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Modulation of T Lymphocytes by Tumor-Released Survivin

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Modulation of T Lymphocytes by Tumor-Released Survivin

by

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A Dissertation submitted in partial satisfaction of
the requirements for the degree of
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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Modulation of T Lymphocytes by Tumor-Released Survivin

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The tumor microenvironment is an area of intense interaction between normal and

malignant cells. Factors and cell types within this environment can play a crucial role in

the progression or regression of the tumor. Of primary interest are tumor-infiltrating T

lymphocytes, which have been shown to have a key role in modifying the dynamics of

the tumor microenvironment to promote or prevent tumor growth. While there is much

in vitro and *in vivo* evidence for a modification of the tumor infiltrating T cell population

toward a pro-tumor environment, what induces these changes within the tumor

microenvironment has remained elusive. Our lab previously identified a role for the

Inhibitor of Apoptosis protein Survivin as a secreted protein in the extracellular milieu,

where it is capable of entering malignant cells and inducing a more aggressive phenotype.

We hypothesized that tumor-secreted Survivin could be responsible for modulation of T

lymphocytes in the tumor microenvironment. We first isolated tumor released Survivin

and confirmed it's ability to be taken up by T cells as it is by malignant cells by confocal

microscopy, flow cytometry and Western blotting. Subsequently, we evaluated

Survivin's affect on T cell proliferation and found that tumor-released Survivin impairs T

cell expansion, but does not alter its activation after exposure to appropriate stimuli.

Assessment of phenotypic changes within the cytotoxic and helper T lymphocyte

populations showed an increase in anti-inflammatory type 2 T cells and a reduction in type 1 T cells, which correlates to what has been observed in cancer patients. Although often modified in the tumor microenvironment in patients, we did not observe and changes in regulatory T cells or Th17 cells in the presence of Survivin. The results of this study provide evidence of Survivin's role as an extracellular mediator of the tumor microenvironment, specifically its role in inducing a pro-tumor type 2 T cell population.

CHAPTER ONE

INTRODUCTION

Cancer

Characteristics of Cancer Cells

Cancer is a complex disease involving the accelerated and uncontrolled proliferation of abnormal cells that invade normal tissues. While each type of cancer has different morphology, treatment and prognosis, several key features remain common throughout. Six hallmarks were originally described by Hanahan and Weinberg in 2000, most of them involving molecular changes within cancer cells themselves (Figure 1.1). The definition of these features and their consolidation into one picture of cancer provided a model through which cancer progresses, eventually gaining these functions to reach the malignant state (Hanahan D and Weinberg RA 2000).

The first of these hallmarks is the ability of cancer cells to replicate without limit, providing them with a never-ending proliferative capacity (Hanahan D and Weinberg RA 2000). While normal human cells have a replicative capacity of approximately 60-70 divisions, malignant cells frequently exceed this limit, providing enough cells to form a tumor despite death of many of the pre-malignant cells along the way (Wyllie AH *et al.* 1980; Bergers G *et al.* 1998). This function of cancer cells to continue to divide past the typical threshold of normal cells was found to be aided by induction of telomere-lengthening functions within these cells. In a normal cell, the chromosome ends, or telomeres, shorten every time the DNA is replicated, until eventually the ends are degraded to the point of inducing apoptosis of the cell (Counter CM *et al.* 1992). By upregulating factors that preserve telomere length (telomerase, ALT) the normal

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proliferative capacity can be greatly exceeded by the malignant cell, resulting in tumor formation (Hanahan D and Weinberg RA 2000).

The next two hallmarks go hand-in-hand to push the unrestrained proliferation allowed by the telomere lengthening mentioned previously: 1. Self-sufficiency in growth signals and 2. Insensitivity to anti-growth signals. In the normal cell, growth is only induced when a need of the body requires it. Some cell populations, like hematopoietic cells and gastric epithelial cells, replicate frequently due to wear and tear, while others replicate in the context of a specific need or after injury. The promotion of this renewal is mediated by growth factors and mitotic signals transmitted through surface and intracellular receptors (Fedi P *et al.* 1997). On the opposing side, anti-growth signals in the extracellular matrix and milieu prevent the proliferation of cells when not needed as well as excessive growth of cells after the necessary divisions have occurred by inhibiting the cell cycle. Malignant cells alter both sides of this control mechanism to allow their unrestrained growth. By inducing their own mitotic signals by either production of growth factors (PDGF, TGF-α) or altering the receptor-signaling cascade (Her2/*neu*, Bcr-Abl) they can continue to proliferate even when exogenous growth factors have been withdrawn. Additionally, by disrupting normal pathways that block the cell cycle, such as the retinoblastoma (pRB) and p15^{INK4B} pathways, their endogenous growth factors push forward unchecked (Fynan TM and Reiss M 1993; Zuo L *et al.* 1996; Datto MB *et al.* 1997). Throughout the process of DNA replication and cellular proliferation, errors frequently occur that must be detected by the cell to maintain cellular integrity (Hanahan D and Weinberg RA 2000). Upon detection, cells are given the chance to repair the mistakes and continue their division or are forced to die if the errors cannot be corrected.
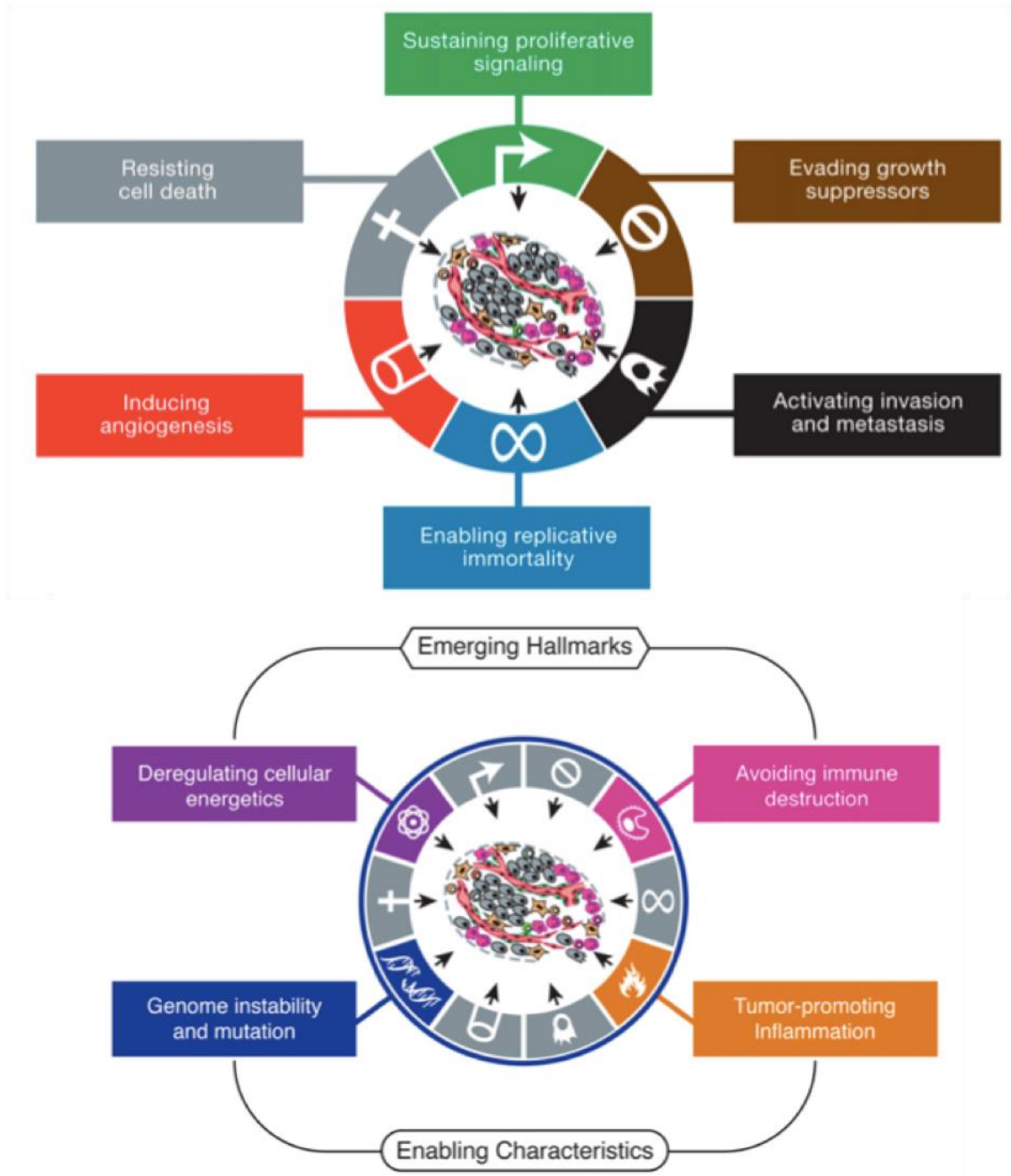
Figure 1.1. The hallmarks of cancer. From Hanahan and Weinberg 2000 and 2011, a schematic of the original (top) and emerging (bottom) characteristics of cancer.

The rapid and uncontrolled proliferation provided by the hallmarks we have discussed thus far provide many opportunities for error that would likely lead to apoptosis in the normal cell. To prevent the likely death that would occur from replication infidelity in the malignant cell mechanisms to inhibit apoptosis are necessary. It has been stated that evasion of apoptosis is the largest barrier a cancer cell must overcome, as studies have shown that induction of the previously mentioned hallmarks alone result in the apoptosis of a large proportion of the cells (Kerr JF *et al.* 1972; Bergers G *et al.* 1998). The evasion of apoptosis is mediated by multiple factors within the cell including the Inhibitor of Apoptosis (IAP) protein and Bcl-2 family members (Hanahan D and Weinberg RA 2000). By interfering with the caspase machinery and mitochondrial-mediated death anti-apoptotic molecules can prevent death of malignant cells in response to replicative errors and cellular stressors.

The final two hallmarks are vital to the advancement of malignancy past the point of a small focal lesion. To continue its growth the tumor must have access to nutrients and the ability to move past tissue barriers. The angiogenic potential allows for the tumor to maintain access to a nutrient blood supply by extending vessels and inducing new vessel growth into the tumor mass (Bergers G *et al.* 1998; Hanahan D and Weinberg RA 2000). This is primarily mediated by increased cancer cell production of pro-angiogenic vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF1/2) and reduced production of anti-angiogenic factors thrombospondin-1 and β-interferon (Bergers G *et al.* 1998). With a nutrient supply available, the only remaining barrier to tumor growth is the boundaries of the tissue compartment it is contained within. Through production of matrix metalloproteinases (MMPs) and decreasing cell-cell adhesion

molecules like E-cadherin, cancer cells can move away from the tumor and past the basement membrane barrier to invade nearby tissues as well as metastasize to distant locations (Coussens LM and Werb Z 1996; Bergers G and Coussens LM 2000). This metastatic function is part of what makes treatment of cancer incredibly difficult, as simple surgical techniques cannot be used systemically to eradicate the malignancy.

As research in the field of cancer progressed, an updated version to this seminal review of cancer was released (Hanahan D and Weinberg RA 2011). In 2011, Hanahan and Weinberg released "Hallmarks of Cancer: The Next Generation," bringing several new players to the forefront of the cancer research world (Figure 1.1). Several of these characteristics revolve around the interaction of the tumor and normal cells within the tumor microenvironment.

The Tumor Microenvironment

The Role of Fibroblasts

Within the body of the tumor there are a variety of cell types, both malignant and normal and as with normal tissue biology it is critical to understand the interactions between the variety of cellular players to understand how it functions. The presence of fibroblasts, endothelial cells and vessels are utilized by the tumor for protection, structure and nutrient support (Hanahan D and Weinberg RA 2011). Fibroblasts play a vital role in normal tissues by production of the extracellular matrix and its ground substance and through the production of cytokines (Whiteside TL 2008). They are also crucial to proper wound healing, with fibroblasts and myofibroblasts initiating wound contracture and closure. In malignancy, fibroblasts help form the structural foundation for the tumor.

Additionally, myofibroblasts, which normally make up only a small portion of normal tissue structure, are increased in tumors. Since myofibroblasts are also increased in chronic inflammation-induced fibrosis, it is thought that inflammation in the tumor microenvironment increases the proportion of myofibroblasts (Liao Y *et al.* 2007; Whiteside TL 2008). The importance of this increase in myofibroblasts has been demonstrated in their ability to enhance tumor metastatic and angiogenic potential (De Wever O *et al.* 2004; Karnoub AE *et al.* 2007).

Immune Cells

The tumor microenvironment is also home to a large number of immune cells, the role of which has been intently investigated. The role of immune cells in the progression or prevention of cancer and within the tumor microenvironment has been intently debated. Both anti-tumor and pro-tumor effects have been characterized and it now appears it is the balance of these factors and the tumor's ability to modulate this balance that determines what part the immune system will play in the development in cancer.

Macrophages

Macrophages are myeloid-derived immune cells that function to phagocytose and kill pathogens and present their antigens to the adaptive immune system. Within the tumor microenvironment macrophages are reprogrammed so that instead of activating the adaptive immune response, they quench it (Liao Y *et al.* 2007). This can be explained by the dual nature of macrophages. As is seen in many immune cell populations, macrophages exist as either M1 or M2 cells. The type 1 or M1 phenotype is primarily

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pro-inflammatory and has an excellent capacity for killing cancer cells, presenting their

antigens and activating an adaptive immune inflammatory response against the tumor

(Liao Y *et al.* 2007). These functions are mediated by production of tumor necrosis

factor alpha (TNF-α) and nitric oxide (NO). However, because these functions require

the presence of oxygen, within the hypoxic tumor microenvironment, they are readily

diminished. The type 2 or M2 phenotype of macrophages plays a more permissive role in

the tumor microenvironment. Their production of growth factors such as VEGF promote

tumor angiogenesis and sustenance (DeNardo DG *et al.* 2009). Release of IL-10 and

prostaglandins by M2 cells diminishes the immune response by Natural Killer (NK) cells

and inflammatory T lymphocytes, providing a safe environment for the tumor (Liao Y *et

al.* 2007; DeNardo DG *et al.* 2009).

Dendritic Cells

Dendritic cells (DCs) are an important driving force in the immune response as

they are the most potent antigen presenting cells. The induction of an immune response

or the production of anergy in adaptive immune cells is partly dependent on their

maturity, with immature cells producing anergy and mature cells potently activating the

adaptive immune response (Liao Y *et al.* 2007). Mature dendritic cells predominate in

most tissues in order to provide activation of the adaptive immune system upon detection

of a pathogen, however, within the tumor microenvironment there is a preponderance of

immature DCs, while mature DCs are a rarity. Increased amounts of VEGF can impair

DC maturation and it is suspected that other cytokines produced in the tumor

microenvironment play an additional role (Liao Y *et al.* 2007).

B Lymphocytes

The role of B lymphocytes has been investigated in several different cancer types including breast, colorectal, melanoma and ovarian cancers (Hansen MH *et al.* 2002; Galon J *et al.* 2006; Milne K *et al.* 2009; Erdag G *et al.* 2012). It is currently unclear if the predominant function of B cells in the tumor microenvironment is pro- or anti-tumor (Linnebacher M and Maletzki C 2012). Studies in breast cancer has shown that greater than 40% of patients have anti-tumor antibody production by B cells that correlate to a more favorable outcome (Hansen MH *et al.* 2002; Kotlan B *et al.* 2005). Additionally, in lung and breast carcinoma, antibodies produced by B cells in the tumor microenvironment may aid in tumor elimination (Kotlan B *et al.* 2005). In contrast, studies in mice have shown that B cells may interfere with T cell eradication of tumor cells (Liao Y *et al.* 2007). Determining the precise role of B cells in other malignancies is an area of intense research with many questions left to be answered, including if they primarily promote or hinder cancer progression.

T Lymphocytes

The role of T lymphocytes in malignancy is complicated at best. Multiple T cell phenotypes have been shown to play a role in the progression or elimination of cancer, depending on the cancer type (Nakayama H *et al.* 2000; Chikamatsu K *et al.* 2007; Schreck S *et al.* 2009). Four main players have emerged in the T cell reaction to malignancy, and the balance of them can alter tumor progression (Figure 1.2). They include Type 1 and Type 2 T lymphocytes, regulatory T lymphocytes (Tregs) and IL-17 secreting T lymphocytes (Th17) (Zhu J and Paul WE 2008; Kryczek I *et al.* 2009).

Type 1 and Type 2 T Lymphocytes. Type 1 T cells are comprised of both CD4^{+}

helper and CD8^{+} cytotoxic populations, Th1 and Tc1, respectively. The defining

characteristic of these cells is their cytokine profile, which consists of tumor necrosis

factor alpha (TNF-α), gamma interferon (IFN-γ), and interleukin 2 (IL-2) (Woodland DL

and Dutton RW 2003). The type 1 T cell cytokine IFN-γ is vital to the anti-tumor

response as it recruits and activates macrophages, as well as increasing the cytotoxic

response by CD8^{+} T cells. Mice deficient in IFN-γ have been shown to spontaneously

develop tumors and have poor tumor clearance, illustrating the importance of IFN-γ in

anti-tumor immunity (Riemensberger J *et al.* 2002). Production of IL-2 by type 1 cells

increases the proliferation of nearby T cells, aiding the immune response by inducing

clonal expansion of tumor-reactive cells.

The antagonists of type 1 cells are the type 2 Tc2 and Th2 cells, which release IL-

4, IL-5, IL-6, IL-10 and IL-13 (Zhu J and Paul WE 2008). Type 2 cytokines prevent the

differentiation of naïve T cells into type 1 cells and oppose the functions of type 1

cytokines. IL-10 decreases the cytotoxic response by decreasing antigen presentation by

macrophages to Th1 cells, while IL-4 leads to decreased type 1 cell differentiation and

IFN-γ production (Wurtz O *et al.* 2004). In cancer, IL-4 has been shown to prevent

apoptosis of malignant cells, as well as increase the metastatic potential of breast cancer

cells through polarization of infiltrating macrophages (DeNardo DG *et al.* 2009).

Peripheral blood and intratumoral T cells from cancer patients frequently have an

imbalance in type 1 and type 2 T cell populations (Filella X *et al.* 2000; Nakayama H *et

al.* 2000; Mainou-Fowler T *et al.* 2003; Murakami H *et al.* 2004; Sharma A *et al.* 2010).

Type 1 responses are typically decreased with a concomitant increase in type 2 cell

numbers and cytokine levels. The potential for metastasis and angiogenesis is increased

by the presence of IL-4 and IL-6, respectively, both of which are produced by type 2 T

cells (Naldini A *et al.* 2003). This shift in the balance of type 1 and type 2 T cells has

been correlated to poor disease progression, as the anti-tumor response is inhibited and a

pro-tumor environment is created.

Regulatory T Cells. Characterized as $CD4^+CD25^+CD127^{low}Foxp3^+$, regulatory

T cells (Tregs) are important in preventing immune responses to autoantigens and over-

activation of the inflammatory response (Liu W *et al.* 2006). Treg cells have been

implicated in promoting tumor growth by suppressing activation of tumor-specific

cytotoxic T cells due to their expression of auto-antigens by production of Transforming

Growth Factor-β (TGF-β), cytolysis, and the induction of anergy (James E *et al.* 2010).

In addition to suppressing the immune response, Tregs are also able to promote cancer

stem cells. The regulation of cancer stem cells by Tregs is mediated by secretion of

TGF-β, which promotes microsphere formation (Penuelas S *et al.* 2009). As with type 2

T cells, increased numbers of Tregs within the tumor correlates with poor prognosis and

decreased responsiveness to immunotherapies (Chattopadhyay S *et al.* 2006; Clarke SL *et al.* 2006; Chikamatsu K *et al.* 2007; James E *et al.* 2010; Sellitto A *et al.* 2011;

Yamagami W *et al.* 2011).

Table 1

Cytokines released by type 1 and type 2 T cells and their functions in malignancy.

	Cytokine	Function
Type 1	Interferon-γ	Differentiation of naïve T cells into type 1 T cells
		Inhibition of the type 2 T cell response
		Increased cytotoxic $CD8^+$ T cell response
		Activation of macrophages
	Interleukin-2	Induction of T cell proliferation
		Clonal expansion of anti-tumor T cells
Type 2	Interleukin-4	Inhibition of the type 1 T cell response
		Reduced interferon-γ production
		Prevention of cancer cell apoptosis
		Polarization of tumor-infiltrating macrophages
	Interleukin-6	Increased metastatic potential
		Increased angiogenic potential
	Interleukin-10	Differentiation of naïve T cells into type 2 T cells
		Decreased cytotoxic $CD8^+$ T cell response
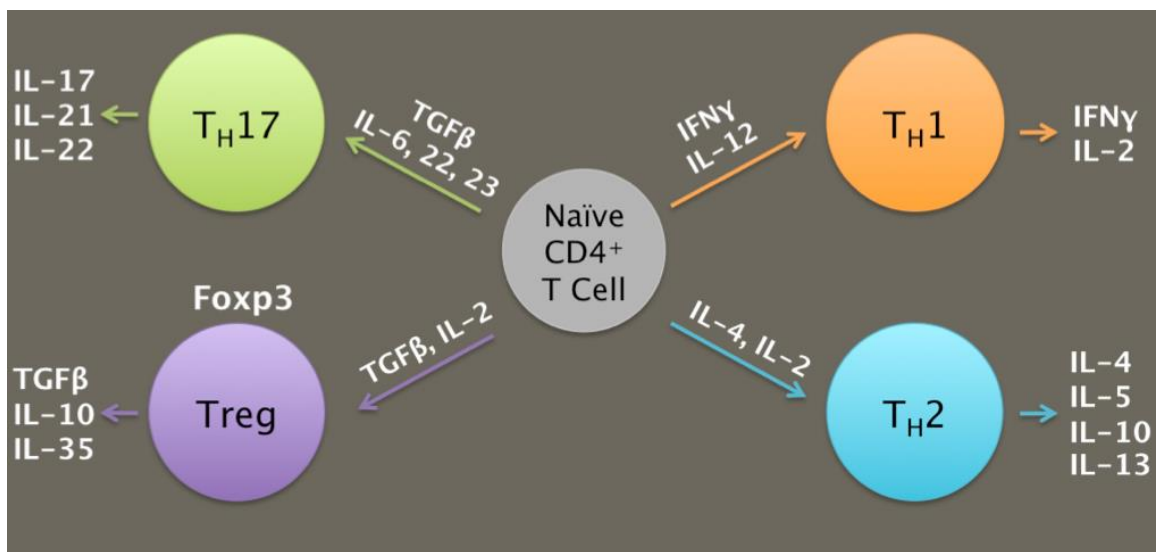
Figure 1.2. The divisions of T cell response. A naïve T cell can become a type 1 (orange), type 2 (blue), Treg (purple) or Th17 (green) cell based on the cytokines it is exposed to when activated. Upon differentiation, each cell type secretes a characteristic cytokine profile.

Th17 Cells. Pro-inflammatory CD4^{+} Th17 cells produce IL-17 to recruit neutrophils and induce the production of pro-inflammatory factors in immune cells (Kryczek I *et al.* 2009; Maniati E *et al.* 2010; Reynolds JM *et al.* 2010). The role of Th17 cells in cancer is still unclear. The inflammatory Th17 response correlates with poor disease outcome in prostate, head and neck and colorectal cancer. This is hypothesized to be due to production of the anti-inflammatory mediator prostaglandin-E2 (PGE2) and pro-angiogenic hypoxia-inducible factor 1-alpha (HIF1α) (Penuelas S *et al.* 2009). However, IL-17 knockout mice exhibited increased susceptibility to metastasis of colon cancer MC38 cells, demonstrating a possible anti-tumor function of Th17 cells (Maniati E *et al.* 2010). Th17 cells reduce Treg cell numbers by lowering TGF-β release and increase cytotoxic T cell function by secretion of IL-21 (Penuelas S *et al.* 2009). Although the precise mechanism of Th17 induction is still unclear, it appears that in malignancies initiated by inflammation, high numbers of Th17 correlate with poor prognosis due to their role in the induction of inflammation and angiogenesis (Yang ZZ *et al.* 2009). In tumors that were not initiated by an inflammatory response, Th17 cells may be beneficial in activating a cytotoxic immune response to eradicate the tumor (Yang ZZ *et al.* 2009).

Although all of these changes that occur to the immune cells in the tumor microenvironment have been documented in patient and animal models, the precise mechanism that causes them is not well understood. While we have evidence for a role for hypoxia in skewing the tumor-infiltrating macrophage population, the same cannot be said for T cells. There are several hypotheses, one of which is the idea that one or more

factors released by the tumor itself is likely a cause for this change in the T lymphocyte

population.

Survivin

Survivin as a Member of the Inhibitor of Apoptosis Family

Survivin is a 142 amino acid protein and member of the Inhibitor of Apoptosis

(IAP) protein family. As a member of this family it contains a baculoviral inhibitor of

apoptosis repeat (BIR) domain, so named because the original members of the IAP

family (Cp-IAP and Op-IAP) were found to use this domain to bind and inhibit caspases

in baculoviruses (Crook NE *et al.* 1993; Clem RJ and Miller LK 1994). In humans,

Survivin and its other IAP family members (XIAP, c-IAP1, c-IAP2, and NAIP) have

been shown to inhibit programmed cell death (Liston P *et al.* 1996; Ambrosini G *et al.*

1997). However, unlike its other family members, Survivin does not contain the typical

RING zinc finger or the caspase-recruitment domain (CARD) necessary for their anti-

apoptotic function. Despite lacking these domains, Survivin is capable of preventing

apoptosis signaled by TNF-α and Fas/FasL.

Anti-Apoptotic Function and Role in Cancer

Members of the IAP family typically prohibit programmed cell death through

direct interaction with caspases, particularly activated caspase-3 and -7 (Deveraux QL *et

al.* 1997; Roy N *et al.* 1997). With Survivin, the mechanism by which it inhibits

apoptosis is not as clear. Some research has suggested that Survivin inhibits caspases-3

and -7 directly (Tamm I *et al.* 1998; Shin S *et al.* 2001), while others have suggested that

there is no interaction with caspase-3 (Banks DP *et al.* 2000). Still others have suggested Survivin's anti-apoptotic effect is through inhibition of caspase-9 due to its ability to bind caspase-9 on the mitotic spindle and its ability to prevent staurosporine-induced death (O'Connor DS *et al.* 2000; Chandele A *et al.* 2004). While the precise mechanism by which Survivin prevents cell death is uncertain, it is known that Survivin can prevent Bax- and Fas-induced apoptosis (Ambrosini G *et al.* 1997; Tamm I *et al.* 1998).

Survivin's overexpression has been documented in most, if not all, types of cancer, and high levels of it have been linked to poorer prognosis in small cell lung cancer, non-small cell lung cancer, hepatocellular carcinoma, and colorectal carcinoma (Kawasaki H *et al.* 1998; Monzo M *et al.* 1999; Ikeguch M *et al.* 2002; Ikehara M *et al.* 2002). Survivin expression is also detectable in normal fetal development. *In situ* hybridization in chick embryos found that Survivin is expressed in the majority of fetal tissues, including lung, kidney and dorsal root ganglion. In mouse embryos, Survivin expression was ubiquitous throughout all tissues(Adida C *et al.* 1998). However, after fetal development Survivin expression is dramatically reduced or absent in mature human tissues. It is likely that through demethylation of the *survivin* gene promoter, malignant and pre-malignant cells are able to overexpress Survivin and evade apoptosis to go on to produce disease (Hattori M *et al.* 2001).

Survivin's Function in Cell Cycle Regulation

Survivin expression can be detected both in the cytoplasm and nucleus throughout the cell cycle, but its expression peaks during the G_2/M phase of the cell cycle (Li F *et al.* 1998). This nuclear and cell-cycle regulated expression led to the idea that Survivin may

play a role in cell proliferation. Indeed, upon further investigation it was found that Survivin is a component of the chromosomal passenger complex (CPC) along with Aurora-B and Borealin (Lens SM *et al.* 2006; Canovas PM and Guadagno TM 2007). Its function in the cell cycle includes organization of the central spindle and correction of spindle attachment errors to allow cell division. This function is seen not only in malignant cells, but also in T lymphocytes. Upon T cell activation and clonal expansion, Survivin is upregulated to aid in the rapid proliferation that occurs (Song J *et al.* 2005).

Survivin in Immune Cell Modulation

Survivin has a well-documented role in malignancy, but recent research shows it has an additional function in inflammation and autoimmunity. The amount of Survivin in the synovial fluid of rheumatoid arthritis patients has been correlated to a more severe disease progression (Bokarewa M *et al.* 2005). It is thought that synoviocytes, which resemble fibroblasts, may be the source of Survivin production in patients with rheumatoid arthritis (Ahn JK *et al.* 2010). Additionally, when recombinant Survivin was added to immune cell cultures, it was found to upregulate adhesion molecules CD11b and CD11c on granulocytes and monocytes. Similar adhesion molecule profiles were observed in rheumatoid arthritis patients with high levels of synovial fluid Survivin (Mera S *et al.* 2008).

Survivin as an Exosomal Protein

In addition to its cytosolic (Fortugno P *et al.* 2002), nuclear (Fortugno P *et al.* 2002) and mitochondrial (Dohi T *et al.* 2004) localization, Survivin has also been found

in the extracellular milieu and in exosomes secreted by cancer cells (Khan S *et al.* 2009; Khan S *et al.* 2011). Survivin has been shown to colocalize with Hsp70 in exosomes isolated from cancer cell-conditioned medium, demonstrating a novel pool of Survivin. Extracellular and exosomal Survivin has been shown to be taken up by malignant cells, resulting in altered function and increased aggressiveness (Khan S *et al.* 2009). Upon uptake into cancer cell lines, extracellular Survivin is able to induce proliferation and invasive potential as well as increase their resistance to UV radiation and chemotherapy. The exact mechanism that allows localization of Survivin into the exosome and its subsequent uptake into nearby malignant cells is not completely understood and is currently under investigation. However, it has been shown that increased cellular stress by sublethal doses of proton radiation enhances packaging of Survivin in exosomes, resulting in increased release of Survivin into the tumor microenvironment (Khan S *et al.* 2011).

Purpose of Study

While the role of T cells in cancer and their phenotypic alteration has been thoroughly assessed, the driving force behind this alteration is still not clear. Because of Survivin's extracellular release by exosomes and its ability to modulate leukocytes in autoimmune disease, we propose that Survivin within the tumor microenvironment could modify the T cell response to promote tumor growth. In order to evaluate this hypothesis we first sought to confirm that Survivin could be taken up by T lymphocytes in a similar manner as observed in malignant cells. Secondly we defined the effect of Survivin on cytotoxic $CD8^+$ T lymphocyte cytokine release and cytotoxic function. Lastly, we

evaluated Survivin's ability to skew normal T lymphocytes toward a type 2 phenotype,

which would aid cancer cells by decreasing anti-tumor inflammation.

CHAPTER TWO

ISOLATION AND T CELL UPTAKE OF TUMOR-RELEASED

SURVIVIN

Introduction

Survivin is a member of the Inhibitor of Apoptosis (IAP) protein family that has been shown to prevent cell death induced by the Fas/FasL, Bax and caspase pathways(Ambrosini G *et al.* 1997; Tamm I *et al.* 1998). This multi-functional protein also acts as a cell cycle regulator and is expressed in the G2/M phase to aid in proliferation. The proliferative and anti-apoptotic bi-functionality of Survivin makes it an essential component in the life of a cancer cell. Survivin has been found in the cytosol, mitochondria and nucleus of the cell (Fortugno P *et al.* 2002; Dohi T *et al.* 2004), as well as extracellularly in exosomes (Khan S *et al.* 2009; Khan S *et al.* 2011). The packaging of Survivin into exosomes allows its release into the tumor microenvironment where it is taken up by other cancer cells (Khan S *et al.* 2009; Khan S *et al.* 2011). This uptake of Survivin by malignant cells results in increased proliferative and invasive potential as well as resistance to chemotherapy (Khan S *et al.* 2009).

In addition to cancer cells, fibroblasts and immune cells populate the tumor microenvironment. While cancer cells take up extracellular Survivin, normal fibroblasts and epithelial cells, which normally do not express Survivin, show no uptake (Khan S *et al.* 2009). Some normal cells, such as T lymphocytes, do express low levels of Survivin causing us to question the ability of these normal cells to take up Survivin from the extracellular space. T lymphocytes are found abundantly within the tumor microenvironment, and frequently are modulated by the tumor to be less cytotoxic,

preventing tumor attack. If Survivin is taken up from the extracellular space by T

lymphocytes it could point towards a role for Survivin in T lymphocyte modulation in the

tumor microenvironment.

We isolated tumor-released Survivin from conditioned medium using the pOZN-

Survivin-WT cell line, which secretes a FLAG-HA-tagged Survivin. Survivin was pulled

down using dual-affinity immunoprecipitation and quantified by Enzyme-Linked

Immunosorbant Assay (ELISA). Evaluation of uptake showed that both malignant cells

and T lymphocytes could indeed take up the purified Survivin. Additionally, because

excess Survivin was observed to be toxic to normal T lymphocytes in large amounts, we

determined the sublethal dose of our purified Survivin that could be used in our future

studies. The results in this chapter provided the preliminary results that lead us to our

findings in the subsequent chapters.

Results & Discussion

Uptake of Purified FLAG-HA-Survivin in T Lymphocytes

Isolation and Quantification of Survivin from Conditioned Medium

We isolated a FLAG-HA tagged Survivin from the conditioned medium of HeLa-

S cells. This was accomplished using the pOZN-Survivin-WT cell line; a cervical

carcinoma HeLa-S cell line engineered to overexpress a FLAG-HA-tagged wild-type

Survivin (Khan S *et al.* 2009). The pOZN-Survivin-WT cells were grown in a bioreactor

and the conditioned medium collected after 24 hours. After eliminating cellular debris by

serial centrifugation, Survivin was isolated from the conditioned medium by

immunoprecepitation (IP) using anti-FLAG and anti-HA antibodies. Quantification of

the purified Survivin was done using a Survivin-specific ELISA and found to be 1.25 pg/μl.

While we had already shown that Survivin from conditioned medium could be taken up by cancer cells (Khan S *et al.* 2009), we needed to evaluate their ability to take up our purified Survivin. HeLa-S cells were cultured with various concentrations of isolated FLAG-HA-Survivin or conditioned medium and Survivin uptake evaluated by immunofluorescence microscopy. Figure 2.1 shows that at 0.625-12.5 pg/ml there is visible uptake of FLAG-HA-Survivin by anti-HA staining, comparable to what was seen in cells cultured with conditioned medium.

Isolation of T Lymphocytes and Determination of Sublethal Dose

Due to the fragile nature of primary T lymphocytes, we sought to determine what dose of extracellular Survivin could be used without causing significant toxicity. $CD3^+$ T lymphocytes were isolated from the peripheral blood of normal volunteers by centrifugation on Ficol-Hypaque solution, followed by magnetic separation. Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy layer and incubated with biotinylated antibodies against CD3. Using streptavidin magnetic beads the T lymphocytes were separated from other PBMCs on a magnetized column and then eluted. These cells were then cultured with 0-10 pg/ml FLAG-HA-Survivin and viability evaluated by 7-AAD staining and flow cytometry. The percentage of cell death increased with increasing doses of Survivin as shown in Figure 2.2. Based on this data we chose to use 1.25 pg/ml in our subsequent experiments.

0 pg/ml   **0.625 pg/ml**   **1.25 pg/ml**

6.25 pg/ml   **12.5 pg/ml**   **Conditioned Medium**
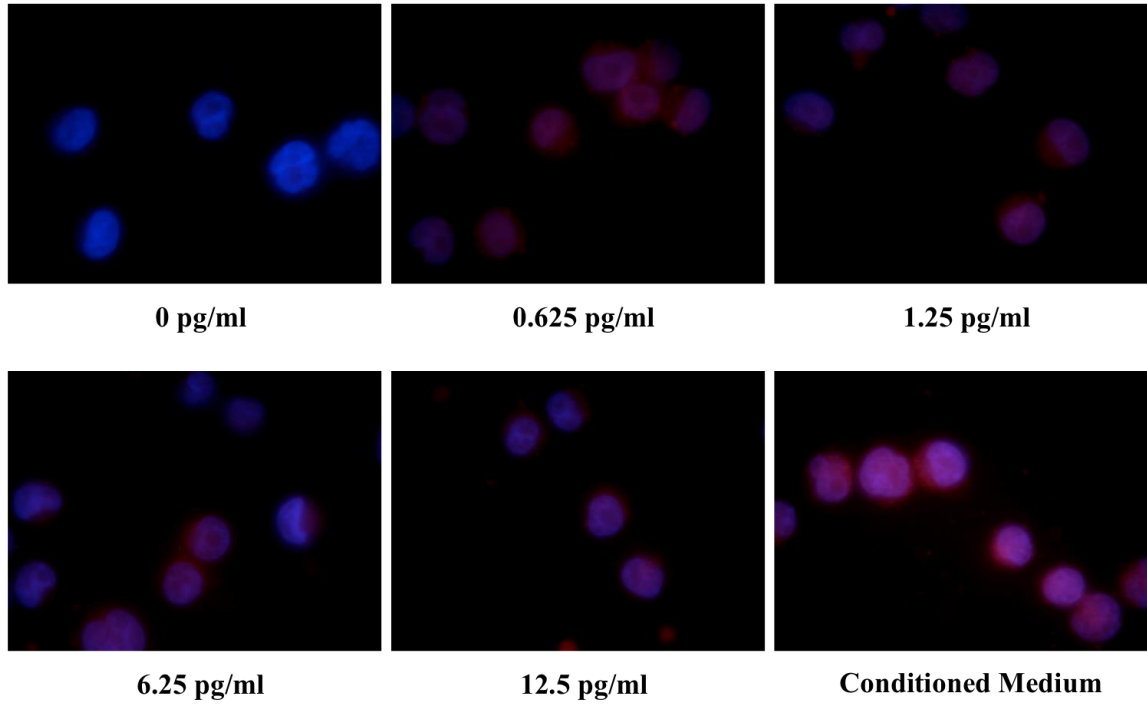
Figure 2.1. Uptake of purified FLAG-HA-Survivin by HeLa-S cells. FLAG-HA-Survivin was isolated by immunoprecipation from conditioned medium and added to cultured HeLa-S cells for 24 hours. Uptake at various concentrations was evaluated using immunofluorescene microscopy. Staining shows uptake at all concentrations equivalent to the conditioned medium positive control (Blue = DAPI; Red = HA).
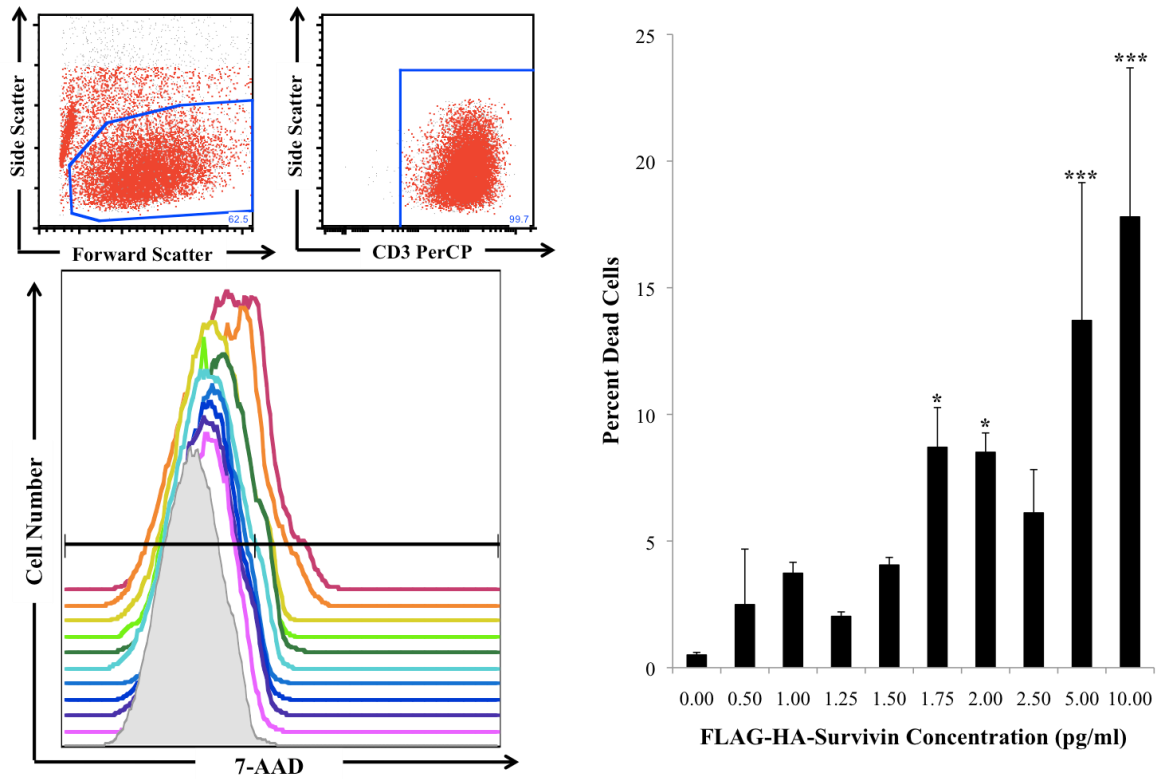
Figure 2.2. Analysis of T lymphocyte death with increasing Survivin concentration. Isolated T lymphocytes were incubated for 24 hours with FLAG-HA-Survivin at various concentrations. Cells were analyzed for death by 7-AAD staining and flow cytometry and showed an increase in cell death with increasing concentrations of Survivin. Gating scheme and percent dead cells are shown (*p< 0.05, *** p<0.001).

Evaluation of Survivin Uptake in T Lymphocytes

To determine if T lymphocytes can take up extracellular Survivin as cancer cells do, our isolated CD3$^+$ T lymphocytes were cultured with 1.25 pg/ml FLAG-HA-Survivin and evaluated for Survivin uptake by confocal microscopy as shown in Figure 2.3. Due to the narrow ring of cytoplasm surrounding each cell, it was difficult to determine the location of Survivin by confocal microscopy alone. The pyknotic staining of Survivin made it appear that it may be on the surface of the cell and not within the cytoplasm. To further assess this, Survivin uptake was evaluated by immunofluorescence microscopy with β-actin colocalization. As shown in Figure 2.4 there are areas of yellow staining where both the β-actin (red) and FLAG-HA-Survivin (green) are colocalized, as well as separate pyknotic green staining areas, showing that Survivin is both within the cytoplasm, as well as on the cell surface.

Further characterization of this uptake was performed using flow cytometry by both surface and intracellularly staining for Survivin and HA from 0-720 minutes after the addition of extracellular Survivin (Figure 2.5A,B). Beginning at 5 minutes, there is a similar amount of Survivin staining on the surface as well as intracellularly, followed by increased intracellular Survivin staining over time. These results indicate an initial surface binding of Survivin on T lymphocytes and a subsequent entry into the cytoplasm of the cell. Additionally, Western blot analysis of FLAG-HA-Survivin uptake in T lymphocytes showed that after entry into the cell, Survivin persists for at least 96 hours as demonstrated in Figure 2.5C.
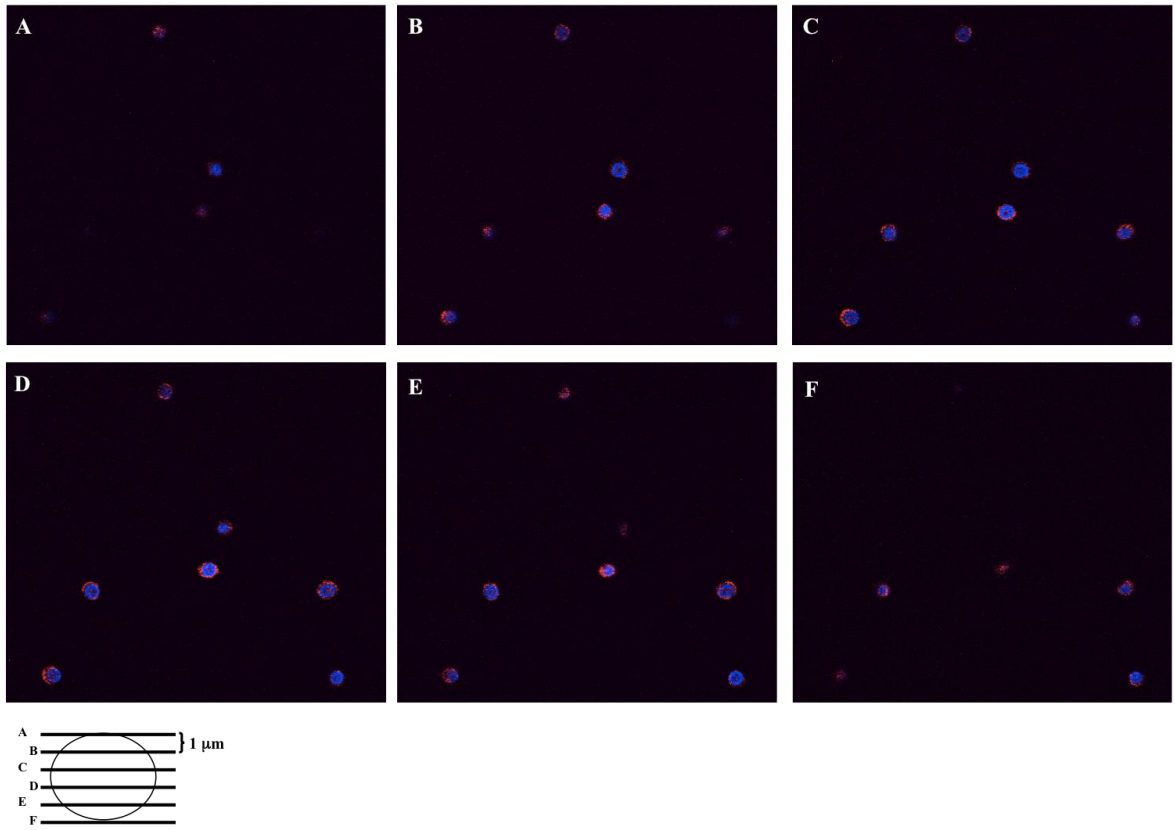
Figure 2.3. Confocal microscopy of Survivin uptake by T lymphocytes. T lymphocytes were incubated with FLAG-HA-Survivin for 24 hours and stained with anti-HA antibodies for confocal microscopy. Images show 1 micron slices through the cells. Pyknotic FLAG-HA-Survivin staining is seen on the T lymphocytes (Blue=DAPI, Red=HA).
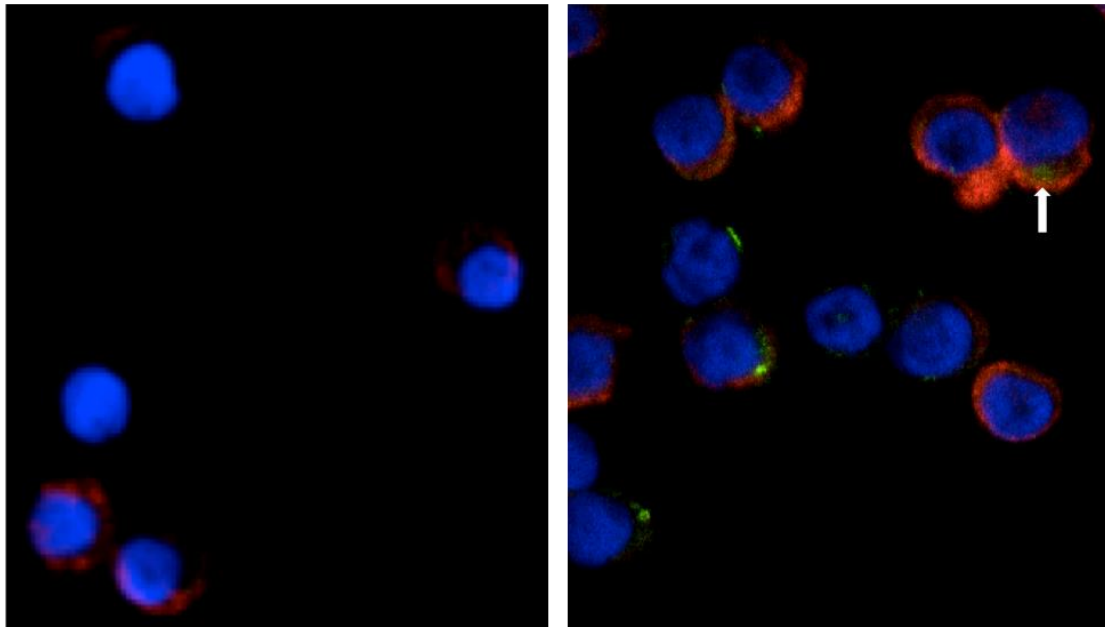
Control   **FLAG-HA-Survivin**

Figure 2.4. Colocalization analysis of Survivin uptake in T lymphocytes. T lymphocytes were cultured with FLAG-HA-Survivin and stained for both FLAG and β-actin. White arrow shows yellow colocalization of β-actin and FLAG, demonstrating the Survivin is in the cytoplasm as well as on the surface (Blue=DAPI, Red= β-actin, Green=FLAG).
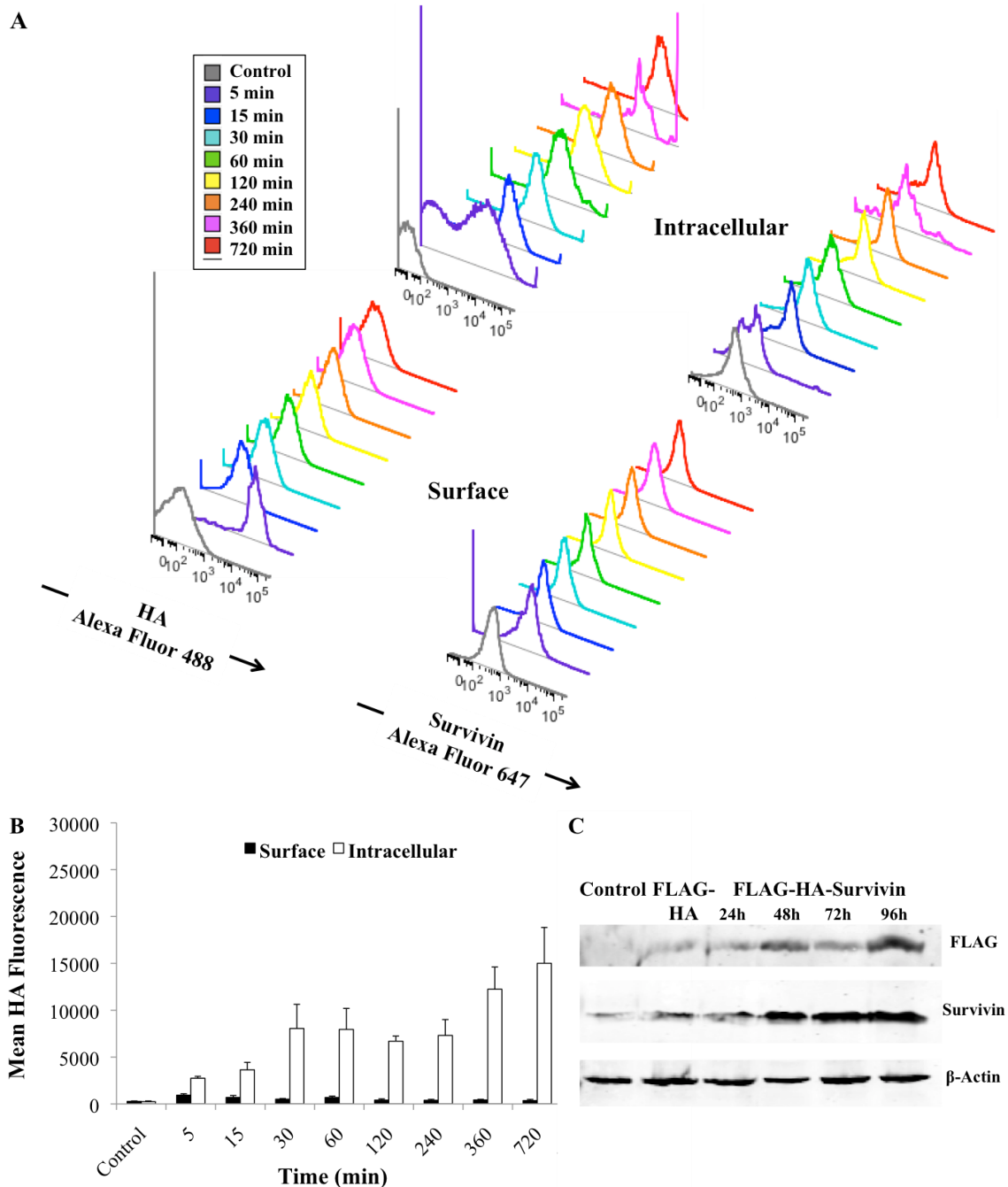
Figure 2.5. Flow cytometric and Western analysis of Survivin uptake in T lymphocytes. A. T lymphocytes were cultured with FLAG-HA-Survivin over time and analyzed for surface binding and intracellular uptake. Some surface binding was observed throughout, while intracellular levels increased over time. B. Graphical representation of flow cytometry data (Mean Intracellular Fluorescence = Total Fluorescence − Surface Fluorescence). C. Western analysis of T lymphocytes shows that FLAG-HA-Survivin persists in the cells even up to 96 hours after removal from culture.

Conclusions

The results of these studies show that indeed our purified FLAG-HA-Survivin can be taken up by malignant cells, just as is seen in conditioned medium. Additionally and most importantly, FLAG-HA-Survivin is able to bind to the surface of T lymphocytes as well as enter into the cells.   This finding, along with the persistence of FLAG-HA-Survivin within the lymphocytes for several days after incubation, points to a potential role for Survivin in immune evasion within the tumor microenvironment.

Materials & Methods

Collection of Conditioned Medium from pOZN-Survivin-WT Cells

FLAG-HA-Survivin pOZN-Survivin-WT cells were grown in a 5L bioreactor at 37°C in Joklik medium supplemented with 10% FCS, L-glutamine and penicillin/streptomycin. After 1 week of growth the conditioned medium was collected and centrifuged at 3,000 x g for 5 minutes at 4°C to remove cells. The supernatant was then centrifuged at 10,000 x g for 10 minutes at 4°C to remove cellular debris.

Isolation of FLAG-HA-Survivin from Conditioned Medium

Anti-FLAG beads (20 μl/ml) were added to the centrifuged conditioned medium and rotated overnight at 4ºC. The beads were pelleted by centrifugation and Survivin eluted from the beads using FLAG peptides. This process was repeated using anti-HA beads (20 μl/ml). Free FLAG and HA peptides were removed from the preparation by dialysis against a 10 mM HEPES buffer (pH 7.5) overnight at 4ºC. The concentration of Survivin was 1.2 ng/ml as determined by ELISA (R&D Systems). Synthesized FLAG

and HA peptides were added to 10mM HEPES buffer at a concentration of 1.2 ng/ml for use as a control.

Culture and Treatment of HeLa-S Cells

HeLa-S cells were cultured in flasks in a 5% CO_2 incubator with DMEM medium supplemented with 10% FCS, L-glutamine and penicillin/streptomycin. To evaluate for purified Survivin uptake, cells were cultured with conditioned medium from pOZN-Survivin-WT cells or with 0.0-12.5 pg/ml FLAG-HA-Survivin for 24 hours.

Isolation of T Lymphocytes from Peripheral Blood

Peripheral blood from normal, healthy donors was collected into potassium EDTA vacuettes as approved by the Institutional Review Board at Loma Linda University. Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy layer following centrifugation on a Ficoll-Hypaque gradient (GE Healthcare). PBMCs were washed with cold Streptavidin Labeling Buffer (PBS with 2mM EDTA pH7.2) and stained with anti-human CD3 biotinylated antibodies (BioLegend) at 4°C. Cells were again washed with cold Streptavidin Labeling Buffer and then incubated with Streptavidin magnetic microbeads. (Miltenyi Biotec) After an additional wash the cells were added to a magnetic column and eluted from the CD3$^-$ cells. The isolated CD3$^+$ cells were cultured in R10 medium (RPMI supplemented with 10% FBS, L-glutamine, penicillin/streptomycin, and 0.1% 2-mercaptoethanol).

Immunofluorescence and Confocal Microscopy

T lymphocytes or HeLa-S cells were cultured in 12-well plates in R10 or FLAG-HA-Survivin medium. Cells were harvested after 24 hours in culture, fixed with 3.7% paraformaldehyde and permeabilized with 0.1% PBS-saponin. Cells were stained with anti-FLAG, anti-HA or anti-Actin primary antibodies and goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 555 secondary antibodies (Invitrogen). Slides were imaged using the Olympus BX50 fluorescent microscope or Zeiss LSM 710 NLO laser scanning confocal imaging system, equipped with an Axio Observer Z1 inverted microscope.

Flow Cytometry

T lymphocytes were cultured in 96-well plates in R10 or FLAG-HA-Survivin medium at a concentration of 1×10^6 cells/ml. Cells were harvested at 5, 15, 30, 60, 120, 240, 360 and 720 minutes and stained for surface-bound or intracellular Survivin using rabbit anti-FLAG, goat anti-rabbit Alexa Fluor 488 secondary (Invitrogen, Carlsbad, CA), and anti-Survivin Alexa Fluor 647 antibodies (Cell Signalling, Danvers, MA). For intracellular staining, cells were fixed and permeabilized using the eBioscience Fixation/Permeabilization Kit (San Diego, CA). Cells were stained for T lymphocyte surface antigens with monoclonal antibodies against CD3, CD4, and CD8 (BioLegend, San Diego, CA). Data was collected on the MACSQuant flow cytometer (Miltenyi Biotec, Auburn, CA) and analyzed with FlowJo analysis software.

Western Blotting

For Western blots of Survivin uptake, T lymphocytes were cultured with FLAG-HA-Survivin, harvested at 24, 48, 72, and 96 hours, and washed with PBS. Whole cell lysates were prepared and separated using 12% Bis-Tris polyacrylamide gels run at 60mA for 45 minutes. Proteins were transferred onto nitrocellulose membranes at 350mA for 75 minutes. After blocking with Odyssey Blocking Buffer, membranes were probed using mouse monoclonal anti-FLAG (Invitrogen), rabbit polyclonal anti-Survivin (Novus Biologicals), and rabbit polyclonal anti-Actin (Cell Signalling). Secondary IR-Dye conjugated goat anti-rabbit and goat anti-mouse antibodies (LICOR) were utilized and bands detected using the Odyssey imaging system (LICOR).

CHAPTER THREE

MODULATION OF T LYMPHOCYTE PROLIFERATION BY

SURVIVIN

Introduction

Humoral and adaptive immune functions are crucial in the prevention of many cancers. Cytotoxic $CD8^+$ T lymphocytes normally aid in the prevention of cancer by destroying abnormal or transformed cells (Rosenberg SA *et al.* 1998). The failure of these responses have been implicated in the development and progression of many cancers, but is exemplified in cancers induced by viral infection such as Burkitt's Lymphoma and nasopharyngeal carcinoma (Li J *et al.* 2007) and increased frequency of malignancy in immunocompromised individuals (Vajdic CM and van Leeuwen MT 2009). Progression of malignancy is in part dependent on the tumor's ability to evade immune detection and cytolytic destruction by T lymphocytes (Hanahan D and Weinberg RA 2011). Indeed, patients with ovarian cancer have significantly improved prognosis when the tumor microenvironment is well infiltrated with cytotoxic T lymphocytes and natural killer cells (Zhang L *et al.* 2003; Sato E *et al.* 2005). However, induction of the T lymphocyte response does not always prevent progression of cancer, even in immunocompetent individuals. The mechanisms by which this occurs have been investigated and linked to reduced induction of T lymphocytes in response to the tumor as well as T regulatory cell inhibition of the T lymphocyte response (Woo EY *et al.* 2001).

Due to Survivin's critical role in cell proliferation, both in malignant cells and T lymphocytes (Altieri DC 2003; Song J *et al.* 2005), and our previous findings that tumor-

released Survivin effects malignant cell proliferation, we sought to evaluate the effects of

tumor-released Survivin on T lymphocyte activation and clonal expansion. In order to

determine the role of Survivin in the tumor immune response, we utilized our isolated

and purified FLAG-HA-Survivin to evaluate T lymphocyte proliferation, activation and

cytotoxic function. We noted a reduction in T lymphocyte proliferation in the presence

of tumor-released Survivin, while activation was not effected. Together this data

demonstrates a role for tumor-released Survivin in the impairment of the anti-tumor

response.

Results & discussion

Reduced Proliferation in Survivin-Exposed T Lymphocytes

To determine if Survivin uptake in T lymphocytes affects their ability to respond

to activating stimuli, we analyzed their proliferation using the cell-cycle-associated

nuclear antigen Ki-67 (Scholzen T and Gerdes J 2000), carboxyfluorescien succinymidyl

ester (CFSE) reduction and BrdU incorporation assays. T lymphocytes were isolated

from healthy, cancer-free donors, stimulated with anti-CD3/CD28 T cell expansion beads

and cultured in control medium, medium containing isolated FLAG and HA peptides, or

medium containing tumor-released FLAG-HA-Survivin. Expression of intracellular Ki-

67 was analyzed at 24, 48, 72 and 96 hours by flow cytometry. Figure 3.1 shows a

representative gating scheme for our flow cytometry data. T cells were selected by

positive CD3 staining and Figure 3.2A shows representative staining of Ki-67 at 72 hours

post-stimulation. At 72 and 96 h, Ki-67 expression was significantly decreased in both

$CD8^+$ and $CD4^+$ T lymphocytes when cultured with FLAG-HA-Survivin compared to

Control and FLAG-HA only medium (Figure 3.2B). This demonstrates decreased T cell

proliferation in the presence of Survivin.
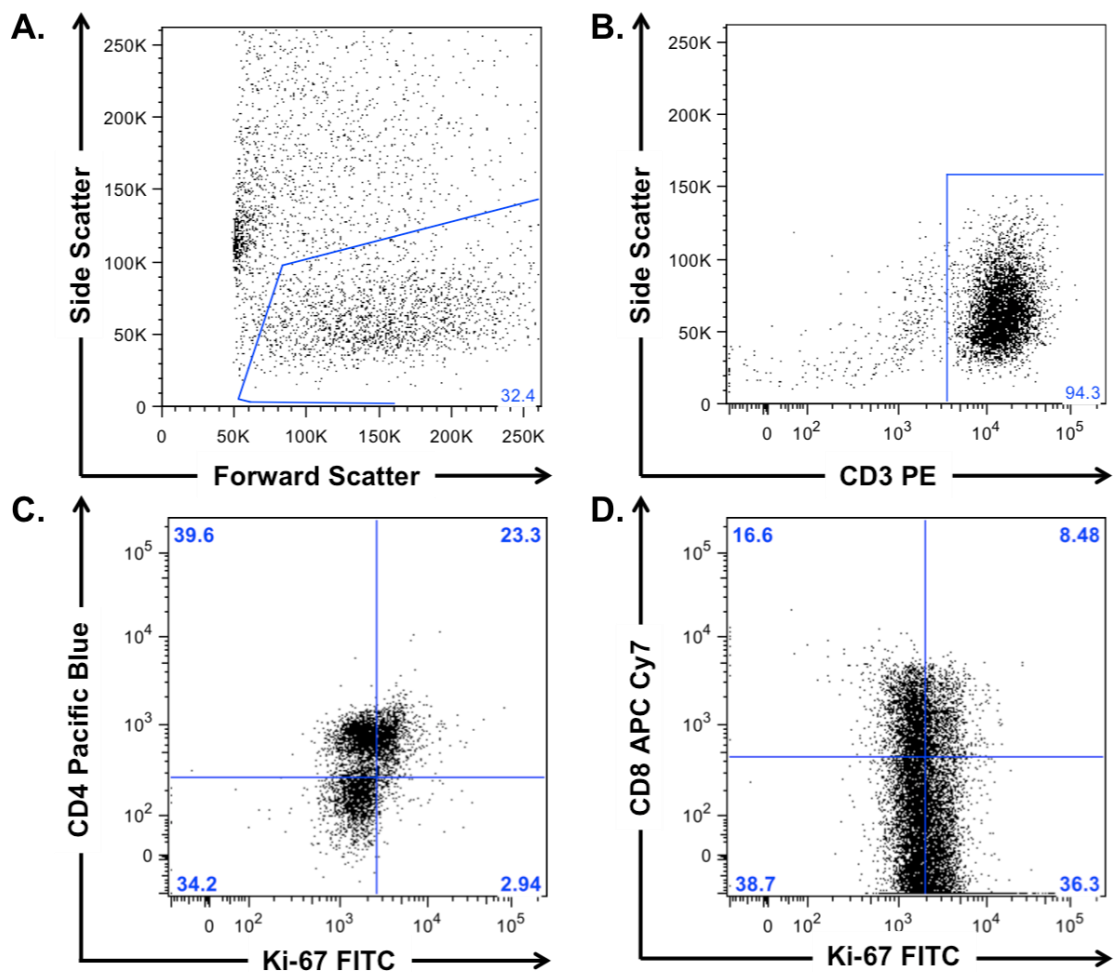
Figure 3.1. Gating scheme for Ki-67 analysis. A. Lymphocytes were selected by their Forward and Side Scatter profiles. B. T lymphocytes were selected by positive staining for CD3. Ki-67 expression was analyzed on both C. CD4 and D. CD8 stained populations.
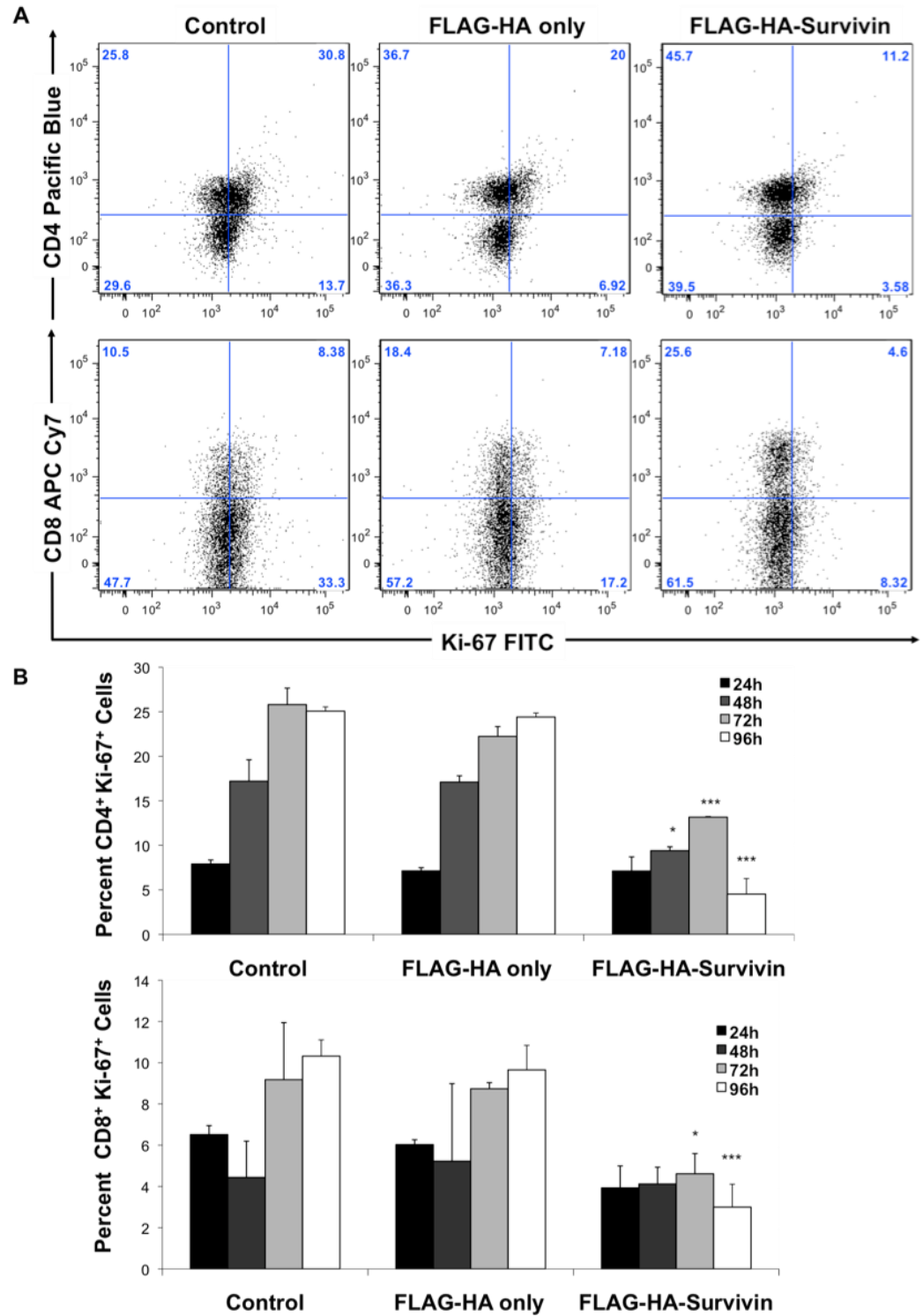
Figure 3.2. Ki-67 expression in T lymphocytes A. Representative gating scheme of T lymphocyte Ki-67 expression. B. Normal T lymphocytes have significantly decreased Ki-67 expression at 72 and 96 hours following incubation with cancer cell-released FLAG-HA-Survivin compared to control and FLAG-HA only medium. *p<0.05, ***p<0.001.

To confirm these results we evaluated CFSE dilution in T lymphocytes. CFSE is a cytosolic fluorescent dye used to detect proliferation, with a dilution of the dye observed with each cell division (Lyons and Parish, 1994). If indeed Survivin is reducing proliferation in these cells, the CFSE fluorescence of cells stimulated in the presence of Survivin should be higher due to reduced dilution through proliferation compared to controls. Helper and cytotoxic T lymphocytes were selected by CD4 and CD8 expression respectively and analyzed for CFSE fluorescence intensity (Figure 3.3). CFSE staining showed dilution in response to stimulation in Control and FLAG-HA only cells, which was prevented by the addition of FLAG-HA-Survivin (Figure 3.4). This confirms are intital Ki-67 data, showing that indeed the presence of tumor-released Survivin prohibits T lymphocyte proliferation in the presence of activating stimuli.

Tertiary confirmation was done by a bromodeoxyuridine (BrdU) incorporation assay. As cells replicate their DNA to prepare for division they incorporate the BrdU, which can be detected by flow cytometry. We expected that in the presence of Survivin, the number of cells that have replicated their DNA for division and have become BrdU-positive would be reduced if Survivin were indeed decreasing proliferation. T lymphocytes were cultured with control medium or that containing FLAG and HA peptides or FLAG-HA-Survivin and BrdU added after 72 hours in culture. Cells were harvested 24 hours later and analyzed for nuclear BrdU by flow cytometry. T lymphocytes cultured with FLAG-HA-Survivin showed significantly reduced percentage of cells with BrdU incorporation (Figure 3.5).

Final confirmation was done by cell counting by flow cytometry to determine if indeed a reduced number of cells was present due to this reduce proliferation observed by

our previous assays. After culture and stimulation total T lymphocyte numbers were significantly decreased in FLAG-HA-Survivin cultures compared to controls (Figure 3.6A). Due to the fact that primary cells are far less resilient to the addition of exogenous materials than malignant cell lines, it was necessary to rule out cell death as a reason for this decreased proliferation and lower cell numbers. To quantify cell death in our populations, we analyzed viability using 7-AAD staining and no significant difference in viability was seen in FLAG-HA-Survivin compared to controls (Figure 3.6B). This data shows that Survivin has an anti-proliferative affect in T lymphocytes.
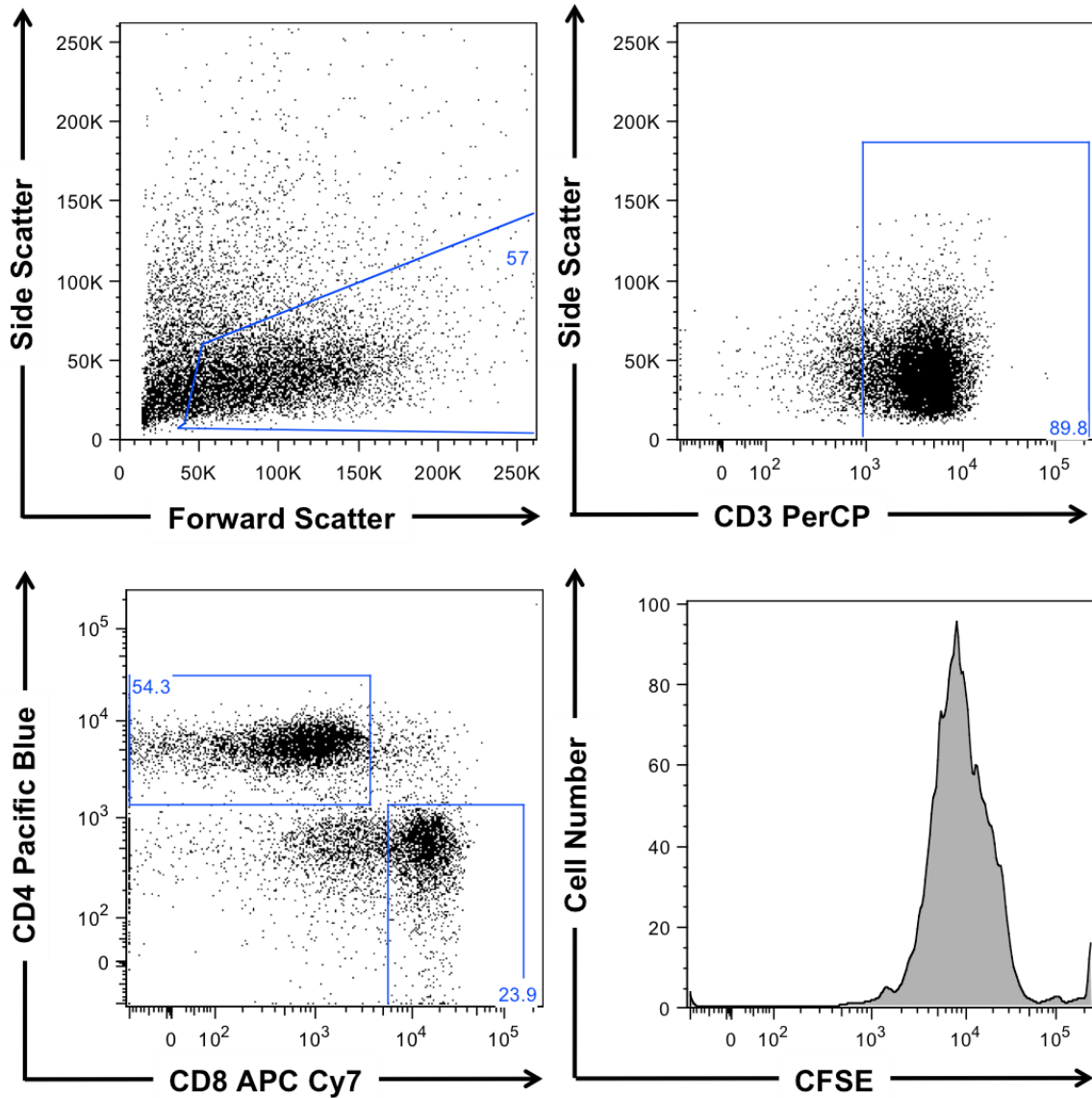
Figure 3.3. Representative gating scheme for CFSE analysis. T lymphocytes were selected by their Forward and Side Scatter profiles and positive staining for CD3. Helper and cytotoxic T lymphocyte populations were selected by CD4 and CD8 staining, respectively.
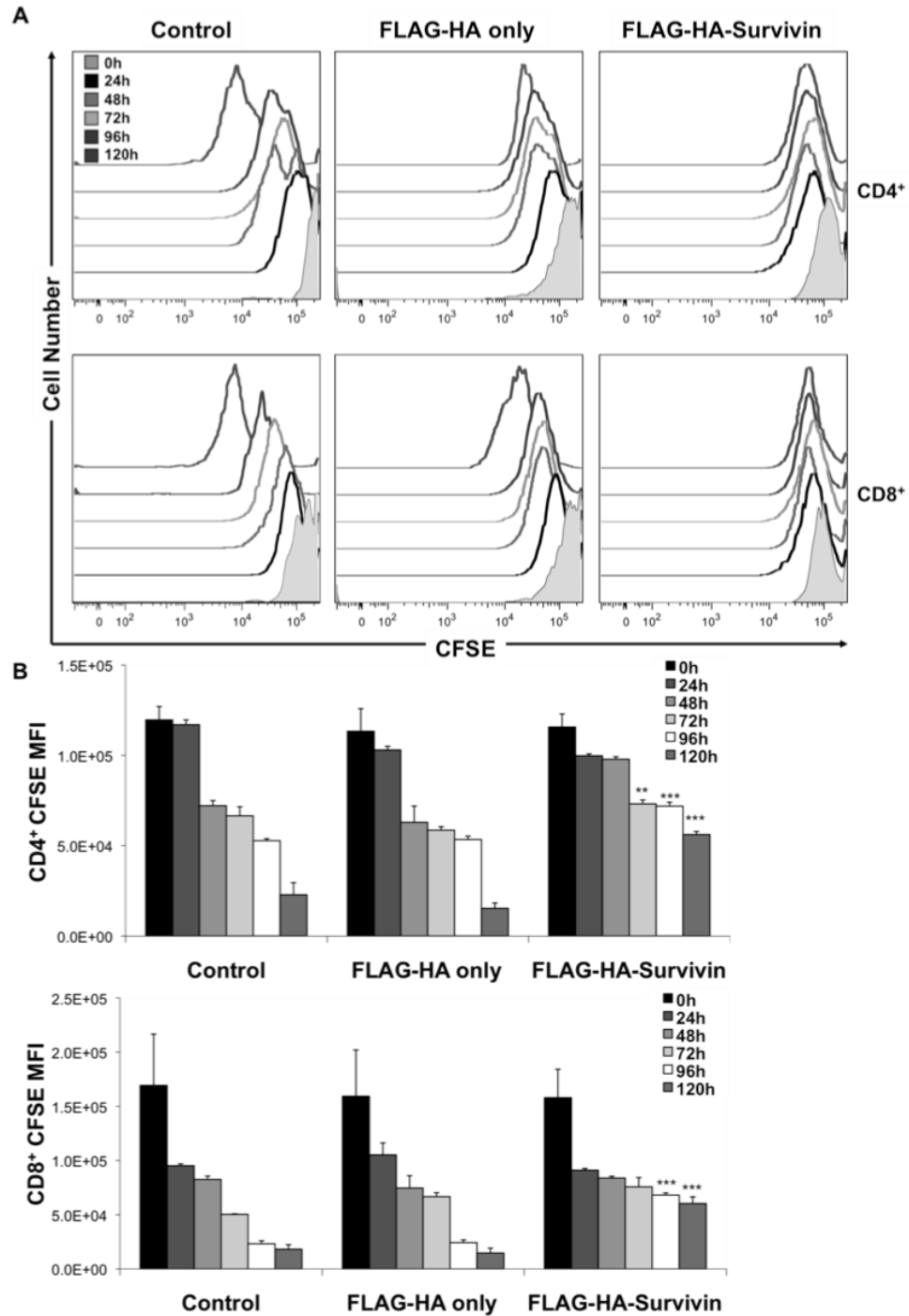
Figure 3.4. CFSE dilution in T lymphocytes. A. Plot of CFSE mean fluorescence intensity. B. T lymphocytes have significantly decreased CFSE expression at 72 and 96 hours after activation in control conditions. CFSE dilution is reduced in cultures containing FLAG-HA-Survivin. $**p<0.01$, $***p<0.001$.
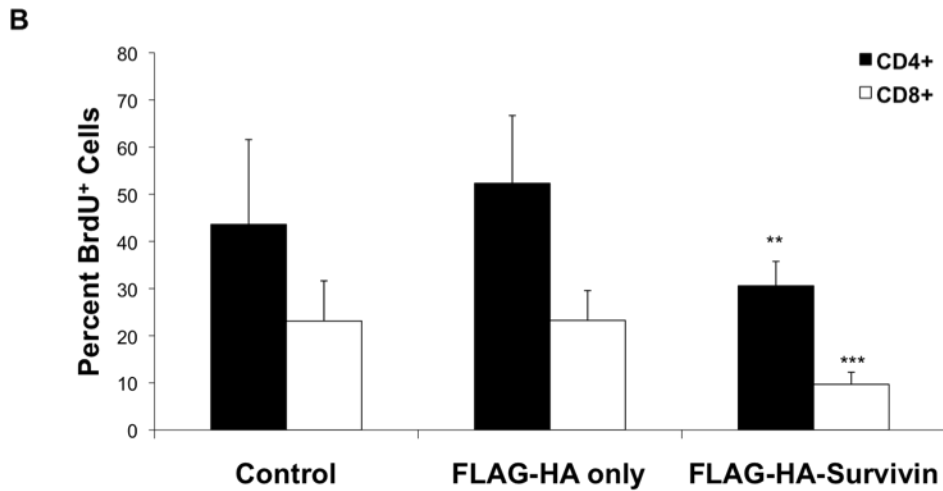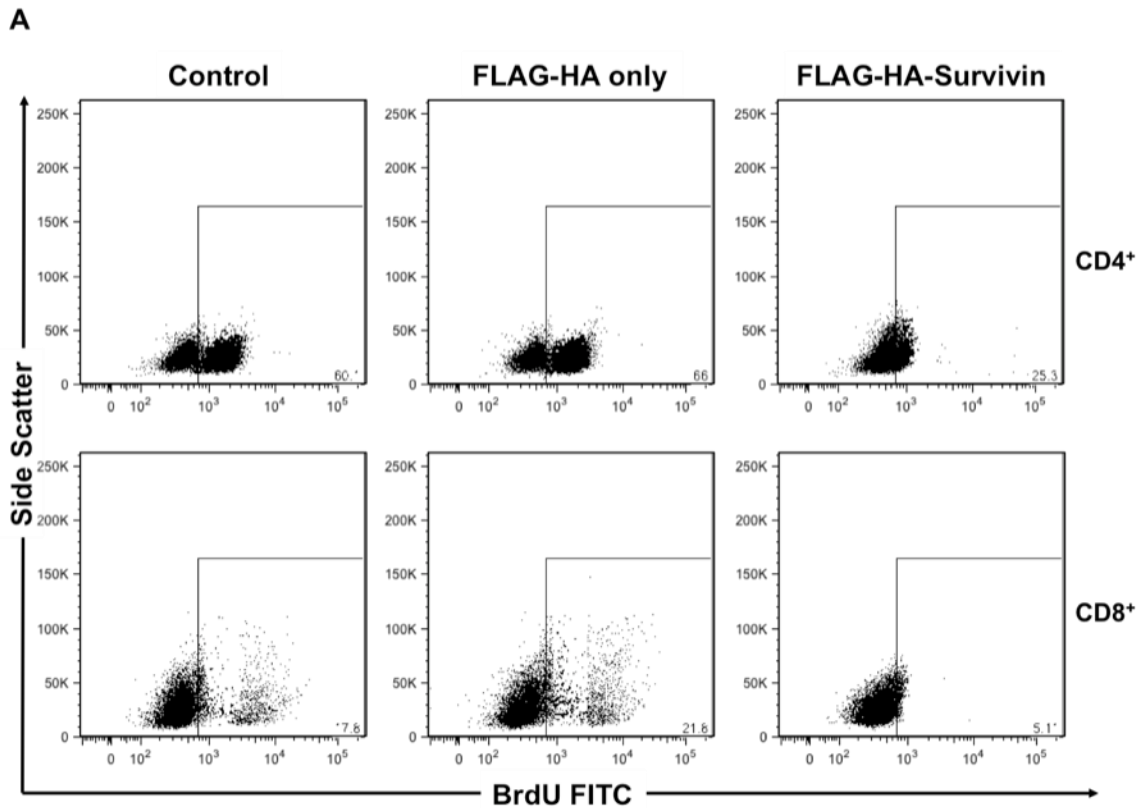
Figure 3.5. BrdU incorporation in T lymphocytes. A. BrdU staining in CD4 and CD8 T lymphocytes. B. T lymphocytes show decreased BrdU incorporation in the presence of Survivin compared to controls. **p<0.01, ***p<0.001.
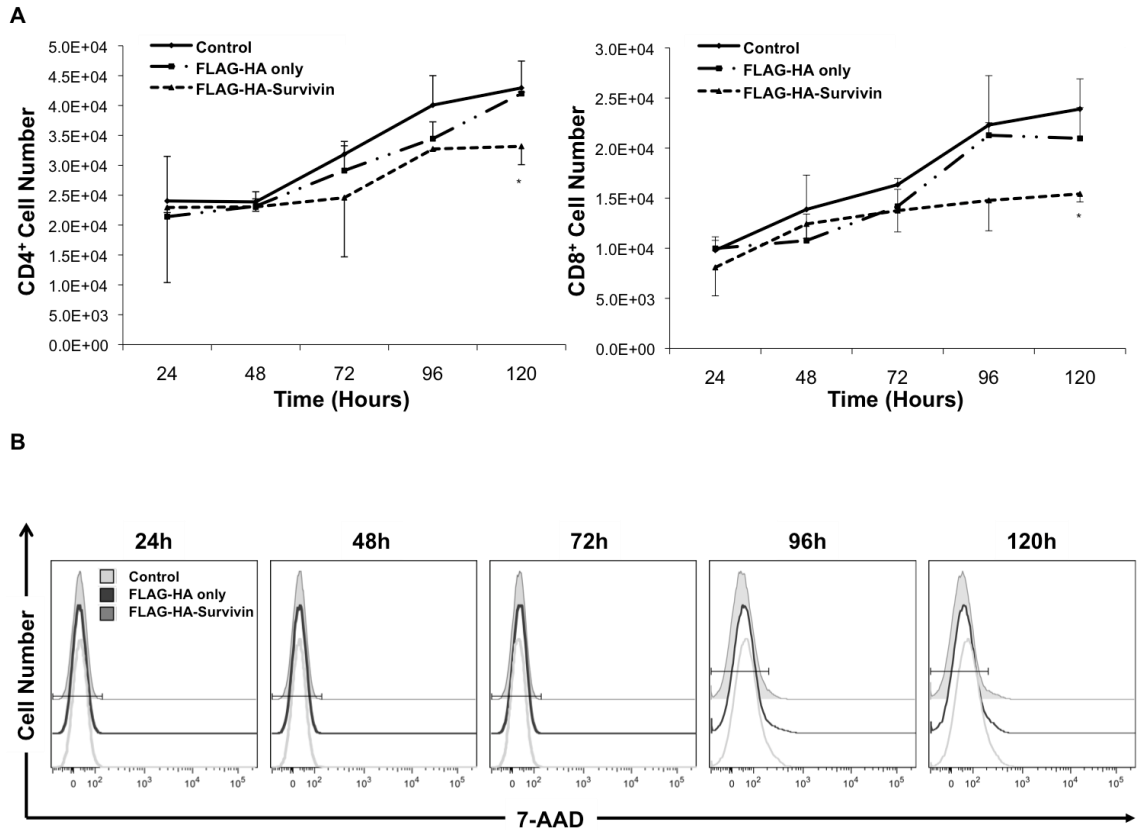
Figure 3.6. Cell number and viability analysis. A. Culture with Survivin results in decreased numbers of both CD4$^+$ and CD8$^+$ T lymphocytes upon stimulation with anti-CD3/anti-CD28. B. Viability analysis by 7-AAD shows no increased cell death in T lymphocytes that are cultured with Survivin.

Survivin's ability to prohibit proliferation in T lymphocytes is in stark contrast to the increased proliferative rate seen in malignant cells after Survivin uptake (Khan S *et al.* 2009). One key difference between T lymphocytes and tumor cell proliferation is the requirement of activation signalling for T lymphocyte clonal expansion (Morris J and Waldmann T 2000; Song J *et al.* 2005). Survivin's anti-proliferative effect on T lymphocytes could be due to an interference with T cell activation.

To examine this possibility, T lymphocytes were stimulated in the presence or absence of extracellular FLAG-HA-Survivin and analyzed for the expression of activation markers CD25 and CD69. CD25, part of the high-affinity interleukin-2 (IL-2) receptor, is expressed on T lymphocytes after activation to aid in the response to the pro-proliferative cytokine IL-2. CD69 is an activation marker that is upregulated on the surface of T lymphocytes 2-4 hours after stimulation through CD3 signaling (Hamann J *et al.* 1993). If Survivin is indeed interfering with the activation process, the expression of these two molecules that require stimulation for expression would be significantly reduced. We found that the addition of FLAG-HA-Survivin during stimulation had no effect on the expression of activation antigens on T lymphocyte cultures (Figure 3.7). This data shows that Survivin's anti-proliferative effects are not mediated by interfering with T cell activation through the T cell receptor-CD3 complex.

T lymphocytes are one of the few adult tissues that normally express Survivin. Indeed, endogenous expression of Survivin is required for proper T cell activation and proliferation (Song J *et al.* 2005). To determine if our exogenous FLAG-HA-Survivin was altering endogenous Survivin levels we measured Survivin expression in stimulated T lymphocytes in the presence of FLAG and HA peptides or FLAG-HA-Survivin by

Western blot. The presence of exogenous FLAG-HA-Survivin does not significantly

alter Survivin protein expression levels after activation (Figure 3.8). From these results

we conclude the inhibition of proliferation by Survivin likely occurs after cells have been

activated and is not due to a reduction in activation-induced Survivin expression.
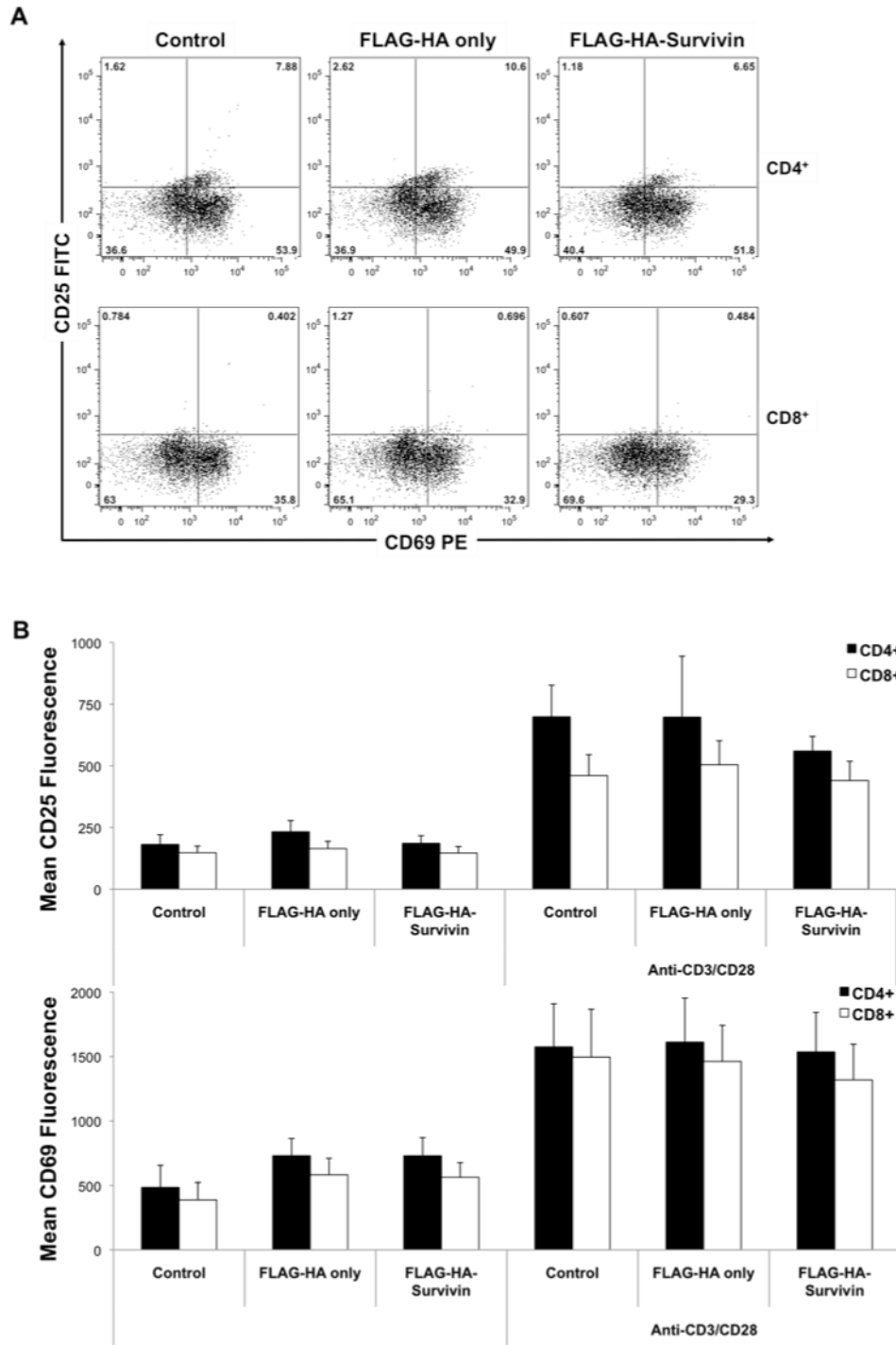
Figure 3.7. Analysis of T lymphocyte activation. A. Flow cytometric analysis of CD25 and CD69 activation markers after stimulation with anti-CD3/anti-CD28. B. No significant difference in CD25 or CD69 expression was seen in T lymphocytes after culture with Survivin.
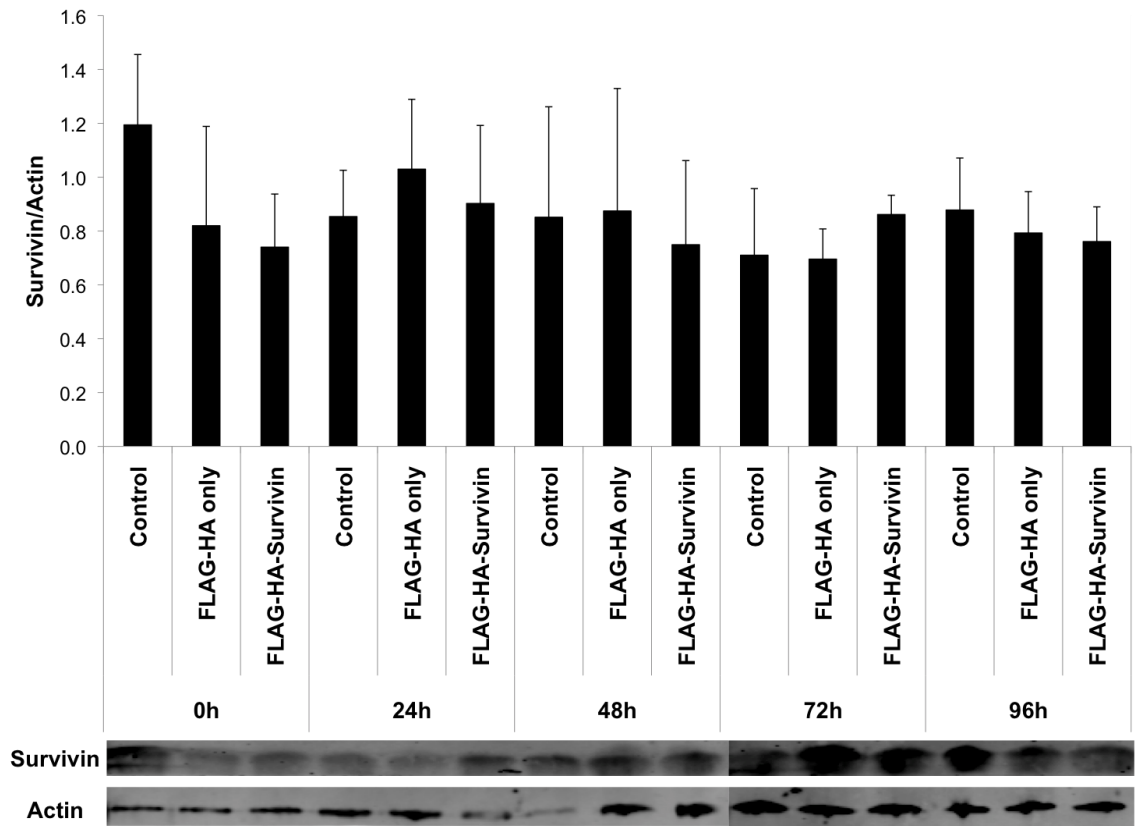
Figure 3.8. Western blot of Survivin expression in activated T lymphocytes. The addition of FLAG-HA-Survivin to T lymphocyte cultures does not significantly affect the expression of endogenous Survivin after activation.

Conclusion

Based on our data, we suggest that Survivin allows malignant cells to evade immune detection be decreasing the ability of T lymphocytes to proliferate in response to activating stimuli. From the evaluation of activation markers CD25 and CD69, Survivin is not inhibiting T lymphocyte stimulation, but is possibly acting downstream of activation signaling. Additionally, no changes in activation-induced endogenous Survivin in the presence of FLAG-HA-Survivin were observed. Due to the significantly diminished proliferation observed in both CD4$^+$ and CD8$^+$ populations from cancer-free donors, we believe a similar effect would be seen in tumor-specific T lymphocyte and overall T lymphocyte populations from cancer patients, though these studies have yet to be accomplished.

Materials & Methods

Isolation of T Lymphocytes from Peripheral Blood

Peripheral blood from normal, healthy donors was collected into potassium EDTA vacuettes as approved by the Institutional Review Board at Loma Linda University. Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy layer following centrifugation on a Ficoll-Hypaque gradient (GE Healthcare). PBMCs were washed with cold Streptavidin Labeling Buffer (PBS with 2mM EDTA pH7.2) and stained with anti-human CD3 biotinylated antibodies (BioLegend) at 4°C. Cells were again washed with cold Streptavidin Labeling Buffer and then incubated with Streptavidin magnetic microbeads. (Miltenyi Biotec) After an additional wash the cells were added to a magnetic column and eluted from the CD3$^-$ cells. The isolated CD3$^+$

cells were cultured in R10 medium (RPMI supplemented with 10% FBS, L-glutamine, penicillin/streptomycin, and 0.1% 2-mercaptoethanol).

T Lymphocyte Proliferation Analysis by CFSE and Ki-67

T lymphocytes were stained with 0.5 μM CellTrace carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Carlsbad, CA) for 15 min at 37°C and the reaction quenched with 5 volumes of cold R10 medium (RPMI supplemented with 10% FBS, L-glutamine, penicillin/streptomycin, and 0.1% 2-mercaptoethanol). Cells were plated in a 96-well plate at 2.5×10^5 cells/well in R10, FLAG-HA, or FLAG-HA-Survivin medium, stimulated with anti-CD3/anti-CD28 T cell expansion beads (6.25 μl/well; Invitrogen, Carlsbad, CA) and harvested at 0, 24, 48, 72, 96 and 120 hours. Cells were stained for surface antigens CD3, CD4, CD8 and for viability using 7-AAD (BioLegend, San Diego, CA). For Ki-67 analysis cells were cultured as above and harvested at 24, 48, 72, and 96 hours. Cells were stained for surface markers, fixed and permeabilized using a Fixation/Permeabilization Kit (eBioscience, San Diego, CA) and stained for intracellular Ki-67.

Analysis of T Lymphocyte Proliferation by BrdU Incorporation

T cells were plated in a 96-well plate at 2.5×10^5 cells/well in R10, FLAG-HA, or FLAG-HA-Survivin medium and stimulated with anti-CD3/anti-CD28 T cell expansion beads (6.25 μl/well; Invitrogen, Carlsbad, CA). At 72 hours in culture, 10 μM BrdU was added to each well and the cells harvested 24 hours after BrdU addition. Cells were stained for surface CD3, CD4 and CD8, fixed and permeabilized using BD

Cytofix/Cytoperm Buffer (BD Biosciences, San Jose, CA), and frozen at -80°C overnight in freezing medium (10% DMSO, 90% heat-inactivated FBS). Cells were thawed, washed using BD Cytofix/Cytoperm and then washed again with BD Perm/Wash Buffer, after which they were re-fixed as before. Cells were treated with DNase (300µg/ml) and stained with anti-BrdU FITC (BD Biosciences, San Jose, CA) before analysis on the MACSQuant flow cytometer.

Analysis of T Lymphocyte Activation

Isolated T lymphocytes were cultured and stimulated as described above. After 24 hours, cells were harvested and stained with monoclonal antibodies against CD3, CD4, CD8 (BioLegend, San Diego, CA), CD25 and CD69 (BD Biosciences, San Jose, CA) and analyzed by flow cytometry.

Western Blotting

To analyze Survivin expression after activation, T lymphocytes were cultured with control, FLAG-HA or FLAG-HA-Survivin medium and activated using anti-CD3/anti-CD28 T cell expansion beads (25 µl/ml; Invitrogen, Carlsbad, CA). Cells were harvested at 0, 24, 48, 72, and 96 hours. Whole cell lysates were prepared and separated using 12% Bis-Tris polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes and probed using rabbit polyclonal anti-Survivin (Novus Biologicals, Littleton, CO) and rabbit polyclonal anti-Actin (Cell Signalling, Danvers, MA). Secondary IR-Dye conjugated goat anti-rabbit antibodies (LICOR, Lincoln, NE) were utilized and bands detected using the Odyssey imaging system (LICOR, Lincoln, NE).

CHAPTER FOUR

ALTERATION OF CYTOTOXIC T LYMPHOCYTES BY

SURVIVIN

Introduction

Peripheral blood and intratumoral T cells from cancer patients frequently exhibit altered T cell populations. Cytotoxic T lymphocyte (CTL) function is frequently diminished as regulatory T cells (Tregs) increase and cytokine profiles are altered. Tregs are a subset of the helper T lymphocyte population and are characterized by their expression of the transcription factor Foxp3 (Chen W *et al.* 2003). Their primary function is to prevent autoimmunity by inducing apoptosis or anergy in lymphocytes that recognized self-antigens. Regulatory T cells have been implicated in promoting tumor growth by suppressing activation of tumor-specific cytotoxic T cells due to their expression of auto-antigens (Clarke SL *et al.* 2006; Sellitto A *et al.* 2011; Wang Z *et al.* 2011; Yamagami W *et al.* 2011). The balance of CTLs and Tregs in the tumor microenvironment is dependent partly on the cytokine environment, as increased levels of Transforming Growth Factor-β (TGF-β) induce naïve helper T cell differentiation into Tregs, which go on to reduce CTL cytotoxic numbers and functions (Chen W *et al.* 2003). Increased numbers of Tregs and decreased numbers of CTLs within the tumor correlates with poor prognosis and decreased responsiveness to immunotherapies (Ke X *et al.* 2008). We hypothesize that Survivin can diminish cytotoxic T cell activity and numbers through modulation of the cytokine environment and the induction of regulatory T cells.

Results & discussion

Cytotoxic Function in CD8⁺ T Lymphocytes

Tumor-specific cytotoxic T lymphocytes (CTLs) are important in the destruction

of cancer cells. As described in chapter three, we have observed decreased proliferation

of CTLs when stimulated in the presence of Survivin, which would likely diminish the

effectiveness of an anti-cancer T lymphocyte response. It is not known if Survivin also

alters the cytotoxic function of CTLs, so we evaluated this using a cytotoxicity assay.

CTLs were isolated from a single donor and cultured with Control, FLAG-HA or FLAG-

HA-Survivin medium and stimulated with PMA and ionomycin for 24 hours. The cells

were then added in culture with pooled peripheral blood mononuclear cells (PBMCs)

from other healthy donors at 5:1, 2.5:1 and 1.25:1 CTL:PBMC ratios. The ability of the

CTLs to kill foreign PBMCs was then evaluated using by lactate dehydrogenase release.

The cytotoxic function of CTLs was diminished at all CTL:PBMC ratios after culture

with FLAG-HA-Survivin compared to control conditions (Figure 4.1). This demonstrates

a possible immune evasion function for tumor-released Survivin by preventing CTL-

mediated killing.
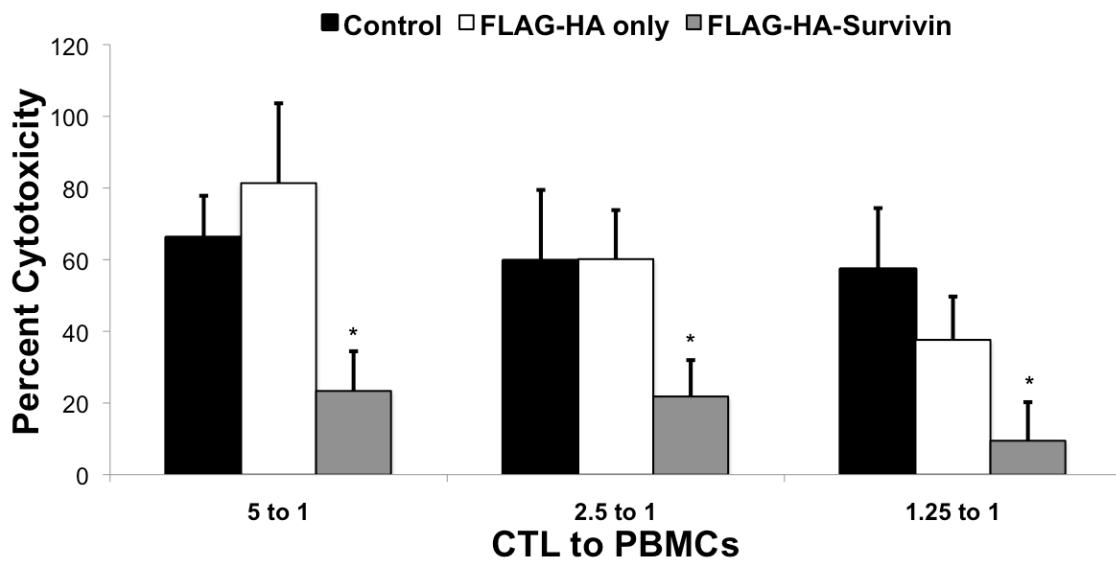
Figure 4.1. Cytotoxicity of CD8^{+} T lymphocytes. Cytotoxic T lymphocyte cytolytic capabilities are reduced by pre-treatment with tumor-released Survivin. CD8^{+} cells from a single donor were cultured with Survivin for 24 h and their cytotoxic function assessed by the CytoTox 96 Non-Radioactive Cytotoxicity Assay. *p<0.05

Regulatory T Lymphocyte Analysis

Evaluation of Regulatory T Lymphocyte Induction

The reduction of cytotoxic function in $CD8^+$ T lymphocytes in the presence of malignancy has been shown to be related to increased regulatory T lymphocytes (Treg) in the tumor microenvironment (Chikamatsu K *et al.* 2007; Ke X *et al.* 2008). To determine if the decreased CTL function observed in the presence of Survivin could be due in part to Tregs, we evaluated the induction of Tregs after culture with Survivin. $CD3^+$ cells were cultured in control or Survivin containing medium with stimulation for 48 hours and analyzed for the number of $CD4^+CD25^+CD127^{low}$ Treg cells by flow cytometry. No differences were seen in the number of Tregs in the presence of Survivin compared to controls (Figure 4.2).

Regulatory T Lymphocyte Function

To analyze the function of Tregs after culture with Survivin, $CD4^+CD127^{low}$ Tregs were isolated by magnetic separation. After 24 hours in culture with control, FLAG-HA, or FLAG-HA-Survivin medium, the cells were washed and plated with PBMCs and anti-CD3/anti-CD28 microbeads for 4 hours. Analysis of CD154 and CD69 levels on non-Treg cells was used to determine the ability of Tregs to suppress activation. We observed that Treg function was not altered by culture with tumor-released Survivin (Figure 4.3). Due to these results, the decreased cytotoxic function of $CD8^+$ cells is likely a direct effect of Survivin, and not through increased Treg numbers or function.
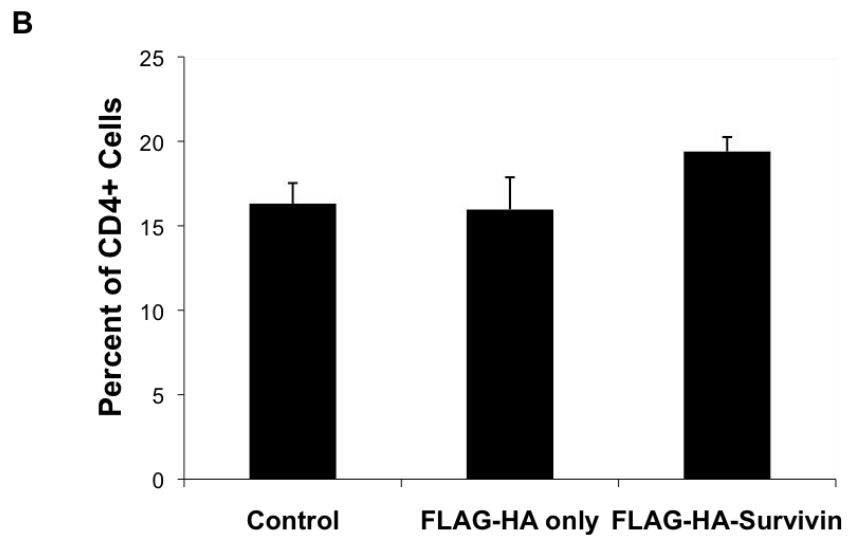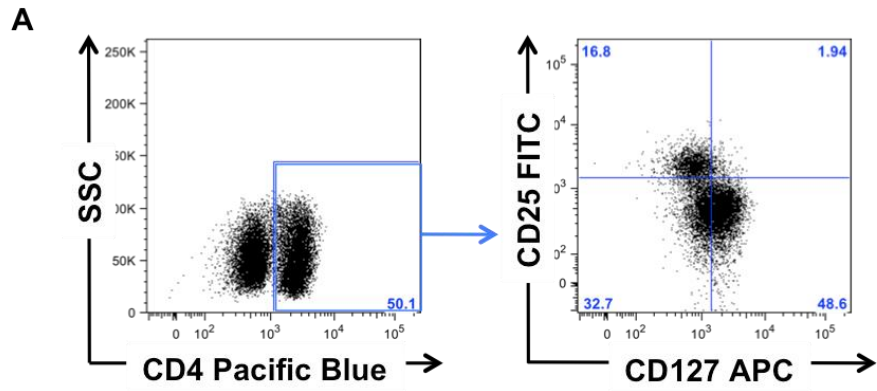
Figure 4.2. Analysis of Regulatory T Lymphocytes   A. Gating scheme for regulatory T cells. B. No significance difference in the amount of regulatory T cells was observed in the presence of Survivin.
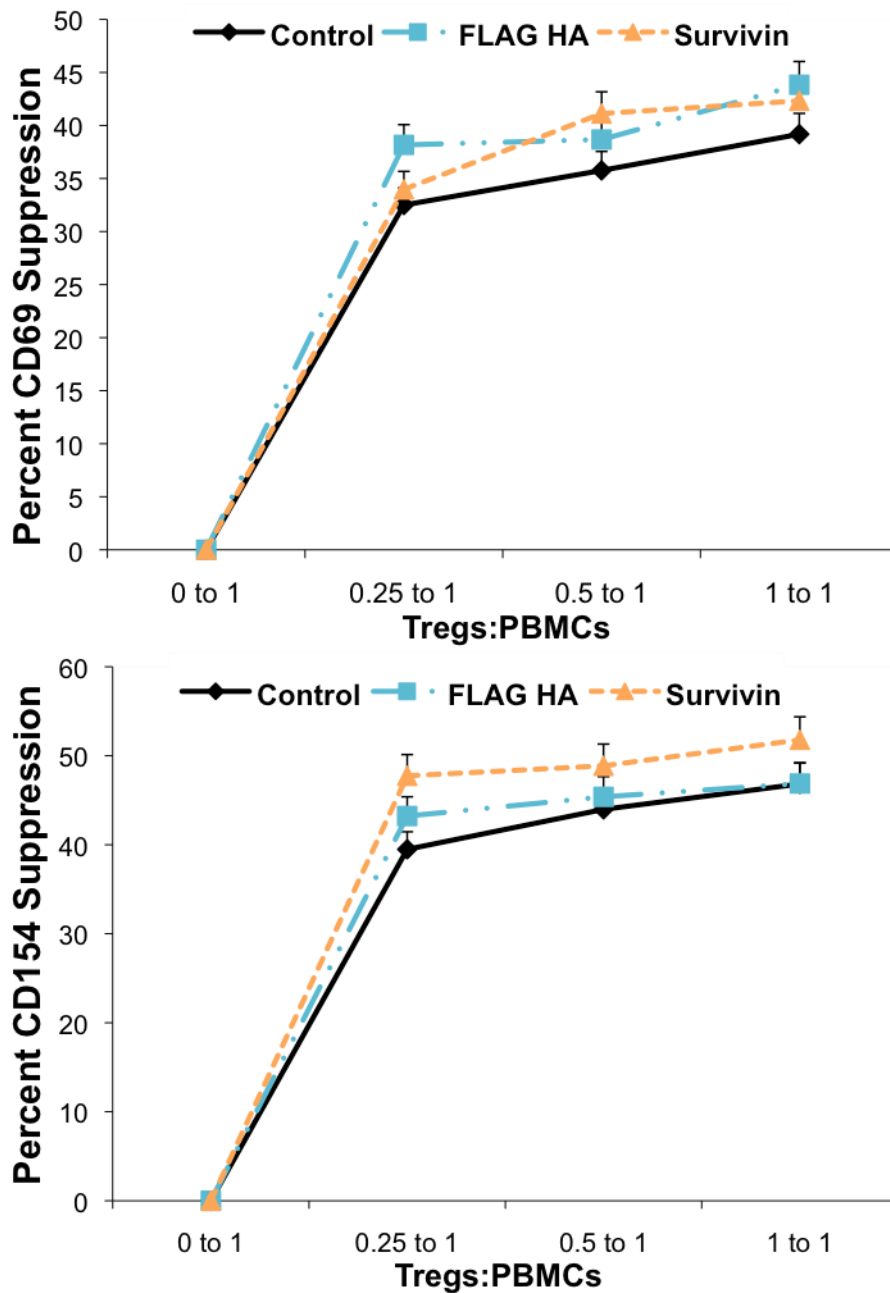
Figure 4.3. Regulatory T Lymphocyte Function Assay. $CD3^+CD4^+CD127^{low}$ Treg suppression capability was evaluated by flow cytometry. No significant difference in Treg function was seen between control and Survivin-treated cells.

Cytotoxic T Lymphocyte Subset Analysis

Intracellular Cytokine Analysis

Although Survivin did not affect Treg function and numbers, CTL function was significantly altered. The proportions of the subsets of cytotoxic T lymphocytes can have a dramatic affect on the inflammatory response to malignant cells. Production of pro-inflammatory cytokines like IFN-γ can enhance CTL function, while anti-inflammatory cytokines can suppress it. The proportion of these cytokines is controlled in part by the proportion of type 1 and type 2 cytotoxic T lymphocytes. Type 1 CTLs (Tc1) are characterized by their expression of IFN-γ and promote the overall cytotoxic functions of surrounding CTLs, while type 2 CTLs (Tc2) release cytokines that blunt CTL function.

To evaluate the relative proportions of Tc1 and Tc2 cells we analyzed intracellular cytokine expression by flow cytometry. Isolated T lymphocytes were cultured in control, FLAG-HA only or FLAG-HA-Survivin medium for 48 hours and then stimulated in the presence of cytokine release inhibitors. After 6 hours of stimulation the cells were stained and evaluated for IFN-γ, IL-4, IL-5, and IL-13 expression. A significant reduction in $CD8^+IFN\text{-}\gamma^+$ Tc1 cells was seen after culture with Survivin, demonstrating a reduction in pro-inflammatory cells (Figure 4.4A,B). Additionally, $CD8^+$ cells expressing the type 2 cytokines IL-5 and IL-13 increased substantially in FLAG-HA-Survivin cultures though the change in IL-4 expressing $CD8^+$ cells was not significant (Figure 4.4B).

Cytokine ELISA

To determine if the effects of Survivin on Tc1 and Tc2 numbers translates to

changes in overall cytokine release by cytotoxic T lymphocytes, we evaluated CTL

cytokine release by ELISA. Isolated CD8$^+$ cells were stimulated with anti-CD3/anti-

CD28 microbeads for 48 hours and PMA and ionomycin added for the final 6 hours. Cell

supernatants were isolated and analyzed for IFN-γ, IL-2, IL-4, IL-5, IL-10 and IL-13

release. Analysis of the cytokine profile showed that pro-inflammatory Tc1 cytokines

IFN-γ and IL-2 were dramatically reduced in Survivin treated cells (Figure 4.5), while

Tc2 cytokines remained unchanged. These changes in inflammatory cytokines may

contribute to the decreased cytotoxic activity observed in the presence of Survivin.
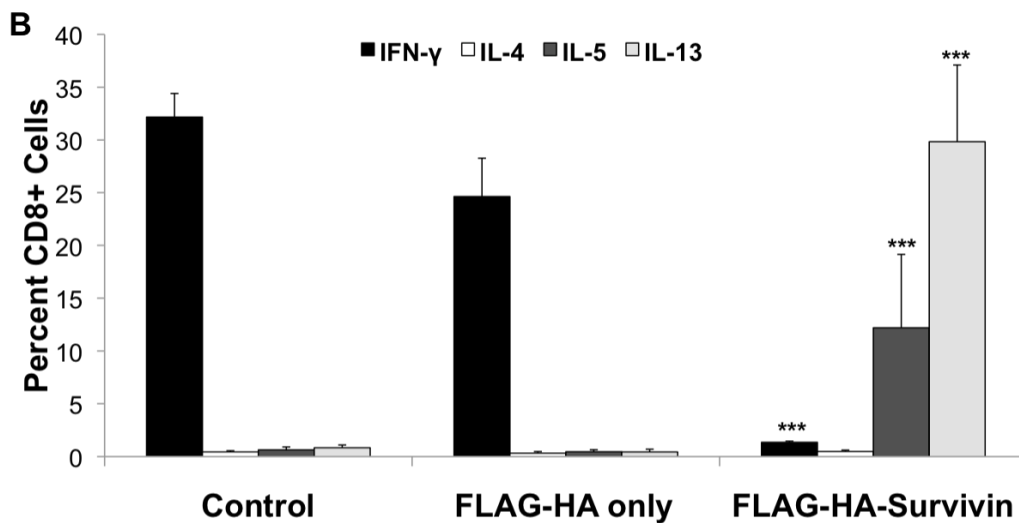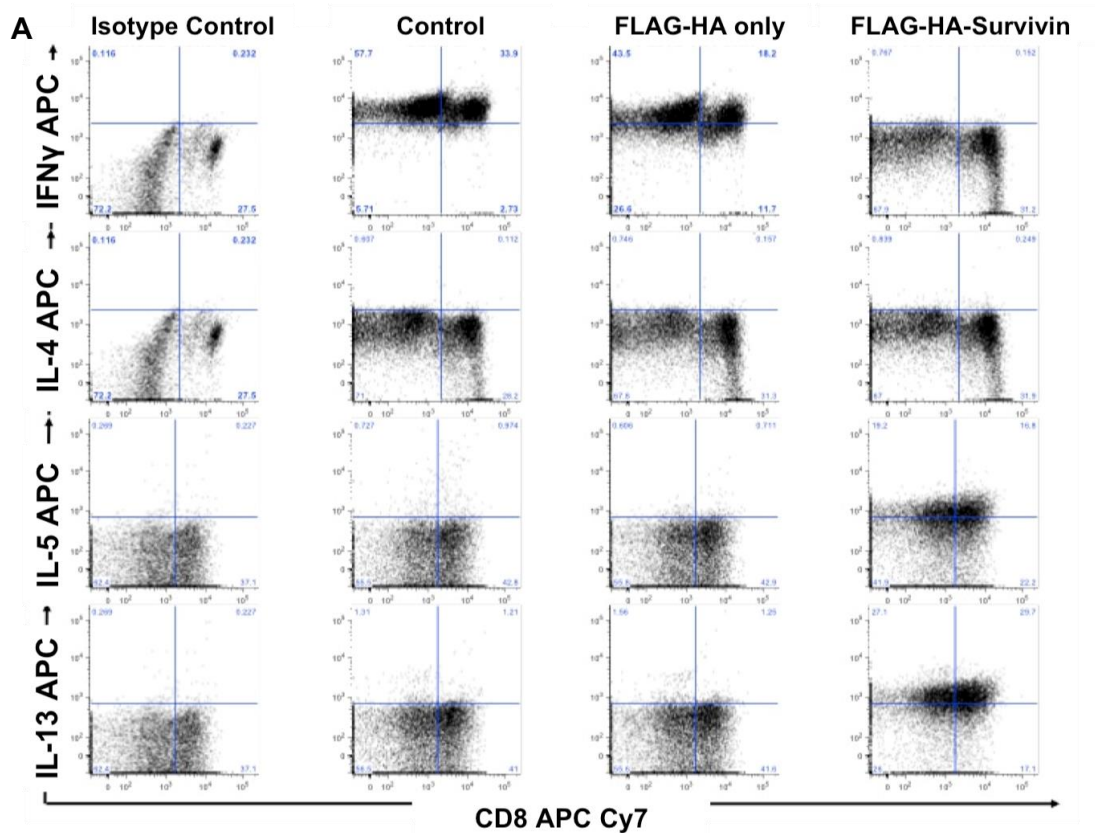
Figure 4.4. Flow Cytometric Analysis of Cytotoxic T Lymphocyte Subsets. A. Gating scheme for $CD8^+$ T cell subsets. B. Survivin significantly decreases $CD8^+IFN-\gamma^+$ cell numbers and increases $CD8^+Il-5^+$ and $CD8^+IL-13^+$ cell numbers. ***$p<0.001$.
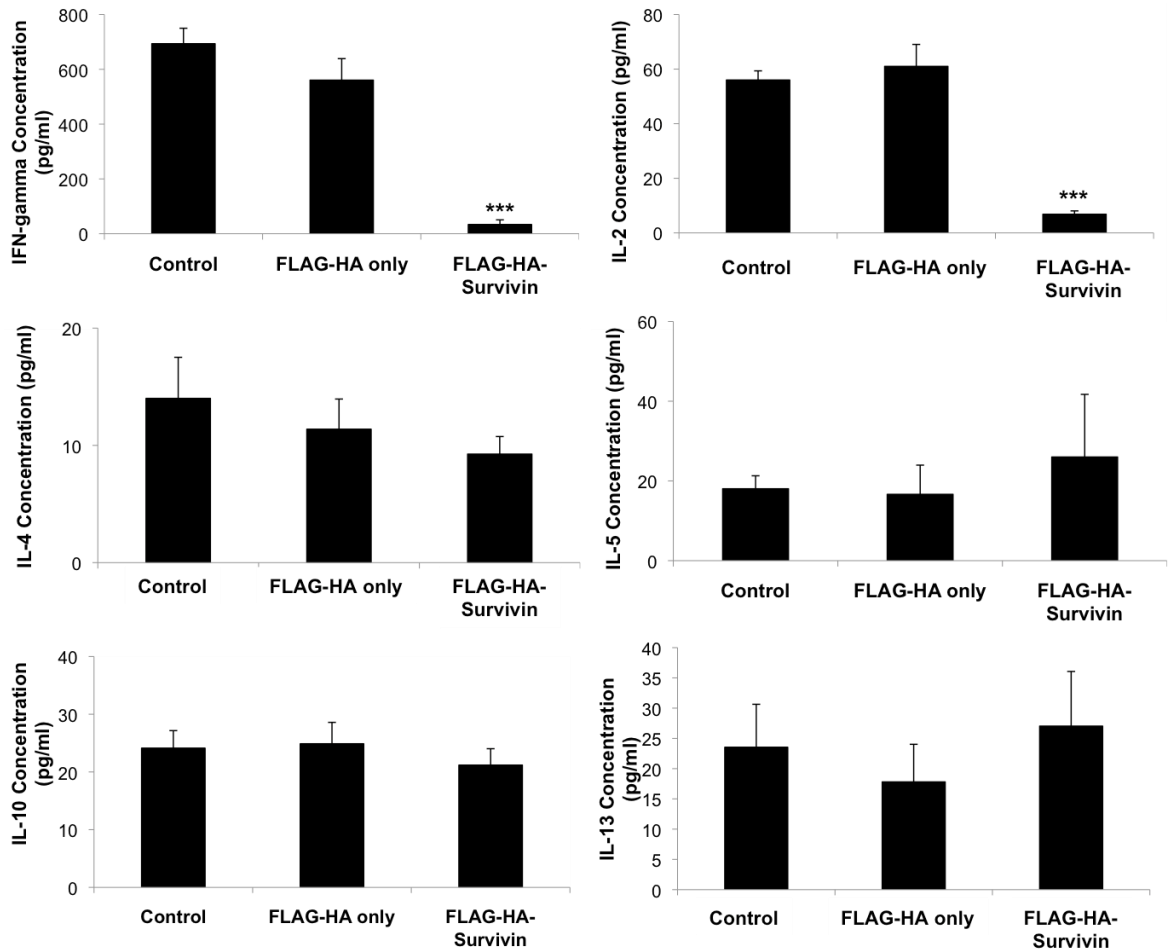
Figure 4.5. ELISA Analysis of Cytotoxic T Cell Cytokines. Type 1 cytokines are decreased and Type 2 cytokines increased by Survivin. Cytokine ELISA shows decreased production of inflammatory cytokines IFN-γ and IL-2 by CD8$^+$ T cells. No change was observed in anti-inflammatory IL-4, IL-5, IL-10 or IL-13 release. ***p<0.001.

Conclusion

The results of our data show a decrease in cytotoxic activity in CTLs in the presence of isolated tumor-released Survivin. While it was hypothesized that this was due to modulation of the regulatory T cell population, we found that Survivin had no significant effect on the numbers of Tregs, nor their ability to suppress activation in response to stimuli. In contrast, it appears the effects on CTL cytotoxic function is more likely to be caused by a shifting of the cytotoxic T lymphocyte population in the presence of Survivin. IFN-γ has been shown to enhance cytotoxic killing functions of CTLs *in vitro* and it is likely that the tremendous reduction in IFN-γ-producing cells and IFN-γ release induced by Survivin is responsible for the decrease in cytotoxic function we observed. Additionally, a similar shift in Tc1 and Tc2 populations and cytokine release has been observed in cancer patients (Filella X *et al.* 2000; Ehi K *et al.* 2008). The release of Survivin into the tumor microenvironment by malignant cells, while not altering the CTL:Treg balance, is likely hampering CTL function by pushing the immune response toward a type 2 and away from the type 1 response. In addition to its anti-proliferative functions in T cells, these findings show that Survivin is capable of altering the immune system towards a pro-tumor environment.

Materials & Methods

Analysis of CTL Cytotoxic Function

CD8$^+$ CTLs were isolated from a single healthy donor using the MACS anti-PE magnetic separation kit (Miltenyi Biotec, Auburn, CA) and anti-CD8 PE antibodies (BD Biosciences, San Jose, CA). CTLs were cultured in 12-well plates in control, FLAG-HA

only or FLAG-HA-Survivin medium and stimulated with PMA (10ng/ml) and ionomycin (0.1μg/ml). After 24 hours in culture the cells were washed and harvested. Pooled PBMCs from various healthy donors were put into a 96-well plate with CTLs at 5:1, 2.5:1, and 1.25:1 (CTL:PBMC) ratios. The cytotoxic function of the CTLs was analyzed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Fitchburg, WI), which measures the release of lactate dehydrogenase upon cell lysis.

Regulatory T Lymphocyte Analysis

CD3$^+$ T lymphocytes were isolated and plated in 96-well in control, FLAG-HA only or FLAG-HA-Survivin medium and stimulated for 48 hours with anti-CD3/anti-CD28 T cell expansion beads (6.25μl/well; Invitrogen, Carlsbad, CA). Cells were stained for CD3, CD4, CD8 (BioLegend, San Diego, CA), CD25 and CD127 (BD Biosciences, San Jose, CA) and CD4$^+$CD25$^+$CD127^{low} Treg populations analyzed by flow cytometry.

Regulatory T Lymphocyte Activity Assay

T regulatory cells were isolated from PBMCs using the EasySep Human CD4$^+$CD127^{low} T Cell Enrichment Kit (Stem Cell Technologies, Vancouver, BC, Canada) and cultured in 12-well plates at 1x10^6 cells/ml in control, FLAG-HA, or FLAG-HA-Survivin medium. After 24 hours, cells were washed, harvested and added to PBMCs at a 0:1, 0.25:1 or 0.5:1 (Treg:PBMC) concentration. Inhibition of cell activation by Tregs was evaluated using the BD FastImmune Human Regulatory T Cell Function Kit (BD Biosciences, San Jose, CA) and measuring the suppression of CD154 and CD69 induction by flow cytometry.

Intracellular Cytokine Analysis

CD3$^+$ T lymphocytes were isolated, plated in 96-well plates as described above and stimulated with PMA (10ng/ml) and ionomycin (0.1μg/ml) in the presence of 3 μM monensin to inhibit cytokine release. After 6 hours of activation, the cells were harvested and stained for T lymphocyte surface markers. Cells were fixed and permeabilized and intracellularly stained with biotinylated antibodies against IFN-γ, IL-4, IL-5, or IL-13 followed by streptavidin-APC (eBioscience, San Diego, CA). Stained cells were evaluated using the MACSQuant flow cytometer.

Cytokine ELISA

Isolated CD8$^+$ cells were stimulated with anti-CD3/anti-CD28 microbeads for 48 hours and PMA and ionomycin added for the final 6 hours. Cell supernatants were isolated and analyzed using Ready-Set-Go! ELISAs (eBioscience) for concentrations of IFN- γ, IL-2, IL-4, IL-5, IL-10, and IL-13.

CHAPTER FIVE

ALTERATION OF HELPER T LYMPHOCYTES BY

SURVIVIN

Introduction

The tumor microenvironment is characterized by the infiltration of T cells (Liao Y *et al.* 2007; Whiteside TL 2008). Investigation into the role of T cells in tumor progression has demonstrated the importance of the inflammatory T cell response in elimination of neoplastic cells (Hamai A *et al.* 2010). The importance of T cell subsets in the anti-tumor response is of interest as imbalances in these cells and the cytokines they produce have been shown to alter the ability of the immune system to eradicate the tumor (Whiteside TL 2008). Of particular interest are the type 1 and type 2 T cells, which are characterized by production of IFN-γ and IL-2 or IL-4 and IL-10, respectively (Zhu J and Paul WE 2008).

Peripheral blood and intratumoral T cells from cancer patients frequently exhibit altered T cell populations. Type 1 responses are typically decreased with a concomitant increase in type 2 cell numbers and cytokine levels (Filella X *et al.* 2000; Ito N *et al.* 2001; Mainou-Fowler T *et al.* 2003; Ehi K *et al.* 2008). The potential for metastasis and angiogenesis is increased by the presence of IL-4 and IL-6, respectively, and the type 2 polarization increases both of these cytokines (Naldini A *et al.* 2003; DeNardo DG *et al.* 2009). This shift is correlated to poor disease progression, as the anti-tumor response is inhibited and a pro-tumor environment is created (Filella X *et al.* 2000; Murakami H *et al.* 2004; Ehi K *et al.* 2008). Th17 cells, which release pro-inflammatory cytokines IL-17 and IL-21, have been implicated in the progression of malignancy in prostate and

colorectal cancers(Liu J *et al.* 2011) (Sfanos KS *et al.* 2008). However, in breast cancer a reduced number of Th17 cells is linked to poor prognosis (Yang L *et al.* 2012). The exact role of these cells in tumor progression seems to be cancer-type specific. While altered T cell populations in the tumor have been well documented from a clinical aspect, what leads to these changes has not been fully elucidated. Here we propose that Survivin could play a role in this helper T lymphocyte shift to a predominately type 2 phenotype.

Results & Discussion

Helper T Lymphocyte Subset Analysis

Intracellular Cytokine Analysis

After 6 hours of stimulation the cells were stained and evaluated for IFN-γ, IL-4, IL-5, and IL-13 expression. A significant reduction in $CD8^+IFN-\gamma^+$ Tc1 cells was seen after culture with Survivin, demonstrating a reduction in pro-inflammatory cells (Figure 4.4A,B). Additionally, $CD8^+$ cells expressing the type 2 cytokines IL-5 and IL-13 increased substantially in FLAG-HA-Survivin cultures though the change in IL-4 expressing $CD8^+$ cells was not significant (Figure 4.4B).

To evaluate the effects of tumor-released Survivin in T cell population skewing, intracellular cytokine staining was used to quantify the number of Th1, Th2, and Th17 cells (Figure 5.1). Isolated T lymphocytes were cultured in control, FLAG-HA only or FLAG-HA-Survivin medium for 48 hours and then stimulated in the presence of cytokine release inhibitors. Figure 5.1A shows representative gating of intracellular cytokine analysis for $CD4^+$ T cells. As was observed in the cytotoxic T cell population, numbers of Th1 ($CD4^+IFN-\gamma^+$) cells were significantly decreased in Survivin-containing cultures

(Figure 5.1B). Additionally, Th2 cells expressing the cytokines IL-4, IL-5 and IL-13 increased substantially in FLAG-HA-Survivin cultures (Figure 5.1B). Evaluation of Th17 cells by expression of IL-17 showed no difference in cell numbers in the presence of Survivin (Figure 5.2). Due to the variable role of Th17 cells in cancer it is not surprising that the ubiquitously expressed Survivin does not have a substantial affect on this cell population.

Cytokine ELISA

To determine if this adjustment in cell numbers correlates to changes in cytokine release, isolated $CD4^+$ cells were plated in control, FLAG-HA only or FLAG-HA-Survivin medium for 48 hours, and the culture supernatants evaluated by ELISA (Figure 5.3). Production of the Th1 cytokines IFN-γ and IL-2 was significantly reduced, which correlates well with our observation that Th1 cell numbers were significantly reduced in Survivin-containing cultures.   Because IFN-γ is necessary for perpetuating the differentiation of naïve T cells into Tc1 and Th1 cells, this reduction in IFN-γ could enhance what we previously observed in the cytotoxic T cells, resulting in blunted cytotoxic activity of CTLs. Additionally, the significant drop in IL-2 could play a part in the decreased proliferation following activation of T cells, as IL-2 is necessary in inducing cell division and clonal expansion of activated T cells.

Secretion of the Th2 cell cytokines showed variable changes in release after culture with Survivin. Although the number of cells expressing IL-4, IL-5 and IL-13 all increased based on intracellular cytokine flow cytometry, only the quantities of IL-4 and IL-13 release was significantly increased when cultured with FLAG-HA-Survivin (Figure

65

5.3). No significant change was seen in IL-5 or IL-10 levels (Figure 5.3). This shows that Survivin does mediate a shift toward a Th2-dominant population, but does not increase the levels of all Th2 cytokines.
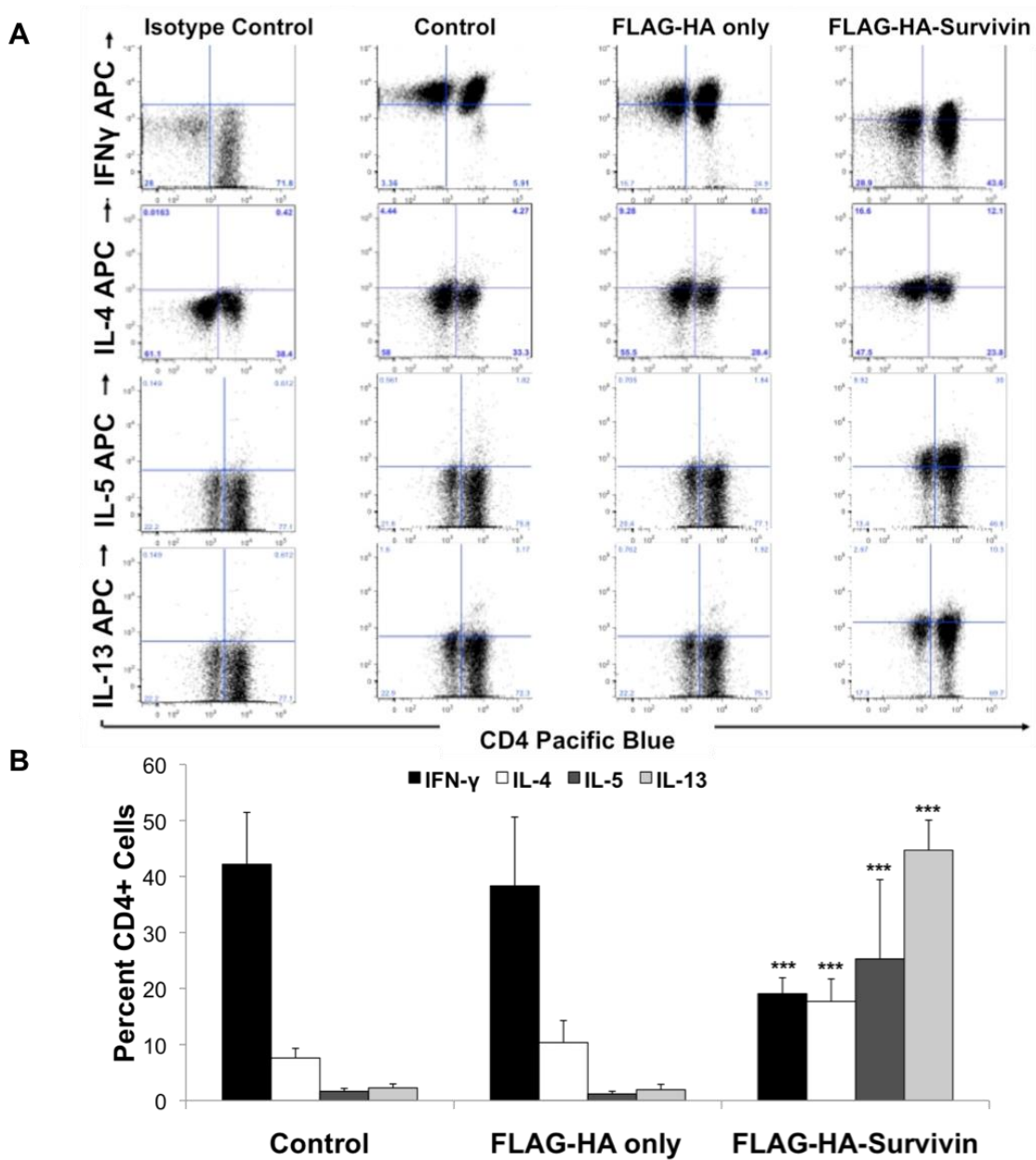
Figure 5.1. Flow Cytometric Analysis of Helper T Lymphocyte Subsets. A. Gating scheme for CD4$^+$ T cell subsets. B. Survivin significantly decreases CD4$^+$IFN-γ$^+$ cell numbers and increases CD4$^+$Il-5$^+$, CD4$^+$IL-13$^+$, and CD4$^+$IL-4$^+$ cell numbers. *p<0.05, **p<0.01 and ***p<0.001.
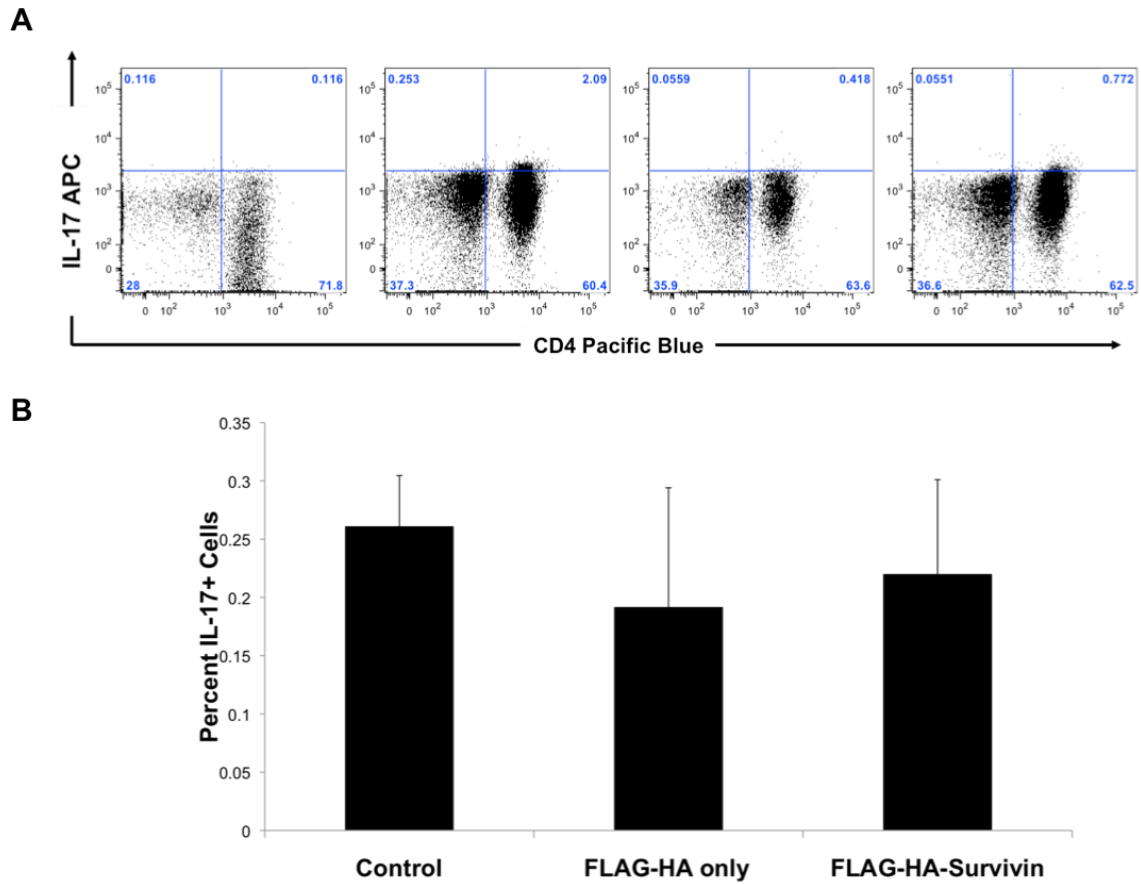
Figure 5.2. Analysis of IL-17 expression in T lymphocytes. A. Representative gating scheme. B. Percent IL-17 expressing CD4^{+} T lymphocytes were not altered in the presence of FLAG-HA-Survivin.
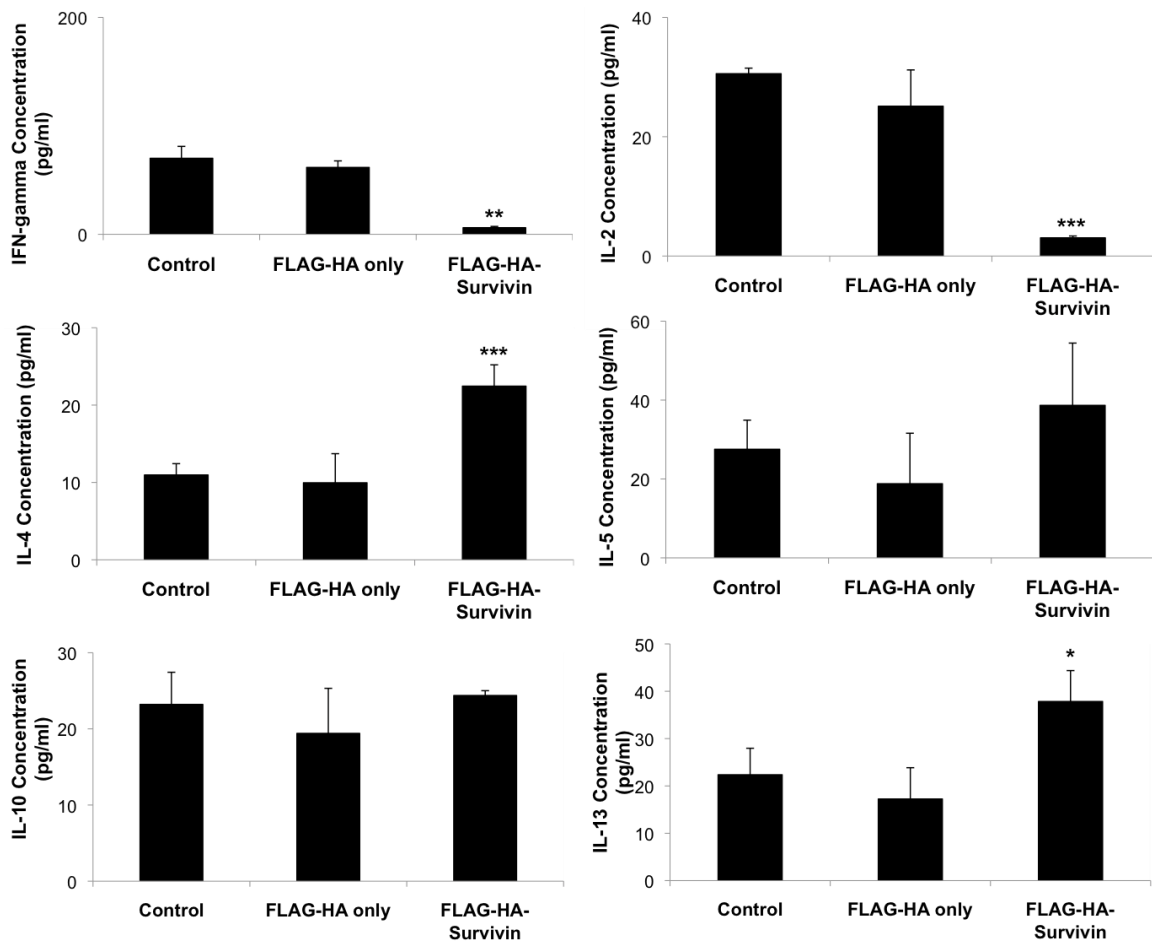
Figure 5.3. ELISA Analysis of Helper T Cell Cytokines. Type 1 cytokines are decreased and Type 2 cytokines increased by Survivin. Cytokine ELISA shows decreased production of inflammatory cytokines IFN-γ and IL-2 by CD4$^+$ T cells and increased IL-4 and IL-13 production in the presence of Survivin. No change was observed in IL-5 or IL-10 release. *p<0.05, **p<0.01 and ***p<0.001.

Conclusion

Alteration of the immune response toward the type 2 phenotype has been demonstrated in many types of cancer, including lymphoma, breast, prostate and lung cancer. Despite this important role in clinical malignancy the mechanism behind this shift has remained elusive. In these studies we showed that the polarization of the helper T cell response leading to a hindered pro-inflammatory response could be mediated by tumor-released Survivin. The overall shift from a type 1 to a type 2 response was shown both by the decrease in IFN-γ^+ T cells and the increase in IL-4$^+$, IL-5$^+$ and IL-13$^+$ T cells in response to Survivin. These findings demonstrate a role for Survivin in mediating the shift from a type 1 to a type 2 T cell response in the tumor microenvironment.

Materials & Methods

Intracellular Cytokine Analysis

CD3$^+$ T lymphocytes were isolated, plated in 96-well plates in control, FLAG-HA only or FLAG-HA-Survivin medium and stimulated with PMA (10ng/ml) and ionomycin (0.1μg/ml) in the presence of 3 μM monensin to inhibit cytokine release. After 6 hours of activation, the cells were harvested and stained for T lymphocyte surface markers. Cells were fixed and permeabilized and intracellularly stained with biotinylated antibodies against IFN-γ, IL-4, IL-5, IL-13, or IL-17 followed by streptavidin-APC (eBioscience, San Diego, CA). Stained cells were evaluated using the MACSQuant flow cytometer.

Cytokine ELISA

Isolated CD4$^+$ cells were stimulated with anti-CD3/anti-CD28 microbeads for 48

hours and PMA and ionomycin added for the final 6 hours in the presence or absence of

Survivin. Cell supernatants were isolated and analyzed using Ready-Set-Go! ELISAs

(eBioscience) for concentrations of IFN- γ, IL-2, IL-4, IL-5, IL-10, IL-13 and IL-17.

CHAPTER SIX

CONCLUSIONS

Summary of Findings

Our studies isolated FLAG-HA tagged Survivin released from malignant cells and characterized its effects on normal T lymphocytes. Evaluation of Survivin's effects on T cells demonstrated its ability to diminish their proliferation without altering their ability to be activated by the proper stimuli. Additionally, Survivin diminishes cytotoxic T cell function by altering the cytokine environment. The large reduction in inflammatory T cells and cytokines, as well as the increase in type 2 T cells by Survivin coincides with what has been seen in cancer patients and supports our hypothesis that extracellular Survivin acts as an immune mediator. From our data we can conclude that: 1) Extracellular Survivin can bind to and be taken up into normal T lymphocytes; 2) Uptake of Survivin diminishes clonal expansion of T lymphocytes; and 3) Survivin shifts the T cell population toward a type 2 phenotype, but does not alter the regulatory T or Th17 populations.

Tumor-Released Survivin Alters the T Lymphocyte Population

In chapter 2 we demonstrated that the tumor-released protein Survivin could be isolated from conditioned medium, and upon addition to T cell cultures, is able to bind to and be taken up by T cells. Like that described by Mera *et al.* (2008) using recombinant protein, tumor-released Survivin is able to bind to the surface of T cells, and as demonstrated in our studies, is also taken up into T cells. This data supports our hypothesis that Survivin uptake could occur within the tumor microenvironment as a

means of modulating T lymphocyte function. The inhibition of T lymphocyte division by Survivin detailed in chapter 3 is in contrast to its effects on malignant cells (Khan S *et al.* 2009). While in malignant cells uptake of Survivin induced proliferation, T cell division is hindered. Based on the evaluation of activation markers CD25 and CD69, Survivin is not inhibiting T cell stimulation, but is possibly acting downstream of activation signaling. Additionally, no changes in activation-induced endogenous Survivin in the presence of FLAG-HA-Survivin were observed. As discussed in chapters 4 and 5, the dramatic changes in IL-2 production in the presence of Survivin it is possible that the mechanism of decreased proliferation is related to cytokine effects.

In addition to their decreased proliferative capacity, Survivin-exposed CD8$^+$ T cells exhibited decreased cytotoxic function compared to controls. Many previous studies have demonstrated the inhibition of tumor-specific CTL function by T regulatory (Treg) cells (Chattopadhyay S *et al.* 2006; James E *et al.* 2010). However in our studies, Survivin did not significantly alter Treg numbers or function. Because of these observations, we hypothesize that Survivin's inhibition of cytotoxic function is independent of Treg-mediated CTL suppression.   Based on our data in chapters 4 and 5, we believe changes to the cytokine profile and decreased IFN-γ production is likely responsible for this suppression. The release of Survivin may be one mechanism by which tumors evade T cell-mediated cytotoxicity.

Although Treg populations were not affected by extracellular Survivin, type 1 and type 2 T cell populations were significantly altered. The decrease in IFN-γ and IL-2 production by T cells, combined with the significant drop in IFN-γ$^+$ cells demonstrates an inhibition of the anti-tumor response. The importance of IFN-γ to immune surveillance

has been shown previously in spontaneous malignancy in IFN-γ deficient mice. Additionally, it is frequently decreased in cancer patients (Filella X *et al.* 2000; Ellyard JI *et al.* 2007; Ehi K *et al.* 2008; Hamai A *et al.* 2010). The overall shift from a type 1 to a type 2 response was shown both by the decrease in IFN-γ^+ T cells and the increase in IL-4^+, IL-5^+ and IL-13^+ T cells in response to tumor-released Survivin.

The importance of CD4$^+$IL-4$^+$cells in malignancy was recently demonstrated in a mouse mammary carcinoma model, in which production of IL-4 by helper T cells was able to polarize macrophages, resulting in increased lung metastasis (DeNardo DG *et al.* 2009). Similarly, increased plasma levels of IL-13 in patients with pancreatic, gastric and esophageal cancers was positively correlated to increased numbers of myeloid derived suppressor cells (Gabitass RF *et al.* 2011). Both these studies showed important downstream effects of the shift to a type 2 response, but did not examine the preliminary cause of the shift. Importantly, when combined with this study and our previous work showing Survivin-associated invasiveness (Khan S *et al.* 2009), a more complete picture emerges with Survivin inducing the type 2 response, which can then lead to increased metastasis and complete polarization of the immune response.

Alteration of the immune response toward the type 2 phenotype has been demonstrated in many types of cancer, including lymphoma, breast, prostate and lung cancer, though the reason for this shift was undetermined (Filella X *et al.* 2000; Nakayama H *et al.* 2000; Ito N *et al.* 2001; Mainou-Fowler T *et al.* 2003; Ehi K *et al.* 2008). Similar studies have been performed looking at Th17 cells in cancer patients (Sfanos KS *et al.* 2008; Kryczek I *et al.* 2009; Yang ZZ *et al.* 2009; Maniati E *et al.* 2010). Unlike the Th1/Th2 shift, which is common across patients with a variety of

cancer types, the role of Th17 cells appear to be cancer-type specific. In prostate and ovarian cancers, increased numbers of Th17 cells in the tumor correlated with better patient outcome, demonstrating an anti-tumor role (Sfanos KS *et al.* 2008; Kryczek I *et al.* 2009). Conversely, in colorectal carcinoma, higher levels of serum IL-17 correlated to poor patient prognosis (Liu J *et al.* 2011). In our studies, no change in IL-17-expressing cells was observed after exposure to Survivin (Supplementary Figure 7), unlike our observed type 1 and type 2 T cell shift.

Clinical Implications

In this study we show that the polarization of the T cell response leading to a pro-tumor response could be mediated in part by tumor-released Survivin. The ability of Survivin to decrease the proliferation and cytotoxicity of T cells and alter the T cell response to malignant cells, as well as positively affect tumor growth and aggressiveness, makes it an important therapeutic target for the treatment of cancer.

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