also unknown. Interestingly, PIK3IP1 is a newly discovered protein reported to negatively regulate the PI3K/AKT pathway by binding to and inhibiting the p110 catalytic subunit of PI3K (244). In a study using mice prone to developing hepatocellular carcinoma (HCC), overexpression of PIK3IP1 in hepatocytes significantly suppressed the development of spontaneous liver cancer (245). Moreover, human HCC samples were shown to have decreased levels of PIK3IP1 protein compared to matched, adjacent normal tissue controls (245). Altogether, these results suggest that PIK3IP1 may act as a negative regulator of proliferation and tumorigenesis by attenuating the AKT/PI3K pathway. Thus, repression of a negative regulator of the PI3K/AKT pathway may represent a novel mechanism by which IL-2 promotes cell growth and proliferation.

Collectively, many of the genes we have identified to be repressed by IL-2 have documented negative roles in cell survival, growth and/or proliferation, and thus the repression of these genes by the IL-2R may play an important role in lymphocyte proliferation. The biological significance of some of these repressed genes in proliferative signaling by IL-2 is currently being evaluated in our lab.

It is clear from these and other studies that the mechanisms of IL-2 signaling are complex. It is remarkable that signaling through the IL-2R alone can regulate such an intricate, coordinated, and diverse wave of proteins that ultimately results in proliferation.

These events are initiated by, and are critically dependent on the Shc and STAT5 pathways, which cooperate to regulate genes that are required for IL-2 induced proliferation. The mechanisms by which Shc and STAT5 coordinate gene expression of positive and negative regulators of proliferation are still not well understood, but multiple mechanisms are at play. For example, it seems that IL-2 can promote proliferation by upregulating cell cycle and proliferation genes (e.g., *cyclins*, *c-myc*), and by repressing genes that negatively regulate proliferation (e.g., *mxd4*, *bbc3*). However, at the same time, IL-2 also attenuates proliferation by inducing expression of negative regulators of proliferation, such as the suppressors of cytokine signaling (SOCS) proteins (74). Thus, IL-2 both represses and induces negative regulators of proliferation. Why and how IL-2 represses some negative regulators, while inducing others, is just one small part of the complex network that is IL-2 signaling.

Chapter 4: Cytokine-mediated reprogramming of polyfunctional CD8+ T-cell responses in the ovarian cancer microenvironment

Eric Tran[1,2], Julie S. Nielsen[2], Darin A. Wick[2], Alvin V. Ng[2], Nancy J. Nesslinger[2], Elissa McMurtrie[3], John R. Webb[1,2], and Brad H. Nelson[1,2,4]

[1]Department of Microbiology and Biochemistry, University of Victoria, Victoria BC, Canada, [2]Trev and Joyce Deeley Research Centre, British Columbia Cancer Agency, Victoria BC, Canada, [3]BC Cancer Agency, Victoria, BC, Canada, and [4]Department of Biology, University of Victoria, Victoria, BC, Canada, and Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada.

ET designed research, performed research, collected data, analyzed and interpreted data, and wrote the manuscript

JSN performed research, and analyzed and interpreted data

DAW performed research

AVN performed research

NJN performed research

EM contributed vital biospecimens and clinical data

JRW designed research

BHN designed research, analyzed and interpreted data, and wrote the manuscript

4.1 Abstract

Tumor-infiltrating CD8+ T cells are associated with increased survival in epithelial ovarian cancer (EOC), but their functional status and signaling requirements remain poorly understood. In viral and malignant diseases, T cells capable of multiple effector functions ("polyfunctional" T cells) are associated with improved outcomes. Therefore, we evaluated the polyfunctional status of tumor-associated CD4+ and CD8+ T cells in EOC by measuring expression of IFN-γ, TNF-α, IL-2, CCL4, and CD107a upon T-cell receptor stimulation. Compared to medium, 100% ascites fluid greatly reduced T-cell proliferation and polyfunctionality within bulk ascites cell preparations in most patient samples. Addition of IL-2, IL-7, IL-12, IL-15, IL-18, and IL-21, alone or in various combinations, restored T-cell proliferation and IFN-γ production to varying degrees, whereas other cytokines (IL-6, IFN-α, IFN-γ, 4-1BBL, and OX-40L) had negligible effects. Remarkably, the combination of IL-2 + IL-12 + IL-18 overcame the immunosuppressive effect of ascites to potently enhance T-cell proliferation and polyfunctionality in all patient samples. The specific polyfunctional profile induced by this cytokine combination is associated with protective immunity in other human diseases. Thus, exogenous cytokines can override the varied immunosuppressive mechanisms in EOC to reprogram polyfunctional T-cell responses toward a protective phenotype.

4.2 Introduction

Each year over 190,000 women worldwide are affected by ovarian cancer (International Agency for Research on Cancer). The most lethal form of ovarian cancer is the high-grade serous subtype (hereafter referred to as EOC), which represents approximately 40% of cases. Standard treatments induce clinical remission in the vast majority of patients, but unfortunately 60-70% of patients relapse within 5 years and ultimately succumb to chemotherapy-resistant disease (246, 247).

Accumulating evidence indicates that the immune system actively influences clinical outcomes in EOC. Specifically, the presence of CD8+ T cells in tumor epithelium has been associated with prolonged disease-free and overall survival in numerous studies (246, 247). However, the ovarian tumor environment also contains many immunosuppressive cell types that are associated with poor survival, including regulatory T cells (Tregs) and tumor associated macrophages (TAMs) (246, 247). Many soluble immunosuppressive factors are also found in ascites, including IL-10, TGF-β, VEGF, B7-H1/PD-L1, B7-H4, SDF-1, EBAG9/RCAS1, Fas ligand, and soluble IL-2 receptor (246). Thus, the ovarian tumor environment contains numerous inhibitory factors that can disable anti-tumor T-cell responses. The heterogeneity of immunosuppressive mechanisms in EOC presents a challenge for immunotherapy, as it suggests that unimodal interventions may be only partially successful. For example, Treg depletion is being evaluated as a means to enhance immunity against several human cancers (70), including EOC (248), but at best would only reverse one mechanism of immunosuppression. A more pragmatic clinical approach would be to deliver factors that

can broadly override immunosuppression in the tumor environment, irrespective of the underlying mechanism.

It is becoming increasingly evident that "polyfunctional" CD4+ and CD8+ T cells, i.e., T cells that can simultaneously perform multiple functions, are an important index of protective immunity (249). Indeed, the presence of polyfunctional T cells is correlated with favorable outcomes in a variety of disease settings, including HIV/AIDS (250-260), hepatitis C (261), lymphocytic choriomeningitis (262), and cancer (263-267). Polyfunctional T-cell responses are also associated with protective immunity after vaccination against smallpox (vaccinia virus) (268), yellow fever (269), tuberculosis (270, 271), and leishmaniasis (272). Despite their importance in disease control, relatively little is known about how polyfunctional T-cell responses are affected by the tumor microenvironment and how best to modulate these responses toward a protective phenotype. Here, we show that the ovarian tumor environment generally suppresses polyfunctional T-cell responses, but that defined combinations of cytokines can override immunosuppression to generate polyfunctional T-cell profiles associated with protective immunity. Our findings suggest new ways to enhance the natural host T-cell response to EOC, which ultimately may lead to improved clinical outcomes for this challenging disease.

4.3 Materials and Methods

Study subjects and sample processing. Specimens were obtained from newly diagnosed patients with high-grade serous EOC who gave written informed consent under protocols approved by the Research Ethics Board of the BC Cancer Agency and

the University of British Columbia. Patients were 40-70 years of age (median, 61 years). Tumor tissue and ascites were obtained at the time of primary surgery prior to any other treatment. Ascites was centrifuged at 300 g for 10 min at 4 °C, and supernatants (ascites fluid) were stored at -80 °C. Ascites cell (AC) pellets containing large quantities of red blood cells were treated with ACK lysis buffer. AC pellets were cryopreserved in liquid nitrogen. Upon thawing, ascites cells were rested in complete RPMI (RPMI 1640 with 10% FBS, 25 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μm β-mercaptoethanol) for 4 h at 37 °C prior to experiments.

Antibodies and cytokines. The following fluorochrome-conjugated antibodies (BD Biosciences) were used for flow cytometry: anti-CD3 (FITC, PECy5), CD4 (FITC, PE), CD8 (PECy5, APC-H7), CD14 (PE), CD19 (PE), CD25 (PECy5), CD56 (PE), CD107a (PECy5), Ki-67 (FITC), CCL4, formerly known as Mip-1β, (PE), IL-2 (APC), TNF-α (PECy7), and IFN-γ (PE, AlexaFluor 700). Anti-Foxp3 (PE) was from eBioscience. The following recombinant human cytokines (Peprotech) were used at 100 ng/ml: IL-6, IL-7, IL-12, IL-15, IL-21, 4-1BBL, and OX-40L. IL-18 (R&D Systems) was used at 100 ng/ml, and IFN-γ (NIAID, NIH) was used at 100 U/ml. Clinical grade IL-2 (Proleukin) and IFN-α (Intron-A) were used at 100 U/ml and 1000 U/ml, respectively. TGF-β (latent and active) in ascites fluid was quantified by ELISA (eBioscience).

Proliferation assays. Ascites cells (AC) were seeded in triplicate in 96-well flat bottom plates at 1 x 10^5 cells per well. AC were resuspended in medium or 100% ascites fluid and were left unstimulated or stimulated with plate bound α-CD3ε antibody (clone OKT3, eBioscience) previously coated at 100 ng/well for 2h at 37 °C, in the presence or

absence of cytokines. T-cell proliferation was measured by detection of Ki-67 using flow cytometry. Cells were washed with FACS buffer (1% FBS in PBS), permeabilized with ice-cold 100% methanol and incubated at -20 °C for at least 1 h. Cells were then washed with FACS buffer and stained with pre-titered antibodies to Ki-67, CD4, and CD8 for at least 30 min at room temperature in the dark. Cells were washed and analyzed with a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star Inc.).

IFN-γ ELISA. Supernatants from unstimulated or stimulated cultures were harvested and stored at -80 °C until analysis. IFN-γ in supernatants was quantified by ELISA (BD OptEIA Human IFN-γ ELISA Set; BD Biosciences) as directed by manufacturer, using Maxisorp 96-well plates (Nunc, Roskilde, Denmark). IFN-γ values were interpolated from a standard curve.

Cell stimulation and staining to assess polyfunctionality. Bulk ascites cells were washed with serum-free RPMI and resuspended at 5 x 10^5 cells/ml in complete RPMI or autologous ascites fluid. Cells were plated in 48-well plates at 3 x 10^5 cells/well and stimulated with or without plate bound α-CD3ε antibody. Cytokines and α-CD107a were added to appropriate wells, and cells were incubated for 48 h at 37 °C. As a positive control, PMA plus ionomycin (both from Sigma) were used at 40 ng/ml and 1.5 μg/ml, respectively. GolgiStop (BD Bioscience, 1:1500 dilution) and brefeldinA (Sigma, 5 μg/ml) were added for the final 6 h of culture to inhibit cytokine secretion. Cells were then harvested, labeled with α-CD4 and CD8 antibodies, fixed and permeabilized with Cytofix/Cytoperm buffer (BD Bioscience) according to manufacturer's instructions,

washed with Perm/Wash buffer, and stored in FACS buffer overnight at 4 °C. Cells were washed again with Perm/Wash buffer, and intracellular staining was performed using antibodies against CCL4, IL-2, TNF-α, and IFN-γ. Cells were fixed with 2% formaldehyde and stored in FACS buffer overnight at 4°C. Samples were analyzed with a BD Bioscience FACSVantage DIVA modified with the Octagon array. SSC Area vs. SSC W were used to gate out doublets. Data were analyzed with FlowJo software (TreeStar) and exported to PESTLE v1.6.1 (Mario Roederer, NIH) for further data analysis.

4.4 Results

Effect of ovarian ascites on T-cell proliferation and polyfunctionality.

To determine the effects of the ovarian tumor environment on T-cell function, we collected primary ascites specimens from a cohort of patients with high-grade serous EOC (Table 8). To model the native tumor microenvironment as best as possible, we analyzed bulk ascites cell pellets, which in addition to CD8+ and CD4+ T cells, contained tumor cells, Tregs, B cells, NK cells, and CD14+ cells (Table 9). Moreover, cells were cultured in 100% ascites fluid to include any soluble factors, such as TGF-β (Table 9), that may impact T-cell function. Because of the lack of well-defined T-cell antigens in ovarian cancer, we used a polyclonal T-cell stimulus (α-CD3 antibody) to stimulate all T-cell types, including both effector and regulatory T cells, within bulk ascites cells. Thus, each patient's T cells were analyzed in the presence of the full

complement of naturally occurring immunosuppressive cell types and soluble factors from that patient.

An important measure of T-cell function is proliferative capacity. To assess proliferation, cultures were stimulated with α-CD3, and T-cell proliferation was assessed 48 h later by staining for the proliferation marker Ki-67. Ascites fluid had highly variable effects on T-cell proliferation, ranging from strong inhibition (1/5 patients) to enhancement of proliferation (2/5 patients) (Fig. 16A, B). In general, similar trends were seen between CD4+ and CD8+ T cells.

The polyfunctional status of T cells was assessed by flow cytometric analysis of five commonly used markers: IFN-γ, TNF-α, IL-2, CCL4, and CD107a (249-252, 268). Polyfunctionality was defined as the simultaneous expression of two or more of these parameters (249, 251, 272). In the majority of patient samples, ascites fluid caused a dramatic inhibition of polyfunctionality (Fig. 16C, D). This was largely attributable to inhibition of CD107a, IFN-γ, and CCL4 (Fig. 16E, F). Intriguingly, the effects of ascites on T-cell polyfunctionality (Fig. 16C-F) and proliferation (Fig. 16A, B) were largely uncoupled. For example, with patient sample IROC008, ascites fluid enhanced T-cell proliferation but inhibited polyfunctionality (Fig. 16A-F). Moreover, there was no significant correlation between the levels of the immunosuppressive cytokine TGF-β or regulatory T cells (Table 9), and the degree of inhibition on T-cell proliferation and polyfunctionality (Pearson correlation, $p > 0.05$, data not shown). Thus, ascites fluid has widely variable effects on T-cell proliferation and polyfunctionality between patient samples, in accord with the heterogenous nature of EOC.

Table 8. Clinical characteristics of patients

Patient ID	Age at diagnosis	Pathologic diagnosis	Grade	FIGO Staging	TNM Staging
IROC008	70	Papillary serous carcinoma	3/3	4	pT3c, pN1
IROC028	61	Papillary serous carcinoma	3/3	3C	pT3c, NX, MX
IROC034	64	Papillary serous carcinoma	3/3	N/A	T3c
IROC036	60	Papillary serous carcinoma	3/3	N/A	T3c, NX, MX
IROC038	40	Papillary serous carcinoma	3/3	3B	pT3b

Median Age: 61; Mean Age: 59; Age Range: 40-70
N/A, not assessed

Table 9. Cellular composition and TGF-β levels in the ascites compartment of high grade serous ovarian cancer patients.

Data is expressed as a percentage of total live cells unless otherwise stated.

Parameter	IROC Patient Sample				
	008	**028**[A]	**034**	**036**	**038**
Lymphocytes[B]	28.5	74.6	78.0	20.6	40.2
CD3+	19.8	59.0	58.0	11.9	21.9
CD3+CD4+	6.5	41.1	26.8	4.5	6.2
CD3+CD8+	12.4	16.9	28.1	6.6	13.0
CD3+CD56+	3.8	1.6	10.1	1.0	3.2
CD56+	2.7	12.0	9.7	3.0	4.6
Tregs[C] (CD4+CD25+FoxP3+)	5.2	6.8	2.9	3.3	2.5
CD25+FoxP3+ in CD4+	12.4	12.1	12.1	6.8	10.7
B cells[C] (CD19+)	8.1	1.4	6.5	4.5	5.0
CD14+	55.3	11.2	1.6	50.5	30.9
TGF-β (pg/ml)	6.3	N/D[D]	33.3	N/D	N/D

[A] Sample contained many large tumor rafts not detectable by FACS. Therefore, values are inflated since visual inspection and IHC staining shows this sample is comprised mainly of tumor cells.

[B] As determined by side and forward scatter.

[C] Tregs and B cells expressed as a percentage of total live lymphocytes

[D] N/D, not detectable

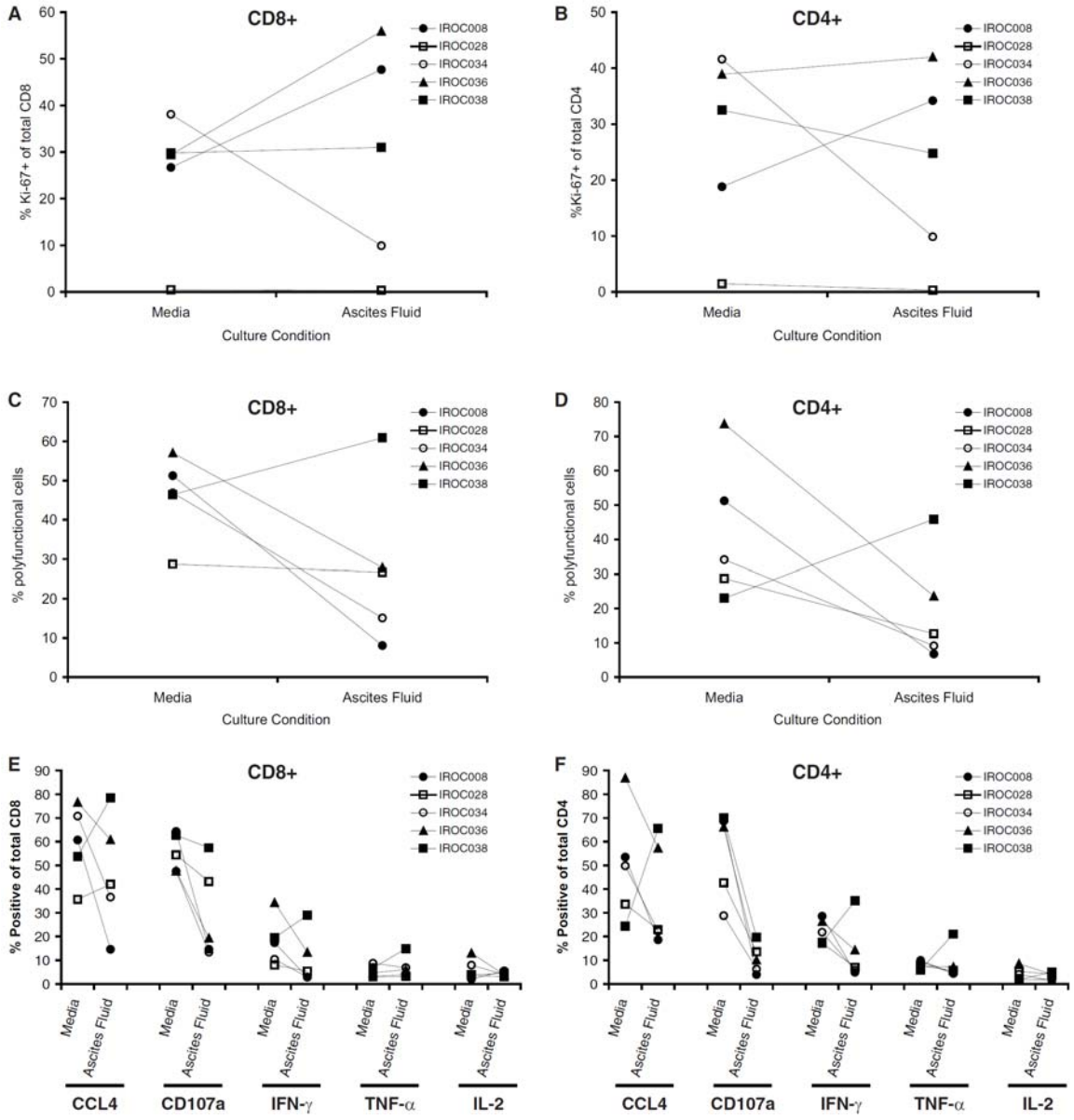


Figure 16. Impact of ascites fluid on T-cell proliferation and polyfunctionality. Bulk ascites cells were stimulated with plate bound α-CD3 in medium or 100% autologous ascites fluid for 48 h. Proliferation of (**A**) CD8+ and (**B**) CD4+ T cells was assessed by measuring expression of the proliferation marker Ki-67 by flow cytometry. The number of polyfunctional (**C**) CD8+ and (**D**) CD4+ T cells was assessed by measuring the expression of CD107a, IFN-γ, IL-2, CCL4, and TNF-α using flow

cytometry. “Polyfunctional” was defined as simultaneous expression of two or more of the above parameters. The effect of ascites on individual functions in CD8+ and CD4+ T cells is shown in (**E**) and (**F**), respectively.

Rescue of T-cell proliferation and function by exogenous cytokines.

A large number of factors have been shown to inhibit T-cell proliferation and function in the ovarian tumor environment, and strategies have been proposed to circumvent these factors on an individual basis. A more pragmatic clinical approach would be to identify immune stimulatory factors that broadly override immunosuppression, regardless of the underlying mechanism. Toward this end, we evaluated a large panel of cytokines with known, direct effects on CD8+ T-cell function, including IL-2, IL-6, IL-7, IL-12, IL-15, IL-18, IL-21, IFN-α, IFN-γ, 4-1BBL, and OX-40L, for the ability to rescue T-cell proliferation and function in the tumor environment. For this survey, we used IFN-γ expression as a surrogate marker for polyfunctionality, as the two are correlated (249). Thus, bulk ascites cells were stimulated with α-CD3 in 100% ascites fluid in the presence or absence of exogenous cytokines, and T-cell proliferation and IFN-γ production were measured 48 h later.

IL-2 and IL-15 were the most potent cytokines for restoring CD8+ and CD4+ T-cell proliferation (Fig. 17A and B). Other cytokines had either a neutral or modestly positive effect, with the exception of IFN-α, which inhibited T-cell proliferation in 2/5 patient samples (Fig. 17A and B). IL-12 was the most potent cytokine for enhancing IFN-γ secretion, while the cytokines IL-15, IL-2, IL-18, and to a lesser extent IL-7, also consistently induced IFN-γ production (Fig. 18). Interestingly, cytokines such as IFN-α, IL-6, and 4-1BBL had variable effects on IFN-γ secretion depending on the patient sample (Fig. 18).

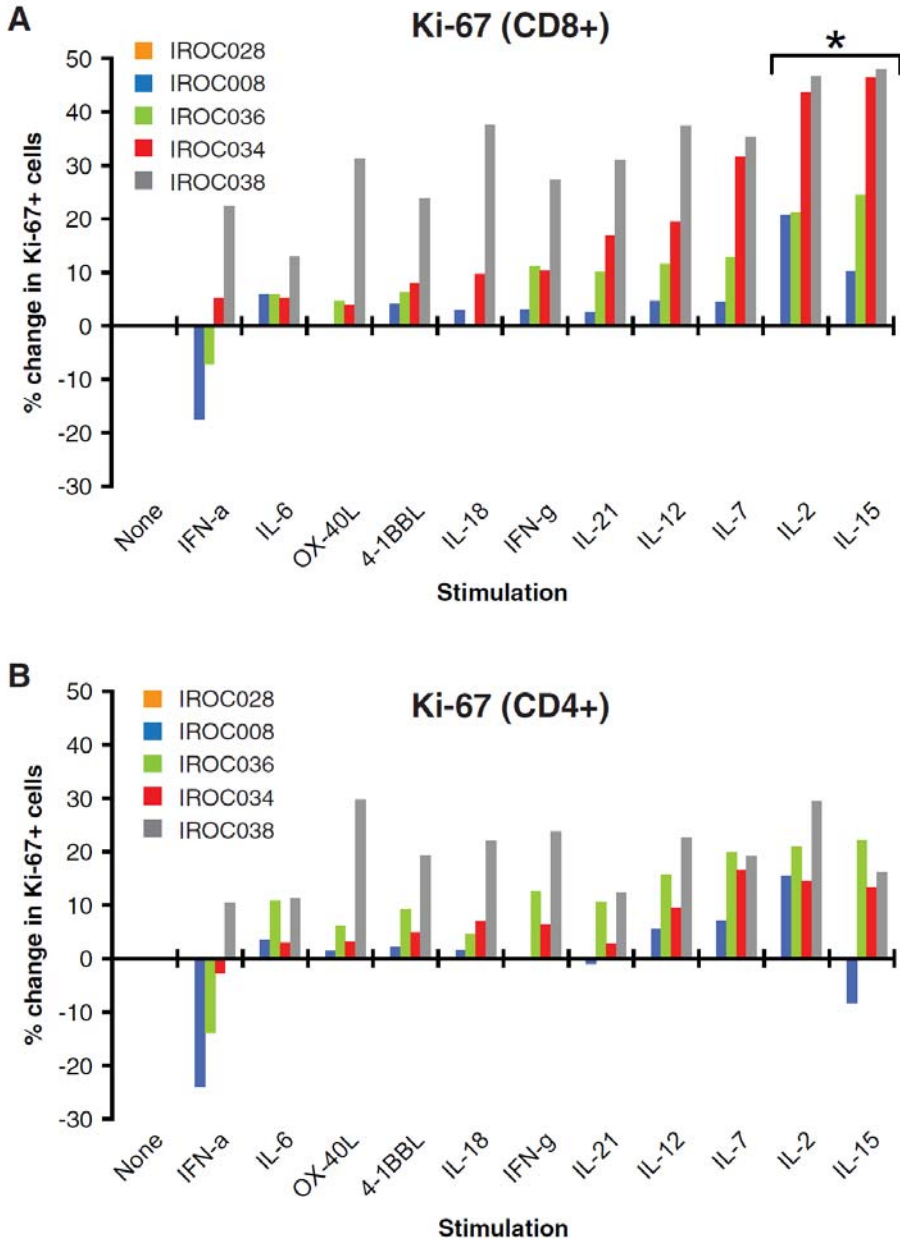


Figure 17. Effects of cytokines on T-cell proliferation.

Bulk ascites cells were plated in triplicate in 96-well flat bottom plates and stimulated with plate bound α-CD3 in 100% autologous ascites fluid for 48 h in the presence or absence of the indicated cytokine. Proliferation of (**A**) CD8+ and **(B)** CD4+ T cells was assessed by measuring expression of the proliferation marker Ki-67 by flow cytometry.

*The effects of IL-2 and IL-15 were significantly greater than all other cytokines (Wilcoxon matched pairs t test, $p < 0.05$). All data in were normalized by subtracting values obtained with ascites fluid alone.

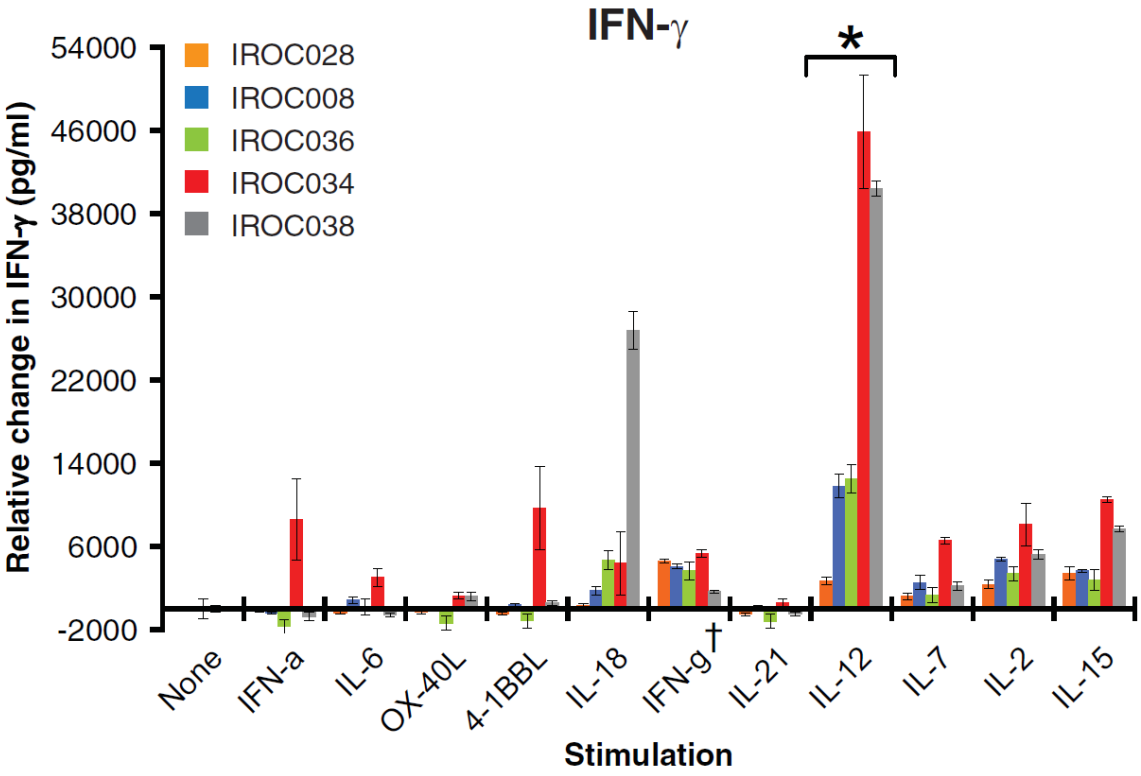


Figure 18. Effects of cytokines on IFN-γ secretion.

Bulk ascites cells were plated in triplicate in 96-well flat bottom plates and stimulated with plate bound α-CD3 in 100% autologous ascites fluid for 48 h in the presence or absence of the indicated cytokine. IFN-γ in the culture supernatants was assayed by IFN-γ ELISA. *The effect of IL-12 was significantly greater than all other cytokines except IL-15 (Wilcoxon matched pairs t test, $p < 0.05$). †Detected IFN-γ may be a result of exogenously added IFN-γ. All data were normalized by subtracting values obtained with ascites fluid alone.

We next tested three different combinations of cytokines based on the single-agent activities seen above, as well as prior reports: a) IL-2 + IL-12, b) IL-2 + IL-12 + IL-18, and c) IL-2 + IL-12 + IL-21. In general, all of the cytokine combinations induced T-cell proliferation (Fig. 19A and B) similar to that seen with IL-2 or IL-15 alone (Fig. 17A and B). Notably however, T cells from IROC028, which failed to proliferate in response to any single cytokine (Fig. 17A and B), responded to all three cytokine combinations (Fig. 19A and B). Consistent with prior literature (273), the combination of IL-2 + IL-12 potently enhanced IFN-γ production in CD8+ T cells (Fig. 20A). The addition of IL-21 to this combination failed to further enhance IFN-γ production, while the addition of IL-18 significantly enhanced both the percentage of IFN-γ-positive CD8+ T cells and the amount of IFN-γ produced on a per-cell basis (as assessed by mean fluorescence intensity, MFI) (Fig. 20A and B). Similar results were seen for CD4+ T cells, although IL-18 did not increase the MFI of IFN-γ (Fig. 20C and D). Collectively, these results revealed IL-2 + IL-12 + IL-18 as the most effective combination for simultaneously enhancing T-cell proliferation and IFN-γ production in the ovarian ascites environment.

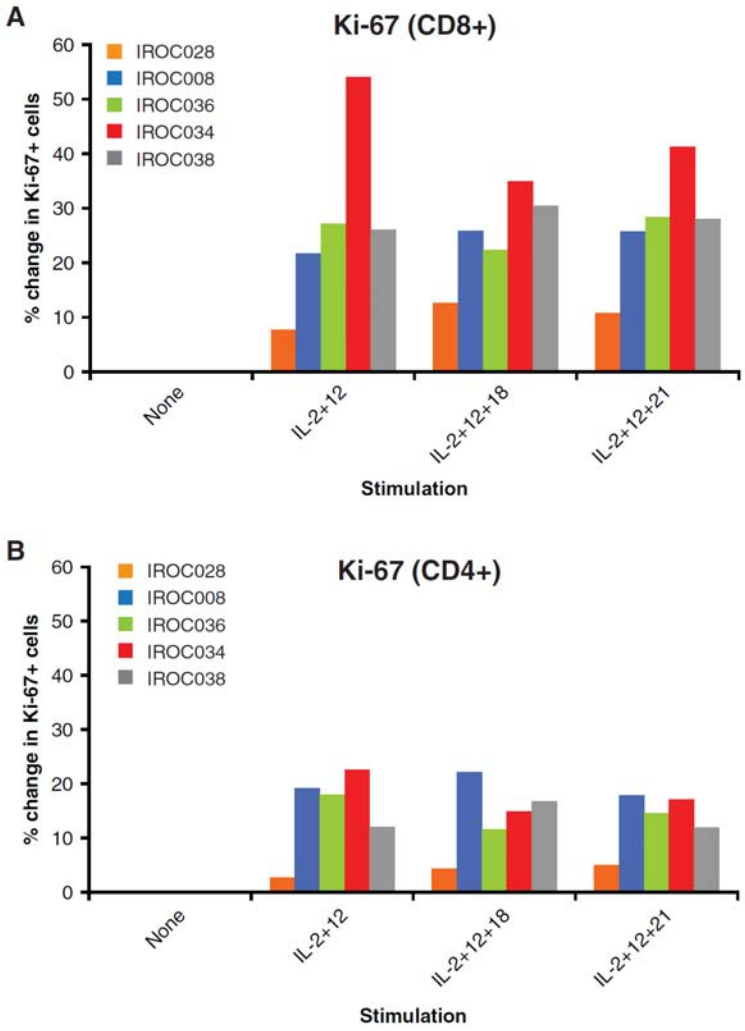


Figure 19. Effects of cytokine combinations on T-cell proliferation.

Bulk ascites cells were plated in triplicate in 96-well flat bottom plates and stimulated with plate bound α-CD3 in 100% autologous ascites fluid for 48 h in the presence or absence of the indicated cytokine combination. Proliferation of (**A**) CD8+ and **(B)** CD4+ T cells was assessed by measuring expression of the proliferation marker Ki-67 by flow cytometry. All data were normalized by subtracting values obtained with ascites fluid alone.

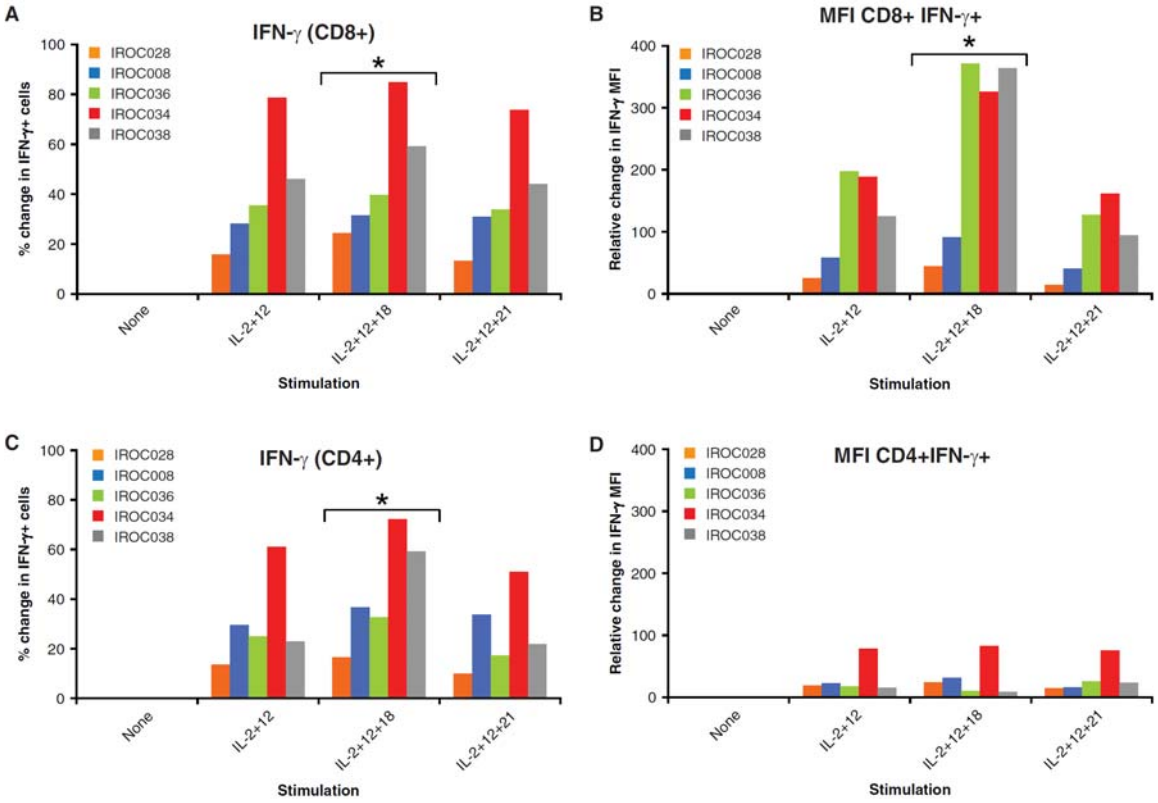


Figure 20. Effects of cytokine combinations on IFN-γ production by T-cells.
(**A**) Percentage and **(B)** mean fluorescence intensity (MFI) of IFN-γ positive CD8+ T cells was determined by intracellular IFN-γ staining. **(C)** Percentage and **(D)** mean fluorescence intensity (MFI) of IFN-γ positive CD4+ T cells was determined by intracellular IFN-γ staining. *The effect of IL-2 + IL-12 + IL-18 was significantly greater than the other two cytokine combinations (Wilcoxon matched pairs t test, $p < 0.05$). All data were normalized by subtracting values obtained with ascites fluid alone.

Potent enhancement of polyfunctional T-cell responses by the combination of IL-2, IL-12, and IL-18.

Having identified several cytokines capable of rescuing T-cell proliferation and IFN-γ expression, we next investigated their effect on T-cell polyfunctionality. When cytokines were tested individually, IL-2 appeared most effective at enhancing CD8+ T-cell polyfunctionality (Fig. 21A). Modest increases in polyfunctionality were seen with double cytokine combinations (Fig. 21A). In particular, the triple combination of IL-2 + IL-12 + IL-18 was most effective at enhancing not only the number of polyfunctional CD8+ T cells (Fig. 21A, $p < 0.05$ for indicated comparisons) but also the magnitude of IFN-γ production on a per-cell basis within the polyfunctional subset (Fig. 22, $p < 0.05$). The effect of cytokines on the polyfunctionality of CD4+ T cells showed similar trends as seen for CD8+ T cells, although the magnitude of enhancement was often lower (Fig. 21B).

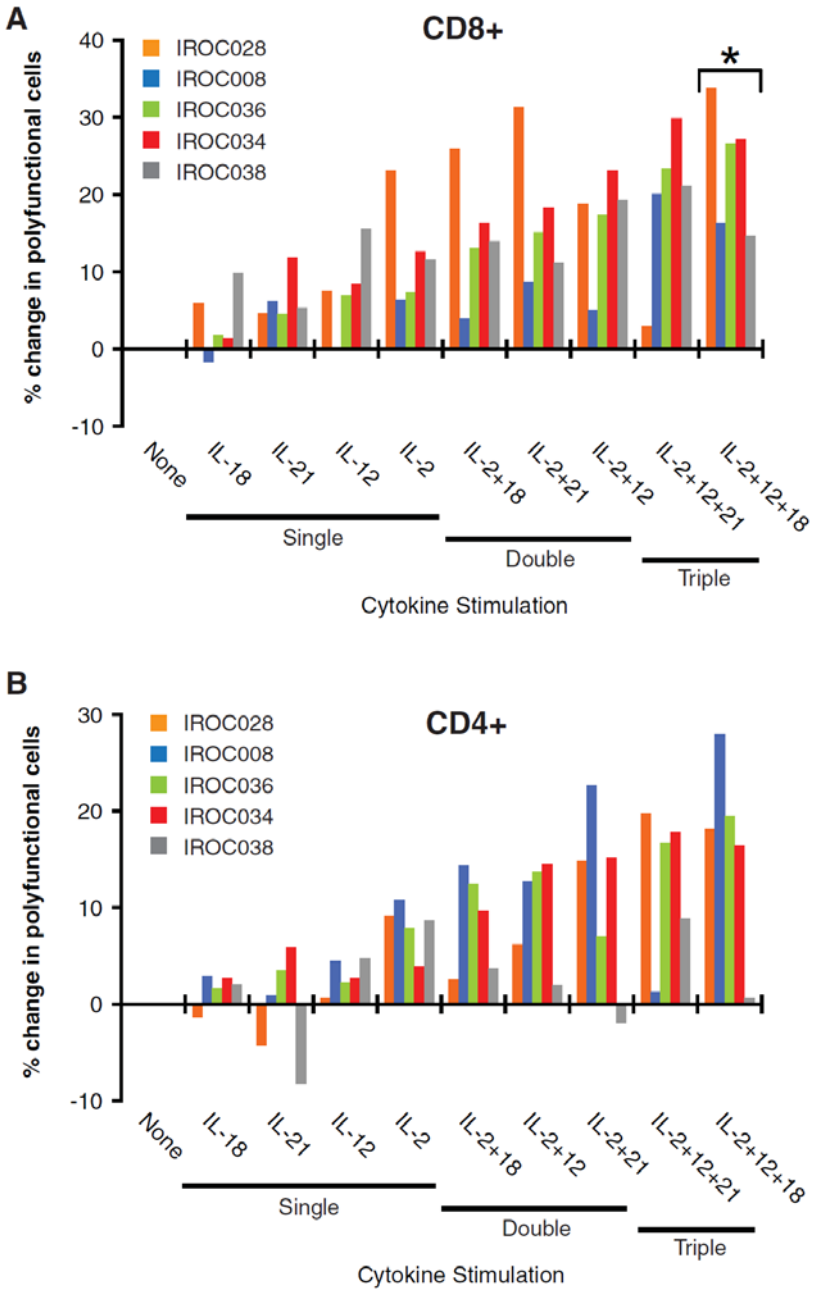


Figure 21. Effects of cytokines on polyfunctional T-cell responses.

Bulk ascites cells were stimulated with plate bound α-CD3 in medium or 100% autologous ascites fluid for 48 h in the presence or absence of the indicated cytokine or

cytokine combination. The number of polyfunctional (**C**) CD8+ and (**D**) CD4+ T cells was assessed as described in Fig. 16. *The effect of IL-2 + IL-12 + IL-18 was significantly greater than all other stimulations except for IL-12, IL-2 + IL-12, and IL-2 + IL-12 + IL-21 (Wilcoxon matched pairs t test, $p < 0.05$).

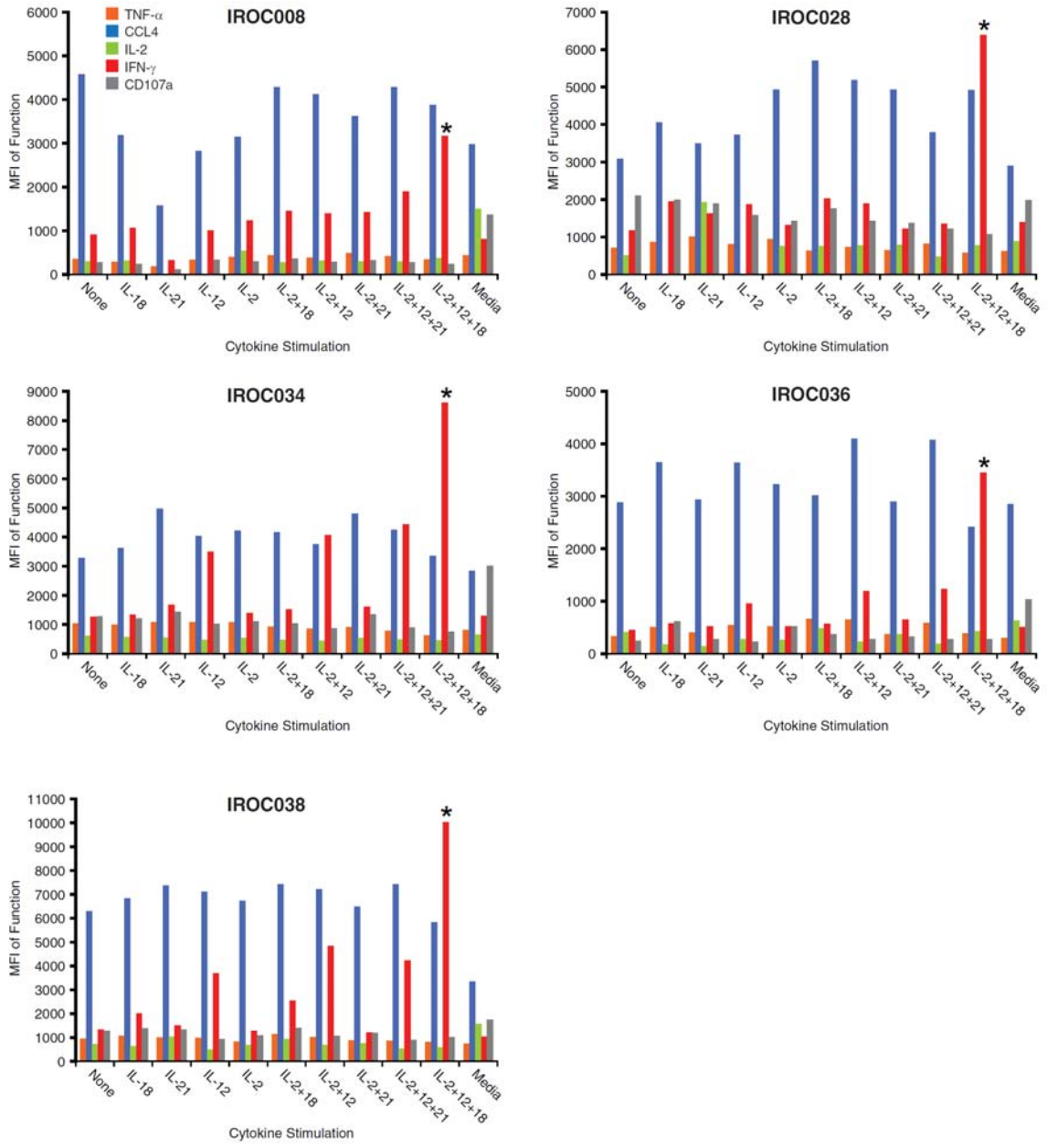


Figure 22. Effects of cytokines on the MFI of functional parameters in polyfunctional CD8+ T-cells.

The MFI corresponding to each function (CD107a, IFN-γ, IL-2, CCL4, and TNF-α) was determined on a per-cell basis for the samples described in Fig. 21 using FlowJo software. *The effect of IL-2 + IL-12 + IL-18 on the MFI of IFN-γ was significantly

greater than all cytokine or cytokine combinations (Wilcoxon matched pairs t test, $p < 0.05$). Data are gated on the 4+ polyfunctional subsets in the CD8+ population. The number of cells positive for a given function ranged from 0-854 events.

Finally, we evaluated the effect of cytokines on different polyfunctional permutations. Strikingly, when bulk ascites cells were stimulated in medium, only four predominant functional permutations were seen among CD8+ T cells: mono-functional T cells expressing either CCL4 or CD107a, bi-functional T cells expressing CD107a and CCL4, and tri-functional T cells expressing CD107a, CCL4, and IFN-γ (Fig. 23, top panel). Moreover, these four permutations were predominant in all five patient samples. In the presence of 100% ascites fluid, three of the four predominant permutations were generally suppressed, while mono-functional T cells expressing CCL4 were spared (Fig. 23, second panel).

For the most part, single cytokines increased the number of polyfunctional CD8+ T cells by modestly enhancing the above functional permutations (Fig. 23, third panel for IL-2 stimulation, and Fig. 24). However, in the presence of combined cytokines, new polyfunctional permutations emerged, all of which included CCL4 and IFN-γ. In particular, the cytokine combination of IL-2 + IL-12 + IL-18 induced the emergence of CD8+ T cells with three distinct permutations: a) CCL4 and IFN-γ; b) CCL4, IFN-γ, and TNF-α; and c) CCL4, IFN-γ, TNF-α, and CD107a (Fig. 23, bottom panel). Notably, these three permutations are induced by a protective smallpox vaccine (268), and permutations (b) and (c) are associated with protective immunity in HIV/AIDS and malignant melanoma (250, 251, 260, 265). Similar polyfunctional permutations and trends were seen for CD4+ T cells, although the effect of cytokines was generally weaker (Fig. 25). Thus, the combination of IL-2 + IL-12 + IL-18 can override the varied immunosuppressive mechanisms in the ovarian tumor environment to not only increase

the number of polyfunctional T cells and magnitude of IFN-γ secretion, but also generate polyfunctional T-cell profiles that are associated with protective immunity.

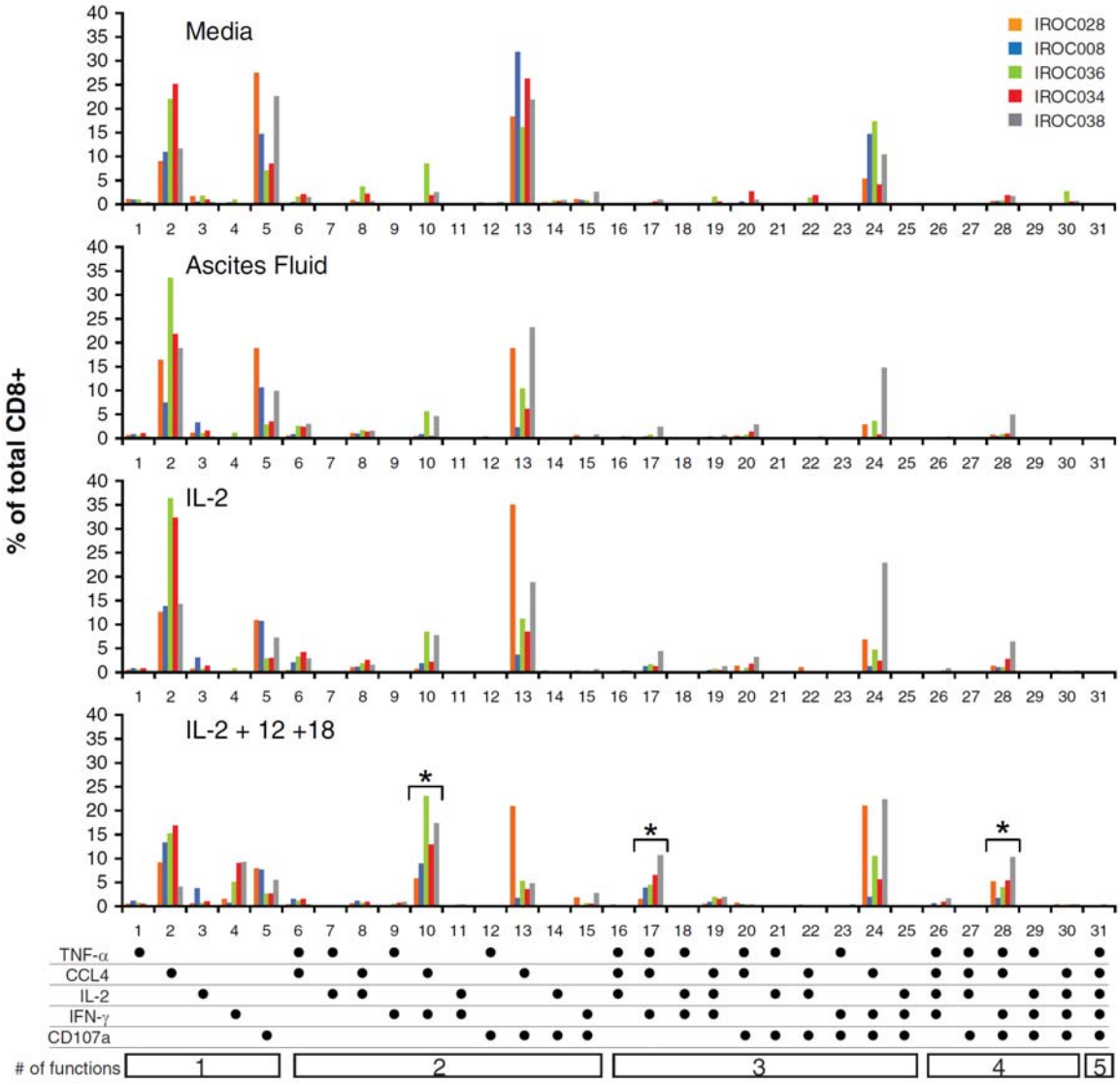


Figure 23. Effects of (select) cytokines on functional permutations of CD8+ T-cells. Boolean gate analysis was performed to quantify the number of T cells expressing each of 31 possible functional permutations. Shown are the results for CD8+ T cells stimulated in medium, ascites fluid, or ascites fluid supplemented with IL-2, or IL-2 + IL-12 + IL-18. The frequency of T cells expressing a given permutation is expressed as a percentage of total CD8+ T cells. *For the indicated permutation, the effect of IL-2 + IL-12 + IL-18

was significantly greater than that seen with medium, ascites fluid, or IL-2 stimulation (shown), as well as other cytokines and cytokine combinations shown in Figure 24 (Wilcoxon matched pairs t test, $p < 0.05$).

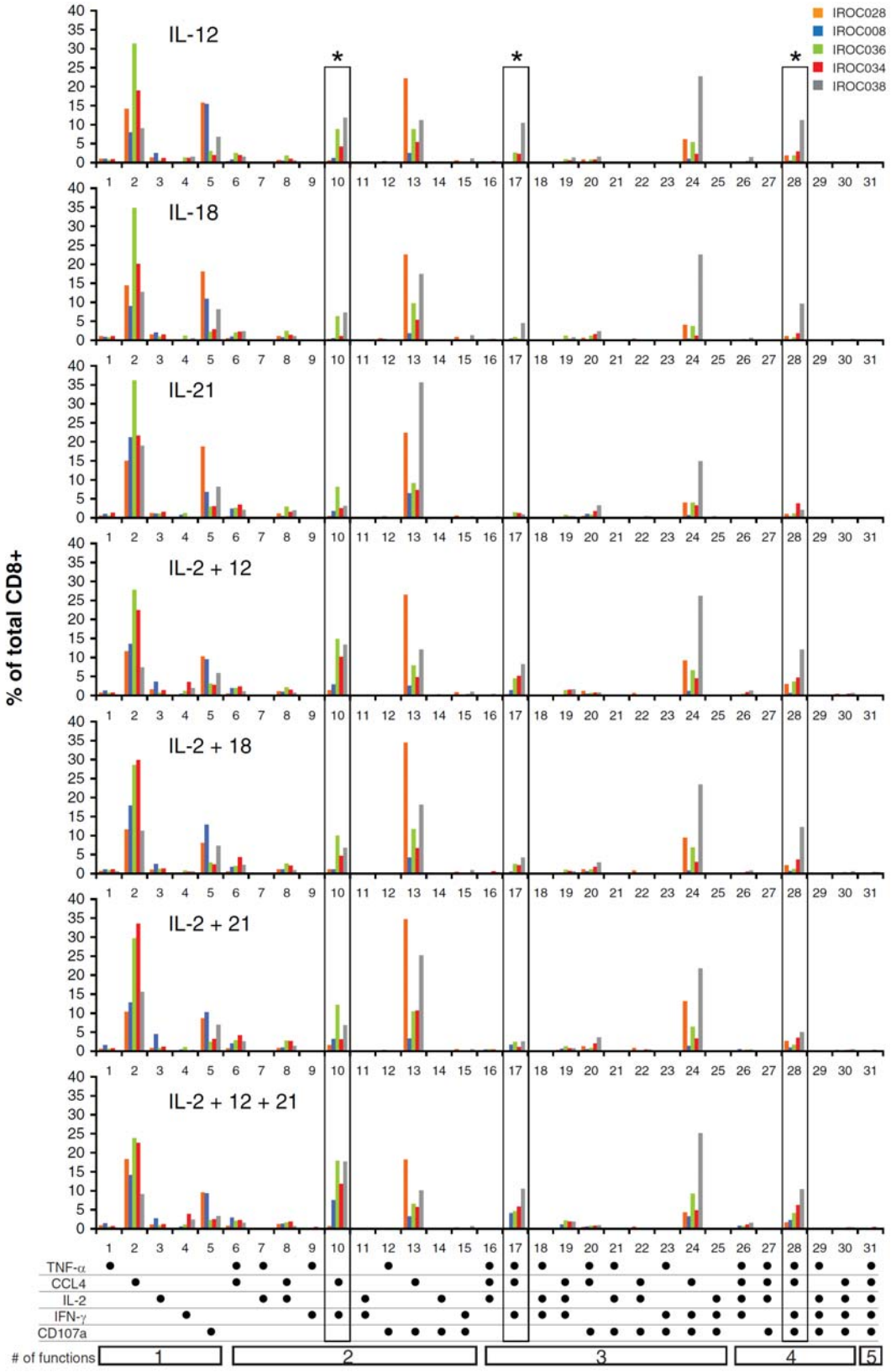
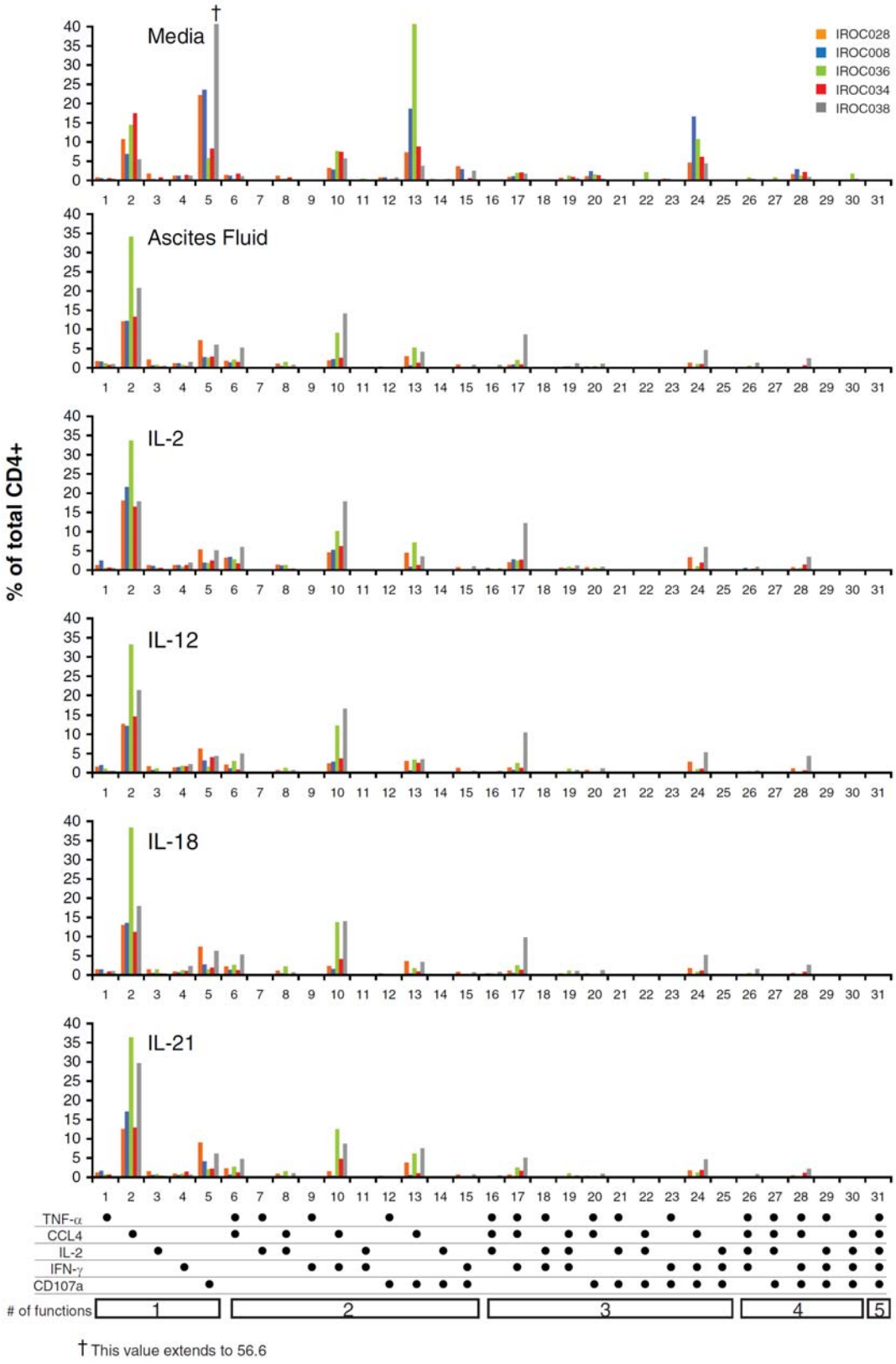

IL-12
IL-18
IL-21
IL-2 + 12
IL-2 + 18
IL-2 + 21
IL-2 + 12 + 21
IROC028
IROC008
IROC036
IROC034
IROC038
% of total CD8+
TNF-α
CCL4
IL-2
IFN-γ
CD107a
# of functions
1
2
3
4
5

Figure 24. Effects of (remaining) cytokines on functional permutations of CD8+ T cells.

Boolean gate analysis of the samples described in Fig. 21 was performed to reveal the presence of all 31 different functional permutations possible when measuring five functions (CD107a, IFN-γ, IL-2, CCL4, and TNF-α). This figure shows the remaining cytokines and cytokine combinations not presented in Fig. 23. *For the indicated permutation, the effect of IL-2 + IL-12 + IL-18 was significantly greater than all other cytokine stimulations except: IL-2 + IL-12 + IL-21 for permutations #10 and #17; and IL-12, IL-2 + IL-12, IL-2 + IL-21, and IL-2 + IL-12 + IL-21 for permutation #28 (Wilcoxon matched pairs t test, $p < 0.05$). The abundance of a given permutation is expressed as a percentage of total CD8+ T cells.


Media
Ascites Fluid
IL-2
IL-12
IL-18
IL-21
IROC028
IROC008
IROC036
IROC034
IROC038
% of total CD4+
TNF-α
CCL4
IL-2
IFN-γ
CD107a
# of functions
1
2
3
4
5
† This value extends to 56.6

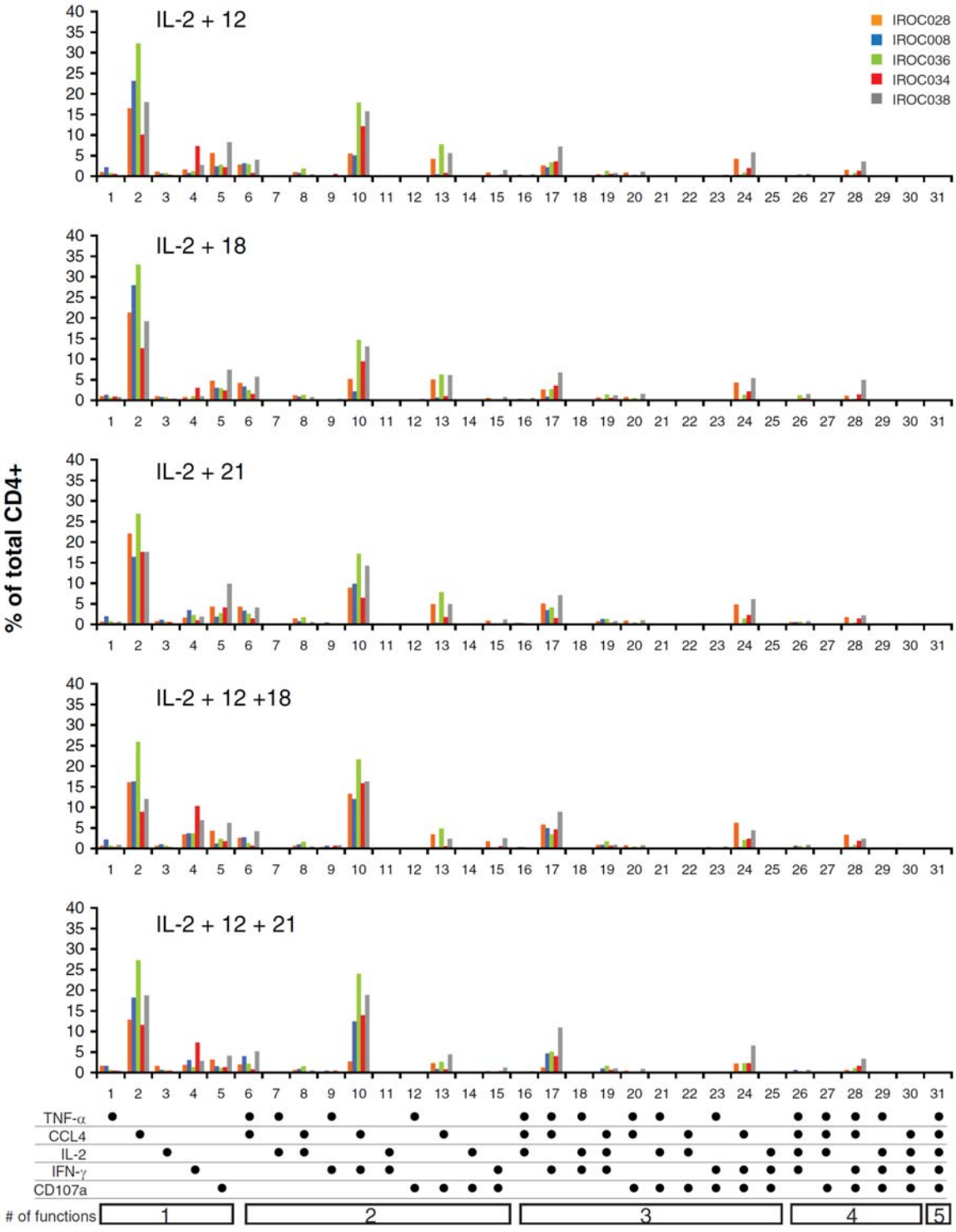

IL-2 + 12
IL-2 + 18
IL-2 + 21
IL-2 + 12 +18
IL-2 + 12 + 21
IROC028
IROC008
IROC036
IROC034
IROC038
% of total CD4+
TNF-α
CCL4
IL-2
IFN-γ
CD107a
# of functions
1
2
3
4
5

Figure 25. Effects of cytokines on functional permutations of CD4+ T cells.
Boolean gate analysis of the samples described in Fig. 21 was performed to reveal the presence of all 31 different functional permutations possible when measuring five functions (CD107a, IFN-γ, IL-2, CCL4, and TNF-α). Data are gated on CD4+ T cells and the abundance of a given permutation is expressed as a percentage of total CD4+ T cells.

4.5 Discussion

The ovarian tumor environment contains a wide variety of immunosuppressive factors that can disable anti-tumor T-cell responses (246, 247). Here we demonstrate that defined combinations of cytokines can override these immunosuppressive effects to enhance polyfunctional T-cell responses. In particular, the combination of IL-2 + IL-12 + IL-18 promoted T-cell proliferation, markedly increased the number of polyfunctional T cells, potently enhanced the amount of IFN-γ secreted by individual T cells, and generated new polyfunctional T-cell permutations that have been associated with protective immunity in other disease settings, including cancer. Together, these results suggest that, given the right signals, T cells can become highly polyfunctional effectors even in the malignant ovarian ascites environment, which offers promise for the development of effective T-cell based therapies for EOC.

Despite the growing recognition that polyfunctional T cells are important for effective immunity, relatively little is known about the factors that induce this functional state. Vaccination studies have identified antigen, adjuvant, and the route of delivery as important factors for generating polyfunctional T cells (253, 257-259, 267-272, 274-280). In a murine model of chronic LCMV infection, IL-21 promoted protective immunity, in part by inducing polyfunctional T cells (262), while treatment of hepatitis C patients with IFN-α early in infection rescued the polyfunctionality of virus-specific memory T cells, which was correlated with resolution of acute disease (261). In contrast to these results, we found that IL-21 had modest effects on T-cell polyfunctionality (Fig. 21 and Fig. 24), and IFN-α was generally ineffective at enhancing T-cell proliferation and function (Fig.

17 and Fig. 18). This discrepancy could reflect differences in disease setting, the differentiation state of T cells, or other factors.

It is intriguing that all five patients showed strikingly similar polyfunctional T-cell profiles in both the presence and absence of cytokine stimulation (Fig. 23). Notably, two of the four main functional permutations induced in the absence of cytokine stimulation (monofunctional CD107a, and bi-functional CD107a and CCL4 expressing CD8+ T cells) are elevated in HIV progressors compared to non-progressors, suggesting that these permutations may represent functionally exhausted T cells (250, 251). Notably, the cytokine combination of IL-2 + IL-12 + IL-18 significantly decreased the number of CD8+ T cells exhibiting these "exhausted" functional permutations and promoted the emergence of three new polyfunctional permutations: a) CCL4 and IFN-γ; b) CCL4, IFN-γ, and TNF-α; and c) CCL4, IFN-γ, TNF-α, and CD107a (Fig. 23). While the relevance of these functional permutations in EOC remains unknown, they are associated with protective immunity in other disease settings. For example, a protective vaccine against smallpox induced CD8+ T cells with these three polyfunctional permutations (268). In HIV/AIDS, tetra-functional (CCL4, IFN-γ, TNF-α, and CD107a) CD8+ T cells are found at significantly higher frequencies in HIV long-term non-progressors compared to progressors (250, 251). Moreover, CD8+ T cells simultaneously expressing both IFN-γ and TNF-α display enhanced cytotoxicity over cells expressing IFN-γ alone, and cells expressing both these cytokines preferentially kill HIV-infected cells (250, 260). Finally, a higher proportion of tumor-reactive T cells co-expressing CCL4, IFN-γ, and TNF-α were detected in melanoma patients with favorable clinical responses to α-CTLA-4

treatment (265). Thus, the polyfunctional permutations induced by the cytokine combination of IL-2 + IL-12 + IL-18 may have therapeutic value.

Our findings shed new light on previous clinical trials in EOC involving single agent IL-2 or IL-12. Intraperitoneal injection of IL-2 alone resulted in an approximately 25 % overall response rate in two separate studies (78, 79), while IL-2 treatment in combination with retinoic acid showed a significantly prolonged progression free- and overall survival (281). By contrast, IL-12 showed little therapeutic efficacy in EOC (282, 283). In the present study, it is intriguing that IL-2 was generally more effective than IL-12 at enhancing T-cell proliferation and polyfunctionality. Trials with IFN-α (284) and IFN-γ (104, 105) have met with mixed results, but for the most part, major increases in overall survival were not seen. The negative clinical trial results with IFN-α could reflect the dual role of this cytokine in regulating T-cell proliferation; in some contexts IFN-α can promote T-cell expansion (285), while our data (Fig. 17) and that from prior studies (286, 287), show that IFN-α can also inhibit T-cell proliferation.

Although the combination of IL-2 + IL-12 + IL-18 was most potent at enhancing T-cell proliferation and polyfunctionality, this combination of cytokines is predicted to have serious side effects if given systemically to patients (107, 288, 289). By optimizing dose and route of delivery, it may be possible to achieve therapeutic efficacy with acceptable toxicity (107, 290). Alternatively, it may be possible to use this cytokine combination *in vitro* to 'imprint' tumor-reactive T cells with specific polyfunctional phenotypes prior to adoptive immunotherapy. Yet another possibility is to engineer T cells to express chimeric cytokine receptors that generate essential signals from the IL-2,

IL-12, and IL-18 receptors but are triggered by well-tolerated cytokines, as we have demonstrated with chimeric GM-CSF/IL-2 receptors (291, 292).

4.6 Acknowledgements

We thank the patients who generously donated specimens; Dr. Peter Watson, Jodi LeBlanc, Rebecca Barnes, Sindy Babinszky, and Kristy Dillon for specimen and clinical data collection; Dr. Julian Lum, David McPhee, Dr. Cecile LePage, Rob Sahota, and Katy Milne for insightful comments; and Dr. Mario Roederer for kindly providing the PESTLE v1.6.1 software.

Chapter 5: Concluding Remarks

Bennett and Beeson gave birth to the cytokine field in the 1950s, when they described a fever-inducing agent present in lymphocyte supernatants and extracts (6). The field has come a long way since then. Several hundred cytokines have now been identified (3), each with a unique, but sometimes overlapping, set of functions that promote both protective and pathological immune responses. Our mechanistic understanding of how cytokines "work" at the molecular level has also grown tremendously since the first description of JAKs and STATs approximately (only) 20 years ago.

5.1 Summary

The biochemical mechanisms by which IL-2 induces T-cell proliferation remains an active area of research. Prior to this thesis work, there was a paradox in the IL-2 signaling field. On the one hand, receptor mutagenesis studies provided evidence for a redundant role for the Shc and STAT5 pathways for inducing proliferation. On the other hand, T cells deficient of STAT5 were completely non-proliferative upon IL-2 stimulation, suggesting that the Shc pathway is unable to induce proliferation in the absence of STAT5. Work done in Chapter 2 of this thesis resolved this paradox by demonstrating that a prior receptor mutant thought to activate the Shc pathway alone in fact activated a low, but functionally significant level of STAT5. Using a more stringent receptor mutant that activated a more "pure" Shc signal, we were able to demonstrate that a Shc signal alone was unable to significantly promote lymphocyte proliferation. This

lack of proliferation was correlated with an inability of the Shc pathway to sustain activation of the Akt/p70S6K pathway. Addition of STAT5 signaling to the Shc signal rescued activation of the Akt/p70S6K pathway and lymphocyte proliferation. Thus, the paradox was solved: STAT5 is essential for IL-2 induced lymphocyte proliferation, and this operates in part by enhancing Shc signaling.

The resolution of this paradox brought about another question: how does STAT5 enhance Shc signaling to promote proliferation? Although the answer to this question remains elusive, work done in Chapter 3 provided some insight. First, we found that there was a moderate level of temporal plasticity for proliferation mediated by the Shc and STAT5 pathways, in that proliferation was not significantly affected as long as both pathways were activated within ~3 h of each other. Since maximal lymphocyte proliferation occurs only when both Shc and STAT5 are activated, we searched for and identified novel genes that were either cooperatively up-regulated or down-regulated upon co-activation of Shc and STAT5, reasoning that genes important for proliferation would be found in these categories. We found that Shc and STAT5 cooperate to up-regulate genes, such as *hbegf*, *id-1*, and *lrp12*, which have been associated with proliferation, but have not yet been reported to be regulated by IL-2. We also found that Shc and STAT5 cooperate to repress genes such as *bbc3*, *mxd4*, *pik3ip1*, and *klhl24*, which may be involved in negatively regulating viability and proliferation. Current efforts in our lab will help determine the functional significance of some of these genes in proliferative signaling by the IL-2R.

The work described in Chapter 4 was more translational in nature and explored important aspects of cytokine therapy for ovarian cancer. Since the immunosuppressive tumor microenvironment represents a major barrier for immunotherapies, we wanted to answer one main question: can a cytokine or combination of cytokines promote effective T-cell responses even in the face of the immunosuppressive tumor environment? We found that the tumor environment had highly variable effects on T-cell proliferation, while it generally inhibited polyfunctional T-cell responses. However, the combination of IL-2 + IL-12 + IL-18 overcame immunosuppression to potently enhance both T-cell proliferation and polyfunctionality in all patient samples tested. Thus, given the right cytokine signals, T cells within a "hostile" immunosuppressive tumor environment can be induced to proliferate and become polyfunctional effectors. From a clinical perspective, these results provide a rationale for pursuing the use of this cytokine combination for cancer therapy. Alternatively, to bypass the significant toxicities associated with systemic cytokine therapy, it may be possible to pre-program T cells with this combination *in vitro* prior to adoptive T cell therapy, or genetically engineer T cells with chimeric receptors that deliver essential signals from these cytokines, but that are under the control of a less toxic cytokine.

5.2 Perspectives and future directions

There is extreme complexity to cytokine signaling networks. Fully characterizing and evaluating the functional significance of the signaling pathways and gene expression programs activated by even a single cytokine such as IL-2 remains a formidable challenge. As described in this thesis, we used both microarray and QPCR methods to

identify a large number of genes associated with IL-2 mediated lymphocyte proliferation. However, the list of genes was relatively large and functionally evaluating each gene for its role in proliferation (using knock-down and over-expression techniques) would require substantial resources. In future, several strategies could be pursued to narrow down the number of genes to a manageable size for functional evaluation. First, the gene expression signature of different T-cell lines and primary T cells stimulated with IL-2 could be assessed. If a gene really is important for IL-2 induced proliferation, then its expression pattern should be consistent among different types of T cells. Using primary T cells is important since, in the end, these are the cells that are physiologically relevant. Second, since proteins ultimately carry out the functions of a cell, future efforts could be directed toward evaluating the protein expression signature upon IL-2 stimulation (using, for example, mass spectrometry or protein array techniques, or Western blotting if antibodies are available). Finally, phospho-kinase arrays could be used on protein lysates from our dual receptor system (as described in Chapters 2 and 3). Since the rescue of the PI3K/AKT pathway and lymphocyte proliferation only occurs when both Shc and STAT5 are activated, then evaluating the phosphorylation state of a large number of kinases when either Shc or STAT5 are activated (poor proliferation) compared to when both are activated (normal proliferation) may provide important clues into how the Shc and STAT5 pathways integrate signals to mediate lymphocyte proliferation.

In the end, identifying the mechanisms by which IL-2 induces cell proliferation is not only rewarding from a basic science standpoint, since this information will provide us with a deeper understanding of how cytokines work in general, but may also be clinically

relevant, since mitogenic pathways are dysregulated in a many cancers. Thus, understanding how these proliferative pathways work at the molecular level may allow for the development of therapies that target key molecules involved in tumor cell proliferation. However, it should be noted that most, if not all, cell signaling studies are carried out in relatively controlled settings *in vitro.* The complexity of cytokine signaling increases exponentially in physiological settings, where a given lymphocyte is often exposed to a large number of cytokines (and other factors) at varying concentrations, each activating different, but potentially redundant, pathways to different extents. Coupled to this are signals delivered through other cell surface receptors, such as antigen, co-stimulatory and adhesion receptors. The differentiation state of a cell may also influence its sensitivity to cytokines. Thus, an important question comes to mind: how relevant is it to study cytokine and cytokine signaling in artificial, controlled *in vitro* settings? From a basic biology standpoint, it is absolutely critical to study the mechanisms of cytokine signaling in controlled *in vitro* settings, and indeed, this is how the very foundation of all cytokine signaling has been built. However, by staying *in vitro*, we may miss biologically relevant signaling pathway interactions that can only be observed in physiological settings. Thus, greater insight into how cytokines work may be gained from developing techniques and strategies that allow for the study cytokine signaling *in vivo*.

The past 30 years has taught us many lessons in the use of cytokines for treating human cancers. Perhaps most importantly, history has shown that despite extremely promising pre-clinical data, single agent cytokine therapy in humans has largely been

ineffective. Thus, the full potential of cytokines in cancer therapy will likely be realized only when used in combination with other cytokines or therapies. Indeed, Chapter 4 of this thesis demonstrated that combinations of cytokines were superior to single agent cytokines for enhancing T-cell responses in the tumor environment, suggesting that delivering combinations of cytokines to patients may lead to improved outcomes. Thus, future efforts should be directed toward assessing the effectiveness of cytokine combinations, such as the one described in Chapter 4, in *in vivo* tumor settings. Given the toxic nature of cytokines at high doses, careful consideration should be placed on dosage and location of cytokine delivery. Cytokines could also be combined with some forms of chemotherapy. Since some chemotherapeutic regimens elicit anti-tumor immune responses, exogenously delivered cytokines could theoretically help promote the anti-tumor immune responses induced by chemotherapy.

Cytokines also hold great promise for improving the adoptive T-cell therapy (ACT) of cancer. An important parameter for the successful ACT of cancer is generating the "right" type of T cells, and this is predominantly influenced by cytokines. For example, IL-2 has been the gold standard cytokine for the *in vitro* expansion of T cells for ACT in humans. However, long term culture of T cells with IL-2 leads to significant telomere shortening and the generation of "exhausted" T cells that do not persist or function well upon re-infusion into cancer patients. To some degree, this obstacle can be overcome by shortening the *in vitro* culture time of T cells, and indeed, it is now clear that younger (less differentiated) T cells are more effective at mediating tumor regression. Alternatively, other cytokines such as IL-15, which unlike IL-2 does not

promote activation induced cell death (AICD), could be assessed for the ability to generate young and/or more functional T cells. And perhaps one could also use another cytokine or cytokine combination to "program" these T cells into more potent tumor killers. Presently, it is unknown which cytokine or cytokine combination is optimal for expanding and "programming" anti-tumor T cells toward a protective phenotype. However, as demonstrated in Chapter 4, the cytokine combination of IL-2, IL-12, and IL-18 potently induced polyfunctional T-cell responses within the tumor environment, which provides hope that this combination could be used therapeutically, or as a "programming" cocktail to imprint T cells prior to adoptive T cell therapy. The true utility of this cytokine combination in these settings will have to be demonstrated in more pre-clinical models before entering the clinic. Ultimately, identifying the optimal cytokines for expanding and programming anti-tumor T cells could translate to improved outcomes for patients undergoing ACT.

The age of the gene modified T cell is upon us. It is now possible to genetically engineer T cells *in vitro* and then infuse them into cancer patients for therapeutic benefit. T cells engineered to express tumor-antigen specific T-cell receptors, chimeric antigen receptors (which are antibody-T-cell receptor chimeras), and even cytokines such as IL-2, have made it to human clinical trials. One promising gene modification that has not yet made it to clinical trials is the chimeric cytokine receptor, which is composed of a desired extracellular cytokine receptor domain fused to desired intracellular signaling modules. The extracellular domain could be a cytokine receptor whose ligand is non-toxic (e.g. G-CSF), or a receptor that binds to a tumor-derived factor (e.g. VEGF), which would

theoretically allow for controlled receptor activation. The intracellular domains could be signaling pathways that induce T-cell proliferation and/or activation. This strategy would by-pass the systemic toxicities associated with many cytokines such as IL-2, as control of T-cell activation would be under a non-toxic cytokine, or locally produced tumor-derived factor. The main question that has to be answered now is: "which signaling pathways are required for eliciting the strongest anti-tumor T-cell response?" Once answered, a chimeric cytokine receptor incorporating these signaling modules could be introduced into anti-tumor T cells, infused into cancer patients, and then activated with the appropriate cytokine to become potent effector T cells which subsequently destroy the tumor.

The large number of immunosuppressive factors found in the ovarian cancer environment presents a major challenge for immunotherapy. However, rational design of new, multi-pronged therapies involving cytokines could overcome immunosuppression and may drastically improve patient outcomes. For example, weeks prior to surgery, peripheral blood mononuclear cells (PBMCs) could be harvested from an ovarian cancer patient and frozen down for later genetic modification. The ovarian cancer patient would then undergo neo-adjuvant chemotherapy with a combination of taxanes, platinum-based agents, and/or anthracyclines, which would: 1) decrease tumor burden; and 2) stimulate the immune system and enhance T-cell function and infiltration into tumors. Cytoreductive surgery would yield tumor-infiltrating lymphocytes (TIL) containing tumor-reactive T cells, which could be genetically engineered with chimeric cytokine receptors containing an extracellular G-CSFR domain and the intracellular signaling

domains from the IL-12 and IL-18 receptors, and then expanded *in vitro* using IL-15, instead of IL-2 (to prevent AICD and telomere shortening). Concurrently, naive T cells from the PBMC compartment could be genetically engineered with antigen receptors specific for a tumor antigen commonly overexpressed by ovarian cancer, such as mucin-1 and folate-binding protein. Since these genetically modified T cells are relatively naïve (have not yet seen antigen), they could then be properly programmed and expanded to become polyfunctional effectors *in vitro* with the cytokine combination of IL-2, IL-12, and IL-18. The preparation of two different T-cell populations (one from PBMC and the other from TIL) serves two important functions. First, TIL currently can not be expanded from all tumor samples, and thus preparing two different T-cell products ensures that at least one can be used for ACT. Second, in the ideal case where both T-cell preparations are generated, combining these two populations for ACT could increase the repertoire of tumor-reactive T cells, and thus increase the likelihood of tumor eradication. For example, although a large number of (potentially uncharacterized) tumor antigens could be recognized by expanded TIL, many tumor antigens are overexpressed self antigens, and so functional T cells specific for these antigens may not exist in the patient (i.e., are deleted during thymic selection, or are not of high enough avidity). Thus, use of T cells genetically engineered with highly avid antigen receptors specific for these self antigens would compliment the expanded TIL to target a larger number of tumor antigens.

After recovery from surgery, the ovarian cancer patient could undergo standard lymphodepleting chemotherapy, followed by ACT of the two different genetically engineered T-cell products. Chemotherapy-induced lymphodepletion would decrease

both the number of immunosuppressive cells (e.g. Tregs) in the tumor environment, and the number of endogenous lymphocytes that would compete with the adoptively transferred T cells for limited amounts of pro-survival homeostatic cytokines. Administration of G-CSF would enhance the anti-tumor activity of T cells engineered with the chimeric receptor, while at the same time treat the neutropenia associated with chemotherapy. The T cells genetically engineered with tumor antigen receptors would likely be potent anti-tumor effectors in the absence of exogenous cytokine stimulation, since proper programming of T cells *in vitro* can obviate the need for *in vivo* cytokine support. Although relatively complex, this multi-pronged therapy overrides many of the immunosuppressive mechanisms employed by ovarian cancer, which hopefully would translate into greatly improved outcomes for this devastating disease.

In summary, cytokines have earned a place in modern-day cancer immunotherapy. However, it is clear that the overall effectiveness of unimodal cytokine therapy is low in the majority of cancer patients. Thus, the future of cytokine therapy lies primarily in combining cytokines with other cytokines and standard- and immuno-therapies; identifying the optimal cytokines for expanding and programming T cells; and genetically engineering T cells with chimeric cytokine receptors. Together, these cytokine strategies hold great promise for improving the lives of cancer patients.

Bibliography

1. Feldmann, M. 2008. Many cytokines are very useful therapeutic targets in disease. *J Clin Invest* 118:3533-3536.

2. Oppenheim, J. J. 2001. Cytokines: past, present, and future. *Int J Hematol* 74:3-8.

3. Vilcek, J., and M. Feldmann. 2004. Historical review: Cytokines as therapeutics and targets of therapeutics. *Trends Pharmacol Sci* 25:201-209.

4. Margolin, K. 2008. Cytokine therapy in cancer. *Expert Opin Biol Ther* 8:1495-1505.

5. Donnelly, R. P., H. A. Young, and A. S. Rosenberg. 2009. An overview of cytokines and cytokine antagonists as therapeutic agents. *Ann N Y Acad Sci* 1182:1-13.

6. Bennett, I. L., Jr., and P. B. Beeson. 1953. Studies on the pathogenesis of fever. II. Characterization of fever-producing substances from polymorphonuclear leukocytes and from the fluid of sterile exudates. *J Exp Med* 98:493-508.

7. Levi-Montalcini, R. a. H., V. 1953. A diffusible agent of mouse sarcoma producing hyperplasia of sympathetic ganglia and hyperneurotization of viscera in the chick embryo. *J. Exp. Zool.* 123:223-288.

8. Isaacs, A., and J. Lindenmann. 1987. Virus interference. I. The interferon. By A. Isaacs and J. Lindenmann, 1957. *J Interferon Res* 7:429-438.

9. Smith, K. A., and J. D. Griffin. 2008. Following the cytokine signaling pathway to leukemogenesis: a chronology. *J Clin Invest* 118:3564-3573.

10. Nowell, P. C. 1960. Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes. *Cancer Res* 20:462-466.

11. Hirschhorn, K., F. Bach, R. L. Kolodny, I. L. Firschein, and N. Hashem. 1963. Immune Response and Mitosis of Human Peripheral Blood Lymphocytes in Vitro. *Science* 142:1185-1187.

12. Bach, F., and K. Hirschhorn. 1964. Lymphocyte Interaction: A Potential Histocompatibility Test in Vitro. *Science* 143:813-814.

13. Bain, B., and L. Lowenstein. 1964. Genetic Studies on the Mixed Leukocyte Reaction. *Science* 145:1315-1316.

14. Pearmain, G., R. R. Lycette, and P. H. Fitzgerald. 1963. Tuberculin-induced mitosis in peripheral blood leucocytes. *Lancet* 1:637-638.

15. Kasakura, S., and L. Lowenstein. 1965. A factor stimulating DNA synthesis derived from the medium of leukocyte cultures. *Nature* 208:794-795.

16. Gordon, J., and L. D. MacLean. 1965. A lymphocyte-stimulating factor produced in vitro. *Nature* 208:795-796.

17. Dumonde, D. C., R. A. Wolstencroft, G. S. Panayi, M. Matthew, J. Morley, and W. T. Howson. 1969. "Lymphokines": non-antibody mediators of cellular immunity generated by lymphocyte activation. *Nature* 224:38-42.

18. David, J. R. 1966. Delayed hypersensitivity in vitro: its mediation by cell-free substances formed by lymphoid cell-antigen interaction. *Proc Natl Acad Sci U S A* 56:72-77.

19. Bloom, B. R., and B. Bennett. 1966. Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. *Science* 153:80-82.

20. Ruddle, N. H., and B. H. Waksman. 1967. Cytotoxic effect of lymphocyte-antigen interaction in delayed hypersensitivity. *Science* 157:1060-1062.

21. Granger, G. A., and T. W. Williams. 1968. Lymphocyte cytotoxicity in vitro: activation and release of a cytotoxic factor. *Nature* 218:1253-1254.

22. Nathan, C. F., M. L. Karnovsky, and J. R. David. 1971. Alterations of macrophage functions by mediators from lymphocytes. *J Exp Med* 133:1356-1376.

23. Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci U S A* 72:3666-3670.

24. Gery, I., R. K. Gershon, and B. H. Waksman. 1971. Potentiation of cultured mouse thymocyte responses by factors released by peripheral leucocytes. *J Immunol* 107:1778-1780.

25. Morgan, D. A., F. W. Ruscetti, and R. Gallo. 1976. Selective in vitro growth of T lymphocytes from normal human bone marrows. 193:1007-1008.

26. Gillis, S., and K. A. Smith. 1977. Long term culture of tumour-specific cytotoxic T cells. *Nature* 268:154-156.

27. Baker, P. E., S. Gillis, and K. A. Smith. 1979. Monoclonal cytolytic T-cell lines. *J Exp Med* 149:273-278.

28. Robb, R. J., A. Munck, and K. A. Smith. 1981. T cell growth factor receptors. Quantitation, specificity, and biological relevance. 154:1455-1474.

29. Smith, K. A., K. J. Gilbride, and M. F. Favata. 1980. Lymphocyte activating factor promotes T-cell growth factor production by cloned murine lymphoma cells. *Nature* 287:853-855.

30. Smith, K. A., L. B. Lachman, J. J. Oppenheim, and M. F. Favata. 1980. The functional relationship of the interleukins. *J Exp Med* 151:1551-1556.

31. Taniguchi, T., S. Ohno, Y. Fujii-Kuriyama, and M. Muramatsu. 1980. The nucleotide sequence of human fibroblast interferon cDNA. *Gene* 10:11-15.

32. Nagata, S., H. Taira, A. Hall, L. Johnsrud, M. Streuli, J. Ecsodi, W. Boll, K. Cantell, and C. Weissmann. 1980. Synthesis in E. coli of a polypeptide with human leukocyte interferon activity. *Nature* 284:316-320.

33. Gray, P. W., D. W. Leung, D. Pennica, E. Yelverton, R. Najarian, C. C. Simonsen, R. Derynck, P. J. Sherwood, D. M. Wallace, S. L. Berger, A. D. Levinson, and D. V. Goeddel. 1982. Expression of human immune interferon cDNA in E. coli and monkey cells. *Nature* 295:503-508.

34. Taniguchi, T., H. Matsui, T. Fujita, C. Takaoka, N. Kashima, R. Yoshimoto, and J. Hamuro. 1983. Structure and expression of a cloned cDNA for human interleukin-2. *Nature* 302:305-310.

35. Gray, P. W., B. B. Aggarwal, C. V. Benton, T. S. Bringman, W. J. Henzel, J. A. Jarrett, D. W. Leung, B. Moffat, P. Ng, L. P. Svedersky, and et al. 1984. Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. *Nature* 312:721-724.

36. Pennica, D., G. E. Nedwin, J. S. Hayflick, P. H. Seeburg, R. Derynck, M. A. Palladino, W. J. Kohr, B. B. Aggarwal, and D. V. Goeddel. 1984. Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature* 312:724-729.

37. Derynck, R., J. A. Jarrett, E. Y. Chen, D. H. Eaton, J. R. Bell, R. K. Assoian, A. B. Roberts, M. B. Sporn, and D. V. Goeddel. 1985. Human transforming growth

factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature* 316:701-705.

38. March, C. J., B. Mosley, A. Larsen, D. P. Cerretti, G. Braedt, V. Price, S. Gillis, C. S. Henney, S. R. Kronheim, K. Grabstein, and et al. 1985. Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature* 315:641-647.

39. Hirano, T., K. Yasukawa, H. Harada, T. Taga, Y. Watanabe, T. Matsuda, S. Kashiwamura, K. Nakajima, K. Koyama, A. Iwamatsu, and et al. 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* 324:73-76.

40. Leonard, W. J., J. M. Depper, G. R. Crabtree, S. Rudikoff, J. Pumphrey, R. J. Robb, M. Kronke, P. B. Svetlik, N. J. Peffer, and T. A. Waldmann. 1984. Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. 311:626-631.

41. Rosenberg, S. A., M. T. Lotze, L. M. Muul, S. Leitman, A. E. Chang, S. E. Ettinghausen, Y. L. Matory, J. M. Skibber, E. Shiloni, J. T. Vetto, and et al. 1985. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N Engl J Med* 313:1485-1492.

42. Lotze, M. T., A. E. Chang, C. A. Seipp, C. Simpson, J. T. Vetto, and S. A. Rosenberg. 1986. High-dose recombinant interleukin 2 in the treatment of patients with disseminated cancer. Responses, treatment-related morbidity, and histologic findings. *Jama* 256:3117-3124.

43. Rosenberg, S. A., J. C. Yang, S. L. Topalian, D. J. Schwartzentruber, J. S. Weber, D. R. Parkinson, C. A. Seipp, J. H. Einhorn, and D. E. White. 1994. Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. *Jama* 271:907-913.

44. Rosenberg, S. A. 2007. Interleukin 2 for patients with renal cancer. *Nat Clin Pract Oncol* 4:497.

45. Wilks, A. F. 1989. Two putative protein-tyrosine kinases identified by application of the polymerase chain reaction. *Proc Natl Acad Sci U S A* 86:1603-1607.

46. Wilks, A. F., A. G. Harpur, R. R. Kurban, S. J. Ralph, G. Zurcher, and A. Ziemiecki. 1991. Two novel protein-tyrosine kinases, each with a second phosphotransferase-related catalytic domain, define a new class of protein kinase. *Mol Cell Biol* 11:2057-2065.

47. Firmbach-Kraft, I., M. Byers, T. Shows, R. Dalla-Favera, and J. J. Krolewski. 1990. tyk2, prototype of a novel class of non-receptor tyrosine kinase genes. *Oncogene* 5:1329-1336.

48. Johnston, J. A., M. Kawamura, R. A. Kirken, Y. Q. Chen, T. B. Blake, K. Shibuya, J. R. Ortaldo, D. W. McVicar, and J. J. O'Shea. 1994. Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2. *Nature* 370:151-153.

49. Velazquez, L., M. Fellous, G. R. Stark, and S. Pellegrini. 1992. A protein tyrosine kinase in the interferon alpha/beta signaling pathway. *Cell* 70:313-322.

50. Witthuhn, B. A., F. W. Quelle, O. Silvennoinen, T. Yi, B. Tang, O. Miura, and J. N. Ihle. 1993. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell* 74:227-236.

51. Argetsinger, L. S., G. S. Campbell, X. Yang, B. A. Witthuhn, O. Silvennoinen, J. N. Ihle, and C. Carter-Su. 1993. Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. *Cell* 74:237-244.

52. Watling, D., D. Guschin, M. Muller, O. Silvennoinen, B. A. Witthuhn, F. W. Quelle, N. C. Rogers, C. Schindler, G. R. Stark, J. N. Ihle, and et al. 1993. Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-gamma signal transduction pathway. *Nature* 366:166-170.

53. Muller, M., J. Briscoe, C. Laxton, D. Guschin, A. Ziemiecki, O. Silvennoinen, A. G. Harpur, G. Barbieri, B. A. Witthuhn, C. Schindler, and et al. 1993. The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction. *Nature* 366:129-135.

54. Silvennoinen, O., B. A. Witthuhn, F. W. Quelle, J. L. Cleveland, T. Yi, and J. N. Ihle. 1993. Structure of the murine Jak2 protein-tyrosine kinase and its role in interleukin 3 signal transduction. *Proc Natl Acad Sci U S A* 90:8429-8433.

55. Schindler, C., K. Shuai, V. R. Prezioso, and J. E. Darnell, Jr. 1992. Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science* 257:809-813.

56. Fu, X. Y., D. S. Kessler, S. A. Veals, D. E. Levy, and J. E. Darnell, Jr. 1990. ISGF3, the transcriptional activator induced by interferon alpha, consists of multiple interacting polypeptide chains. *Proc Natl Acad Sci U S A* 87:8555-8559.

57. Schindler, C., X. Y. Fu, T. Improta, R. Aebersold, and J. E. Darnell, Jr. 1992. Proteins of transcription factor ISGF-3: one gene encodes the 91-and 84-kDa ISGF-3 proteins that are activated by interferon alpha. *Proc Natl Acad Sci U S A* 89:7836-7839.

58. Fu, X. Y., C. Schindler, T. Improta, R. Aebersold, and J. E. Darnell, Jr. 1992. The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in signal transduction. *Proc Natl Acad Sci U S A* 89:7840-7843.

59. Zhong, Z., Z. Wen, and J. E. Darnell, Jr. 1994. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* 264:95-98.

60. Zhong, Z., Z. Wen, and J. E. Darnell, Jr. 1994. Stat3 and Stat4: members of the family of signal transducers and activators of transcription. *Proc Natl Acad Sci U S A* 91:4806-4810.

61. Wakao, H., F. Gouilleux, and B. Groner. 1994. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. *Embo J* 13:2182-2191.

62. Hou, J., U. Schindler, W. J. Henzel, T. C. Ho, M. Brasseur, and S. L. McKnight. 1994. An interleukin-4-induced transcription factor: IL-4 Stat. *Science* 265:1701-1706.

63. Ratsimandresey, R. A., J. Rappaport, and J. F. Zagury. 2009. Anti-cytokine therapeutics: history and update. *Curr Pharm Des* 15:1998-2025.

64. Taylor, P. C., and M. Feldmann. 2009. Anti-TNF biologic agents: still the therapy of choice for rheumatoid arthritis. *Nat Rev Rheumatol* 5:578-582.

65. Tayal, V., and B. S. Kalra. 2008. Cytokines and anti-cytokines as therapeutics--an update. *Eur J Pharmacol* 579:1-12.

66. Brennan, F. M., and I. B. McInnes. 2008. Evidence that cytokines play a role in rheumatoid arthritis. *J Clin Invest* 118:3537-3545.

67. Malek, T. R., and A. L. Bayer. 2004. Tolerance, not immunity, crucially depends on IL-2. *Nat Rev Immunol* 4:665-674.

68. Nelson, B. H. 2004. IL-2, regulatory T cells, and tolerance. *J Immunol* 172:3983-3988.

69. Malek, T. R., A. Yu, V. Vincek, P. Scibelli, and L. Kong. 2002. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. *Immunity* 17:167-178.

70. Zou, W. 2006. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 6:295-307.

71. Cesana, G. C., G. DeRaffele, S. Cohen, D. Moroziewicz, J. Mitcham, J. Stoutenburg, K. Cheung, C. Hesdorffer, S. Kim-Schulze, and H. L. Kaufman. 2006. Characterization of CD4+CD25+ regulatory T cells in patients treated with high-dose interleukin-2 for metastatic melanoma or renal cell carcinoma. *J Clin Oncol* 24:1169-1177.

72. Schindler, C., D. E. Levy, and T. Decker. 2007. JAK-STAT signaling: from interferons to cytokines. *J Biol Chem* 282:20059-20063.

73. Schindler, C. W. 2002. Series introduction. JAK-STAT signaling in human disease. *J Clin Invest* 109:1133-1137.

74. Shuai, K., and B. Liu. 2003. Regulation of JAK-STAT signalling in the immune system. *Nat Rev Immunol* 3:900-911.

75. Reich, N. C., and L. Liu. 2006. Tracking STAT nuclear traffic. *Nat Rev Immunol* 6:602-612.

76. O'Shea, J. J., M. Gadina, and R. D. Schreiber. 2002. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell* 109 Suppl:S121-131.

77. Nelson, B. H., and D. M. Willerford. 1998. Biology of the interleukin-2 receptor. *Adv Immunol* 70:1-81.

78. Edwards, R. P., W. Gooding, B. C. Lembersky, K. Colonello, R. Hammond, C. Paradise, C. D. Kowal, A. J. Kunschner, M. Baldisseri, J. M. Kirkwood, and R. B. Herberman. 1997. Comparison of toxicity and survival following intraperitoneal recombinant interleukin-2 for persistent ovarian cancer after platinum: twenty-four-hour versus 7-day infusion. *J Clin Oncol* 15:3399-3407.

79. Vlad, A. M., R. A. Budiu, D. E. Lenzner, Y. Wang, J. A. Thaller, K. Colonello, P. A. Crowley-Nowick, J. L. Kelley, F. V. Price, and R. P. Edwards. 2010. A phase II trial of intraperitoneal interleukin-2 in patients with platinum-resistant or platinum-refractory ovarian cancer. *Cancer Immunol Immunother* 59:293-301.

80. Capitini, C. M., T. J. Fry, and C. L. Mackall. 2009. Cytokines as Adjuvants for Vaccine and Cellular Therapies for Cancer. *Am J Immunol* 5:65-83.

81. Sabatino, M., S. Kim-Schulze, M. C. Panelli, D. Stroncek, E. Wang, B. Taback, D. W. Kim, G. Deraffele, Z. Pos, F. M. Marincola, and H. L. Kaufman. 2009. Serum vascular endothelial growth factor and fibronectin predict clinical response to high-dose interleukin-2 therapy. *J Clin Oncol* 27:2645-2652.

82. Atkins, M., M. Regan, D. McDermott, J. Mier, E. Stanbridge, A. Youmans, P. Febbo, M. Upton, M. Lechpammer, and S. Signoretti. 2005. Carbonic anhydrase IX expression predicts outcome of interleukin 2 therapy for renal cancer. *Clin Cancer Res* 11:3714-3721.

83. Yang, J. C., S. L. Topalian, D. J. Schwartzentruber, D. R. Parkinson, F. M. Marincola, J. S. Weber, C. A. Seipp, D. E. White, and S. A. Rosenberg. 1995. The use of polyethylene glycol-modified interleukin-2 (PEG-IL-2) in the treatment of patients with metastatic renal cell carcinoma and melanoma. A phase I study and a randomized prospective study comparing IL-2 alone versus IL-2 combined with PEG-IL-2. *Cancer* 76:687-694.

84. Shanafelt, A. B., Y. Lin, M. C. Shanafelt, C. P. Forte, N. Dubois-Stringfellow, C. Carter, J. A. Gibbons, S. L. Cheng, K. A. Delaria, R. Fleischer, J. M. Greve, R. Gundel, K. Harris, R. Kelly, B. Koh, Y. Li, L. Lantz, P. Mak, L. Neyer, M. J. Plym, S. Roczniak, D. Serban, J. Thrift, L. Tsuchiyama, M. Wetzel, M. Wong, and A. Zolotorev. 2000. A T-cell-selective interleukin 2 mutein exhibits potent antitumor activity and is well tolerated in vivo. *Nat Biotechnol* 18:1197-1202.

85. Lode, H. N., R. Xiang, J. C. Becker, S. D. Gillies, and R. A. Reisfeld. 1998. Immunocytokines: a promising approach to cancer immunotherapy. *Pharmacol Ther* 80:277-292.

86. Boyman, O., M. Kovar, M. P. Rubinstein, C. D. Surh, and J. Sprent. 2006. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science* 311:1924-1927.

87. Sportes, C., R. E. Gress, and C. L. Mackall. 2009. Perspective on potential clinical applications of recombinant human interleukin-7. *Ann N Y Acad Sci* 1182:28-38.

88. Pellegrini, M., T. Calzascia, A. R. Elford, A. Shahinian, A. E. Lin, D. Dissanayake, S. Dhanji, L. T. Nguyen, M. A. Gronski, M. Morre, B. Assouline, K. Lahl, T. Sparwasser, P. S. Ohashi, and T. W. Mak. 2009. Adjuvant IL-7 antagonizes multiple cellular and molecular inhibitory networks to enhance immunotherapies. *Nat Med* 15:528-536.

89. Sportes, C., R. R. Babb, M. C. Krumlauf, F. T. Hakim, S. M. Steinberg, C. K. Chow, M. R. Brown, T. A. Fleisher, P. Noel, I. Maric, M. Stetler-Stevenson, J. Engel, R. Buffet, M. Morre, R. J. Amato, A. Pecora, C. L. Mackall, and R. E. Gress. Phase I study of recombinant human interleukin-7 administration in subjects with refractory malignancy. *Clin Cancer Res* 16:727-735.

90. Sportes, C., F. T. Hakim, S. A. Memon, H. Zhang, K. S. Chua, M. R. Brown, T. A. Fleisher, M. C. Krumlauf, R. R. Babb, C. K. Chow, T. J. Fry, J. Engels, R. Buffet, M. Morre, R. J. Amato, D. J. Venzon, R. Korngold, A. Pecora, R. E. Gress, and C. L. Mackall. 2008. Administration of rhIL-7 in humans increases in vivo TCR repertoire diversity by preferential expansion of naive T cell subsets. *J Exp Med* 205:1701-1714.

91. Rosenberg, S. A., C. Sportes, M. Ahmadzadeh, T. J. Fry, L. T. Ngo, S. L. Schwarz, M. Stetler-Stevenson, K. E. Morton, S. A. Mavroukakis, M. Morre, R. Buffet, C. L. Mackall, and R. E. Gress. 2006. IL-7 administration to humans leads to expansion of CD8+ and CD4+ cells but a relative decrease of CD4+ T-regulatory cells. *J Immunother* 29:313-319.

92. Waldmann, T. A. 2006. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol* 6:595-601.

93. Bhave, N. S., and W. E. Carson, 3rd. 2009. Immune modulation with interleukin-21. *Ann N Y Acad Sci* 1182:39-46.

94. Parrish-Novak, J., S. R. Dillon, A. Nelson, A. Hammond, C. Sprecher, J. A. Gross, J. Johnston, K. Madden, W. Xu, J. West, S. Schrader, S. Burkhead, M. Heipel, C. Brandt, J. L. Kuijper, J. Kramer, D. Conklin, S. R. Presnell, J. Berry, F. Shiota, S. Bort, K. Hambly, S. Mudri, C. Clegg, M. Moore, F. J. Grant, C. Lofton-Day, T. Gilbert, F. Rayond, A. Ching, L. Yao, D. Smith, P. Webster, T. Whitmore, M. Maurer, K. Kaushansky, R. D. Holly, and D. Foster. 2000. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature* 408:57-63.

95. Thompson, J. A., B. D. Curti, B. G. Redman, S. Bhatia, J. S. Weber, S. S. Agarwala, E. L. Sievers, S. D. Hughes, T. A. DeVries, and D. F. Hausman. 2008. Phase I study of recombinant interleukin-21 in patients with metastatic melanoma and renal cell carcinoma. *J Clin Oncol* 26:2034-2039.

96. Davis, I. D., B. K. Skrumsager, J. Cebon, T. Nicholaou, J. W. Barlow, N. P. Moller, K. Skak, D. Lundsgaard, K. S. Frederiksen, P. Thygesen, and G. A. McArthur. 2007. An open-label, two-arm, phase I trial of recombinant human

interleukin-21 in patients with metastatic melanoma. *Clin Cancer Res* 13:3630-3636.

97. Davis, I. D., B. Brady, R. F. Kefford, M. Millward, J. Cebon, B. K. Skrumsager, U. Mouritzen, L. T. Hansen, K. Skak, D. Lundsgaard, K. S. Frederiksen, P. E. Kristjansen, and G. McArthur. 2009. Clinical and biological efficacy of recombinant human interleukin-21 in patients with stage IV malignant melanoma without prior treatment: a phase IIa trial. *Clin Cancer Res* 15:2123-2129.

98. Borden, E. C. 2006. Interferons. In *Cancer Medicine 7*, 7th ed. D. W. Kufe, et al., ed. BC Decker. 733-743.

99. Borden, E. C., G. C. Sen, G. Uze, R. H. Silverman, R. M. Ransohoff, G. R. Foster, and G. R. Stark. 2007. Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov* 6:975-990.

100. Ikeda, H., L. J. Old, and R. D. Schreiber. 2002. The roles of IFN gamma in protection against tumor development and cancer immunoediting. *Cytokine Growth Factor Rev* 13:95-109.

101. Shustik, C. 1998. Interferon in the Treatment of Multiple Myeloma. *Cancer Control* 5:226-234.

102. Jonasch, E., and F. G. Haluska. 2001. Interferon in oncological practice: review of interferon biology, clinical applications, and toxicities. *Oncologist* 6:34-55.

103. Miller, C. H., S. G. Maher, and H. A. Young. 2009. Clinical Use of Interferon-gamma. *Ann N Y Acad Sci* 1182:69-79.

104. Windbichler, G. H., H. Hausmaninger, W. Stummvoll, A. H. Graf, C. Kainz, J. Lahodny, U. Denison, E. Muller-Holzner, and C. Marth. 2000. Interferon-gamma in the first-line therapy of ovarian cancer: a randomized phase III trial. *Br J Cancer* 82:1138-1144.

105. Alberts, D. S., C. Marth, R. D. Alvarez, G. Johnson, M. Bidzinski, D. R. Kardatzke, W. Z. Bradford, J. Loutit, D. H. Kirn, M. C. Clouser, and M. Markman. 2008. Randomized phase 3 trial of interferon gamma-1b plus standard carboplatin/paclitaxel versus carboplatin/paclitaxel alone for first-line treatment of advanced ovarian and primary peritoneal carcinomas: results from a prospectively designed analysis of progression-free survival. *Gynecol Oncol* 109:174-181.

106. Giannopoulos, A., C. Constantinides, E. Fokaeas, C. Stravodimos, M. Giannopoulou, A. Kyroudi, and A. Gounaris. 2003. The immunomodulating

effect of interferon-gamma intravesical instillations in preventing bladder cancer recurrence. *Clin Cancer Res* 9:5550-5558.

107. Weiss, J. M., J. J. Subleski, J. M. Wigginton, and R. H. Wiltrout. 2007. Immunotherapy of cancer by IL-12-based cytokine combinations. *Expert Opin Biol Ther* 7:1705-1721.

108. Del Vecchio, M., E. Bajetta, S. Canova, M. T. Lotze, A. Wesa, G. Parmiani, and A. Anichini. 2007. Interleukin-12: biological properties and clinical application. *Clin Cancer Res* 13:4677-4685.

109. Colombo, M. P., and G. Trinchieri. 2002. Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine Growth Factor Rev* 13:155-168.

110. Lacy, M. Q., S. Jacobus, E. A. Blood, N. E. Kay, S. V. Rajkumar, and P. R. Greipp. 2009. Phase II study of interleukin-12 for treatment of plateau phase multiple myeloma (E1A96): a trial of the Eastern Cooperative Oncology Group. *Leuk Res* 33:1485-1489.

111. Daud, A. I., R. C. DeConti, S. Andrews, P. Urbas, A. I. Riker, V. K. Sondak, P. N. Munster, D. M. Sullivan, K. E. Ugen, J. L. Messina, and R. Heller. 2008. Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma. *J Clin Oncol* 26:5896-5903.

112. Robertson, M. J., J. M. Kirkwood, T. F. Logan, K. M. Koch, S. Kathman, L. C. Kirby, W. N. Bell, L. M. Thurmond, J. Weisenbach, and M. M. Dar. 2008. A dose-escalation study of recombinant human interleukin-18 using two different schedules of administration in patients with cancer. *Clin Cancer Res* 14:3462-3469.

113. Robertson, M. J., J. W. Mier, T. Logan, M. Atkins, H. Koon, K. M. Koch, S. Kathman, L. N. Pandite, C. Oei, L. C. Kirby, R. C. Jewell, W. N. Bell, L. M. Thurmond, J. Weisenbach, S. Roberts, and M. M. Dar. 2006. Clinical and biological effects of recombinant human interleukin-18 administered by intravenous infusion to patients with advanced cancer. *Clin Cancer Res* 12:4265-4273.

114. Tarhini, A. A., M. Millward, P. Mainwaring, R. Kefford, T. Logan, A. Pavlick, S. J. Kathman, K. H. Laubscher, M. M. Dar, and J. M. Kirkwood. 2009. A phase 2, randomized study of SB-485232, rhIL-18, in patients with previously untreated metastatic melanoma. *Cancer* 115:859-868.

115. Anaya, J. P., and J. J. Sias. 2005. The use of interleukin-2 in human immunodeficiency virus infection. *Pharmacotherapy* 25:86-95.

116. Eklund, J. W., and T. M. Kuzel. 2005. Interleukin-2 in the treatment of renal cell carcinoma and malignant melanoma. *Cancer Treat Res* 126:263-287.

117. Smith, K. A. 1988. Interleukin-2: inception, impact, and implications. *Science* 240:1169-1176.

118. Leonard, W. J., and J. J. O'Shea. 1998. Jaks and STATs: biological implications. *Annu Rev Immunol* 16:293-322.

119. Friedmann, M. C., T. S. Migone, S. M. Russell, and W. J. Leonard. 1996. Different interleukin 2 receptor beta-chain tyrosines couple to at least two signaling pathways and synergistically mediate interleukin 2-induced proliferation. *Proc Natl Acad Sci U S A* 93:2077-2082.

120. Gaffen, S. L., S. Y. Lai, M. Ha, X. Liu, L. Hennighausen, W. C. Greene, and M. A. Goldsmith. 1996. Distinct tyrosine residues within the interleukin-2 receptor beta chain drive signal transduction specificity, redundancy, and diversity. *J Biol Chem* 271:21381-21390.

121. Evans, G. A., M. A. Goldsmith, J. A. Johnston, W. Xu, S. R. Weiler, R. Erwin, O. M. Howard, R. T. Abraham, J. J. O'Shea, W. C. Greene, and et al. 1995. Analysis of interleukin-2-dependent signal transduction through the Shc/Grb2 adapter pathway. Interleukin-2-dependent mitogenesis does not require Shc phosphorylation or receptor association. *J Biol Chem* 270:28858-28863.

122. Ravichandran, K. S., and S. J. Burakoff. 1994. The adapter protein Shc interacts with the interleukin-2 (IL-2) receptor upon IL-2 stimulation. *J Biol Chem* 269:1599-1602.

123. Fujii, H., Y. Nakagawa, U. Schindler, A. Kawahara, H. Mori, F. Gouilleux, B. Groner, J. N. Ihle, Y. Minami, T. Miyazaki, and et al. 1995. Activation of Stat5 by interleukin 2 requires a carboxyl-terminal region of the interleukin 2 receptor beta chain but is not essential for the proliferative signal transmission. *Proc Natl Acad Sci U S A* 92:5482-5486.

124. Gaffen, S. L., S. Y. Lai, W. Xu, F. Gouilleux, B. Groner, M. A. Goldsmith, and W. C. Greene. 1995. Signaling through the interleukin 2 receptor beta chain activates a STAT-5-like DNA-binding activity. *Proc Natl Acad Sci U S A* 92:7192-7196.

125. Izquierdo Pastor, M., K. Reif, and D. Cantrell. 1995. The regulation and function of p21ras during T-cell activation and growth. *Immunol Today* 16:159-164.

126. Lord, J. D., B. C. McIntosh, P. D. Greenberg, and B. H. Nelson. 1998. The IL-2 receptor promotes proliferation, bcl-2 and bcl-x induction, but not cell viability through the adapter molecule Shc. *J Immunol* 161:4627-4633.

127. Gadina, M., C. Sudarshan, R. Visconti, Y. J. Zhou, H. Gu, B. G. Neel, and J. J. O'Shea. 2000. The docking molecule gab2 is induced by lymphocyte activation and is involved in signaling by interleukin-2 and interleukin-15 but not other common gamma chain-using cytokines. *J Biol Chem* 275:26959-26966.

128. Gu, H., H. Maeda, J. J. Moon, J. D. Lord, M. Yoakim, B. H. Nelson, and B. G. Neel. 2000. New role for Shc in activation of the phosphatidylinositol 3-kinase/Akt pathway. *Mol Cell Biol* 20:7109-7120.

129. Nishida, K., Y. Yoshida, M. Itoh, T. Fukada, T. Ohtani, T. Shirogane, T. Atsumi, M. Takahashi-Tezuka, K. Ishihara, M. Hibi, and T. Hirano. 1999. Gab-family adapter proteins act downstream of cytokine and growth factor receptors and T- and B-cell antigen receptors. *Blood* 93:1809-1816.

130. Bader, A. G., S. Kang, L. Zhao, and P. K. Vogt. 2005. Oncogenic PI3K deregulates transcription and translation. *Nat Rev Cancer* 5:921-929.

131. Luo, J., B. D. Manning, and L. C. Cantley. 2003. Targeting the PI3K-Akt pathway in human cancer: rationale and promise. *Cancer Cell* 4:257-262.

132. Cantley, L. C. 2002. The phosphoinositide 3-kinase pathway. *Science* 296:1655-1657.

133. Cully, M., H. You, A. J. Levine, and T. W. Mak. 2006. Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat Rev Cancer* 6:184-192.

134. Vivanco, I., and C. L. Sawyers. 2002. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* 2:489-501.

135. Seger, R., and E. G. Krebs. 1995. The MAPK signaling cascade. *Faseb J* 9:726-735.

136. Crawley, J. B., J. Willcocks, and B. M. Foxwell. 1996. Interleukin-7 induces T cell proliferation in the absence of Erk/MAP kinase activity. *Eur J Immunol* 26:2717-2723.

137. Moon, J. J., and B. H. Nelson. 2001. Phosphatidylinositol 3-kinase potentiates, but does not trigger, T cell proliferation mediated by the IL-2 receptor. *J Immunol* 167:2714-2723.

138. Brennan, P., J. W. Babbage, B. M. Burgering, B. Groner, K. Reif, and D. A. Cantrell. 1997. Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. *Immunity* 7:679-689.

139. Lali, F. V., J. Crawley, D. A. McCulloch, and B. M. Foxwell. 2004. A late, prolonged activation of the phosphatidylinositol 3-kinase pathway is required for T cell proliferation. *J Immunol* 172:3527-3534.

140. Moon, J. J., E. D. Rubio, A. Martino, A. Krumm, and B. H. Nelson. 2004. A permissive role for phosphatidylinositol 3-kinase in the Stat5-mediated expression of cyclin D2 by the interleukin-2 receptor. *J Biol Chem* 279:5520-5527.

141. Mui, A. L., H. Wakao, N. Harada, A. M. O'Farrell, and A. Miyajima. 1995. Interleukin-3, granulocyte-macrophage colony-stimulating factor, and interleukin-5 transduce signals through two forms of STAT5. *J Leukoc Biol* 57:799-803.

142. Wittig, I., and B. Groner. 2005. Signal transducer and activator of transcription 5 (STAT5), a crucial regulator of immune and cancer cells. *Curr Drug Targets Immune Endocr Metabol Disord* 5:449-463.

143. Debierre-Grockiego, F. 2004. Anti-apoptotic role of STAT5 in haematopoietic cells and in the pathogenesis of malignancies. *Apoptosis* 9:717-728.

144. Desrivieres, S., C. Kunz, I. Barash, V. Vafaizadeh, C. Borghouts, and B. Groner. 2006. The biological functions of the versatile transcription factors STAT3 and STAT5 and new strategies for their targeted inhibition. *J Mammary Gland Biol Neoplasia* 11:75-87.

145. Yoshimura, A., M. Ichihara, I. Kinjyo, M. Moriyama, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, T. Hara, and A. Miyajima. 1996. Mouse oncostatin M: an immediate early gene induced by multiple cytokines through the JAK-STAT5 pathway. *Embo J* 15:1055-1063.

146. Matsumoto, A., M. Masuhara, K. Mitsui, M. Yokouchi, M. Ohtsubo, H. Misawa, A. Miyajima, and A. Yoshimura. 1997. CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. *Blood* 89:3148-3154.

147. John, S., C. M. Robbins, and W. J. Leonard. 1996. An IL-2 response element in the human IL-2 receptor alpha chain promoter is a composite element that binds Stat5, Elf-1, HMG-I(Y) and a GATA family protein. *Embo J* 15:5627-5635.

148. Lord, J. D., B. C. McIntosh, P. D. Greenberg, and B. H. Nelson. 2000. The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. *J Immunol* 164:2533-2541.

149. Moriggl, R., D. J. Topham, S. Teglund, V. Sexl, C. McKay, D. Wang, A. Hoffmeyer, J. van Deursen, M. Y. Sangster, K. D. Bunting, G. C. Grosveld, and J. N. Ihle. 1999. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. *Immunity* 10:249-259.

150. Dumon, S., S. C. Santos, F. Debierre-Grockiego, V. Gouilleux-Gruart, L. Cocault, C. Boucheron, P. Mollat, S. Gisselbrecht, and F. Gouilleux. 1999. IL-3 dependent regulation of Bcl-xL gene expression by STAT5 in a bone marrow derived cell line. *Oncogene* 18:4191-4199.

151. Nosaka, T., T. Kawashima, K. Misawa, K. Ikuta, A. L. Mui, and T. Kitamura. 1999. STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. *Embo J* 18:4754-4765.

152. Nyga, R., C. Pecquet, N. Harir, H. Gu, I. Dhennin-Duthille, A. Regnier, V. Gouilleux-Gruart, K. Lassoued, and F. Gouilleux. 2005. Activated STAT5 proteins induce activation of the PI 3-kinase/Akt and Ras/MAPK pathways via the Gab2 scaffolding adapter. *Biochem J* 390:359-366.

153. Santos, S. C., V. Lacronique, I. Bouchaert, R. Monni, O. Bernard, S. Gisselbrecht, and F. Gouilleux. 2001. Constitutively active STAT5 variants induce growth and survival of hematopoietic cells through a PI 3-kinase/Akt dependent pathway. *Oncogene* 20:2080-2090.

154. Rosa Santos, S. C., S. Dumon, P. Mayeux, S. Gisselbrecht, and F. Gouilleux. 2000. Cooperation between STAT5 and phosphatidylinositol 3-kinase in the IL-3-dependent survival of a bone marrow derived cell line. *Oncogene* 19:1164-1172.

155. Van Parijs, L., Y. Refaeli, J. D. Lord, B. H. Nelson, A. K. Abbas, and D. Baltimore. 1999. Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. *Immunity* 11:281-288.

156. Wang, D., D. Stravopodis, S. Teglund, J. Kitazawa, and J. N. Ihle. 1996. Naturally occurring dominant negative variants of Stat5. *Molecular and cellular biology* 16:6141-6148.

157. Oda, A., H. Wakao, and H. Fujita. 2002. Calpain is a signal transducer and activator of transcription (STAT) 3 and STAT5 protease. *Blood* 99:1850-1852.

158. Schuster, B., L. Hendry, H. Byers, S. F. Lynham, M. A. Ward, and S. John. 2007. Purification and identification of the STAT5 protease in myeloid cells. *The Biochemical journal* 404:81-87.

159. Ramos, H. L., J. J. O'Shea, and W. T. Watford. 2007. STAT5 isoforms: controversies and clarifications. *The Biochemical journal* 404:e1-2.

160. Mui, A. L., H. Wakao, T. Kinoshita, T. Kitamura, and A. Miyajima. 1996. Suppression of interleukin-3-induced gene expression by a C-terminal truncated Stat5: role of Stat5 in proliferation. *The EMBO journal* 15:2425-2433.

161. Moriggl, R., V. Gouilleux-Gruart, R. Jahne, S. Berchtold, C. Gartmann, X. Liu, L. Hennighausen, A. Sotiropoulos, B. Groner, and F. Gouilleux. 1996. Deletion of the carboxyl-terminal transactivation domain of MGF-Stat5 results in sustained DNA binding and a dominant negative phenotype. *Molecular and cellular biology* 16:5691-5700.

162. Yamashita, H., H. Iwase, T. Toyama, and Y. Fujii. 2003. Naturally occurring dominant-negative Stat5 suppresses transcriptional activity of estrogen receptors and induces apoptosis in T47D breast cancer cells. *Oncogene* 22:1638-1652.

163. Socolovsky, M., A. E. Fallon, S. Wang, C. Brugnara, and H. F. Lodish. 1999. Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. *Cell* 98:181-191.

164. Satoh, T., Y. Minami, T. Kono, K. Yamada, A. Kawahara, T. Taniguchi, and Y. Kaziro. 1992. Interleukin 2-induced activation of Ras requires two domains of interleukin 2 receptor beta subunit, the essential region for growth stimulation and Lck-binding domain. *J Biol Chem* 267:25423-25427.

165. Hatakeyama, M., H. Mori, T. Doi, and T. Taniguchi. 1989. A restricted cytoplasmic region of IL-2 receptor beta chain is essential for growth signal transduction but not for ligand binding and internalization. *Cell* 59:837-845.

166. Chatterjee-Kishore, M., K. L. Wright, J. P. Ting, and G. R. Stark. 2000. How Stat1 mediates constitutive gene expression: a complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene. *Embo J* 19:4111-4122.

167. Gunning, P., J. Leavitt, G. Muscat, S. Y. Ng, and L. Kedes. 1987. A human beta-actin expression vector system directs high-level accumulation of antisense transcripts. *Proc Natl Acad Sci U S A* 84:4831-4835.

168. Onishi, M., T. Nosaka, K. Misawa, A. L. Mui, D. Gorman, M. McMahon, A. Miyajima, and T. Kitamura. 1998. Identification and characterization of a constitutively active STAT5 mutant that promotes cell proliferation. *Molecular and cellular biology* 18:3871-3879.

169. Kitamura, T., K. Hayashida, K. Sakamaki, T. Yokota, K. Arai, and A. Miyajima. 1991. Reconstitution of functional receptors for human granulocyte/macrophage colony-stimulating factor (GM-CSF): evidence that the protein encoded by the AIC2B cDNA is a subunit of the murine GM-CSF receptor. *Proc Natl Acad Sci U S A* 88:5082-5086.

170. Nelson, B. H., J. D. Lord, and P. D. Greenberg. 1996. A membrane-proximal region of the interleukin-2 receptor gamma c chain sufficient for Jak kinase activation and induction of proliferation in T cells. *Mol Cell Biol* 16:309-317.

171. Ravichandran, K. S., V. Igras, S. E. Shoelson, S. W. Fesik, and S. J. Burakoff. 1996. Evidence for a role for the phosphotyrosine-binding domain of Shc in interleukin 2 signaling. *Proc Natl Acad Sci U S A* 93:5275-5280.

172. Fukada, T., M. Hibi, Y. Yamanaka, M. Takahashi-Tezuka, Y. Fujitani, T. Yamaguchi, K. Nakajima, and T. Hirano. 1996. Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. *Immunity* 5:449-460.

173. Lai, C. F., J. Ripperger, K. K. Morella, Y. Wang, D. P. Gearing, G. H. Fey, and H. Baumann. 1995. Separate signaling mechanisms are involved in the control of STAT protein activation and gene regulation via the interleukin 6 response element by the box 3 motif of gp130. *J Biol Chem* 270:14847-14850.

174. Burchill, M. A., J. Yang, C. Vogtenhuber, B. R. Blazar, and M. A. Farrar. 2007. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *Journal of immunology (Baltimore, Md.* 178:280-290.

175. Harir, N., C. Pecquet, M. Kerenyi, K. Sonneck, B. Kovacic, R. Nyga, M. Brevet, I. Dhennin, V. Gouilleux-Gruart, H. Beug, P. Valent, K. Lassoued, R. Moriggl, and F. Gouilleux. 2006. Constitutive activation of Stat5 promotes its cytoplasmic localization and association with PI 3-kinase in myeloid leukemias.

176. Moriggl, R., V. Sexl, L. Kenner, C. Duntsch, K. Stangl, S. Gingras, A. Hoffmeyer, A. Bauer, R. Piekorz, D. Wang, K. D. Bunting, E. F. Wagner, K. Sonneck, P. Valent, J. N. Ihle, and H. Beug. 2005. Stat5 tetramer formation is associated with leukemogenesis. *Cancer cell* 7:87-99.

177. Price, D. J., R. A. Nemenoff, and J. Avruch. 1989. Purification of a hepatic S6 kinase from cycloheximide-treated Rats. *J Biol Chem* 264:13825-13833.

178. Du, K., and P. N. Tsichlis. 2005. Regulation of the Akt kinase by interacting proteins. *Oncogene* 24:7401-7409.

179. Sarbassov, D. D., D. A. Guertin, S. M. Ali, and D. M. Sabatini. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307:1098-1101.

180. Maira, S. M., I. Galetic, D. P. Brazil, S. Kaech, E. Ingley, M. Thelen, and B. A. Hemmings. 2001. Carboxyl-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane. *Science* 294:374-380.

181. Jones, S. M., R. Klinghoffer, G. D. Prestwich, A. Toker, and A. Kazlauskas. 1999. PDGF induces an early and a late wave of PI 3-kinase activity, and only the late wave is required for progression through G1. *Curr Biol* 9:512-521.

182. Lockyer, H. M., E. Tran, and B. H. Nelson. 2007. STAT5 is essential for Akt/p70S6 kinase activity during IL-2-induced lymphocyte proliferation. *Journal of immunology (Baltimore, Md.* 179:5301-5308.

183. Kovanen, P. E., L. Young, A. Al-Shami, V. Rovella, C. A. Pise-Masison, M. F. Radonovich, J. Powell, J. Fu, J. N. Brady, P. J. Munson, and W. J. Leonard. 2005. Global analysis of IL-2 target genes: identification of chromosomal clusters of expressed genes. *Int Immunol* 17:1009-1021.

184. Kovanen, P. E., A. Rosenwald, J. Fu, E. M. Hurt, L. T. Lam, J. M. Giltnane, G. Wright, L. M. Staudt, and W. J. Leonard. 2003. Analysis of gamma c-family cytokine target genes. Identification of dual-specificity phosphatase 5 (DUSP5) as a regulator of mitogen-activated protein kinase activity in interleukin-2 signaling. *J Biol Chem* 278:5205-5213.

185. Beadling, C., and K. A. Smith. 2002. DNA array analysis of interleukin-2-regulated immediate/early genes. *Med Immunol* 1:2.

186. Zhang, Z., A. Martino, and J. L. Faulon. 2007. Identification of expression patterns of IL-2-responsive genes in the murine T cell line CTLL-2. *J Interferon Cytokine Res* 27:991-995.

187. Mzali, R., L. Seguin, C. Liot, A. Auger, P. Pacaud, G. Loirand, C. Thibault, J. Pierre, and J. Bertoglio. 2005. Regulation of Rho signaling pathways in interleukin-2-stimulated human T-lymphocytes. *Faseb J* 19:1911-1913.

188. Gatzka, M., R. Piekorz, R. Moriggl, J. Rawlings, and J. N. Ihle. 2006. A role for STAT5A/B in protection of peripheral T-lymphocytes from postactivation apoptosis: insights from gene expression profiling. *Cytokine* 34:143-154.

189. John, S., C. M. Robbins, and W. J. Leonard. 1996. An IL-2 response element in the human IL-2 receptor alpha chain promoter is a composite element that binds STAT5, Elf-1, HMG-I(Y) and a GATA family protein. 15:5627-5635.

190. Dautry, F., D. Weil, J. Yu, and A. Dautry-Varsat. 1988. Regulation of pim and myb mRNA accumulation by interleukin 2 and interleukin 3 in murine hematopoietic cell lines. *J Biol Chem* 263:17615-17620.

191. Allen, J. D., E. Verhoeven, J. Domen, M. van der Valk, and A. Berns. 1997. Pim-2 transgene induces lymphoid tumors, exhibiting potent synergy with c-myc. *Oncogene* 15:1133-1141.

192. Lord, J. D., B. C. McIntosh, P. D. Greenberg, and B. H. Nelson. 2000. The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. *Journal of Immunology.* 164:2533-2541.

193. Martino, A., J. H. t. Holmes, J. D. Lord, J. J. Moon, and B. H. Nelson. 2001. Stat5 and Sp1 regulate transcription of the cyclin D2 gene in response to IL-2. *Journal of immunology (Baltimore, Md.* 166:1723-1729.

194. Turner, J. M. 1993. IL-2-dependent induction of G□1□ cyclins in primary T cells is not blocked by rapamycin or cyclosporin A. *Int.Immunol.* 5:1199-1209.

195. Stern, J. B., and K. A. Smith. 1986. Interleukin-2 induction of T-cell G1 progression and c-myb expression. 233:203-206.

196. Liu, C. C., S. Rafii, A. Granelli-Piperno, J. A. Trapani, and J. D. Young. 1989. Perforin and serine esterase gene expression in stimulated human T cells. Kinetics, mitogen requirements, and effects of cyclosporin A. *J Exp Med* 170:2105-2118.

197. Matsumoto, A., M. Masuhara, K. Mitsui, M. Yokouchi, M. Ohtsubo, H. Misawa, A. Miyajima, and A. Yoshimura. 1997. CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. *Blood* 89:3148-3154.

198. Sporri, B., P. E. Kovanen, A. Sasaki, A. Yoshimura, and W. J. Leonard. 2001. JAB/SOCS1/SSI-1 is an interleukin-2-induced inhibitor of IL-2 signaling. *Blood* 97:221-226.

199. Massague, J., and R. R. Gomis. 2006. The logic of TGFbeta signaling. *FEBS Lett* 580:2811-2820.

200. Xue, H. H., P. E. Kovanen, C. A. Pise-Masison, M. Berg, M. F. Radovich, J. N. Brady, and W. J. Leonard. 2002. IL-2 negatively regulates IL-7 receptor alpha chain expression in activated T lymphocytes. *Proc Natl Acad Sci U S A* 99:13759-13764.

201. Lindemann, M. J., Z. Hu, M. Benczik, K. D. Liu, and S. L. Gaffen. 2008. Differential regulation of the IL-17 receptor by gammac cytokines: inhibitory signaling by the phosphatidylinositol 3-kinase pathway. *J Biol Chem* 283:14100-14108.

202. Tinel, M., M. A. Robin, J. Doostzadeh, M. Maratrat, F. Ballet, N. Fardel, J. el Kahwaji, P. Beaune, M. Daujat, G. Labbe, and et al. 1995. The interleukin-2 receptor down-regulates the expression of cytochrome P450 in cultured rat hepatocytes. *Gastroenterology* 109:1589-1599.

203. McMurray, H. R., E. R. Sampson, G. Compitello, C. Kinsey, L. Newman, B. Smith, S. R. Chen, L. Klebanov, P. Salzman, A. Yakovlev, and H. Land. 2008. Synergistic response to oncogenic mutations defines gene class critical to cancer phenotype. *Nature* 453:1112-1116.

204. Moon, J. J., and B. H. Nelson. 2001. Phosphatidylinositol 3-kinase potentiates, but does not trigger, T cell proliferation mediated by the IL-2 receptor. *Journal of Immunology.* 167:2714-2723.

205. Brennen, P., J. W. Babbage, B. M. Burgering, B. Groner, K. Reif, and D. A. Cantrell. 1997. Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. *Immunity* 7:679-689.

206. Garcia, Z., A. Kumar, M. Marques, I. Cortes, and A. C. Carrera. 2006. Phosphoinositide 3-kinase controls early and late events in mammalian cell division. *Embo J* 25:655-661.

207. Chambard, J. C., R. Lefloch, J. Pouyssegur, and P. Lenormand. 2007. ERK implication in cell cycle regulation. *Biochim Biophys Acta* 1773:1299-1310.

208. Moriggl, R., D. J. Topham, S. Teglund, V. Sexl, C. McKay, D. Wang, A. Hoffmeyer, J. van Deursen, M. Y. Sangster, K. D. Bunting, G. C. Grosveld, and J. N. Ihle. 1999. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. *Immunity* 10:249-259.

209. Hoskin, D. W., J. J. Butler, D. Drapeau, S. M. Haeryfar, and J. Blay. 2002. Adenosine acts through an A3 receptor to prevent the induction of murine anti-CD3-activated killer T cells. *Int J Cancer* 99:386-395.

210. Garnis, C., B. P. Coe, L. Zhang, M. P. Rosin, and W. L. Lam. 2004. Overexpression of LRP12, a gene contained within an 8q22 amplicon identified by high-resolution array CGH analysis of oral squamous cell carcinomas. *Oncogene* 23:2582-2586.

211. Ongusaha, P. P., J. C. Kwak, A. J. Zwible, S. Macip, S. Higashiyama, N. Taniguchi, L. Fang, and S. W. Lee. 2004. HB-EGF is a potent inducer of tumor growth and angiogenesis. *Cancer Res* 64:5283-5290.

212. Nanba, D., and S. Higashiyama. 2004. Dual intracellular signaling by proteolytic cleavage of membrane-anchored heparin-binding EGF-like growth factor. *Cytokine Growth Factor Rev* 15:13-19.

213. Higashiyama, S., H. Iwabuki, C. Morimoto, M. Hieda, H. Inoue, and N. Matsushita. 2008. Membrane-anchored growth factors, the epidermal growth factor family: beyond receptor ligands. *Cancer Sci* 99:214-220.

214. Nanba, D., A. Mammoto, K. Hashimoto, and S. Higashiyama. 2003. Proteolytic release of the carboxy-terminal fragment of proHB-EGF causes nuclear export of PLZF. *J Cell Biol* 163:489-502.

215. Kinugasa, Y., M. Hieda, M. Hori, and S. Higashiyama. 2007. The carboxyl-terminal fragment of pro-HB-EGF reverses Bcl6-mediated gene repression. *J Biol Chem* 282:14797-14806.

216. Blotnick, S., G. E. Peoples, M. R. Freeman, T. J. Eberlein, and M. Klagsbrun. 1994. T lymphocytes synthesize and export heparin-binding epidermal growth factor-like growth factor and basic fibroblast growth factor, mitogens for vascular cells and fibroblasts: differential production and release by CD4+ and CD8+ T cells. *Proc Natl Acad Sci U S A* 91:2890-2894.

217. Peoples, G. E., S. Blotnick, K. Takahashi, M. R. Freeman, M. Klagsbrun, and T. J. Eberlein. 1995. T lymphocytes that infiltrate tumors and atherosclerotic plaques produce heparin-binding epidermal growth factor-like growth factor and basic fibroblast growth factor: a potential pathologic role. *Proc Natl Acad Sci U S A* 92:6547-6551.

218. Ling, M. T., X. Wang, X. Zhang, and Y. C. Wong. 2006. The multiple roles of Id-1 in cancer progression. *Differentiation* 74:481-487.

219. Wong, Y. C., X. Wang, and M. T. Ling. 2004. Id-1 expression and cell survival. *Apoptosis* 9:279-289.

220. Lee, J. Y., M. B. Kang, S. H. Jang, T. Qian, H. J. Kim, C. H. Kim, Y. Kim, and G. Kong. 2009. Id-1 activates Akt-mediated Wnt signaling and p27(Kip1) phosphorylation through PTEN inhibition. *Oncogene* 28:824-831.

221. Kim, D., X. C. Peng, and X. H. Sun. 1999. Massive apoptosis of thymocytes in T-cell-deficient Id1 transgenic mice. *Mol Cell Biol* 19:8240-8253.

222. Qi, Z., and X. H. Sun. 2004. Hyperresponse to T-cell receptor signaling and apoptosis of Id1 transgenic thymocytes. *Mol Cell Biol* 24:7313-7323.

223. Yang, Y., H. C. Liou, and X. H. Sun. 2006. Id1 potentiates NF-kappaB activation upon T cell receptor signaling. *J Biol Chem* 281:34989-34996.

224. Baker, S. J. 2003. Small unstable apoptotic protein, an apoptosis-associated protein, suppresses proliferation of myeloid cells. *Cancer Res* 63:705-712.

225. Kelley, K. D., K. R. Miller, A. Todd, A. R. Kelley, R. Tuttle, and S. J. Berberich. YPEL3, a p53-regulated gene that induces cellular senescence. *Cancer Res* 70:3566-3575.

226. Hosono, K., T. Sasaki, S. Minoshima, and N. Shimizu. 2004. Identification and characterization of a novel gene family YPEL in a wide spectrum of eukaryotic species. *Gene* 340:31-43.

227. Kim, J. H., S. Yoon, M. Won, S. H. Sim, J. J. Ko, S. Han, K. A. Lee, K. Lee, and J. Bae. 2009. HIP1R interacts with a member of Bcl-2 family, BCL2L10, and induces BAK-dependent cell death. *Cell Physiol Biochem* 23:43-52.

228. Hyun, T. S., D. S. Rao, D. Saint-Dic, L. E. Michael, P. D. Kumar, S. V. Bradley, I. F. Mizukami, K. I. Oravecz-Wilson, and T. S. Ross. 2004. HIP1 and HIP1r stabilize receptor tyrosine kinases and bind 3-phosphoinositides via epsin N-terminal homology domains. *J Biol Chem* 279:14294-14306.

229. Porpaczy, E., M. Bilban, G. Heinze, M. Gruber, K. Vanura, I. Schwarzinger, S. Stilgenbauer, B. Streubel, C. Fonatsch, and U. Jaeger. 2009. Gene expression signature of chronic lymphocytic leukaemia with Trisomy 12. *Eur J Clin Invest* 39:568-575.

230. Jeffers, J. R., E. Parganas, Y. Lee, C. Yang, J. Wang, J. Brennan, K. H. MacLean, J. Han, T. Chittenden, J. N. Ihle, P. J. McKinnon, J. L. Cleveland, and G. P.

Zambetti. 2003. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell* 4:321-328.

231. Ekert, P. G., A. M. Jabbour, A. Manoharan, J. E. Heraud, J. Yu, M. Pakusch, E. M. Michalak, P. N. Kelly, B. Callus, T. Kiefer, A. Verhagen, J. Silke, A. Strasser, C. Borner, and D. L. Vaux. 2006. Cell death provoked by loss of interleukin-3 signaling is independent of Bad, Bim, and PI3 kinase, but depends in part on Puma. *Blood* 108:1461-1468.

232. Fischer, S. F., G. T. Belz, and A. Strasser. 2008. BH3-only protein Puma contributes to death of antigen-specific T cells during shutdown of an immune response to acute viral infection. *Proc Natl Acad Sci U S A* 105:3035-3040.

233. You, H., M. Pellegrini, K. Tsuchihara, K. Yamamoto, G. Hacker, M. Erlacher, A. Villunger, and T. W. Mak. 2006. FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. *J Exp Med* 203:1657-1663.

234. Grandori, C., S. M. Cowley, L. P. James, and R. N. Eisenman. 2000. The Myc/Max/Mad network and the transcriptional control of cell behavior. *Annu Rev Cell Dev Biol* 16:653-699.

235. Hooker, C. W., and P. J. Hurlin. 2006. Of Myc and Mnt. *J Cell Sci* 119:208-216.

236. Iritani, B. M., J. Delrow, C. Grandori, I. Gomez, M. Klacking, L. S. Carlos, and R. N. Eisenman. 2002. Modulation of T-lymphocyte development, growth and cell size by the Myc antagonist and transcriptional repressor Mad1. *Embo J* 21:4820-4830.

237. Rudolph, B., A. O. Hueber, and G. I. Evan. 2001. Expression of Mad1 in T cells leads to reduced thymic cellularity and impaired mitogen-induced proliferation. *Oncogene* 20:1164-1175.

238. Laezza, F., T. J. Wilding, S. Sequeira, F. Coussen, X. Z. Zhang, R. Hill-Robinson, C. Mulle, J. E. Huettner, and A. M. Craig. 2007. KRIP6: a novel BTB/kelch protein regulating function of kainate receptors. *Mol Cell Neurosci* 34:539-550.

239. Cahalan, M. D., and K. G. Chandy. 2009. The functional network of ion channels in T lymphocytes. *Immunol Rev* 231:59-87.

240. Pacheco, R., T. Gallart, C. Lluis, and R. Franco. 2007. Role of glutamate on T-cell mediated immunity. *J Neuroimmunol* 185:9-19.

241. Levite, M. 2008. Neurotransmitters activate T-cells and elicit crucial functions via neurotransmitter receptors. *Curr Opin Pharmacol* 8:460-471.

242. Boldyrev, A. A., D. O. Carpenter, and P. Johnson. 2005. Emerging evidence for a similar role of glutamate receptors in the nervous and immune systems. *J Neurochem* 95:913-918.

243. Lombardi, G., C. Dianzani, G. Miglio, P. L. Canonico, and R. Fantozzi. 2001. Characterization of ionotropic glutamate receptors in human lymphocytes. *Br J Pharmacol* 133:936-944.

244. Zhu, Z., X. He, C. Johnson, J. Stoops, A. E. Eaker, D. S. Stoffer, A. Bell, R. Zarnegar, and M. C. DeFrances. 2007. PI3K is negatively regulated by PIK3IP1, a novel p110 interacting protein. *Biochem Biophys Res Commun* 358:66-72.

245. He, X., Z. Zhu, C. Johnson, J. Stoops, A. E. Eaker, W. Bowen, and M. C. DeFrances. 2008. PIK3IP1, a negative regulator of PI3K, suppresses the development of hepatocellular carcinoma. *Cancer Res* 68:5591-5598.

246. Nelson, B. H. 2008. The impact of T-cell immunity on ovarian cancer outcomes. *Immunol Rev* 222:101-116.

247. Chu, C. S., S. H. Kim, C. H. June, and G. Coukos. 2008. Immunotherapy opportunities in ovarian cancer. *Expert Rev Anticancer Ther* 8:243-257.

248. Barnett, B., I. Kryczek, P. Cheng, W. Zou, and T. J. Curiel. 2005. Regulatory T cells in ovarian cancer: biology and therapeutic potential. *Am J Reprod Immunol* 54:369-377.

249. Seder, R. A., P. A. Darrah, and M. Roederer. 2008. T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* 8:247-258.

250. Betts, M. R., M. C. Nason, S. M. West, S. C. De Rosa, S. A. Migueles, J. Abraham, M. M. Lederman, J. M. Benito, P. A. Goepfert, M. Connors, M. Roederer, and R. A. Koup. 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 107:4781-4789.

251. Duvall, M. G., M. L. Precopio, D. A. Ambrozak, A. Jaye, A. J. McMichael, H. C. Whittle, M. Roederer, S. L. Rowland-Jones, and R. A. Koup. 2008. Polyfunctional T cell responses are a hallmark of HIV-2 infection. *Eur J Immunol* 38:350-363.

252. Almeida, J. R., D. A. Price, L. Papagno, Z. A. Arkoub, D. Sauce, E. Bornstein, T. E. Asher, A. Samri, A. Schnuriger, I. Theodorou, D. Costagliola, C. Rouzioux, H. Agut, A. G. Marcelin, D. Douek, B. Autran, and V. Appay. 2007. Superior control

of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J Exp Med* 204:2473-2485.

253. Almeida, J. R., D. Sauce, D. A. Price, L. Papagno, S. Y. Shin, A. Moris, M. Larsen, G. Pancino, D. C. Douek, B. Autran, A. Saez-Cirion, and V. Appay. 2009. Antigen sensitivity is a major determinant of CD8+ T-cell polyfunctionality and HIV suppressive activity. *Blood* 113:6351-6360.

254. Ferre, A. L., P. W. Hunt, J. W. Critchfield, D. H. Young, M. M. Morris, J. C. Garcia, R. B. Pollard, H. F. Yee, Jr., J. N. Martin, S. G. Deeks, and B. L. Shacklett. 2009. Mucosal immune responses to HIV-1 in elite controllers: a potential correlate of immune control. *Blood* 113:3978-3989.

255. Rehr, M., J. Cahenzli, A. Haas, D. A. Price, E. Gostick, M. Huber, U. Karrer, and A. Oxenius. 2008. Emergence of polyfunctional CD8+ T cells after prolonged suppression of human immunodeficiency virus replication by antiretroviral therapy. *J Virol* 82:3391-3404.

256. Kannanganat, S., B. G. Kapogiannis, C. Ibegbu, L. Chennareddi, P. Goepfert, H. L. Robinson, J. Lennox, and R. R. Amara. 2007. Human immunodeficiency virus type 1 controllers but not noncontrollers maintain CD4 T cells coexpressing three cytokines. *J Virol* 81:12071-12076.

257. Bansal, A., B. Jackson, K. West, S. Wang, S. Lu, J. S. Kennedy, and P. A. Goepfert. 2008. Multifunctional T-cell characteristics induced by a polyvalent DNA prime/protein boost human immunodeficiency virus type 1 vaccine regimen given to healthy adults are dependent on the route and dose of administration. *J Virol* 82:6458-6469.

258. Valor, L., J. Navarro, J. Carbone, C. Rodriguez-Sainz, J. Gil, F. Lopez, and E. Fernandez-Cruz. 2008. Immunization with an HIV-1 immunogen induces CD4+ and CD8+ HIV-1-specific polyfunctional responses in patients with chronic HIV-1 infection receiving antiretroviral therapy. *Vaccine* 26:2738-2745.

259. Winstone, N., A. Guimaraes-Walker, J. Roberts, D. Brown, V. Loach, N. Goonetilleke, T. Hanke, and A. J. McMichael. 2009. Increased detection of proliferating, polyfunctional, HIV-1-specific T cells in DNA-modified vaccinia virus Ankara-vaccinated human volunteers by cultured IFN-gamma ELISPOT assay. *Eur J Immunol* 39:975-985.

260. Lichterfeld, M., X. G. Yu, M. T. Waring, S. K. Mui, M. N. Johnston, D. Cohen, M. M. Addo, J. Zaunders, G. Alter, E. Pae, D. Strick, T. M. Allen, E. S. Rosenberg, B. D. Walker, and M. Altfeld. 2004. HIV-1-specific cytotoxicity is

preferentially mediated by a subset of CD8(+) T cells producing both interferon-gamma and tumor necrosis factor-alpha. *Blood* 104:487-494.

261. Badr, G., N. Bedard, M. S. Abdel-Hakeem, L. Trautmann, B. Willems, J. P. Villeneuve, E. K. Haddad, R. P. Sekaly, J. Bruneau, and N. H. Shoukry. 2008. Early interferon therapy for hepatitis C virus infection rescues polyfunctional, long-lived CD8+ memory T cells. *J Virol* 82:10017-10031.

262. Yi, J. S., M. Du, and A. J. Zajac. 2009. A Vital Role for Interleukin-21 in the Control of a Chronic Viral Infection. *Science* 324:1572-1576.

263. Imai, N., H. Ikeda, I. Tawara, and H. Shiku. 2009. Tumor progression inhibits the induction of multifunctionality in adoptively transferred tumor-specific CD8+ T cells. *Eur J Immunol* 39:241-253.

264. Imai, N., H. Ikeda, I. Tawara, L. Wang, L. Wang, H. Nishikawa, T. Kato, and H. Shiku. 2009. Glucocorticoid-induced tumor necrosis factor receptor stimulation enhances the multifunctionality of adoptively transferred tumor antigen-specific CD8 T cells with tumor regression. *Cancer Sci* 100:1317-1325.

265. Yuan, J., S. Gnjatic, H. Li, S. Powel, H. F. Gallardo, E. Ritter, G. Y. Ku, A. A. Jungbluth, N. H. Segal, T. S. Rasalan, G. Manukian, Y. Xu, R. A. Roman, S. L. Terzulli, M. Heywood, E. Pogoriler, G. Ritter, L. J. Old, J. P. Allison, and J. D. Wolchok. 2008. CTLA-4 blockade enhances polyfunctional NY-ESO-1 specific T cell responses in metastatic melanoma patients with clinical benefit. *Proc Natl Acad Sci U S A* 105:20410-20415.

266. Carpenito, C., M. C. Milone, R. Hassan, J. C. Simonet, M. Lakhal, M. M. Suhoski, A. Varela-Rohena, K. M. Haines, D. F. Heitjan, S. M. Albelda, R. G. Carroll, J. L. Riley, I. Pastan, and C. H. June. 2009. Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proc Natl Acad Sci U S A* 106:3360-3365.

267. Perales, M. A., J. Yuan, S. Powel, H. F. Gallardo, T. S. Rasalan, C. Gonzalez, G. Manukian, J. Wang, Y. Zhang, P. B. Chapman, S. E. Krown, P. O. Livingston, S. Ejadi, K. S. Panageas, M. E. Engelhorn, S. L. Terzulli, A. N. Houghton, and J. D. Wolchok. 2008. Phase I/II study of GM-CSF DNA as an adjuvant for a multipeptide cancer vaccine in patients with advanced melanoma. *Mol Ther* 16:2022-2029.

268. Precopio, M. L., M. R. Betts, J. Parrino, D. A. Price, E. Gostick, D. R. Ambrozak, T. E. Asher, D. C. Douek, A. Harari, G. Pantaleo, R. Bailer, B. S. Graham, M. Roederer, and R. A. Koup. 2007. Immunization with vaccinia virus induces

polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J Exp Med* 204:1405-1416.

269. Gaucher, D., R. Therrien, N. Kettaf, B. R. Angermann, G. Boucher, A. Filali-Mouhim, J. M. Moser, R. S. Mehta, D. R. Drake, 3rd, E. Castro, R. Akondy, A. Rinfret, B. Yassine-Diab, E. A. Said, Y. Chouikh, M. J. Cameron, R. Clum, D. Kelvin, R. Somogyi, L. D. Greller, R. S. Balderas, P. Wilkinson, G. Pantaleo, J. Tartaglia, E. K. Haddad, and R. P. Sekaly. 2008. Yellow fever vaccine induces integrated multilineage and polyfunctional immune responses. *J Exp Med* 205:3119-3131.

270. Beveridge, N. E., D. A. Price, J. P. Casazza, A. A. Pathan, C. R. Sander, T. E. Asher, D. R. Ambrozak, M. L. Precopio, P. Scheinberg, N. C. Alder, M. Roederer, R. A. Koup, D. C. Douek, A. V. Hill, and H. McShane. 2007. Immunisation with BCG and recombinant MVA85A induces long-lasting, polyfunctional Mycobacterium tuberculosis-specific CD4+ memory T lymphocyte populations. *Eur J Immunol* 37:3089-3100.

271. Elvang, T., J. P. Christensen, R. Billeskov, T. Thi Kim Thanh Hoang, P. Holst, A. R. Thomsen, P. Andersen, and J. Dietrich. 2009. CD4 and CD8 T cell responses to the M. tuberculosis Ag85B-TB10.4 promoted by adjuvanted subunit, adenovector or heterologous prime boost vaccination. *PLoS ONE* 4:e5139.

272. Darrah, P. A., D. T. Patel, P. M. De Luca, R. W. Lindsay, D. F. Davey, B. J. Flynn, S. T. Hoff, P. Andersen, S. G. Reed, S. L. Morris, M. Roederer, and R. A. Seder. 2007. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat Med* 13:843-850.

273. Trinchieri, G., and P. Scott. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions. *Research in immunology* 146:423-431.

274. Speiser, D. E., D. Lienard, N. Rufer, V. Rubio-Godoy, D. Rimoldi, F. Lejeune, A. M. Krieg, J. C. Cerottini, and P. Romero. 2005. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. *J Clin Invest* 115:739-746.

275. Wille-Reece, U., B. J. Flynn, K. Lore, R. A. Koup, A. P. Miles, A. Saul, R. M. Kedl, J. J. Mattapallil, W. R. Weiss, M. Roederer, and R. A. Seder. 2006. Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates. *J Exp Med* 203:1249-1258.

276. Smith, C., L. Beagley, and R. Khanna. 2009. Acquisition of polyfunctionality by Epstein-Barr virus-specific CD8+ T cells correlates with increased resistance to galectin-1 mediated suppression. *J Virol* 83:192-198.

277. Appay, V., D. C. Douek, and D. A. Price. 2008. CD8+ T cell efficacy in vaccination and disease. *Nat Med* 14:623-628.

278. Cayabyab, M. J., B. Korioth-Schmitz, Y. Sun, A. Carville, H. Balachandran, A. Miura, K. R. Carlson, A. P. Buzby, B. F. Haynes, W. R. Jacobs, and N. L. Letvin. 2009. Recombinant BCG prime-recombinant adenovirus boost vaccination in rhesus monkeys elicits robust polyfunctional SIV-specific T cell responses. *J Virol* 83:5505-5513.

279. Dubie, R. A., S. Maksaereekul, B. L. Shacklett, D. Lemongello, K. S. Cole, F. Villinger, S. A. Blozis, P. A. Luciw, and E. E. Sparger. 2009. Co-immunization with IL-15 enhances cellular immune responses induced by a vif-deleted simian immunodeficiency virus proviral DNA vaccine and confers partial protection against vaginal challenge with SIVmac251. *Virology* 386:109-121.

280. Trumpfheller, C., M. Caskey, G. Nchinda, M. P. Longhi, O. Mizenina, Y. Huang, S. J. Schlesinger, M. Colonna, and R. M. Steinman. 2008. The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. *Proc Natl Acad Sci U S A* 105:2574-2579.

281. Recchia, F., G. Saggio, A. Cesta, G. Candeloro, A. Nuzzo, M. Lombardo, G. Carta, and S. Rea. 2005. Interleukin-2 and 13-cis retinoic acid as maintenance therapy in advanced ovarian cancer. *Int J Oncol* 27:1039-1046.

282. Lenzi, R., R. Edwards, C. June, M. V. Seiden, M. E. Garcia, M. Rosenblum, and R. S. Freedman. 2007. Phase II study of intraperitoneal recombinant interleukin-12 (rhIL-12) in patients with peritoneal carcinomatosis (residual disease < 1 cm) associated with ovarian cancer or primary peritoneal carcinoma. *J Transl Med* 5:66.

283. Hurteau, J. A., J. A. Blessing, S. L. DeCesare, and W. T. Creasman. 2001. Evaluation of recombinant human interleukin-12 in patients with recurrent or refractory ovarian cancer: a gynecologic oncology group study. *Gynecol Oncol* 82:7-10.

284. Hall, G. D., J. M. Brown, R. E. Coleman, M. Stead, K. S. Metcalf, K. R. Peel, C. Poole, M. Crawford, B. Hancock, P. J. Selby, and T. J. Perren. 2004. Maintenance treatment with interferon for advanced ovarian cancer: results of the Northern and Yorkshire gynaecology group randomised phase III study. *Br J Cancer* 91:621-626.

285. Curtsinger, J. M., J. O. Valenzuela, P. Agarwal, D. Lins, and M. F. Mescher. 2005. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. *J Immunol* 174:4465-4469.

286. Erickson, S., O. Sangfelt, J. Castro, M. Heyman, S. Einhorn, and D. Grander. 1999. Interferon-alpha inhibits proliferation in human T lymphocytes by abrogation of interleukin 2-induced changes in cell cycle-regulatory proteins. *Cell Growth Differ* 10:575-582.

287. Klimpel, G. R., A. J. Infante, J. Patterson, C. B. Hess, and M. Asuncion. 1990. Virus-induced interferon alpha/beta (IFN-alpha/beta) production by T cells and by Th1 and Th2 helper T cell clones: a study of the immunoregulatory actions of IFN-gamma versus IFN-alpha/beta on functions of different T cell populations. *Cell Immunol* 128:603-618.

288. Osaki, T., J. M. Peron, Q. Cai, H. Okamura, P. D. Robbins, M. Kurimoto, M. T. Lotze, and H. Tahara. 1998. IFN-gamma-inducing factor/IL-18 administration mediates IFN-gamma- and IL-12-independent antitumor effects. ***J.Immunol.*** 160:1742-1749.

289. Carson, W. E., J. E. Dierksheide, S. Jabbour, M. Anghelina, P. Bouchard, G. Ku, H. Yu, H. Baumann, M. H. Shah, M. A. Cooper, J. Durbin, and M. A. Caligiuri. 2000. Coadministration of interleukin-18 and interleukin-12 induces a fatal inflammatory response in mice: critical role of natural killer cell interferon-gamma production and STAT-mediated signal transduction. *Blood* 96:1465-1473.

290. Osaki, T., W. Hashimoto, A. Gambotto, H. Okamura, P. D. Robbins, M. Kurimoto, M. T. Lotze, and H. Tahara. 1999. Potent antitumor effects mediated by local expression of the mature form of the interferon-gamma inducing factor, interleukin-18 (IL-18). *Gene therapy* 6:808-815.

291. Cheng, L. E., C. Ohlen, B. H. Nelson, and P. D. Greenberg. 2002. Enhanced signaling through the IL-2 receptor in CD8+ T cells regulated by antigen recognition results in preferential proliferation and expansion of responding CD8+ T cells rather than promotion of cell death. *Proc Natl Acad Sci U S A* 99:3001-3006.

292. Nelson, B. H., J. D. Lord, and P. D. Greenberg. 1994. Cytoplasmic domains of the interleukin-2 receptor beta and gamma chains mediate the signal for T-cell proliferation. *Nature* 369:333-336.