The involvement of STAT proteins in cytokine-mediated regulation of CD28 surface expression on antigen-specific CD8+ T cells expanded for adoptive cell therapy

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Abstract

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Metastatic melanoma is an aggressive cancer responsive to adoptive cell therapy (ACT) with tumor-reactive CD8+ T lymphocytes. ACT efficacy correlates with persistence and proliferation of infused T cells; literature suggests strategies to inhibit terminal differentiation of tumor-reactive CD8+ T cells during expansion increases their anti-tumor effect. IL-21 suppresses effector T cell differentiation and promotes memory qualities, such as CD62L and CD28 expression, in human antigen-specific CD8+ T cells. Compared to common gamma chain cytokines IL-2, IL-7 and IL-15, IL-21 is unique in its ability to support CD28 surface expression on activated CD8+ T cells. Although the role of CD28 in T cell-mediated immunity is well understood, mechanisms that regulate surface CD28 expression are not well defined. We investigated the ability of cytokines to support CD28 surface expression on human CD8+ T cells via STAT protein activation. We found IL-21 promotes sustained STAT3 activation; IL-21-

mediated STAT3 activation is required for expansion of MART-1-specific CD8+ T cells characterized by CD28 surface expression. Using chromatin immunoprecipitation (ChIP) analysis, we found IL-21-activated STAT3 associates with a consensus STAT binding sequence within the human *CD28* promoter, supporting a role for STAT3 as a *CD28* transcriptional activator. In contrast to IL-21, conventional STAT3-activating cytokine IL-6 was less efficient at sustaining STAT3 activation, and supported only low levels of CD28 surface expression. In contrast to IL-21, IL-15 actively inhibits CD28 surface expression on activated CD8+ T cells. ChIP analysis demonstrated IL-15-mediated STAT5 associates with the *CD28* promoter at the same site as STAT3, supporting a role for STAT5 as a *CD28* transcriptional inhibitor. Our observations suggest antagonism between STAT3 and STAT5 for transcriptional modulation of *CD28* and support IL-21 as the ideal cytokine with which to modulate antigen-specific CD8+ T cells expanded for ACT, based upon the unique ability of IL-21 to support CD28 surface expression.

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List of Abbreviations

γ_c	common gamma chain
ACT	adoptive cell therapy
APC	allophycocyanin
CAR	chimeric antigen receptor
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
ChIP	chromatin immunoprecipitation
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
DC	dendritic cells
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
IL	interleukin
INR	inoperative transcriptional initiator
JAK	janus kinase
MAPK	mitogen-activated protein kinase
MART-1	melanoma-associated antigen recognized by T cells
mRNA	messenger ribonucleic acid
NK	natural killer
NKT	natural killer T
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PI3K	phosphatidylinositol 3-kinase
STAT	signal transducer and activator of transcription
T_{CM}	central memory T cells
TCR	T cell receptor
T_{EM}	effector memory T cells
TIL	tumor infiltrating lymphocytes

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Chapter 1: Introduction

1.1 Overview

Metastatic melanoma is one of the most aggressive human cancers and is a leading cause of cancer deaths in developed countries (Lomas et al., 2008). The administration of *ex vivo* activated and expanded autologous tumor-specific T lymphocytes, known as adoptive cell therapy (ACT), induces clinical responses in patients previously refractory to conventional metastatic melanoma therapy (Dudley et al., 2005; Hunder et al., 2008). Patient response rate is highly correlative with persistence of the infused T cells *in vivo* (Robbins et al., 2004), and proliferative potential of the infused cells is thought to be the key determinant in persistence (Zhou et al., 2005). Increasing evidence suggests that strategies used to inhibit terminal differentiation of tumor-reactive CD8+ T cells during activation and expansion *ex vivo* will lead to an increase in their anti-tumor effect *in vivo* (Gattinoni et al., 2005; Robbins et al., 2004; Zhou et al., 2005). Modulation of specific signaling pathways will prove central to obtaining the cellular phenotype and particular cellular traits most efficacious for ACT; defining the mechanism(s) by which these pathways are executed to promote the desired cellular characteristics will allow optimization of ACT for individuals with metastatic melanoma, and in time can be made applicable to other types of cancers.

1.2 Melanoma

In the United States, one third of all cancers are skin cancers (Lomas et al., 2008). Non-melanoma skin cancer affects the older population, while melanoma generally affects younger patients between the ages of 20 and 45 years old (Jerant et al., 2000). Early-stage melanoma is often curable; surgery is beneficial for primary (localized) melanoma. Malignant melanoma, however, is characterized by aggressive local growth and metastasis; 3 out of 4 skin cancer

related deaths are due to metastatic melanoma (Jerant et al., 2000). Historically, the conventional treatments for metastatic melanoma included chemotherapy and cytokine therapy, both of which resulted in low patient response rates (Khattak et al., 2013). Until recently, there were no effective systemic therapies available for metastatic melanoma. However, the genetic mutations found in melanoma have recently been characterized, and immunotherapy-based strategies have improved; immunotherapy is defined as the treatment of disease by modulating the immune response. As such, the new standard of care now includes drugs that target the mitogen-activated protein kinase (MAPK) pathway (BRAF inhibitor vemurafenib) and activated T cells (anti-CTLA-4 antibody ipilimumab) (Menzies and Long, 2013). Despite being important advances in the management of melanoma, there are limitations to these treatments; the response to vemurafenib is short lived, and its use is restricted to those patients whose tumors are characterized by a BRAF V600 mutation (Lee and Margolin, 2012). Furthermore, although ipilimumab can result in durable responses, complete responses are rare (Hodi et al., 2010; Lee and Margolin, 2012). An alternate approach to melanoma treatment is the use of adoptive cell therapy, wherein the patient's own cells are manipulated *ex vivo* and subsequently re-infused to exert an *in vivo* anti-tumor response. Although labour intensive, highly technical, and possessing the requirement for a more lengthy product (cells) generation time, adoptive cell therapy offers the benefit of potentially higher response rates, and greater durable complete response rates. Trials are currently underway assessing the efficacy of melanoma-reactive CD8+ T cells in adoptive cell therapy.

1.3 Adoptive Cell Therapy

Adoptive cell therapy (ACT) is a branch of immunotherapy that involves the transfer of

autologous cells that have undergone *ex vivo* modulation, back into a patient. Adoptive T cell therapy, specifically, involves the isolation and subsequent *ex vivo* activation and expansion of tumor-specific T cells (to generate the large quantity of T cells required for effective tumor reduction in the patient), followed by re-infusion into the patient to obtain an anti-tumor response. Generally, anti-tumor T cell clones obtained from peripheral blood, or tumor infiltrating lymphocytes (TIL) obtained from a tumor biopsy, are expanded for treatment; newer strategies involve engineering T cells to express modified T cell receptors (TCRs) or chimeric antigen receptors (CARs) that will recognize tumor antigens (Park et al., 2011).

Multiple types of cancers respond to adoptive T cell therapy (Rosenberg et al., 2008); the major hurdle faced by this strategy is the *ex vivo* expansion of the low frequency of native tumor-reactive T cells to the large quantity required for ACT. Cells require multiple rounds of stimulation to reach the number required for ACT; stimulation results in activation and initiation of the differentiation pathway, resulting in a loss of lymphoid homing receptors and eventual T cell exhaustion. For optimal patient response, the pool of T cells generated for re-infusion into the patient should exhibit characteristics of persistence, survival, and self-renewal; these cells should also possess the ability to traffic to the lymph nodes to identify tumor antigen and to initiate an anti-tumor response (Rosenberg et al., 2008). Trials are underway investigating the efficacy of both CD4+ T cells and CD8+ T cells in ACT for various cancers.

1.4 ACT for the Treatment of Metastatic Melanoma

Melanoma is one of the most immunogenic solid cancers. As such, melanoma has become the model in which many immunotherapy strategies have been studied and developments made. Of particular interest in the setting of melanoma is the presence of a relatively high frequency of

naïve MART-1 (melanoma-associated antigen recognized by T cells)-specific CD8+ T cells in the blood of healthy HLA-$A2^+$ donors (Comin-Anduix et al., 2004). The presence of the endogenous population of tumor-specific CD8+ T cells suggests that the immune system has the capacity to identify and eradicate tumor cells, if not overwhelmed by tumor progression, metastasis and invasion. Furthermore, exploitation of the relatively high precursor frequency of MART-1-specific CD8+ T cells in patients allows for the *ex vivo* activation and expansion of biologically relevant quantities of these cells for ACT. For optimal ACT efficacy, cells must possess persistence, proliferative potential, and the ability to home to lymph nodes. Evidence suggests that strategies designed to prevent terminal differentiation and exhaustion of tumor-reactive T cells will produce an enhanced anti-tumor effect (Gattinoni et al., 2005; Robbins et al., 2004; Zhou et al., 2005). Studies involving the use of cytokines as well as other recombinant proteins and chemicals are underway, assessing the ability of these modulators to promote expansion of CD8+ T cells while maintaining a naïve- or central memory-like phenotype. Herein we are focused on cytokines as modulators of tumor-reactive CD8+ T cells for ACT.

Central Memory versus Effector Memory CD8+ T Cells for ACT

Naïve CD8+ T cells, upon encountering antigen, undergo clonal expansion up to as much as 10^5-fold (Mueller et al., 2013). The expanded T cells are driven to differentiate to express cytolytic molecules and cytokines in order to clear the pathogen. Upon pathogen clearance, during the contraction phase, a subset of antigen-specific CD8+ T cells emerge that have modified their transcriptional program to express pro-survival and homing molecules; these cells occupy the memory compartment. Memory cells express CD127 (interleukin-7 receptor α) for cell survival and homeostasis, persist in higher quantities relative to their naïve precursors, and

rapidly recall effector molecules upon antigen re-exposure (Youngblood et al., 2013). Furthermore, transcription factors play a role in memory fate determination. Expression of the transcriptional repressor BCL-6 favours memory generation, while high expression of T-bet and Blimp-1 favours terminally differentiation effector cells (Mueller et al., 2013). Central memory T (T_{CM}) cells express CD62L (L-selectin) and CCR7, home to lymphoid tissue, produce interleukin-2 and proliferate extensively; effector memory T (T_{EM}) cells have lost expression of CD62L and CCR7, populate non-lymphoid tissues, produce effector cytokines, are less proliferative, and are the first to respond to antigen re-exposure (Mueller et al., 2013; Youngblood et al., 2013).

ACT studies in mice have shown that less differentiated subsets of CD8+ T cells, including naïve T and T_{CM} cells, exhibit superior persistence, expansion and anti-tumor activities (Klebanoff et al., 2012). Furthermore, retrospective analyses from human trials of ACT have likewise demonstrated that the adoptive transfer of less differentiated T cells highly correlates with clinical responses (Klebanoff et al., 2012). As such, it is clear that strategies employed to prevent terminal differentiation of T cells during the expansion phase for ACT will produce a cohort of cells most optimal for successful ACT.

Interleukin-2 in ACT

Interleukin (IL)-2 belongs to the common gamma chain (γ_c) receptor cytokine family, along with IL-4, IL-7, IL-9, IL-15 and IL-21. Members of this cytokine family signal through a receptor complex that includes the γ_c receptor subunit. Signaling pathways PI3K/AKT, Ras/MAPK and JAK/STAT are activated by members of this family, and are central to the maintenance of normal immune system function, affecting T cells, B cells, NK cells, mast cells and myeloid and

erythroid progenitors. In humans, mutations within the γ_c leads to X-linked severe combined immunodeficiency, characterized by the diminished ability to mount a humoral or cell-mediated immune response.

IL-2 is mainly produced by activated CD4+ T cells; however, activated CD8+ T cells, dendritic cells (DCs) and natural killer (NK) cells also produce IL-2. IL-2 drives clonal expansion, effector development, and memory T cell recall responses. Low-dose IL-2 has been shown to support the persistence of human melanoma-specific adoptively transferred CD8+ T cells *in vivo* (Yee et al., 2002). Historically, however, high doses of IL-2 have been required to obtain the quantity of cells required for ACT; such IL-2 administration induces rapid T cell differentiation (Gattinoni et al., 2005). Furthermore, the *in vivo* administration of IL-2 can induce activation-induced cell death, as well as promote the development of regulatory T cells, actions that can potentially counteract the anti-tumor response of ACT (Boyman et al., 2007; Waldmann, 2006). As such, IL-2 is not considered the optimal cytokine for modulating tumor-reactive CD8+ T cells for ACT.

1.5 Cytokines as Modulators of CD8+ T Cells Expanded for ACT

The phenotype and function of CD8+ T cells expanded for ACT can be modulated during *ex vivo* culture by the addition of exogenous cytokines (Hinrichs et al., 2008; Klebanoff et al., 2004; Li et al., 2005).

Interleukin-15

The γ_c receptor family includes IL-15. IL-15 is produced by macrophages and DCs and regulates activation and proliferation of NK cells and provides survival signals to memory T cells. Studies

have shown that CD8+ T cells activated in the presence of IL-2 are characterized by an T_{EM}-like phenotype (characterized by loss of CD62L and CCR7 expression, relative to naïve controls) while those activated in the presence of IL-15 polarize to a T_{CM}-like phenotype (characterized by CD62L and CCR7 expression) (Klebanoff et al., 2004; Weninger et al., 2001). Furthermore, these studies demonstrate an enhanced *in vivo* function of murine tumor-reactive CD8+ T cells activated *in vitro* in the presence of IL-15 versus IL-2 (Klebanoff et al., 2004; Weninger et al., 2001). These observations support the superiority of a less-differentiated, CD8+ T_{CM}-like population for optimal ACT, and suggest that IL-15 is a good candidate for modulating tumor-reactive CD8+ T cells for ACT.

Interleukin-21

IL-21 is the newest member of the γ_c receptor family. IL-21 is predominantly expressed by CD4+ T cells and NKT cells, although it can also be expressed by virally-infected CD8+ T cells (Spolski and Leonard, 2008; Williams et al., 2011). IL-21 is a pleiotropic cytokine and plays many roles within the immune system, including regulating plasma cell differentiation, CD8+ T cell and NK cell cytotoxicity (Spolski and Leonard, 2008). IL-21 has been shown to suppress the differentiation of murine naïve CD8+ T cells into cytolytic T cells, and murine T cells expanded in the presence of IL-21 and used for ACT exhibited augmented anti-tumor activity compared to cells expanded in the presence of IL-2 (Hinrichs et al., 2008). Human CD8+ T cells activated in the presence of IL-21 also exhibit a unique early effector/central memory-like phenotype. Similar to observations in the murine model, human CD8+ T cells activated in the presence of IL-21 exhibit high CD62L expression, a hallmark of central memory phenotype (Albrecht et al., 2011; Kaka et al., 2009; van Leeuwen et al., 2005; Wolfl et al., 2011).

Furthermore, human MART-1-specific CD8+ T cells activated in the presence of IL-21 express CCR7 and CD28, receptors responsible for homing to the lymph node and autocrine IL-2 production, respectively (Li et al., 2005).

Studies have also demonstrated that IL-21 confers a distinct genetic program on activated CD8+ T cells, imparting increased CD62L expression upon secondary antigen exposure (in contrast to CD8+ T cells activated in the presence of IL-15 alone, which lose CD62L expression upon re-stimulation) and overall enhanced anti-tumor activity (Hinrichs et al., 2008). Compared to IL-2 and IL-15, anti-melanoma CD8+ T cells activated in the presence of IL-21 were characterized by higher expression of *Lef1*, *Tcf7* and *Sell* (CD62L), all genes associated with an immature effector CD8+ T cell phenotype (Hinrichs et al., 2008). In contrast, CD8+ T cells activated in the presence of IL-2 or IL-15 alone expressed higher *Gzmb*, *Il2ra*, *Ifng*, and *Eomes*, all genes associated with a mature effector CD8+ T cell phenotype (Hinrichs et al., 2008).

Furthermore, combinatorial studies have demonstrated that IL-21 acts synergistically with IL-15, but not with IL-2, to promote melanoma tumor regression in mice (Zeng et al., 2005). CD8+ T cells activated in the presence of IL-15 plus IL-21 exhibit high expression of CD62L; furthermore, these cells exhibit greater expansion and enhanced effector function than CD8+ T cells activated in the presence of either IL-15 or IL-21 alone (Zeng et al., 2005).

We have previously shown that in human CD8+ T cells, IL-21 exposure promotes the development of an antigen-specific CD8+ T cell population with a unique early effector/central memory-like phenotype (CD45RO+CCR7-/+CD28+) and the capacity to generate IL-2 upon antigen-stimulation ('helper-independence') (Li et al., 2005; Li and Yee, 2008). IL-21 is unique in this ability amongst the various γ_c receptor cytokines IL-2, IL-7 and IL-15 (Li et al., 2005). Of particular interest is the ability of IL-21 to support the expression of CD28 on the surface of

antigen-specific CD8+ T cells (Li et al., 2005). Taken together, these observations suggest that IL-21 is a good candidate for modulating tumor-reactive CD8+ T cells for ACT.

Interleukin-6

IL-6 is expressed by a variety of cells, including monocytes, macrophage and T cells and mediates the acute inflammatory response (Wegenka et al., 1993). IL-6 is generally categorized as a proinflammatory cytokine; indeed, improper IL-6 signaling contributes to inflammatory diseases such as inflammatory bowel disease (Naugler and Karin, 2008). However, IL-6 can also mediate anti-inflammatory activities by decreasing the level of proinflammatory cytokines during an immune response (Xing et al., 1998). For instance, IL-6 can inhibit LPS-induced TNF-α production by monocytes (Schindler et al., 1990). IL-6 exerts its effects through STAT3 activation (Wegenka et al., 1993). Interestingly, IL-6 has recently been shown to induce IL-21 production in human CD4+ T cells (Diehl et al., 2012). It is not yet known if IL-6 induces IL-21 expression in human CD8+ T cells; however, CD8+ T cells are capable of IL-21 production under certain conditions such as viral infection (Williams et al., 2011).

Although there does not yet exist a precedent for IL-6 in CD8+ T cell modulation for ACT, based on its abilities to activate STAT3 (Wegenka et al., 1993), to inhibit TNF-α (a known inhibitor of CD28 expression (Bryl et al., 2001)) (Schindler et al., 1990), and to promote IL-21 expression in CD4+ T cells (Diehl et al., 2012), IL-6 holds potential for future use in strategies designed to modulate tumor-reactive CD8+ T cells for ACT.

1.6 CD28

CD28 is a homodimeric 44 kDa membrane glycoprotein expressed on the surface of T cells that

serves as a co-stimulatory receptor for TCR-mediated activation (Williams et al., 1992). Human CD4+ T cells and about half of human CD8+ T cells constitutively express surface CD28 (Riley and June, 2005). Individuals with chronic infection or of advanced age exhibit significantly lower surface CD28 expression on T cells, suggesting that immune senescence correlates with the loss of CD28 expression. Indeed, CD28-CD8+ T cells exhibit significantly shorter telomeres compared to CD28+CD8+ T cells (Monteiro et al., 1996). Contrary to human T cells, CD28 is expressed on the surface of all murine T cells, and is not downregulated with chronic infection or increased age (June et al., 1994). Despite the fact that a great wealth of knowledge exists regarding the role of CD28 in T cell-mediated immunity, the mechanisms that govern the regulation of CD28 surface expression have not been well defined.

Naïve T lymphocyte response to antigen requires the ligation of the T cell receptor (TCR) and a co-stimulatory molecule such as CD28 on the surface of the cell. Both naïve CD8+ T cells and central memory CD8+ T cells are characterized by CD28 surface expression. Ligation of CD28 on the T cell surface by its cognate ligand B7.1/B7.2 (CD80/CD86) results in stabilization of IL-2 messenger RNA (mRNA) which promotes maximal induction of autocrine IL-2 expression for lymphocyte proliferation (Lindstein et al., 1989; Thompson et al., 1989). Following TCR ligation, naïve CD8+ T cells initiate a differentiation program designed to mount a cytotoxic immune response against the antigen. Initiation of the differentiation program results in transient down-regulation of CD28 expression on the surface of the cells, resulting in the inability to produce autocrine IL-2, in effect rendering the cells helper-dependent. Interestingly, recent studies show that the introduction of a constitutively expressed *CD28* gene into human CD28-CD8+ T cells restores IL-2 production (Topp et al., 2003). The ability to produce autocrine IL-2 negates the need for CD4+ helper T cells, resulting in a 'helper-independent'

population of CD8+ T cells able to undergo a proliferative response following antigen recognition (Topp et al., 2003).

The *CD28* promoter contains a functionally singular inoperative transcriptional initiator (INR) element that consists of two motifs, α and β (Vallejo et al., 1998). These two motifs are located in the proximal region of the *CD28* promoter, immediately downstream from the TATA box, and regulate the basal transcription of CD28 (Vallejo et al., 1998). CD28- T cells are characterized by a loss of α- and β-bound protein complexes and exhibit a complete transcriptional block; CD28- cells lack mRNA of all known splice variants of CD28 (Vallejo et al., 1998). Nuclear extracts from CD28- cells did not activate transcription of αβ-initiator DNA templates; however, addition of nuclear extracts from CD28+ cells restored transcription of αβ-initiator DNA templates (Vallejo et al., 2001). Coordinate binding of α- and β-specific transcription factors is required for CD28 expression; mutation or deletion of either α or β motif is sufficient to inactivate the *CD28* promoter (Vallejo et al., 2002). Data are still emerging, although it appears as though the α- and β-specific transcription factors are ubiquitous proteins (Vallejo et al., 2002). Furthermore, CD28GR, a novel promoter element of *CD28*, has been identified (Lin and Tam, 2001). This regulatory element is critical for conferring constitutive transcriptional activation of the *CD28* gene (Lin and Tam, 2001).

Optimal responses to ACT require that cells generated for re-infusion into patients resist both terminal differentiation and exhaustion. Retention of CD28 expression on the surface of CD8+ T cells expanded for ACT contributes to the proliferation and persistence of transferred cells *in vivo*, through CD28-mediated autocrine IL-2 production. As such, defining the mechanisms by which IL-21 mediates CD28 surface expression is critical. Furthermore, defining the mechanisms involved in IL-21-mediated CD28 surface expression will allow for

optimization of alternate cell generation strategies for ACT, such as the expansion of polyclonal TIL (tumor infiltrating lymphocytes), for patients with metastatic melanoma and other types of cancers. We hypothesize that the unique preference of IL-21 for induction of the STAT3 signal transduction pathway is responsible for the ability of IL-21 to promote CD28 expression on activated human CD8+ T cells.

1.7 STAT Signal Transduction

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway transduces signals from the membrane to the nucleus extremely rapidly (Leonard and O'Shea, 1998). There are four mammalian JAK proteins, Jak1, Jak2, Jak3, and Tyk2, which phosphorylate cytokine receptors upon cognate cytokine binding (Leonard and O'Shea, 1998). There are seven mammalian STAT proteins, Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6, which associate with tyrosine residues within the cytokine receptor (or other STAT or JAK proteins) that have been phosphorylated by JAKs (Leonard and O'Shea, 1998). Upon association with phospho-tyrosine residues in the cytokine receptor, STATs are themselves phosphorylated, which promotes homo- or hetero-dimerization of two STAT proteins (Leonard and O'Shea, 1998). The STAT dimers then translocates to the nucleus where they associates with target DNA sequences and modulates gene expression (Leonard and O'Shea, 1998).

Members of the γ_c receptor family such as IL-2, IL-7 and IL-15 induce tyrosine phosphorylation of predominantly STAT5, while IL-21 has been shown to activate predominantly STAT3 (Zeng et al., 2007). The difference in STAT activation is thought to account for the synergy observed between IL-21 and other members of the γ_c receptor family, as outlined above. Indeed, we hypothesize that the difference in STAT protein activation is the

basis for the unique ability of IL-21 to support surface CD28 expression on activated human CD8+ T cells. It is not known if strictly the action of STAT3 alone can mimic IL-21 activity, or if other cytokines that activate STAT3, such as IL-6, can act similarly to IL-21 with respect to CD28 surface expression.

1.8 Remaining Questions

It has been demonstrated in the literature that the most efficacious T lymphocytes for adoptive cell therapy are those that resist terminal differentiation and exhaustion. The optimal characteristics of CD8+ T cells expanded for ACT are outlined above. While most studies have focused on modulating T cells for better outcomes in ACT, less investigation has gone into analyzing the mechanisms by which these modulators exert their effects. Specifically, understanding the mechanism of CD28 retention on the surface of expanded T cells, either antigen-specific or bulk polyclonal infiltrating, will allow current techniques to be more finely tuned. It is clear that IL-21 promotes the expression of CD28 on the surface of human antigen-specific CD8+ T cells; however, the mechanism of IL-21-mediated CD28 surface expression has not yet been elucidated. Furthermore, it is not known if other cytokines that share similar characteristics with IL-21 can also modulate CD28 surface expression. We begin to address these questions in the studies that follow.

Chapter 2: IL-21-mediated maintenance of CD28 on the surface of human antigen-specific CD8+ T cells is STAT3 dependent

Introduction

Although the role of CD28 in T lymphocyte-mediated immunity is well understood, the mechanisms that govern the regulation of CD28 expression on the surface of T lymphocytes are not well defined. CD28 is constitutively expressed on human CD4+ T cells, and on approximately half of human CD8+ T cells; CD28 expression is significantly lower on cells from individuals with chronic infection or advanced age, suggesting that loss of CD28 surface expression correlates with immune senescence (Riley and June, 2005). Furthermore, proinflammatory cytokine tumor necrosis factor (TNF)-α has been shown to reduce the level of cell surface expression of CD28 on human CD4+ T cells by inhibiting the activity of the *CD28* promoter (Bryl et al., 2001). In contrast to human T cells, CD28 is expressed on all murine T cells, and does not undergo downregulation with chronic infection or increasing age (June et al., 1994).

Successful antigen-specific T cell responses require ligation of both the T cell receptor (TCR) as well as a co-stimulatory molecule such as CD28 on the naïve CD8+ T cell. CD28 surface expression is a hallmark of both naïve and central memory T cells, and ligation of CD28 by its cognate ligand B7.1/B7.2 (CD80/CD86) promotes the expression of autocrine IL-2 for subsequent lymphocyte proliferation. Upon TCR ligation, naïve CD8+ T cells initiate a differentiation program designed to mount a cytotoxic response against antigen. The differentiation program results in a progressive loss of CD28 surface expression and diminished ability to produce IL-2.

Adoptive cell therapy (ACT) is the administration of *ex vivo* activated and expanded autologous tumor-specific T lymphocytes. ACT has been shown to induce clinical responses in metastatic melanoma patients previously refractory to conventional therapy (Dudley et al., 2005;

Hunder et al., 2008). Patient response rate is highly correlative with persistence of the infused T cells *in vivo* (Robbins et al., 2004), and proliferative potential of the infused cells is thought to be the key determinant in persistence (Zhou et al., 2005). Although low-dose IL-2 has been shown to support persistence of adoptively transferred T cells *in vivo* (Yee et al., 2002), historically, high-doses of IL-2 have been required to obtain the quantity of cells required for ACT; such IL-2 administration induces rapid terminal T cell differentiation (Gattinoni et al., 2005). Furthermore, *in vivo* administration of IL-2 can induce activation-induced cell death, as well as promote the development of regulatory T cells, actions that potentially counteract the anti-tumor response of ACT (Boyman et al., 2007; Waldmann, 2006). Increasing evidence suggests that strategies used to inhibit terminal differentiation of tumor-reactive CD8+ T cells during activation and expansion *ex vivo* will lead to an increase in their anti-tumor effect *in vivo* (Gattinoni et al., 2005; Robbins et al., 2004; Zhou et al., 2005).

Recent literature has demonstrated that IL-21 suppresses effector T cell differentiation and promotes memory qualities, such as surface CD62L and CD28 expression, in antigen-specific CD8+ T cells (Hinrichs et al., 2008; Li et al., 2005). IL-21 is a newly identified member of the common γ-chain receptor family of cytokines. IL-21 has pleiotropic effects on the immune system, including regulating plasma cell differentiation, CD8+ T cell cytotoxicity, and natural killer (NK) cell cytotoxicity (Spolski and Leonard, 2008). IL-21 is predominantly produced by CD4+ T cells and NKT cells (Spolski and Leonard, 2008). Additionally, virally-infected CD8+ T cells can also express IL-21 (Williams et al., 2011). Similar to other γ-chain cytokines, IL-21 signals through the JAK/STAT pathway; of note is IL-21's predominance for signaling via STAT3, while IL-2, IL-7 and IL-15 favour signal transduction via STAT5. The mechanisms by which IL-21 suppresses effector differentiation and promotes memory qualities

in CD8+ T cells have not yet been investigated. Therefore, we wanted to investigate the mechanism of IL-21-mediated surface CD28 expression on activated human CD8+ T cells.

It has been observed that the presence of IL-21 allows activated CD8+ T cells to retain surface CD28 expression (Li et al., 2005). CD28 surface expression is durable on this population of cells for up to 4 weeks, functional in that it promotes IL-2 production upon ligation, and is retained for various tumor-associated self-antigens (MART-1, gp100, NY-ESO-1) (Li et al., 2005). It is possible that retention of CD28 expression on the surface of cells involved in ACT will play a critical role in proliferation, and thus persistence, of transferred cells in the patient. Therefore, defining the role that IL-21 plays in the maintenance of CD28 on the surface of activated CD8+ T cells will allow optimization of ACT for individuals with metastatic melanoma and other types of cancer.

In this study, we investigate the ability of IL-21 to support CD28 surface expression on activated human CD8+ T cells through the activation of STAT3. We find that IL-21 promotes STAT3 activation in human CD8+ T cells, and that IL-21-mediated STAT3 activation is required both for the expansion of MART-1-specific human CD8+ T cells and for the surface expression of CD28 on MART-1-specific CD8+ T cells. Finally, using chromatin immunoprecipitation analysis, we find that IL-21-activated STAT3 associates with the human *CD28* promoter, supporting a direct role for IL-21 signaling in the expression of surface CD28 on antigen-specific CD8+ T cells expanded for adoptive cell therapy. Taken together, our observations indicate that IL-21 promotes CD28 surface expression on activated human CD8+ T cells through STAT3 activation, suggesting that STAT3 acts as a direct transcriptional activator of the *CD28* gene, and highlights the importance of IL-21 signaling in the design of future strategies for ACT.

Results

IL-21 exposure leads to upregulation of CD28 on human CD8+ T cells

To obtain sufficient numbers of IL-21-derived cytotoxic T lymphocytes (CTL; arising from a naïve population of CD8+ T cells) for downstream studies, we adapted our human CD8+ T cell model to include polyclonal activation. For this protocol, we activated naïve human CD8+ T cells with anti-CD3/anti-CD28 beads in the absence or presence or IL-21 and assayed surface CD28 expression by flow cytometry over time. We observed that CD3/CD28 stimulation in the presence of IL-21 promoted sustained CD28 surface expression on activated cells (Fig. 2-1). Furthermore, we observed that as early as day 4 post-stimulation, IL-21-mediated CD28 was higher on the surface of activated cells compared to those activated in the absence of exogenous cytokine. Consistent with previous results (Li et al., 2005), we observed that IL-21 promoted sustained CD28 surface expression on activated human CD8+ T cells, and by day 7, the majority of CD8+ T cells activated in the presence of IL-21 expressed high surface CD28.

Previous observations demonstrated that the combination of IL-21 plus IL-10 together promoted a memory phenotype in murine CD8+ T cells (Cui et al., 2011). STAT3 activation was achieved through signaling by these two cytokines and was required for memory precursor cells to populate the CD8+ T cell memory pool (Cui et al., 2011). The IL-21/IL-10/STAT3 signaling cascade promoted expression of transcription factors critical during the effector to memory transition of activated CD8+ T cells (Cui et al., 2011). In contrast to the findings in the murine system, in human CD8+ T cells, IL-21 alone promotes the expansion of antigen-specific CD8+ T cells with characteristics of a central memory phenotype, including the retention of CD28 surface expression (Li et al., 2005). In human CD8+ T cells, IL-21 exposure alone led to the enrichment of the antigen-specific CD8+ T cell compartment with a helper-independent

phenotype. Taken together, these observations prompted us to evaluate the ability of IL-21 alone to activate STAT3, and to investigate the mechanism by which IL-21 promotes memory characteristics in human CD8+ T cells.

IL-21 mediates STAT3 activation in human CD8+ T cells

IL-21 predominantly signals via STAT3 whereas other γ-chain cytokines such as IL-2, IL-7 and IL-15 predominantly signal via STAT5. To determine the mechanism(s) by which IL-21 supports CD28 expression on the surface of activated human CD8+ T cells, we evaluated the ability of IL-21 to activate STAT3 in human CD8+ T cells. Naïve human CD8+ T cells were either left untreated or were stimulated with anti-CD3/anti-CD28 beads and cultured in the absence or presence of IL-21. We then assessed active STAT3 by flow cytometry using a STAT3 phospho-specific antibody. As early as 20 minutes following culture with IL-21, we observed STAT3 activation in nearly 90% of human CD8+ T cells (Fig. 2-2A). This observation suggests that STAT3 activity correlates with the ability of IL-21 to sustain CD28 expression on expanded antigen-specific CD8+ T cells.

IL-21 promotes sustained STAT3 activation in human CD8+ T cells

Typically, STAT activation occurs quickly (Leonard and O'Shea, 1998), and this was confirmed in the human CD8+ T cells with the majority of cells possessing active STAT3 after 20 minutes in culture with IL-21 (Fig. 2-2A). Next, we were interested in determining how long STAT3 activation was sustained by IL-21. Flow cytometry analysis demonstrated that IL-21-mediated active STAT3 persisted in the majority of cells at 24 hours following polyclonal CD8+ T cell stimulation (Fig. 2-2B). Of note, cells stimulated for 24 hours in the absence of exogenous

cytokine exhibited low levels of active STAT3 following stimulation, suggesting that CD3/CD28 stimulation alone caused a small induction of STAT3 activity. However, IL-21 substantially enhanced both the percentage of cells possessing active STAT3 and the amount of active STAT3 in the cells at 24 hours post-stimulation, compared to the no cytokine control. Extending the time course to 4 days, we observed that approximately half of the cells stimulated in the presence of IL-21 for 4 days still retained active STAT3 (Fig. 2-2B). These observations indicate that IL-21 promotes sustained STAT3 activation in CD8+ T cells that peaks on or near 24 hours post-stimulation, and remains high for up to 4 days.

STAT3 activation is required for IL-21-mediated expansion of human antigen-specific CD8+ T cells characterized by surface CD28 expression

We were next interested in defining the requirements for active STAT3 for the IL-21-mediated expansion of antigen-specific CD8+ T cells that retain surface expression of CD28. NSC 74859 is a STAT3-specific chemical inhibitor that selectively inhibits STAT3 transcriptional activity (Siddiquee et al., 2007). We first wanted to examine whether NSC 74859 impacts proliferation. CD8+ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and stimulation assays were performed in the presence of increasing concentrations of NSC 74859 (range: 100 – 1000 uM). Based on CFSE dilution, we observed that proliferation of stimulated CD8+ T cells in the presence of NSC 74859 was reduced by only one cell division by day 7, compared to cells stimulated in the absence of the inhibitor (Supplemental Fig. 2-S1). Using flow cytometry, we observed that IL-21-mediated activation of STAT3 was inhibited in cells stimulated in the presence of IL-21 plus NSC 74859 (Fig. 2-2C). Furthermore, the observation that the levels of phospho-STAT3 were reduced to background with the addition of NSC 74859 supports our

previous observation (Fig. 2-2B) that CD3/CD28 stimulation alone induces low levels of STAT3 activity. Taken together, these experiments indicate that IL-21 possesses the ability to promote sustained active STAT3 that is abrogated by the use of a chemical STAT3 inhibitor. The ability to sustain active STAT3 supports the hypothesis that active STAT3 is required for IL-21-mediated CD28 surface expression on expanded antigen-specific CD8+ T cells.

IL-21 promotes the expansion of human antigen-specific CD8+ T cells with sustained CD28 surface expression (Li et al., 2005). To determine if STAT3 activation is required for IL-21-mediated maintenance of CD28 expression on the surface of antigen-specific CD8+ T cells, we assayed CD28 expression on cells expanded in the presence of both IL-21 and the STAT3 inhibitor NSC 74859. We used irradiated autologous mature dendritic cells (DCs) pulsed with MART-1 peptide to stimulate naïve human CD8+ T cells. Following two rounds of stimulation, flow cytometry was used to quantify antigen-specific CD8+ T cells and to assess surface CD28 expression. Consistent with previous observations, we observed that MART-1-specific CD8+ T cells were expanded 5-fold in the presence of IL-21 compared to those stimulated in the absence of exogenous cytokine (Fig. 2-3A; two left panels). Furthermore, we found that cells that underwent antigen-specific stimulation in the presence of both IL-21 and the STAT3 inhibitor NSC 74859 expanded to the same degree (Fig. 2-3A; right panel) compared to the cells stimulated in the absence of exogenous cytokine. These observations suggest that STAT3 activation is indeed required for the enhanced expansion of antigen-specific CD8+ T cells. In addition, we show here that nearly 80% of the antigen-specific CD8+ T cell population retains CD28 surface expression when stimulated in the presence of IL-21, compared to only 4% of the antigen-specific population that expresses surface CD28 when expanded in the absence of exogenous cytokine (Fig. 2-3B; two left panels). Furthermore, the majority of cells in the

antigen-specific CD8+ T cell population present when stimulated in the presence of both IL-21 and NSC 74859 do not retain surface CD28 expression (Fig. 2-3B; right panel). The data support that STAT3 activation is required for the sustained expression of CD28 on the surface of antigen-specific CD8+ T cells. Taken together, these observations indicate that STAT3 activation is critical for both IL-21-mediated expansion of the antigen-specific CD8+ T cell population, as well as for maintenance of CD28 expression on the surface of the antigen-specific population of cells.

IL-21-mediated active STAT3 associates with the human *CD28* promoter

Our data indicate that IL-21 promotes CD28 surface expression through the activation of STAT3. As such, we were interested in determining if this was a direct result of active STAT3 associating with the promoter region of the human *CD28* gene. Using Matinspector software, we determined that the human *CD28* promoter contains three consensus STAT binding sequences (data not shown). The upstream binding site is referred to here as distal, while the second and third sites are referred to here as proximal, due to their proximity to the transcriptional start site. Using chromatin immunoprecipitation (ChIP), we assayed the ability of IL-21-activated STAT3 to associate with the *CD28* promoter at the distal and proximal consensus STAT binding sites. We found that in naïve human CD8+ T cells stimulated for 24 hours in the presence of IL-21, activated STAT3 associates with the *CD28* promoter at the distal consensus STAT binding site (Fig. 2-4a). In contrast, the association of STAT3 with the proximal consensus STAT binding site in CD8+ T cells stimulated in the presence of IL-21 was not significantly different from the amount of STAT3 associated with the same site in CD8+ T cells stimulated in the absence of exogenous cytokine (Fig. 2-4b). These observations suggest that STAT3 association with the

proximal consensus STAT binding site is dispensable for *CD28* transcriptional activation. Furthermore, these observations support a direct role for IL-21-mediated STAT3 in the transcriptional activation of *CD28*, but do not prove conclusively that STAT3 association with the *CD28* promoter results in CD28 expression. Further studies are required to quantify CD28 messenger RNA (mRNA) levels in CD8+ T cells stimulated in the absence or presence of IL-21 in order to correlate STAT3 association with the *CD28* promoter with induction of CD28 transcription. In this case, we would expect to see an increase in CD28 mRNA expression upon stimulation in the presence of IL-21, suggesting that STAT3 association with the *CD28* promoter is responsible for CD28 transcription and subsequent surface expression. However, if we do not observe an increase in CD28 mRNA upon stimulation in the presence of IL-21, it suggests that other factors are driving the surface expression of CD28, such as the existence of intracellular CD28 protein stores or surface CD28 recycling. To address these, we could evaluate CD28 expression in the presence of IL-21 and cycloheximide to determine if *de novo* CD28 protein synthesis is required for IL-21-mediated surface expression, or introduce a fluorescently labeled *CD28* gene into cells to evaluate trafficking of surface CD28 in the presence of IL-21.

IL-21 promotes ACT efficacy

Recent literature strongly supports the hypothesis that less-differentiated T lymphocytes are the most efficacious cell type for adoptive cell therapy for various cancers, including metastatic melanoma. These optimal, less-differentiated cells are central memory-like and are characterized by the ability to proliferate extensively in the patient, produce IL-2 (via CD28 ligation), and express surface CD62L and CCR7 for homing to secondary lymphoid organs. Others have demonstrated that IL-21 has the ability to suppress effector differentiation and

promote memory qualities such as surface CD62L expression (Hinrichs et al., 2008). In addition, it has previously been shown that IL-21 promotes CD28 expression (Li et al., 2005). Here, we demonstrate that the ability of IL-21 to promote CD28 surface expression on tumor-specific cytotoxic CD8+ T cells is dependent upon the activation of STAT3. Furthermore, we show that IL-21-activated STAT3 associates with the distal consensus STAT binding site in the human *CD28* promoter. Together, our results support a direct role for IL-21 in CD28 expression in human CD8+ T cells.

As seen here, IL-21, through activation of STAT3, has the ability to promote expansion of a human tumor-specific cytotoxic CD8+ T cell population with retained surface CD28 expression. This population of cells represents a more effective cytotoxic T lymphocyte population for ACT. Importantly, IL-21-expanded antigen-specific CD8+ T cells with surface CD28 expression do not require any genetic modification to obtain high levels of surface CD28 (Topp et al., 2003). In addition, maintenance of CD28 on the surface of activated cells, mediated by IL-21, promotes a cell population that is helper-independent. That is, these cells are self-propagating due to IL-2 secretion upon CD28 ligation and thus do not need to rely on CD4+ T cell help for IL-2 production. Such characteristics would potentially allow these cells to proliferate, and thus to persist, in the patient. This is a critical goal to achieve, because the response rate of patients to ACT is highly correlated with persistence of infused T cells *in vivo* (Robbins et al., 2004).

IL-21 presents an attractive choice for enhancing ACT strategies for several reasons. These include that IL-21 is effective for promoting CD28 expression on T cells expressing tumor antigens in addition to MART-1 antigen (Li et al., 2005), the addition of IL-21 during the T cell expansion phase is not technically challenging, IL-21 is widely available, and IL-21 is FDA-

approved and is currently being used in clinical trials. Furthermore, this strategy for enhancing the efficacy of antigen-specific CD8+ T cells for ACT through use of IL-21 could also be widely applicable, for example in TIL (tumor infiltrating lymphocytes) generation strategies, promoting the expansion of a polyclonal T cell population that retains CD28 expression.

Chapter 2 Figure Legends

Figure 2-1. IL-21 promotes CD28 expression on human CD8+ T cells. Representative FACS plots of surface CD28 expression on naïve human CD8+ T cells stimulated with anti-CD3/anti-CD28 beads over time (4 – 11 days) in the absence of cytokine or 30 ng/ml IL-21.

Figure 2-2. IL-21-mediated STAT3 activation is abrogated by chemical inhibition in human CD8+ T cells. (a) Representative FACS plots of active STAT3 (pSTAT3) in naïve human CD8+ T cells cultured for 20 minutes in the absence of cytokine (left panel) or 30 ng/ml IL-21 (right panel). (b) Representative FACS plots of active STAT3 in naïve human CD8+ T cells stimulated with anti-CD3/anti-CD28 beads for 24 hours or 4 days (left and right panel, respectively) in the absence of cytokine or 30 ng/ml IL-21. (c) Representative FACS plots of active STAT3 in naïve human CD8+ T cells stimulated with anti-CD3/anti-CD28 beads for 24 hours in the presence of 30 ng/ml IL-21 with and without 500 uM NSC 74859 (chemical inhibitor of STAT3 activation).

Figure 2-3. Antigen-specific expansion of human CD8+ T cells in the presence of IL-21 promotes STAT3-dependent expression of surface CD28. Representative FACS plots of expansion of antigen-specific CD8+ T cells activated from naïve human CD8+ T cells with MART-1 peptide-pulsed autologous mature DCs for 14 days in the presence of 30 ng/ml IL-21 with and without 500 uM STAT3 inhibitor NSC 74859. (a) The population of expanded antigen-specific CD8+ T cells was identified with peptide-MHC-tetramer. Data expressed as percentage of tetramer-positive cells within the lymphocyte population. (b) The population of antigen-specific CD8+ T cells expressing surface CD28. Data expressed as percentage of CD28-expressing cells within the gated tetramer-positive (as seen in (a) above) population.

Figure 2-4. IL-21-mediated active STAT3 associates with the promoter of the human *CD28* gene. Pooled ChIP analysis from three experiments of naïve human CD8+ T cells stimulated with anti-CD3/anti-CD28 beads for 24 hours in the absence of cytokine or 30 ng/ml IL-21. (a) STAT3 association with distal consensus STAT binding site in human *CD28* promoter. (b) STAT3 association with proximal consensus STAT binding site in human *CD28* promoter.

Supplemental Figure 2-S1. STAT3 chemical inhibitor NSC 74859 slows, but does not inhibit, proliferation of stimulated human CD8+ T cells. Representative FACS plots of CFSE dilution in naïve human CD8+ T cells stimulated with anti-CD3/anti-CD28 beads in the presence of various concentrations of STAT3 inhibitor NSC 74859 (range: 100 – 1000 uM) for 2, 5 and 7 days.

Chapter 2 Figures

FIGURE 2-1

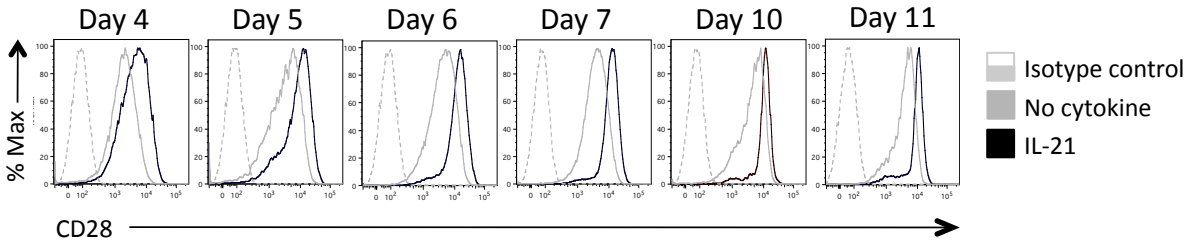

Day 4
Day 5
Day 6
Day 7
Day 10
Day 11
% Max
CD28
Isotype control
No cytokine
IL-21

FIGURE 2-2

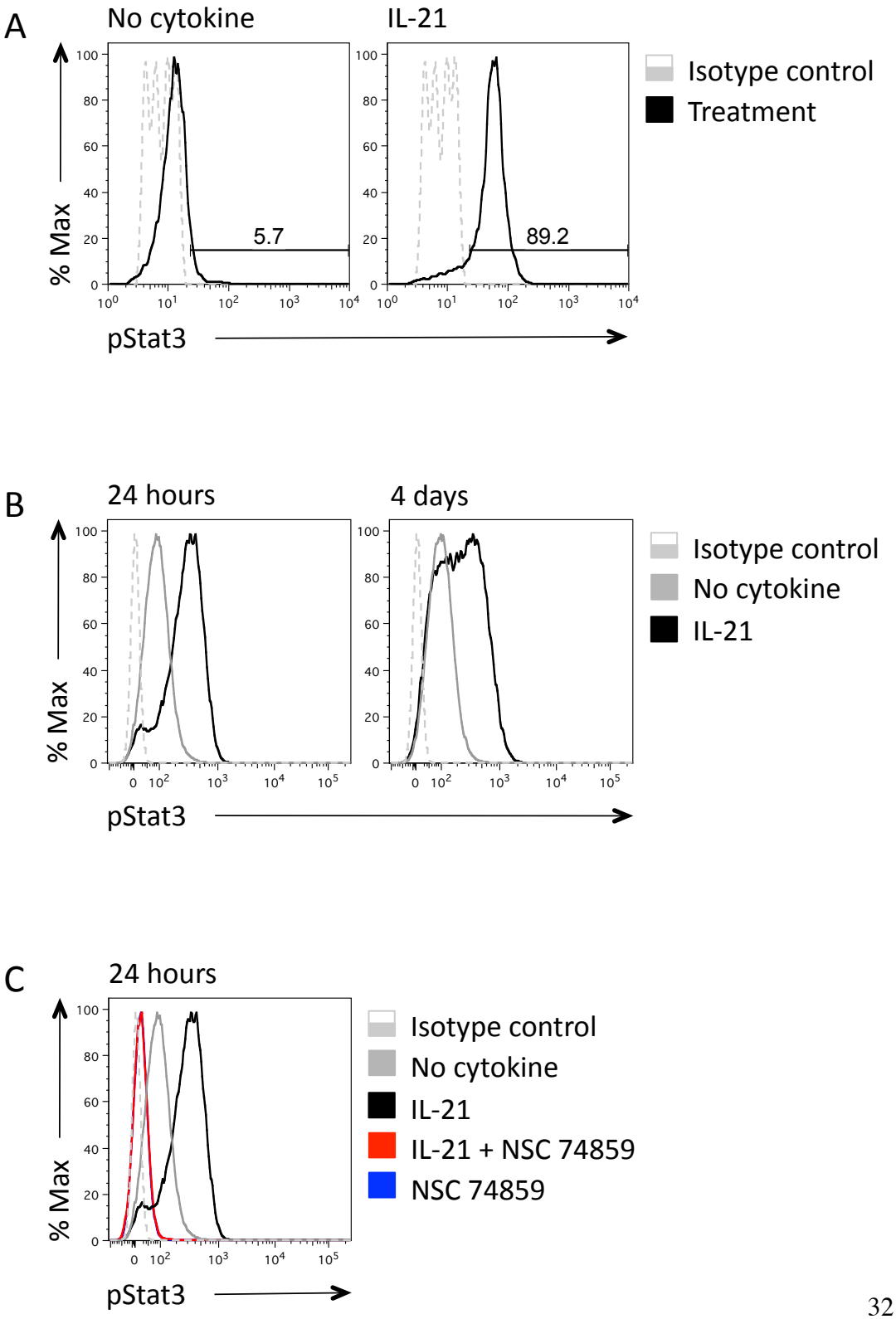

A
No cytokine
IL-21
Isotype control
Treatment
% Max
5.7
89.2
pStat3
B
24 hours
4 days
Isotype control
No cytokine
IL-21
% Max
pStat3
C
24 hours
Isotype control
No cytokine
IL-21
IL-21 + NSC 74859
NSC 74859
% Max
pStat3

FIGURE 2-3

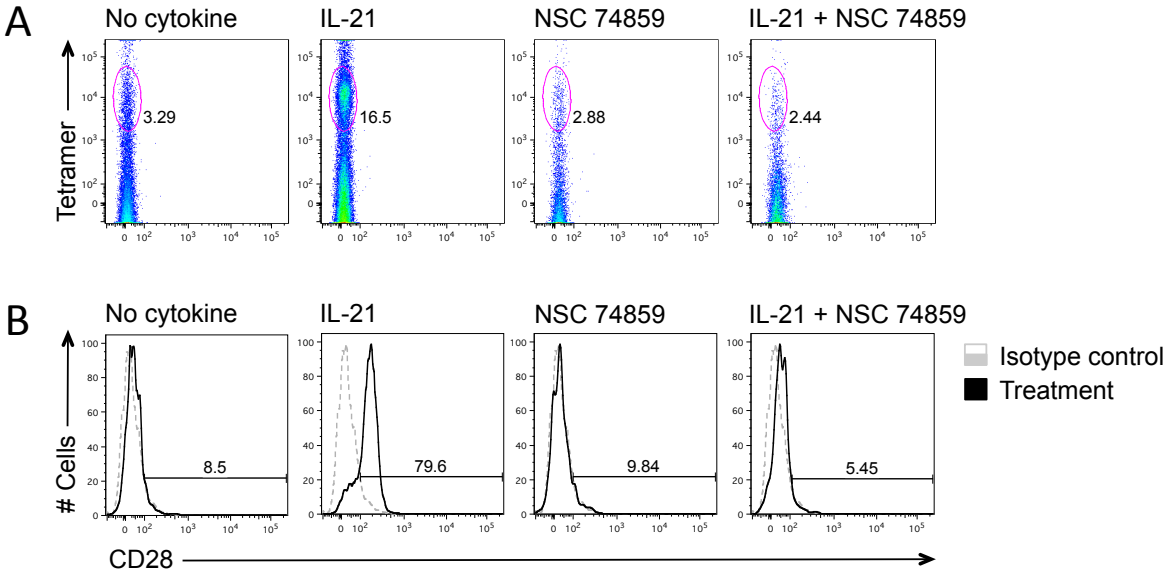
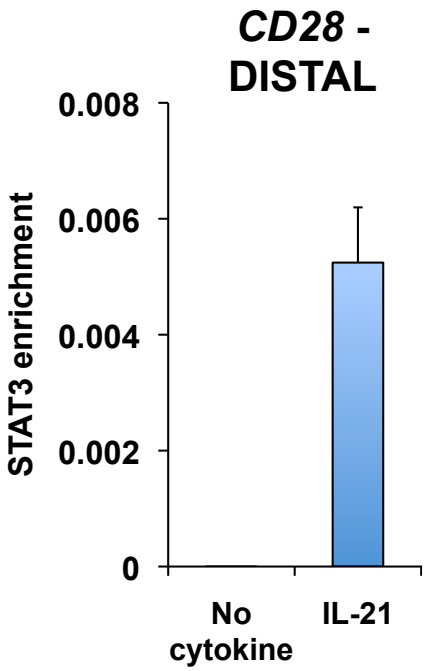
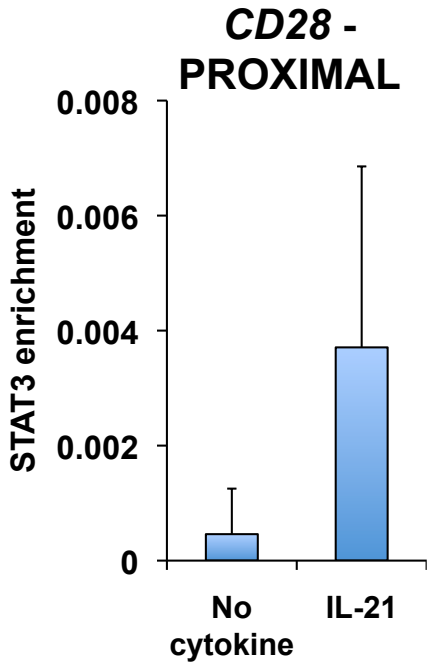

A
No cytokine
IL-21
NSC 74859
IL-21 + NSC 74859
Tetramer
3.29
16.5
2.88
2.44
B
No cytokine
IL-21
NSC 74859
IL-21 + NSC 74859
Cells
8.5
79.6
9.84
5.45
CD28
Isotype control
Treatment

FIGURE 2-4

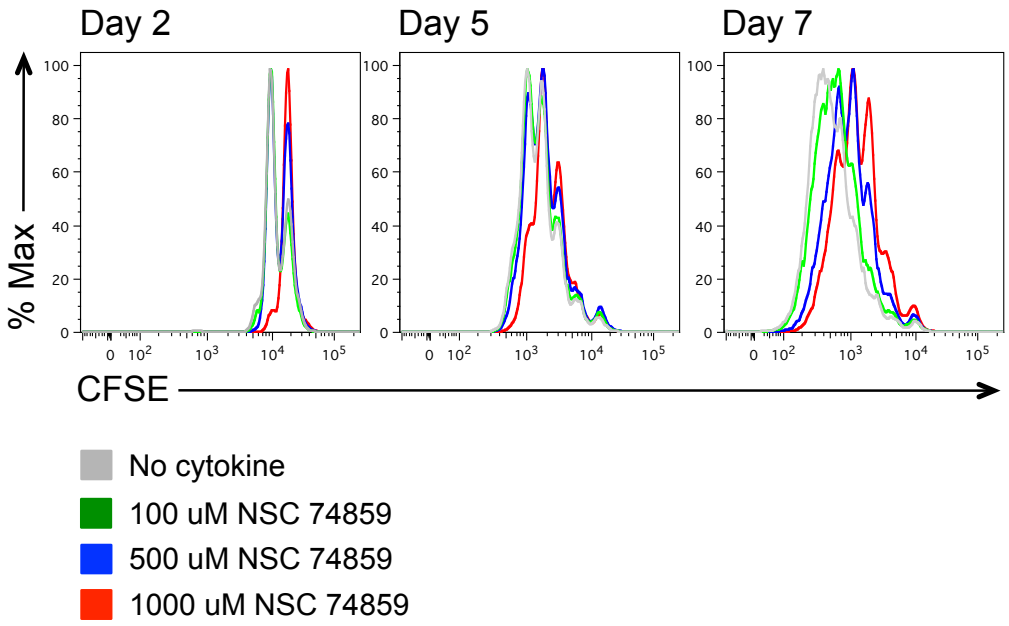
A


CD28 -
DISTAL
STAT3 enrichment
0.008
0.006
0.004
0.002
0
No cytokine
IL-21

B


CD28 -
PROXIMAL
STAT3 enrichment
0.008
0.006
0.004
0.002
0
No cytokine
IL-21

SUPPLEMENTAL FIGURE 2-S1



Chapter 3: Characterizing the roles of IL-6 and IL-15 in regulating CD28 surface expression on activated human CD8+ T cells

Introduction

CD28 expression on the surface of T lymphocytes allows for self-propagation through autocrine interleukin (IL)-2 production upon cognate ligand binding; autocrine IL-2 production promotes T cell persistence *in vivo*. Persistence of T cells *in vivo* correlates with the patient response rate to adoptive T cell therapy (ACT) (Yee et al., 2002). As such, surface CD28 expression is an attractive feature for T cells whose ultimate fate is re-infusion into a patient for ACT. We are investigating strategies to promote CD28 expression on the surface of antigen-specific CD8+ T cells for effective ACT of metastatic melanoma.

The mechanisms that govern the regulation of CD28 expression on the surface of activated human T lymphocytes have not been thoroughly defined. Tumor necrosis factor (TNF)-α has been shown to prevent CD28 expression in T cells by inhibiting the activity of the *CD28* promoter (Bryl et al., 2001); however, much less is known about the mechanisms responsible for induction of CD28 expression. Our recent data indicate that signal transducer and activator of transcription (STAT) 3 activation is required for the expression of surface CD28 on activated human CD8+ T cells (Chapter 2). Furthermore, our data demonstrate that IL-21 signaling is sufficient to provide the STAT3 activation signal necessary for CD28 expression on activated human CD8+ T cells (Chapter 2).

It is unknown if IL-21 exclusively provides the signals required for the induction of surface CD28 expression on human CD8+ T cells, or rather, if other cytokines can perform a similar role in CD28 induction. Arguing in favour of a role for additional cytokines in this process, similar to IL-21, other cytokines are known to activate STAT3. For instance, IL-6 is a conventional STAT3 activating cytokine (Wegenka et al., 1993). IL-6 is a pleiotropic cytokine that is expressed by a variety of cells, including monocytes, macrophages and T cells, and is a

major mediator of the acute inflammatory response (Wegenka et al., 1993). IL-6 is generally considered a proinflammatory cytokine; dysregulated IL-6 signaling has been shown to contribute to inflammatory diseases such as inflammatory bowel disease (Naugler and Karin, 2008). IL-6 can also exhibit anti-inflammatory activities by mitigating the level of proinflammatory cytokines during an immune response; IL-6 has been shown to inhibit LPS-induced TNF-α production in monocytes (Schindler et al., 1990; Xing et al., 1998). Based on its ability to activate STAT3 and to inhibit TNF-α, a known inhibitor of CD28 expression (Bryl et al., 2001), we hypothesized that IL-6 would promote the induction of CD28 surface expression.

Furthermore, the IL-21 receptor shares the common gamma chain (γ_c) subunit with other cytokine receptors from the γ_c cytokine family, including the IL-15 receptor. IL-15 is produced by non-T cells, such as macrophages and dendritic cells (DCs). IL-15 is responsible for a host of immune functions, including promoting activation and proliferation of T cells and natural killer cells (Ma et al., 2006). IL-15 also supports survival of the memory T cell pool (Ma et al., 2006). IL-15 is complexed with the membrane-bound IL-15Rα subunit on the surface of IL-15-expressing cells, and is presented in *trans* to recipient cells (Waldmann, 2006). Recipient cells express the IL-15 receptor complex comprised of IL-15Rβ subunit complexed with the γ_c subunit (Waldmann, 2006). The γ_c subunit is responsible for transducing the cognate cytokine signal. The shared usage of the γ_c subunit between IL-21 and IL-15 receptors leads to the hypothesis that these two cytokines might initiate some similar downstream signaling events upon cognate cytokine binding.

Members of the γ_c cytokine family activate overlapping sets of signal transducer and activator of transcription (STAT) proteins. Both STAT3 and STAT5 are activated by most of the γ_c cytokine family including IL-2, IL-7, IL-15 and IL-21 (Kovanen and Leonard, 2004);

however, IL-21 predominantly signals via STAT3 (Habib et al., 2003) and IL-15 predominantly signals via STAT5 (Johnston et al., 1995). Recent data demonstrates competition between STAT3 and STAT5 for association at various genetic loci, such as at the *Il17* (Yang et al., 2011) and *Bcl6* (Walker et al., 2013) loci.

Intriguingly, IL-15 and IL-21 appear to behave differently with respect to CD28 expression. In contrast to the sustained expression of CD28 observed on activated CD8+ T cells responding to IL-21 (Chapter 2), it was recently demonstrated that human CD28+CD8+ memory T cells actively dividing in the presence of IL-15 lose surface expression of CD28 (Chiu et al., 2006). The loss of CD28 expression was attributed in part to the induction of TNF-α by IL-15 in these cells, implicating a negative feedback loop that downregulates CD28 expression (Bryl et al., 2001; Chiu et al., 2006). Similarly, naïve CD8+ T cells cultured in the presence of IL-15 lose expression of CD28 (Alves et al., 2005). The contribution of T cell receptor (TCR) signaling to CD28 expression in the presence of IL-15 has not been investigated. In light of the above observations, we hypothesized that TCR signals would provide sufficient STAT3 activation to promote CD28 surface expression, despite the presence of IL-15.

In these studies, we investigated the ability of cytokines IL-6 and IL-15 to support CD28 surface expression on activated human CD8+ T cells. We find that IL-6 promotes short-lived STAT3 activation that is not sufficient to support CD28 expression on activated CD8+ T cells. Our observations utilizing chromatin immunoprecipitation (ChIP) analysis suggest that IL-6 is unable to support CD28 expression due to the inability of IL-6 to promote the association of STAT3 with the *CD28* promoter. Furthermore, we find that IL-15 exposure during activation of human CD8+ T cells inhibits the expression of CD28. Our observations suggest that IL-15 actively prevents CD28 expression; ChIP analysis suggests that STAT5 competes with STAT3

for the binding site within the *CD28* promoter, thus inhibiting CD28 expression in the presence of IL-15. Taken together, our observations underscore the superiority of IL-21, compared to both IL-6 and IL-15, in its ability to promote CD28 surface expression on activated human CD8+ T cells; neither IL-6 nor IL-15 possess the ability to support sustained CD28 surface expression, which is a desired trait for cells destined for ACT.

Results

IL-6 promotes short-lived STAT3 activation in human CD8+ T cells

Recent observations discussed in Chapter 2 demonstrated that IL-21-mediated CD28 expression on the surface of activated human CD8+ T cells required STAT3 activation. In our original experiments evaluating IL-21-mediated STAT3 activation, we included IL-6 as a control, as it is a known strong activator of STAT3 (Zhong et al., 1994). We anticipated that human CD8+ T cells cultured in the presence of IL-6 would possess high quantities of active STAT3 for the duration of our experiments. Interestingly, our observations using IL-6 as a control for STAT3 activation diverged from those of IL-21 after early induction of STAT3 activation. As such, we were prompted to further evaluate the ability of IL-6 to support CD28 expression on activated human CD8+ T cells.

Naïve human CD8+ T cells were cultured in the absence of cytokine, IL-6 or IL-21 for 20 minutes. Following, active STAT3 was assessed by flow cytometry using a STAT3 phospho-specific antibody. Consistent with data demonstrated previously in Chapter 2, we observed STAT3 activation in the majority of human CD8+ T cells when cultured in the presence of IL-21 (Fig. 3-1A). Likewise, IL-6 promoted STAT3 activation equivalent to the level induced by IL-21 at 20 minutes (Fig. 3-1A).

We next wanted to assess whether IL-6 could sustain STAT3 activation similar to IL-21. Naïve human CD8+ T cells were stimulated with anti-CD3/anti-CD28 beads and cultured in the absence of cytokine, IL-6 or IL-21. Active STAT3 was assessed by flow cytometry at 24 hours and 4 days. Surprisingly, when we analyzed STAT3 activation over time, we observed that IL-6 and IL-21 differed in their ability to sustain active STAT3. Consistent with data displayed in Chapter 2, CD8+ T cells stimulated in the presence of IL-21 exhibited high active STAT3 at 24 hours that was only moderately reduced by 4 days (Fig. 3-1B). Unlike IL-21, however, IL-6 was less efficient at sustaining active STAT3 in CD8+ T cells stimulated in the presence of the cytokine for 24 hours (Fig. 3-1B). Furthermore, IL-6 was much less efficient at sustaining active STAT3 in CD8+ T cells stimulated in the presence of the cytokine for 4 days; IL-6-mediated active STAT3 levels returned to near baseline by 4 days (Fig. 3-1B). It is important to note that an extensive dose response of IL-6 was not performed in these studies; it is possible that alternate concentrations of IL-6 might more efficiently promote sustained active STAT3 in stimulated CD8+ T cells for longer time periods.

These observations indicate that despite IL-6 and IL-21 individually inducing equivalent STAT3 activation at 20 minutes, IL-6 was less efficiently able to support a sustained pool of active STAT3 in activated human CD8+ T cells. These observations are in contrast to the actions of IL-21, which we observed to sustain active STAT3 in stimulated CD8+ T cells up to 4 days. Taken together, these observations suggest that various STAT3-activating cytokines can exert different temporal effects with regard to STAT3 activation, which might be responsible for the divergent downstream effects observed in human CD8+ T cells.

IL-6 does not support expansion of human antigen-specific CD8+ T cells characterized by surface CD28 expression

Based on the observation that IL-6 promotes STAT3 activation equivalent to that of IL-21 at early (20 minutes) but not late (4 days) time points, we were interested in evaluating the ability of IL-6 to support the expansion of antigen-specific CD8+ T cells characterized by surface CD28 expression. Naïve human CD8+ T cell were stimulated with autologous mature DCs pulsed with MART-1 peptide. Antigen-specific CD8+ T cells were quantified and surface expression of CD28 was assessed using flow cytometry after two rounds of stimulation. Consistent with data demonstrated in Chapter 2, MART-1-specific CD8+ T cells were expanded 4-fold in the presence of IL-21, compared to cells stimulated in the absence of exogenous cytokine (Fig. 3-2A; right and left panel, respectively). In the presence of IL-6, however, MART-1-specific CD8+ T cells did not expand beyond that of the control group with no exogenous cytokine (Fig. 3-2A; middle and left panel, respectively).

Furthermore, IL-6 supported only low levels of CD28 expression on the surface of MART-1-specific CD8+ T cells. Consistent with data from Chapter 2, antigen-specific CD8+ T cells expanded in the presence of IL-21 expressed high surface CD28 (Fig. 3-2B; right panel). In contrast, the small population of antigen-specific CD8+ T cells expanded in the presence of IL-6 expressed low surface CD28 (Fig. 3-2B; middle panel). Taken together, these observations indicate that initial STAT3 activation, induced by both IL-6 and IL-21 (individually) at 20 minutes (as seen in Figure 3-1A), does not correlate with expansion of human antigen-specific CD8+ T cells. Furthermore, initial activation of STAT3, as observed by IL-6, might be sufficient to promote low CD28 surface expression, but sustained STAT3 activation, as observed by IL-21, is required for high CD28 surface expression on human CD8+ T cells.

IL-6-mediated active STAT3 associates inefficiently with the human *CD28* promoter

To further investigate the differences in CD28 expression observed between CD8+ T cells stimulated in the presence of IL-6 (low CD28 expression) versus IL-21 (high CD28 expression), we evaluated the ability of IL-6 to induce the association of STAT3 with the promoter region of the human *CD28* gene using chromatin immunoprecipitation (ChIP) analysis. Naïve human CD8+ T cells were stimulated with anti-CD3/anti-CD28 beads and cultured in the absence of cytokine or in the presence of IL-6 for 24 hours. We then assessed whether STAT3 could associate with the *CD28* promoter at the distal and proximal binding sites in response to IL-6. Observations described in Chapter 2 indicate that IL-21-mediated STAT3 associated with the *CD28* promoter at the distal, but not the proximal, consensus STAT binding sites in CD8+ T cells. In contrast, STAT3 associated inefficiently with the *CD28* promoter at the distal consensus STAT binding sites in CD8+ T cells stimulated in the presence of IL-6 (Fig. 3-3A). IL-6-mediated STAT3 associated even less efficiently at the proximal consensus STAT binding site (Fig. 3-3B).

These observations suggest that IL-6 fails to induce CD28 surface expression on CD8+ T cells because IL-6 cannot efficiently sustain STAT3 activation or the association of STAT3 with the *CD28* promoter. Thus, in contrast to IL-21, IL-6 does not efficiently promote this memory characteristic in activated human CD8+ T cells. Taken together, the observations indicating that IL-6 signaling promotes STAT3 activation equivalent to that of IL-21 at 20 minutes, but does not efficiently promote sustained active STAT3 at later time points, speaks to the temporal nature of STAT3 signaling. These observations support a mechanism(s) by which various cytokines that employ similar signal transduction pathways can result in diverse cellular outcomes. Furthermore, these observations highlight the importance of evaluating signal transduction over

time, as cytokine-induced characteristics that are present immediately following activation of cells might persist for varying amounts of time, as is evidenced in our studies.

IL-15 exposure during activation of human CD8+ T cells suppresses the expression of surface CD28

IL-15 is an attractive candidate for ACT as it promotes the development of a less-differentiated, central memory-like CD8+ T cell population (Klebanoff et al., 2004; Weninger et al., 2001). Furthermore, IL-15 functions to support the survival of the antigen-specific memory T cell pool. Observations by others indicate that IL-15 inhibits CD28 expression in the context of proliferating naïve or memory CD8+ T cells (Alves et al., 2005; Chiu et al., 2006). However, the mechanisms that govern the induction of expression of CD28 on human CD8+ T cells upon activation in the presence of IL-15 have not been fully investigated. Furthermore, as described in Chapter 2, CD8+ T cell stimulation alone (i.e. in the absence of exogenous cytokine) resulted in a small induction of STAT3 activity. As such, we were interested in investigating the expression of CD28 on naïve human CD8+ T cells activated in the presence of IL-15, to determine how the addition of stimulation signals (induction of active STAT3) to IL-15 signals (induction of active STAT5) would affect CD28 expression. We hypothesized that TCR signals would provide sufficient STAT3 activation to promote low levels of CD28 surface expression, even in the presence of IL-15.

Naïve human CD8+ T cells were stimulated with anti-CD3/anti-CD28 beads in the absence of cytokine, IL-15 or IL-21. Surface CD28 expression was assessed by flow cytometry over time. Consistent with data demonstrated in Chapter 2, IL-21 promoted sustained CD28 surface expression on activated human CD8+ T cells; by day 7, the majority of activated CD8+ T

cells expressed high surface CD28 in the presence of IL-21 (Fig. 3-4). Furthermore, CD8+ T cells that received TCR signals in the absence of exogenous cytokine also expressed surface CD28, albeit to lower levels that CD8+ T cells stimulated in the presence of IL-21 (Fig. 3-4). In contrast, IL-15 did not promote surface expression of CD28 on activated CD8+ T cells (Fig. 3-4). Consistent with observations in unstimulated cells cultured in IL-15 (Alves et al., 2005), CD28 levels remained very low in cells stimulated in the presence of IL-15 (Fig. 3-4). Somewhat surprisingly, CD8+ T cells stimulated in the presence of IL-15 expressed even less surface CD28 compared to cells stimulated in the absence of exogenous cytokine. Taken together, these observations suggest that IL-15 is unable to induce expression of CD28 on activated human CD8+ T cells. Furthermore, IL-15 appears to actively prevent the TCR-mediated increase in CD28 surface expression that is attributed to STAT3 activation observed with TCR signals alone (Fig. 3-4).

Polyclonal activation of naïve CD8+ T cells is less clinically relevant to the goal of ACT where expansion of a particular anti-tumor population is desired. As such, we further assayed IL-15 in the context of antigen-specific CD8+ T cell activation to determine how IL-15 influences CD28 expression on the cell surface of antigen-specific cells. Naïve CD8+ T cells were stimulated with MART-1 peptide-pulsed mature DCs in the presence of IL-15, IL-21 or in the absence of cytokine. After two rounds of stimulation, we monitored CD28 expression on the surface of MART-1-specific CD8+ T cells by flow cytometry. Consistent with observations demonstrated in Chapter 2, MART-1-specific CD8+ T cells expanded approximately 5-fold in the presence of IL-21 in comparison to cells stimulated in the absence of exogenous cytokine (Fig. 3-5A; right and left panel, respectively). In contrast, antigen-specific CD8+ T cells stimulated in the presence of IL-15 did not expand much beyond that of the control group with

no exogenous cytokine (Fig. 3-5A; middle and left panel, respectively). These observations suggest that signals provided by IL-15 upon activation are not sufficient to promote antigen-specific CD8+ T cell expansion.

We next wanted to determine if IL-15 could support CD28 expression on the surface of activated human CD8+ T cells. As demonstrated in Chapter 2, antigen-specific CD8+ T cells expanded in the presence of IL-21 displayed high surface CD28 expression (Fig. 3-5B; right panel). However, MART-1-specific CD8+ T cells activated in the presence of IL-15 did not express surface CD28 (Fig. 3-5B; middle panel). In fact, the antigen-specific CD8+ T cells activated in the presence of IL-15 expressed less surface CD28 than the control group expanded in the absence of exogenous cytokine (Fig. 3-5B; middle and left panel, respectively). These data further suggest that IL-15 might actively inhibit CD28 expression on antigen-specific CD8+ T cells. Taken together, these observations indicate that IL-15 does not promote surface CD28 expression on activated human CD8+ T cells. Instead, our data suggest that IL-15 actively inhibits the low level CD28 surface expression induced by TCR signaling. Therefore, IL-21 and IL-15 exert different effects on the expression of surface CD28.

IL-15-mediated STAT5 associates with the human *CD28* promoter

We next wanted to start to define mechanistically why IL-21 and IL-15 display different activated with respect to CD28 surface expression. We hypothesized that the individual STAT proteins activated by IL-15 and IL-21 might compete for the STAT binding sites at the *CD28* locus, resulting in the differential CD28 expression mediated by these two cytokines. To start to address this possibility, we first assessed the ability of IL-15 to promote STAT3 activation in naïve human CD8+ T cells. Naïve CD8+ T cells were cultured in no cytokine, IL-15 or IL-21

for 20 minutes. STAT3 activation was assessed using a phospho-specific antibody and flow cytometry. Consistent with previous observations in Chapter 2, IL-21 promoted STAT3 activation at 20 minutes (Fig. 3-6). In contrast, IL-15 did not activate STAT3 in naïve human CD8+ T cells (Fig. 3-6). The lack of STAT3 activation mediated by IL-15 may contribute to the observation that IL-15 cannot induce the expression of surface CD28 on CD8+ T cells. Our observations indicate that IL-15 does not activate STAT3 in human CD8+ T cells (Fig. 3-6), and that IL-15 does not promote CD28 expression on the surface of human CD8+ T cells (Fig. 3-4 and Fig. 3-5B). In fact, our observations in the antigen-specific stimulation setting suggest that IL-15 might actively inhibit surface expression of CD28 (Fig. 3-5B).

There is evidence that STAT3 and STAT5 compete for binding sites at some genetic loci, and importantly, this competition has been shown to lead to reciprocal expression patterns for these genes (Walker et al., 2013; Yang et al., 2011). As such, we performed a ChIP assay to evaluate the ability of IL-15-activated STAT5 to associate with the human *CD28* promoter, hypothesizing that STAT5 association with the *CD28* promoter would be inhibitory to CD28 surface expression on CD8+ T cells. Naïve human CD8+ T cells were stimulated with anti-CD3/anti-CD28 beads in the absence of cytokine or in the presence of IL-15 for 24 hours. Cells were harvested for ChIP analyses utilizing antibodies to STAT3 or STAT5. In Chapter 2, we observed that IL-21-mediated STAT3 associated with the *CD28* promoter at the distal, but not the proximal, consensus STAT binding sites upon CD8+ T cell stimulation. IL-15 did not promote the association of active STAT3 with either the distal or the proximal consensus STAT binding sites (Fig. 3-7A and Fig. 3-7B, respectively). Instead, IL-15 promoted the association of active STAT5 with both the distal and the proximal consensus STAT binding sites (Fig. 3-7A and Fig. 3-7B, respectively). Taken together, these observations suggest that the STAT3

association with the *CD28* promoter is activating, while the STAT5 association with the *CD28* promoter is inhibitory. As such, IL-15 might directly inhibit the induction of CD28 expression in activated human CD8+ T cells by preventing the association of TCR-mediated active STAT3 with consensus STAT binding sites within the human *CD28* promoter. This observation supports the hypothesis that STAT3 and STAT5 act antagonistically and compete for binding sites within the transcription induction loci of the human *CD28* promoter. Furthermore, this suggests that inhibitory STAT5 out-competes activating STAT3 for the binding site within the *CD28* promoter, thus inhibiting CD28 surface expression on activated human CD8+ T cells in the presence of IL-15.

IL-21 is preferable to IL-6 or IL-15 for ACT

Based on our observations described in Chapter 2, and also here with IL-6 and IL-15, IL-21 appears to be the superior cytokine with which to modulate human CD8+ T cells to express surface CD28 for more efficacious ACT. While IL-6 was able to promote STAT3 activation equivalent to that of IL-21 at early time points (20 minutes), IL-6 did not efficiently promote sustained STAT3 activation as is seen with IL-21. Consequently, IL-6 did not efficiently support CD28 expression on activated human CD8+ T cells. It is likely that sustained STAT3 activation is required for CD28 surface expression, and we showed that IL-21, but not IL-6, could sustain active STAT3 up to 4 days. An extensive dose response of IL-6 was not performed in these studies; it is possible that more frequent dosing and/or higher concentrations of IL-6 might more efficiently mediate longer-lived STAT3 activation, and subsequently support CD28 expression. These possibilities should be investigated in future studies. Furthermore, it is worth noting that the ChIP analysis for IL-6-mediated active STAT3 association with the *CD28* promoter was only

performed at 24 hours, and not at earlier time points. Future studies will be needed to investigate whether IL-6 can more efficiently induce the association of STAT3 with the *CD28* promoter at 20 minutes with the cytokine, a time point at which IL-6 promotes STAT3 phosphorylation equivalent to that of IL-21.

We also showed that IL-15 did not support the expression of surface CD28 on activated human CD8+ T cells. Furthermore, CD8+ T cells activated in the presence of IL-15 expressed even less surface CD28 than CD8+ T cells activated in the absence of exogenous cytokine, suggesting that IL-15 might actively inhibit CD28 expression on activated CD8+ T cells. Further studies are required to test the hypothesis that IL-15-activated STAT5 preferentially binds the *CD28* promoter over IL-21-activated (or TCR-activated) STAT3 to prevent CD28 expression. In addition, future investigation into the pattern of CD28 expression in the presence of both IL-21 and IL-15 will shed light on the issue of competitive binding between IL-21-mediated STAT3 and IL-15-mediated STAT5.

As described in Chapter 2, activation of tumor-specific CD8+ T cells in the presence of IL-21 presents an attractive strategy for expanding cells for ACT. IL-21 itself possesses many characteristics that make its use widely applicable. At this time, IL-6 and IL-15 do not appear to be suitable cytokines for achieving the characteristic surface CD28 that is desired for ACT. It is possible that higher concentrations or more frequent dosing of IL-6 might promote CD28 surface expression, but further studies are required. IL-15 has been shown in the literature to prevent terminal differentiation and to promote a central memory-like CD8+ T cell population optimal for ACT; in our studies, IL-15 appears to actively inhibit CD28 expression. As such, further studies are needed to determine if additional mediators, such as IL-21, can overcome IL-15-mediated inhibition of CD28 surface expression. CD28-mediated autocrine IL-2 production

contributes to the proliferation and persistence of transferred cells *in vivo*; as such, strategies to promote the expression of CD28 on the surface of expanded CD8+ T cells might prove to be the most efficacious approach for successful ACT.

Chapter 3 Figure Legends

Figure 3-1. IL-6 promotes short-lived STAT3 activation in human CD8+ T cells. (a) Representative FACS plot of active STAT3 (pSTAT3) in naïve human CD8+ T cells cultured for 20 minutes in the absence of cytokine, 100 ng/ml IL-6 or 30 ng/ml IL-21. (b) Representative FACS plots of active STAT3 in naïve human CD8+ T cells stimulated with anti-CD3/anti-CD28 beads for 24 hours or 4 days (left panel and right panel, respectively) in the absence of cytokine, 100 ng/ml IL-6 or 30 ng/ml IL-21.

Figure 3-2. IL-6 does not support expansion of human antigen-specific CD8+ T cells characterized by surface CD28 expression. Representative FACS plots antigen-specific CD8+ T cells activated from naïve human CD8+ T cells with MART-1 peptide-pulsed autologous mature DCs for 14 days in the absence of cytokine, 100 ng/ml IL-6 or 30 ng/ml IL-21. (a) The population of expanded antigen-specific CD8+ T cells was identified with MART-1 peptide-MHC-tetramer. Data expressed as percentage of tetramer-positive cells within the lymphocyte population. (b) The population of antigen-specific CD8+ T cells that express surface CD28. Data expressed as percentage of CD28-expressing cells within the gated tetramer-positive (as seen in (a) above) population.

Figure 3-3. IL-6-mediated active STAT3 associates inefficiently with the human *CD28* promoter. Pooled ChIP analysis from three experiments of naïve human CD8+ T cells stimulated with anti-CD3/anti-CD28 beads for 24 hours in the absence of cytokine, 100 ng/ml IL-6 or 30 ng/ml IL-21. (a) STAT3 association with distal consensus STAT binding site in human *CD28* promoter. (b) STAT3 association with proximal consensus STAT binding site in human *CD28* promoter.

Figure 3-4. IL-15 suppresses CD28 surface expression by activated human CD8+ T cells. Representative FACS plots display surface CD28 expression on naïve human CD8+ T cells stimulated with anti-CD3/anti-CD28 beads over time (5 – 13 days) in the absence of cytokine, 30 ng/ml IL-15 or 30 ng/ml IL-21.

Figure 3-5. IL-15 exposure during activation of human antigen-specific CD8+ T cells suppresses the expression of surface CD28. Representative FACS plots MART-1-specific CD8+ T cells activated from naïve human CD8+ T cells with MART-1 peptide-pulsed autologous mature DCs for 14 days in the absence of cytokine, 30 ng/ml IL-15 or 30 ng/ml IL-21. (a) The population of antigen-specific CD8+ T cells was identified with MART-1 peptide-MHC-tetramer. Data expressed as percentage of tetramer-positive cells within the lymphocyte population. (b) The population of antigen-specific CD8+ T cells that express CD28 on the surface. Data expressed as percentage of CD28-expressing cells within the gated tetramer-positive (as seen in (a) above) population.

Figure 3-6. IL-15 does not promote STAT3 activation in human CD8+ T cells. Representative FACS plots of active phospho-STAT3 in naïve human CD8+ T cells cultured for 20 minutes in the absence of cytokine (left panel), 30 ng/ml IL-15 (middle panel) or 30 ng/ml IL-21 (right panel).

Figure 3-7. IL-15-mediated active STAT5 associates with the human *CD28* promoter. Pooled ChIP analysis from three experiments of naïve human CD8+ T cells stimulated with anti-CD3/anti-CD28 beads for 24 hours in the absence of cytokine, 30 ng/ml IL-15 or 30 ng/ml IL-

21. (a) STAT3 and STAT5 association with distal consensus STAT binding site in human *CD28* promoter. (b) STAT3 and STAT5 association with proximal consensus STAT binding site in human *CD28* promoter.

Chapter 3 Figures

FIGURE 3-1

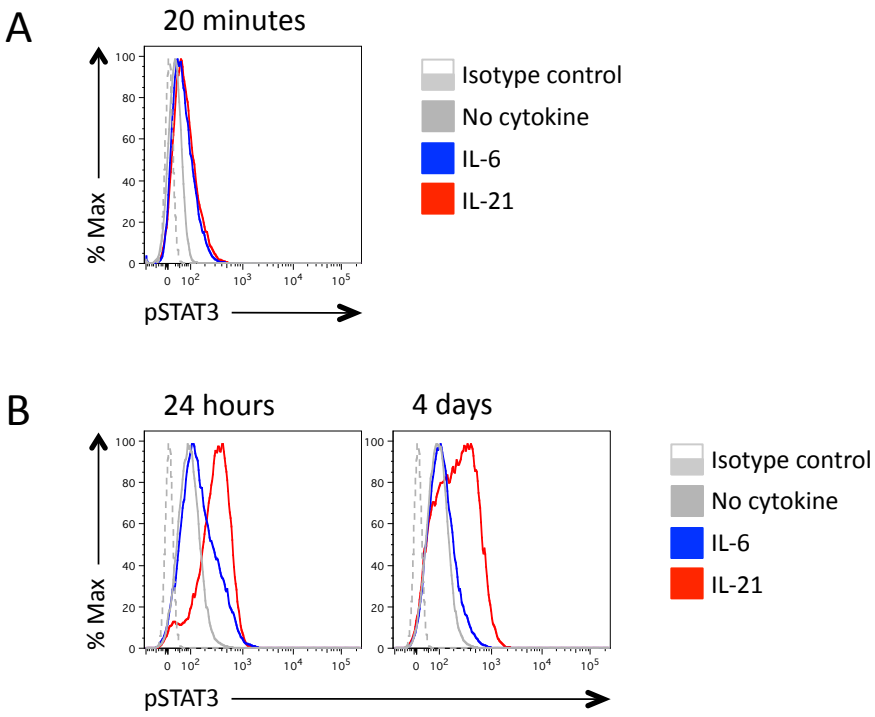

A
20 minutes
% Max
pSTAT3
Isotype control
No cytokine
IL-6
IL-21
B
24 hours
4 days
% Max
pSTAT3
Isotype control
No cytokine
IL-6
IL-21

FIGURE 3-2

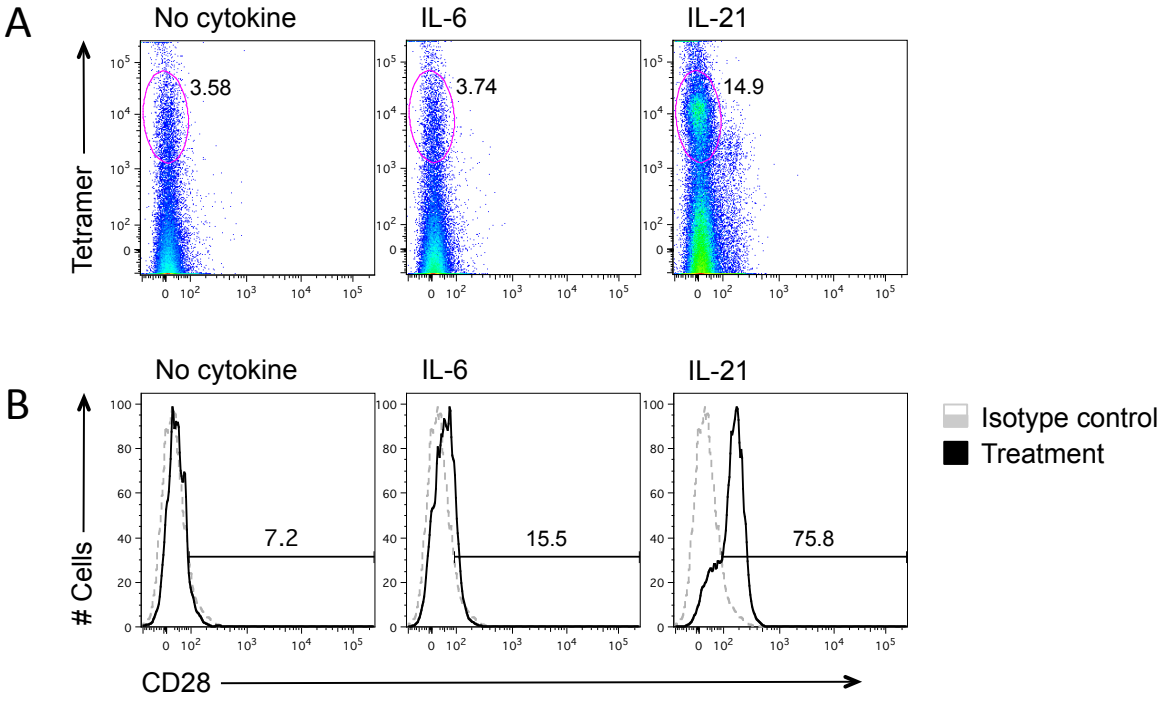
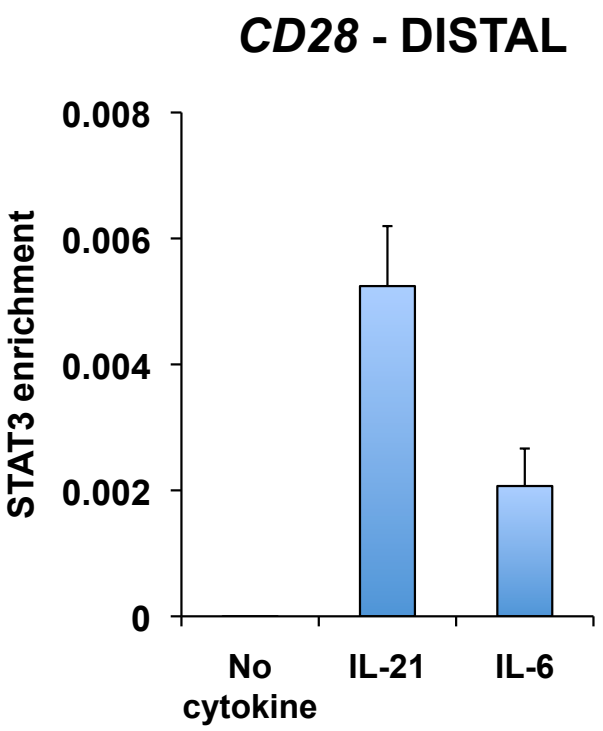

A
No cytokine
IL-6
IL-21
Tetramer
3.58
3.74
14.9
B
No cytokine
IL-6
IL-21
Cells
7.2
15.5
75.8
CD28
Isotype control
Treatment

FIGURE 3-3

A


CD28 - DISTAL
STAT3 enrichment
0.008
0.006
0.004
0.002
0
No cytokine
IL-21
IL-6

B

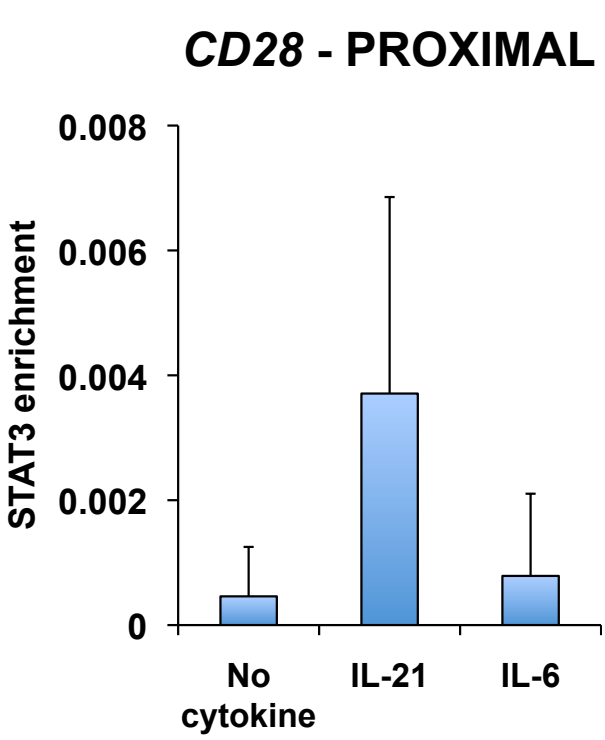

CD28 - PROXIMAL
STAT3 enrichment
0.008
0.006
0.004
0.002
0
No cytokine
IL-21
IL-6

FIGURE 3-4

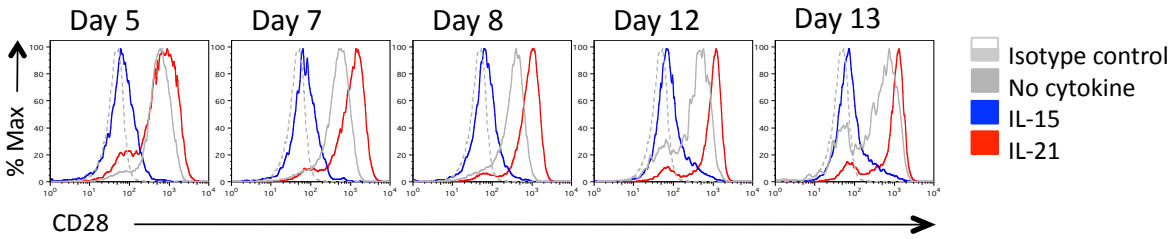

Day 5
Day 7
Day 8
Day 12
Day 13
% Max
CD28
Isotype control
No cytokine
IL-15
IL-21

FIGURE 3-5

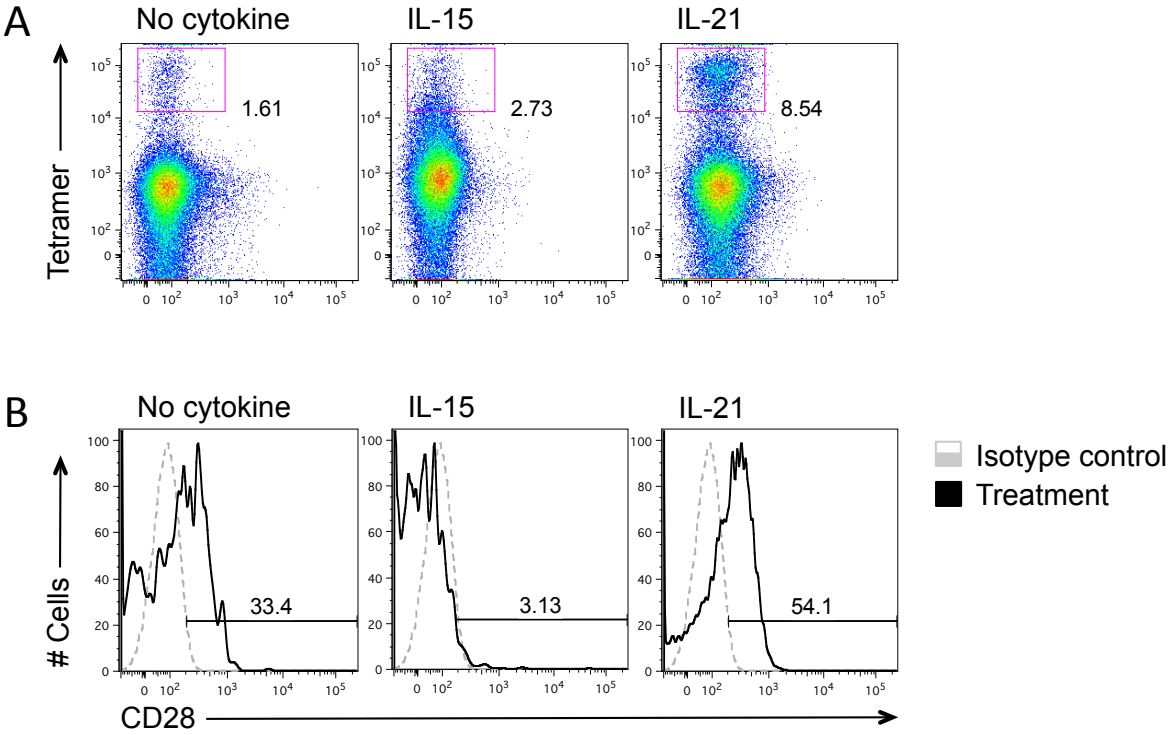

A
No cytokine
IL-15
IL-21
Tetramer
1.61
2.73
8.54
B
No cytokine
IL-15
IL-21
Cells
CD28
33.4
3.13
54.1
Isotype control
Treatment

FIGURE 3-6

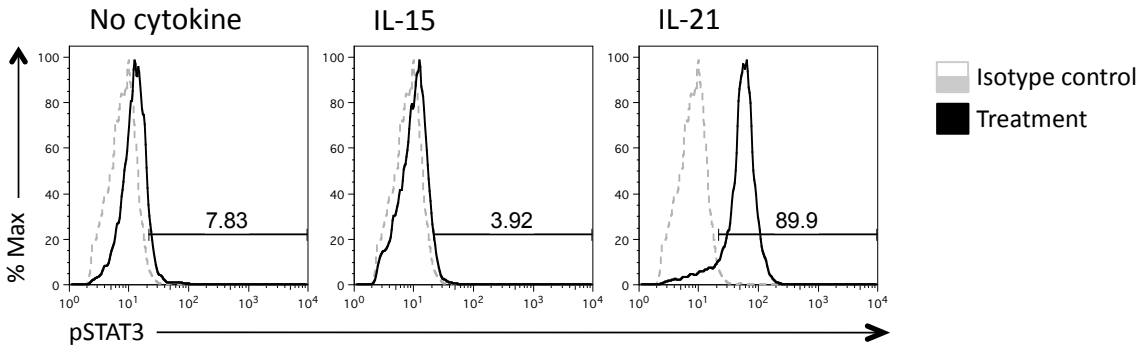
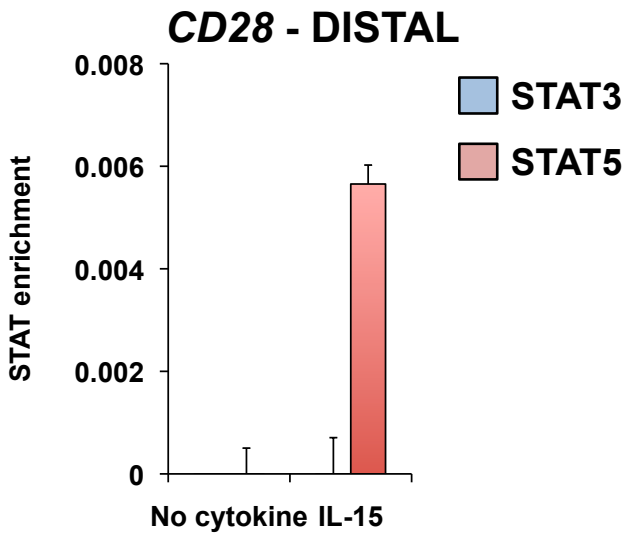
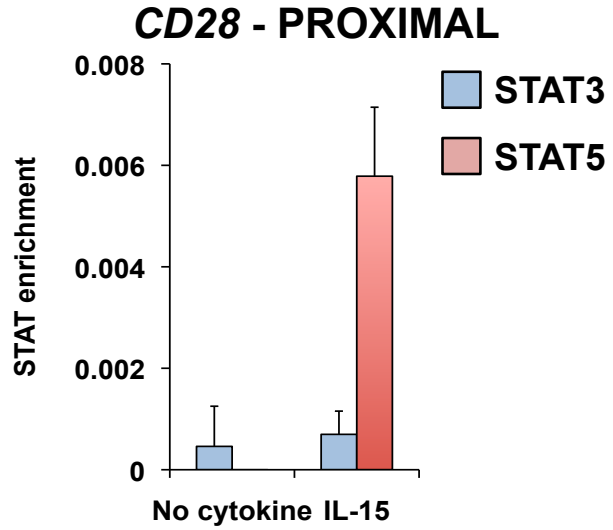

No cytokine
IL-15
IL-21
% Max
pSTAT3
7.83
3.92
89.9
Isotype control
Treatment

FIGURE 3-7

A


CD28 - DISTAL
STAT3
STAT5
STAT enrichment
0.008
0.006
0.004
0.002
0
No cytokine
IL-15

B


CD28 - PROXIMAL
STAT3
STAT5
STAT enrichment
0.008
0.006
0.004
0.002
0
No cytokine
IL-15

Chapter 4: Concluding Remarks and Outstanding Questions

The work described herein has investigated the role of several cytokines in the modulation of CD28 surface expression on activated human CD8+ T cells. Understanding the contribution of cytokine modulators to the phenotype and cellular characteristics of cells expanded for adoptive cell therapy is of great interest, due to studies showing the increased efficacy of ACT with cells that have resisted terminal differentiation (Gattinoni et al., 2005; Robbins et al., 2004; Zhou et al., 2005). T cells that retain a less-differentiated phenotype have been shown to demonstrate enhanced *in vivo* tumor reactivity (Klebanoff et al., 2004; Weninger et al., 2001). As such, strategies are under investigation to modulate cells during expansion for ACT to prevent terminal differentiation. Despite evidence indicating that IL-21 promotes surface CD28 expression on activated human CD8+ T cells, the mechanisms responsible for IL-21-mediated CD28 surface expression had not yet been investigated. Here we show that STAT3 activation is required for IL-21-mediated CD28 surface expression.

Ligation of surface CD28 promotes the stabilization of IL-2 mRNA, which promotes the maximal induction of autocrine IL-2 expression for T cell proliferation (Lindstein et al., 1989; Thompson et al., 1989). As such, the maintenance of surface CD28 on cells destined for ACT is desirable to support persistence of T cells in patients. Previous studies strongly support a correlation between persistence of infused T cells *in vivo* and patient response rate to ACT (Yee et al., 2002). Proliferative potential of the infused cells is considered to be the main contributor to persistence (Gattinoni et al., 2005). Despite its ability to promote persistence of adoptively transferred T cells *in vivo* at low concentrations, IL-2 is required at high concentrations to obtain the quantity of cells required for ACT (Boyman et al., 2007; Waldmann, 2006). High dose IL-2 administration has been shown to induce rapid T cell terminal differentiation (Boyman et al., 2007). Taken together, these observations suggest that IL-2 is not the most suitable cytokine for

modulation of cells for ACT.

Investigation into the ability of IL-15 to promote the expansion of optimal T cells for ACT have shown that CD8+ T cells activated in the presence of IL-15 polarize to a central memory-like phenotype (Klebanoff et al., 2004; Weninger et al., 2001). Furthermore, studies demonstrated that murine tumor-reactive CD8+ T cells activated *in vitro* in the presence of IL-15 exhibited enhanced *in vivo* function, compared to cells activated in the presence of IL-2 (Klebanoff et al., 2004; Weninger et al., 2001). In contrast, studies have shown that IL-15 induces the loss of CD28 from the surface of both naïve and memory CD8+ T cells cultured in the presence of the cytokine (Alves et al., 2005; Chiu et al., 2006). Based on the ability of IL-15 to promote a less-differentiated, CD8+ T_{CM}-like population, these observations support IL-15 as a suitable candidate for modulating cells for ACT. However, studies had not yet investigated the effect of IL-15 on CD28 expression on TCR-triggered CD8+ T cells.

Here we show that IL-15 actively inhibits CD28 surface expression, and suggest that this inhibition occurs through transcriptional inhibition of the *CD28* gene. IL-15 did not promote STAT3 activation, and did not induce CD28 expression on the surface of human CD8+ T cells stimulated in a polyclonal or antigen-specific setting. Furthermore, the level of surface CD28 expression in the presence of IL-15 was actually lower than the level on cells stimulated in the absence of exogenous cytokine. This observation was somewhat surprising, and suggests that IL-15 actively inhibits surface expression of CD28 induced by TCR-triggering. Supporting this hypothesis, we observe that IL-15-activated STAT5 associates with the human *CD28* promoter at distal and proximal consensus STAT binding sequences. STAT3 was observed to associate with the distal consensus STAT binding sequence in cells stimulated in the presence of IL-21, a cytokine that is able to induce the expression of CD28 (discussed below). These observations

support the hypothesis that IL-15 actively inhibits *CD28* transcriptional induction by preventing association of TCR-triggered STAT3 with the *CD28* promoter, in effect preventing (TCR-triggered) basal levels of CD28 expression on cells stimulated in the presence of IL-15.

The IL-15-mediated observations in our studies were based on the addition of exogenous IL-15 alone; IL-15 was not assayed in combination with IL-21 in our studies. As such, it remains to be determined if stronger/longer-lived (than TCR-triggered) STAT3 activation (i.e. by IL-21) can successfully compete with IL-15-mediated STAT5 for association at the distal consensus STAT binding sequence within the *CD28* promoter, in effect negating the inhibitory effect of IL-15 on CD28 expression. Preliminary data from our lab indicates that human CD8+ T cells stimulated in a polyclonal fashion in the presence of both IL-15 and IL-21 together exhibit a level of surface CD28 expression equivalent to the level on cells stimulated in the presence of IL-21 alone (data not shown). We hypothesize that cells stimulated in the presence of both IL-15 and IL-21 will exhibit STAT3, and not STAT5, association with the distal consensus STAT binding sequence within the human *CD28* promoter. We hypothesize that, like in cells stimulated in the presence of IL-15 alone, STAT5 will associate with the proximal consensus STAT binding sequence in cells stimulated in the presence of IL-15 and IL-21 together. However, if STAT5 remains associated with the distal consensus STAT binding sequence even in the presence of exogenous IL-21 (and thus, STAT3), it may suggest that STAT3 is required to initiate *CD28* transcriptional activation, but that STAT5 is required to maintain transcription. Although there is no precedent for such a scenario in the literature, rather the literature indicates competition between STAT3 and STAT5, further studies would be needed to assess the kinetics of STAT association and *CD28* transcriptional activation.

Our studies indicate that STAT3 is required for CD28 expression, and that STAT3

associates with the distal consensus STAT binding sequence, suggesting the requirement of this site for CD28 expression. In the presence of IL-15, CD28 expression is inhibited and, instead of STAT3, STAT5 associates with the distal consensus STAT binding sequence. Furthermore, in the presence of IL-15, STAT5 also associates with the proximal consensus STAT binding sequence within the *CD28* promoter. The biological relevance of the association of STAT5 with the proximal consensus STAT binding sequence is unknown. Future studies are required to determine if STAT5 association with both the distal and proximal consensus STAT binding sequences are required for CD28 expression inhibition.

The newest member of the γ_c receptor family, IL-21, has been shown to suppress the terminal differentiation of T cells (Albrecht et al., 2011; Hinrichs et al., 2008; Kaka et al., 2009; Li et al., 2005; van Leeuwen et al., 2005; Wolfl et al., 2011). Furthermore, murine T cells expanded in the presence of IL-21 for ACT demonstrated augmented anti-tumor activity compared to cells expanded in the presence of IL-2 (Hinrichs et al., 2008). The mechanisms by which IL-21 modulates the phenotype and cellular characteristics of cells destined for ACT had not yet been thoroughly investigated.

Here we show that IL-21 mediates CD28 surface expression through STAT3 activation. In our studies, IL-21 induces the activation of STAT3 in human CD8+ T cells cultured for 20 minutes. Interestingly, we found that IL-21 is able to sustain STAT3 activation in these cells up to 4 days. Using a STAT3-specific chemical inhibitor, we further showed that STAT3 activation is required for IL-21-mediated expansion of human antigen-specific CD8+ T cells characterized by surface CD28 expression. Indeed, STAT3 activation was required for both expansion of the antigen-specific population, as well as for surface CD28 expression on the antigen-specific population. We hypothesized that STAT3 was involved in direct transcriptional activation of the

CD28 gene. Supporting this hypothesis, we found that IL-21-mediated STAT3 associates with the distal consensus STAT binding sequence within the human *CD28* promoter. IL-21-mediated STAT3 did not associate with the proximal consensus STAT binding sequence, suggesting this sequence is dispensable for *CD28* transcriptional activation.

The IL-21-mediated observations in our studies were based on experiments performed with only one concentration of IL-21; this concentration was previously established in our lab as the optimal concentration for IL-21-mediated antigen-specific CD8+ T cell expansion (Li et al., 2005). Dose response assays for IL-21 showed equivalent STAT3 activation at 20 minutes for IL-21 concentrations ranging from 3 – 100 ng/ml (data not shown). However, it remains to be investigated if alternate concentrations of IL-21 can further augment the surface expression of CD28. Based on equivalent STAT3 activation, we hypothesize that one log lower or 3-fold higher IL-21 concentrations will not affect the level of CD28 surface expression. However, it is possible that a dose response of IL-21 will correlate with the level of CD28 surface expression, and thus should be tested. It is important to keep in mind that an upper limit of IL-21 concentration (100 ng/ml) exists whereby expansion of antigen-specific CD8+ T cells is negatively affected by the presence of the cytokine (Li et al., 2005). Ideally, the concentration of IL-21 used to expand antigen-specific CD8+ T cells characterized by surface CD28 will promote optimal expansion while concurrently supporting optimal CD28 surface expression.

The levels of CD28 transcript were monitored in two preliminary experiments. We hypothesized that the CD28 transcript level would correlate with surface expression of CD28. However, in CD8+ T cells activated in the presence of IL-21 for 24 hours, only a minimal increase in CD28 mRNA was detected compared to cells activated in the absence of cytokine (data not shown). Based on these preliminary observations, further evaluation of the level of IL-

21-mediated CD28 transcript expression over time is needed. We hypothesize that IL-21-mediated STAT3 promotes the transcriptional activation of the *CD28* gene at earlier time points than 24 hours. If we observe CD28 transcript levels that do not correlate with surface expression, it will suggest that mechanisms other than transcriptional regulation are responsible for the surface expression of CD28 mediated by IL-21. Such mechanisms could include post-transcriptional or post-translational modifications, as well as CD28 recycling/trafficking to the surface.

Interestingly, we observed that IL-21-mediated STAT3 associates with only the distal consensus STAT binding sequence, and much more modestly, if at all, with the proximal consensus STAT binding sequence. It is clear from our data that STAT3 association with the distal consensus STAT binding sequence is sufficient for CD28 surface expression. What is not clear from our data, however, is what role, if any, the proximal consensus STAT binding sequence plays in the expression of *CD28*. Future investigation into the contribution of the proximal consensus STAT binding sequence will shed light onto this sequence as a potential activating or inhibitory sequence; it is possible that alternate activating STAT proteins are recruited to this sequence or that alternate STAT proteins associated with CD28 inhibition are recruited to this site. We did not investigate what other STAT proteins are activated by IL-21 in human CD8+ T cells. It is possible that STAT1 is activated by IL-21 in these cells, and thus associates with the proximal consensus STAT binding site. We hypothesize that IL-21 will promote STAT1 activation, albeit to levels lower than STAT3 activation. As discussed above, it is possible that STAT3 is required to induce *CD28* transcriptional activation, while an additional STAT protein is required to maintain *CD28* transcriptional activation. ChIP assays for STAT1 should be performed if STAT1 is found to be activated by IL-21 in human CD8+ T cells to

determine if STAT1 associates with the proximal consensus STAT binding sequence.

Data in the literature indicate that cells expanded in the presence of IL-15 or in the presence of IL-21 for ACT will exhibit a central memory-like phenotype (Albrecht et al., 2011; Kaka et al., 2009; Klebanoff et al., 2004; Li et al., 2005; van Leeuwen et al., 2005; Weninger et al., 2001; Wolfl et al., 2011). Our observations indicate that cells expanded in the presence of IL-15 will lack surface expression of CD28, while cells expanded in the presence of IL-21 will maintain surface expression of CD28. As such, cells expanded in IL-15 will be rendered helper-dependent, while cells expanded in IL-21 will remain helper-independent. Exogenous IL-2 that is required to support survival of IL-15-expanded CD8+ T cells *in vivo* has potential negative effects, such as supporting regulatory T cells. IL-21-expanded CD8+ T cells, however, can produce autocrine IL-2 and as such do not require exogenous IL-2. Autocrine IL-2 may be produced and consumed at physiological levels, potentially alleviating the draw back of regulatory T cell support. Furthermore, our data and that of others (Li et al., 2005) show that antigen-specific CD8+ T cells stimulated in the presence of IL-21 undergo greater expansion than cells stimulated in the presence of IL-15. From a feasibility standpoint, culturing cells for a shorter amount of time is more cost efficient. Also, less time in culture correlates with a more favourable outcome for ACT. Taken together, these observations suggest that IL-21 is the superior cytokine in which to expand cells for ACT. However, further investigation is required to determine if in some scenarios cells expanded in the presence of IL-15 might be favourable to cells expanded in the presence of IL-21. Interestingly, combinatorial studies have demonstrated that IL-21 acts synergistically with IL-15, promoting greater expansion of, and enhanced effector function in, CD8+ T cells stimulated in the presence of the two cytokines together, compared to CD8+ T cells expanded in either cytokine alone (Zeng et al., 2005). Our preliminary studies are

consistent with others (Alves et al., 2005), showing that surface expression of CD28 is equivalent in cells expanded in IL-21 and IL-15 together, compared to IL-21 alone (data not shown). Further studies are required to determine the ultimate cytokine, or combination thereof, that will promote the greatest expansion of antigen-specific cells while maintaining the least terminally differentiated phenotype. We hypothesize that this may be achieved using a combinatorial approach, whereby one cytokine makes up for the shortcomings of the other(s), as is the case for IL-21-mediated CD28 expression in the presence of IL-15, which normally inhibits CD28 expression. Understanding the mechanisms by which these cytokines regulate the desired proteins (CD28, etc.) will help to achieve this goal.

IL-6 is a conventional STAT3 activator (Wegenka et al., 1993) and studies have shown that IL-6 can inhibit LPS-induced TNF-α production by monocytes (Schindler et al., 1990). These observations suggest that IL-6 might promote the expression of surface CD28, however the activities of IL-6 had not yet been assessed in ACT strategies. In our studies, IL-6 promotes the activation of STAT3 in human CD8+ T cells cultured for 20 minutes. However, much less IL-6-mediated active STAT3 is present at 24 hours, and only baseline levels are detectable by 4 days. Compared to IL-21 and its ability to sustain active STAT3 up to 4 days with concurrent CD28 surface expression, we concluded that IL-6 was unable to efficiently sustain STAT3 activation for the duration of time required to promote subsequent CD28 surface expression. Furthermore, IL-6 does not efficiently promote association of STAT3 with the human *CD28* promoter. This was consistent with the observation that low levels of IL-6-mediated active STAT3 are observed at 24 hours. Future investigation into the ability of IL-6 to mediate STAT3 association with the human *CD28* promoter at an earlier time point (such as 20 minutes when IL-6 exhibits STAT3 phosphorylation equivalent to that of IL-21) will address whether IL-6 acts as

a mediator of CD28 surface expression in some circumstances.

Our studies with IL-6 did not employ an extensive dose response, and as such it is possible that we did not observe efficient IL-6-mediated sustained STAT3 activation due to concentrations of IL-6 that were too low (or too high, see below). Furthermore, we did not assess the ability of IL-6 to inhibit TNF-α production by activated CD8+ T cells in our studies. It is possible that IL-6 possesses the ability to inhibit TCR-triggered TNF-α, similar to its ability to inhibit LPS-induced TNF-α (Schindler et al., 1990). Indeed, IL-6-mediated TNF-α inhibition supports the hypothesis that IL-6 could promote CD28 surface expression through inhibition of a negative regulator of CD28 expression. However, at the concentration and time points assayed, we did not observe IL-6 to possess the ability to efficiently promote CD28 expression. As outlined above, a more extensive dose response with IL-6 is required to test whether there is a threshold concentration required to sustain STAT3 activation, or, coupled with TNF-α inhibition, there is a threshold concentration required that could result in high levels of CD28 surface expression on human CD8+ T cells activated in the presence of IL-6.

IL-6 does not efficiently support sustained STAT3 activation due to the induction of SOCS3 (suppressor of cytokine signaling 3) expression (Yasukawa et al., 2003). *SOCS3* gene expression is induced by STAT3 activation (Kubo et al., 2003), and SOCS3 negatively regulates the IL-6-mediated STAT3 signal (Vanden Berghe et al., 2000); IL-6 signal transduction induces the expression of a protein that inhibits further IL-6 signal transduction. This is of interest to us, as we hypothesize that IL-21 would similarly induce the expression of SOCS3 through STAT3 activation. Indeed, preliminary data from our lab indicates that IL-21 induces SOCS3 mRNA (data not shown). This suggests that *de novo* IL-21-mediated SOCS3 protein expression could negatively affect further IL-21 signal transduction, and inhibit sustained IL-21-mediated STAT3

activation (24 hours and 4 days), as viewed with IL-6. However, this possibility is in contrast to the sustained STAT3 activation we observe with IL-21. Data in the literature indicate that the receptor for other STAT3-activating cytokines, such as IL-10, show a decreased affinity for SOCS3 binding (Yasukawa et al., 2003). For these cytokines, cytokine-mediated activation of STAT3, which leads to subsequent SOCS3 expression, results in less inhibition of the cytokine signal transduction pathway. It has not yet been investigated, but we hypothesize that the IL-21 receptor similarly exhibits a decreased affinity for SOCS3 binding, and thus the STAT3 signal is sustained with IL-21, rather than inhibited by SOCS3. Future studies should investigate the ability of SOCS3 to bind the IL-21 receptor.

In conclusion, our studies demonstrate the requirement for sustained STAT3 activation in the expression of CD28 on the surface of human antigen-specific CD8+ T cells. IL-21 is able to promote sustained STAT3 activation, but IL-6 is not able to efficiently sustain STAT3 activation. Consequently, IL-21 mediates high levels of surface CD28 expression on activated human CD8+ T cells. Furthermore, IL-15 appears to actively inhibit *CD28* transcriptional activation through the association of active STAT5 with the human *CD28* promoter. In closing, our studies have demonstrated that despite similarities in signal transduction pathways or receptor components, various cytokines differentially modulate CD28 surface expression. These observations can contribute to the optimization of ACT strategies for patients with metastatic melanoma and other cancers.

Chapter 5: Materials and Methods

Human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were obtained from leukapheresis of healthy volunteers typed by the HLA Typing Laboratory at the Puget Sound Blood Center (Seattle, WA).

Naïve CD8+ T cell enrichment

CD8+ T cells were isolated first by positive selection from human PBMCs utilizing the Dynal CD8 Positive Isolation Kit (Life Technologies). Following, the naïve compartment of CD8+ T cells was purified utilizing negative selection with CD45RO Microbeads (Miltenyi Biotec Inc.). The purity of the naïve CD8+ T cells was greater than 95% as determined by flow cytometry.

Generation of human monocyte-derived dendritic cells

PBMCs were incubated in RPMI 1640 media (Life Technologies) for 2-3 hours at 37°C and washed gently with warm phosphate buffered saline (PBS). The remaining adherent cells were culture in AIM-V media (Life Technologies) containing IL-4 (500 U/ml) and GM-CSF (800 U/ml) added on days 0, 3 and 5 to generate immature DCs. Following, the immature DCs were cultured in AIM-V media containing IL-1β (2 ng/ml), IL-6 (1000 U/ml), TNF-α (10 ng/ml) and prostaglandin E2 (PGE_2; 1 μg/ml) for 2 days to generate mature DCs. All cytokines from R & D Systems; PGE_2 from Sigma-Aldrich.

Polyclonal stimulation of CD8+ T cells

Naïve human CD8+ T cells were suspended at 10^6/ml in cytotoxic T lymphocyte (CTL) media containing RPMI 1640, 10% fetal bovine serum (FBS; Tissue Culture Biologicals), 50 U/ml penicillin (Life Technologies), and 50 mg/ml streptomycin (Life Technologies); 2 x 10^6 naïve

CD8+ T cells were added to each well of a 24 well tissue culture plate (Nunc). Polyclonal T cell activation was initiated using Dynabeads® Human T-Activator CD3/CD28 for T-Cell Expansion and Activation (Life Technologies) at a bead:cell ratio of 1:1. Cytokine (30 ng/ml IL-21 from Biotrend Chemicals, LLC; 100 ng/ml IL-6 from R & D Systems; 30 ng/ml IL-15 from R & D Systems) or STAT3 inhibitor (500 uM NSC 74859; Santa Cruz Biotechnology Inc.) was added individually, or together as indicated, to each well of the tissue culture plate immediately following bead addition to cells. For subsequent cellular analysis at indicated time points, T cells were harvested and beads were removed from T cells using a magnet; T cells were washed twice to remove beads for downstream analysis.

Expansion of human antigen-specific CD8+ T cells

Melanoma M27-specific CD8+ T cells were generated as follows. Naïve human CD8+ T cells were suspended at 10^6/ml in CTL media; 10 x 10^6 naïve CD8+ T cells were added to TC25 tissue culture flasks (Corning). Mature DCs were harvested and 2 x 10^6 cells/ml pulsed for 4 hours at room temperature with 40 μg/ml synthetic peptide (PolyPeptide Group) in the presence of 3 μg/ml β2-microglobulin (Scripps Laboratories) in PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich). Following pulse, mature DCs were washed with PBS and suspended in CTL media at 10^6/ml. Mature DCs were irradiated (5000 rads) prior to culture with naïve CD8+ T cells. Peptide-pulsed, irradiated mature DCs were co-cultured with naïve CD8+ T cells at a ratio of 1:10; 10^6 DCs were added to each TC25 tissue culture flask containing 10 x 10^6 naïve CD8+ T cells. Cytokine (30 ng/ml IL-21 from Biotrend Chemicals, LLC; 100 ng/ml IL-6 from R & D Systems; 30 ng/ml IL-15 from R & D Systems) or chemical STAT3 inhibitor (500 uM NSC 74859; Santa Cruz Biotechnology Inc.) was added individually,

or together as indicated, to each tissue culture flask immediately following co-culture initiation. The first round of stimulation occurred for 1 week. The second round of stimulation, following the same protocol, occurred on day 8. IL-2 (50 U/ml; R & D Systems) and IL-7 (10 ng/ml; R & D Systems) were added after the second round of stimulation, on day 9, to support further expansion of antigen-specific CD8+ T cells. On day 14, following two rounds of stimulation, T cells were harvested for subsequent tetramer and CD28 analysis by flow cytometry.

Chromatin immunoprecipitation

Naïve CD8+ T cells were stimulated in a polyclonal setting with anti-CD3/anti-CD28 beads at a 1:1 bead:cell ratio in a 24 well tissue culture plate. Cytokine (30 ng/ml IL-21; 100 ng/ml IL-6; 30 ng/ml IL-15) was added at culture induction. Following 24 hours of stimulation, beads were removed from the culture and cells were crosslinked in 1% (final concentration) formaldehyde (Sigma-Aldrich) for 10 minutes at room temperature. Cell pellets were harvested and washed 2X in ice cold PBS containing protein inhibitor cocktail (Roche), and then snap frozen in liquid nitrogen. Samples were stored at -80°C until further use. Chromatin immunoprecipitation was performed with a ChIP Assay Kit (Millipore) as per the manufacturer's instructions, employing a mixture of antibodies to total STAT3 and total STAT5 (Santa Cruz Biotechnology, Inc.) and phospho-STAT3 and phospho-STAT5 (Cell Signaling Technology).

Peptide-MHC tetramer staining

Allophycocyanin (APC)-labeled M27-MHC-tetramer was produced in the immune monitoring laboratory at the Fred Hutchinson Cancer Research Center. Following harvest, cells were first labeled with tetramer-APC for 1 hour at room temperature, followed by incubation with anti-

CD28-PerCPCy5.5 (BD Biosciences) for 20 minutes at room temperature. Cells were analyzed for expansion of the antigen-specific compartment and CD28 surface expression via flow cytometry.

Assay for active STAT3

Naïve CD8+ T cells were cultured alone, or stimulated in a polyclonal setting with anti-CD3/anti-CD28 beads at a 1:1 bead:cell ratio, in a 24 well tissue culture plate. Cytokine (30 ng/ml IL-21; 100 ng/ml IL-6; 30 ng/ml IL-15) or chemical STAT3 inhibitor NSC 74859 (500 uM) was added at culture induction. At indicated time points (20 minutes, 24 hours or 4 days), cells were harvested (beads removed via magnet when necessary) and fixed with 2% formaldehyde for 10 minutes at 37°C. Following, cells were permeabilized with 90% methanol for 30 minutes on ice. After washing cells with FACS buffer (5% FBS in PBS), cells with incubated with R-phycoerythrin (PE)-conjugated phospho-STAT3 (Y705) antibody (BD Biosciences) in FACS buffer for 1 hour at room temp and then analyzed for active STAT3 content via flow cytometry.

CFSE dilution assay

Naïve CD8+ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE; CellTrace™ CFSE Cell Proliferation Kit from Life Technologies), as per the manufacturer's instructions. Following, naïve CD8+ T cells were stimulated in a polyclonal setting with anti-CD3/anti-CD28 beads at a 1:1 bead:cell ratio in a 24 well tissue culture plate. Chemical STAT3 inhibitor NSC 74859 (range: 100 – 1000 uM) was added at culture induction. At indicated time points (2, 5 and 7 days), cells were harvested (beads removed via magnet) and directly analyzed

for CFSE dilution via flow cytometry.

Flow cytometry

All FACS data were acquired via an LSR II flow cytometer and analyzed via FlowJo software (Tree Star, Inc.).

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