Regulation of T Cell Death in Alloimmune-Mediated Vascular Rejection

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Abstract

Organ transplantation remains the most important therapeutic option for end-stage organ failure, but success of this procedure is limited by the eventual rejection of almost all grafts. T cells reject grafts by recognizing genetic differences between recipient and donor. Activation of T cells by donor antigens drives clonal expansion of graft-reactive T cells. The magnitude of the response depends on a balance between T cell activation and death. Understanding the mechanisms that regulate cell death may result in new approaches to prevent graft failure. I found that human CD8 T cells activated in the presence of IL-1, IL-6, and IL-23 were protected against activation-induced cell death (AICD) as a result of increases in c-FLIP$_S$ expression induced by IL-6. I then used an aortic interposition model of transplant arteriosclerosis (TA), a form of arterial rejection that is the main cause of heart transplant failure, to determine the effect of IL-6 on T cell death. Grafted arteries that lacked IL-6 developed less severe rejection as compared to IL-6$^{+/+}$ arteries, and this correlated with an increase in T cell death. These findings indicate that IL-6 inhibits T cell death after organ transplantation.

I also examined the intrinsic regulation of T cell responses by Bcl-2 family proteins. Bim is a pro-apoptotic protein known to down-regulate immune responses. Besides inducing T cell death, I found that Bim was unexpectedly required for proliferation of T cells in response to alloantigen stimulation *in vitro*. A partial reduction in Bim expression was sufficient to attenuate activation whereas a complete elimination of Bim was required to prevent CD4 T cell death in response to cytokine withdrawal. *In vivo*, there was significantly less vascular rejection in Bim$^{+/-}$, but not Bim$^{-/-}$, graft recipients. T cell proliferation in response to allograft arteries was significantly reduced in both Bim$^{+/-}$ and Bim$^{-/-}$ mice, but cell death was attenuated only in Bim$^{-/-}$ animals. These findings indicate that Bim regulates not only T cell death but also allogeneic T cell activation.

In summary, my work has provided insight into the mechanisms by which extrinsic and intrinsic regulators of cell death affect immune responses in transplantation. These pathways are potential targets in the development of novel therapies to manage TA.

Keywords: T Cell; Apoptosis; Transplant Arteriosclerosis; Bim; Fas; c-FLIP; IL-6

I dedicate this thesis to my lab family who provided endless support, friendship, laughter, and who made this journey truly enjoyable

.

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unending passion for medical research is contagious and has been a continuous source of inspiration for me.

Table of Contents

List of Figures

List of Acronyms

AICD	Activation-induced cell death
APAF-1	Apoptotic protease activating factor-1
APC	Antigen presenting cell
Bim	Bcl-2 Interacting mediator of cell death
CARD	Caspase-recruitment domain
CDICD	Cytokine deprivation-induced cell death
c-FLIP	Cellular FLICE-inhibitory protein
c-FLIP$_L$	Cellular FLICE-inhibitory protein (long isoform)
c-FLIPs	Cellular FLICE-inhibitory protein (short isoform)
CFSE	Carboxyfluorescein succinimidyl ester
CNI	Calcineurin inhibitor
DISC	Death-inducing signalling complex
EC	Endothelial cell
HLA	Human leukocyte antigen
IFNɣ	Interferon-gamma
IL-#	Interleukin-#
IUVS	Intravascular ultrasound
MCL-1	Induced myeloid cell leukemia-1
MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin
PI	Propidium Iodide
SMC	Smooth muscle cell
TA	Transplant arteriosclerosis
TCR	T cell receptor
Treg	Regulatory T cell

Chapter 1. Introduction

1.1. Organ Transplantation

Organ transplantation remains the only therapeutic option for end-stage organ failure. However, the rejection of transplanted organs by the immune system of the recipient remains a formidable hurdle to the overall success of this procedure. Over the last two decades, advancements in the formulation of immunosuppressive drugs, has resulted in greatly improved short-term allograft survival (85-95% survival >1 year) (Lechler, Sykes et al. 2005). However, the rate of long-term graft loss has not diminished, and there are life-altering and life-threatening complications associated with the continual use of non-specific immunosuppressants. These include opportunistic infections, viral-induced tumours, increased risk for cardiovascular diseases, and malignancy (Penn 1988; Dantal, Hourmant et al. 1998; Fishman and Rubin 1998; Lechler, Sykes et al. 2005). The current mission in the field of transplantation immunology is to harness the power of physiological mechanisms that maintain self-tolerance, to induce and maintain transplantation tolerance. Tolerance is defined as a state in which the immune system does not mount a pathogenic response to alloantigens, without the use of non-specific immunosuppressants, while maintaining immunological responses to all other immunogens (Li, Wells et al. 2000; Lechler, Sykes et al. 2005). In order to achieve this, it is essential to understand the basic mechanisms by which immune responses are activated and cause rejection in transplantation.

Organ transplant rejection is mediated by T cells. CD4 T helper cells become activated when they encounter foreign major histocompatibility complex **(MHC)**-peptide, or self-MHC displaying foreign peptide. Activated CD4 T cells can then go on to cause tissue damage by producing inflammatory cytokines, and can also support the activation

and differentiation of effector CD8 cytotoxic T cells that cause direct damage to the graft. CD4 T cells also drive the production of alloreactive antibodies that damage the graft through complement-dependent and -independent mechanisms (Rocha, Plumb et al. 2003). Other cells of the immune system, such as macrophages also contribute to organ transplant rejection *via* secretion of cytokines and pro-inflammatory factors that result in up-regulation of adhesion molecules on endothelial cells **(ECs)** and recruitment of leukocytes to the graft (Rocha, Plumb et al. 2003).

T cells are directly activated by donor-derived antigen-presenting cells **(APCs)**, which process antigens and display peptide fragments in the context of MHC on their surface for antigen presentation to T cells. This "direct recognition" of foreign MHC-peptide on donor derived APCs, which are carried into the host *via* the graft, triggers a strong immune response resulting in acute and chronic rejection (Kreisel, Krasinskas et al. 2004). While in a regular "physiological' immune response the proportion of T cells that is activated towards pathogens is low (~1/100,000), in a transplant setting *via* direct recognition, this proportion is much larger ~1/100-1/10 (Suchin, Langmuir et al. 2001). The magnitude of this response is based on the high level of polymorphism in human leukocyte antigen **(HLA)** genes that encode MHC. A large repertoire of CD8 T cells can respond to foreign MHC class I molecules, which are cell-surface membrane glycoproteins specifically involved in antigen presentation; these are expressed on most cells. In contrast, MHC class II molecules are expressed mainly on APCs and present antigens to CD4 T cells. The number, duration, and severity of acute rejection episodes, as well as level of MHC mismatch, are independent risk factors of graft failure due to rejection (Taylor, Edwards et al. 2006; Colvin-Adams and Agnihotri 2011).

Following the initial phase of allo-recognition *via* the "direct" pathway, the recipient's immune system clears many of the donor-derived APCs, and the "indirect" pathway of allo-recognition becomes the predominant mechanism of T cell activation (Weiss, Madsen et al. 2008). The "indirect" pathway involves the recognition of donor antigens that are engulfed and presented to T cells by recipient APCs. A "semi-direct" pathway has also been described in which recipient APCs acquire donor MHC molecules from donor cells; in this way, recipient APCs activate T cells that recognize foreign MHC molecules (Herrera, Golshayan et al. 2004). If the donor and the recipient

have identical MHCs, graft rejection is caused by minor histocompatibility antigens, which are peptides of polymorphic cellular proteins that differ between donor and recipient, and are presented on MHC molecules and recognized as foreign by T cells (den Haan, Meadows et al. 1998).

1.2. Transplant Arteriosclerosis

Chronic rejection is one of the main challenges for the success of transplantation. Transplant arteriosclerosis **(TA)** is the main cause of chronic heart transplant rejection, it is characterized by a diffuse, aggressive, accelerated form of sclerosis that results from alloimmune rejection of allograft arteries (Mitchell and Libby 2007). In TA, the recipient's immune system targets foreign ECs and smooth muscle cells **(SMCs)** of allograft arteries, as shown in Figure 1. This triggers a reparative response that results in the accumulation of SMCs and aberrant extracellular matrix **(ECM)** deposition, which eventually leads to luminal narrowing, ischemia and graft failure.(Billingham 1992; Libby and Pober 2001; Mitchell and Libby 2007; Angelini, Castellani et al. 2014).

Healthy arteries consist of three defined layers. The outermost layer, the adventitia, contains fibroblasts and immune-system cells, and has roles in regulating immune responses and vasomotor tone (Gonzalez, Arribas et al. 2001; Laflamme, Roberge et al. 2006). The middle layer, the media, is composed of SMCs bound by internal and external layers of elastic laminae, and is responsible for constricting and dilating the artery (Havelka and Kibbe 2011). The inner most layer, the intima, is found in the space between the endothelium and internal elastic laminae. The main feature of TA involves hyperplastic thickening of the intima in the space between these two structures. Development of TA is a slow, biphasic process consisting of early intimal thickening and late inward remodeling (Tsutsui, Ziada et al. 2001).

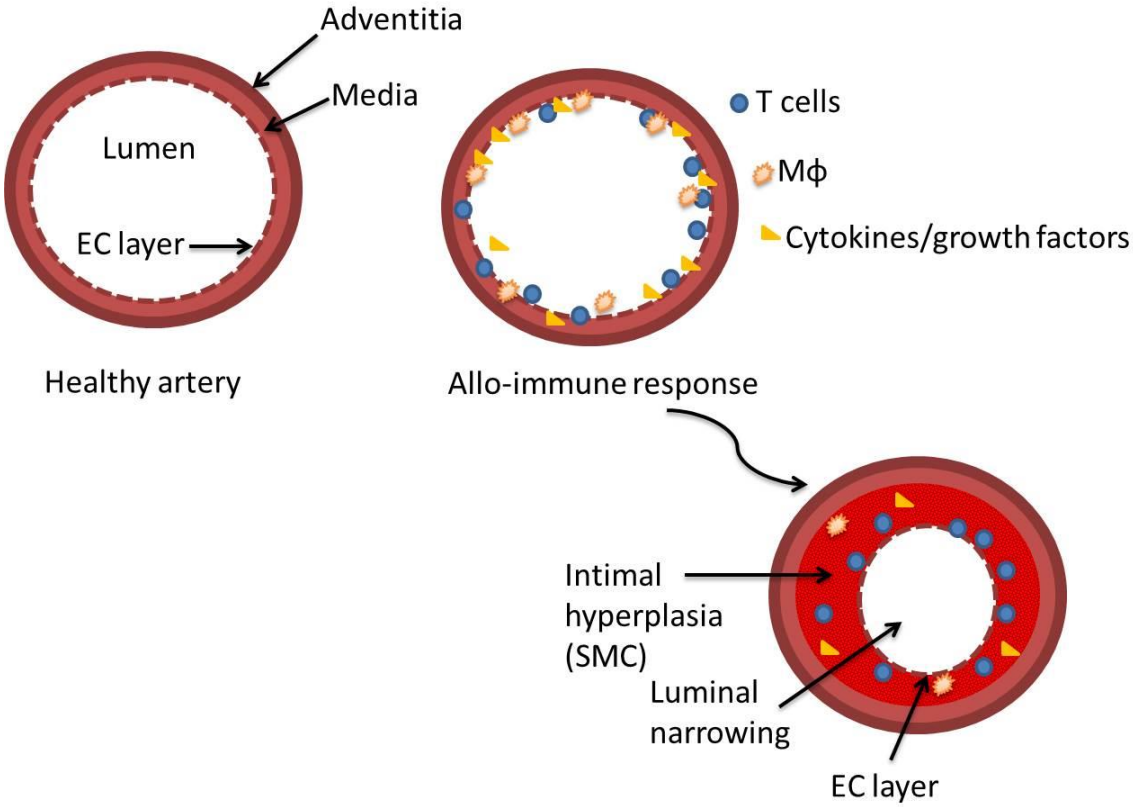

Figure 1. In TA, an alloimmune response results in intimal hyperplasia and luminal narrowing.

Healthy arteries consist of three layers: the adventitia, the media that is composed of SMCs and the intima, which consists of a single EC layer. During an alloimmune response, cells of the recipient's immune system target the foreign tissue resulting in expansion of the intima that eventually results in occlusion of the lumen and graft dysfunction.

TA can manifest as early as weeks following organ transplantation. At this time lesions are characterized by mild intimal thickening and fibrosis, and a small accumulation of lipids and ECM proteins (Johnson, Gao et al. 1989). During these early stages the size of the lumen is often preserved due to compensatory outward expansion of the vessel (Tsutsui, Ziada et al. 2001). As lesions progress, fibrous and fibrofatty plaques with cholesterol clefts develop, usually without necrotic cores (Gao, Alderman et al. 1988; Johnson, Gao et al. 1989; Tellides and Pober 2007). At the later stages of disease, compensatory outward expansion becomes compromised and inward constriction is observed (Tsutsui, Ziada et al. 2001)

The majority of cells in the expanded intima are vascular SMCs. T cells and macrophages are also found and usually located just beneath the EC lining (Salomon, Hughes et al. 1991). SMCs in the intima also tend to produce an abnormally large amount of ECM, which is often elastin poor and collagen rich, a feature that likely contributes to vessel dysfunction (Pober, Jane-wit et al. 2014). Evidence of dysfunction is identified when afflicted arteries fail to dilate in response to acetylcholine (Hollenberg, Klein et al. 2001). Aggregates of B and T cells, and myeloid cells are commonly found in the adventitia in TA (Wehner, Fox-Talbot et al. 2010), while the media tends to be unaffected (Pober, Jane-wit et al. 2014). Understanding how these vascular responses are regulated is essential for the development of novel therapies for TA.

1.3. Cellular Effectors and Regulators of Alloimmune Vascular Rejection and TA.

While immune and non-immune factors contribute to the development and pathology of TA, immune factors are the most important contributors since TA occurs only in donor arteries and not in the native arteries of the recipient (Weiss, Madsen et al. 2008; Nath, Basha et al. 2010). Therefore, this thesis focuses primarily on alloimmune cellular effectors of vascular rejection and TA.

1.3.1. Immunological Mediators of TA

Innate Immunity

The main innate immune cells involved in the development of TA are natural killer cells and macrophages (Millington and Madsen 2010). Natural killer cells can recognize allogeneic tissue since they express receptors that recognize the presence of self MHC class I on the cell surface. This mechanism results in what is called “activation to absent self”, which is known to play a major role in rejection of non-self hematopoietic stem cells and their progeny after bone marrow transplantation (Benjamin, Gill et al. 2010). NK cells can also be a source of cytokines such as interferonɣ **(IFNɣ)**, which serves as a mitogen for SMCs as is further discussed in Section 1.3.2 below. Furthermore, other roles for NK cells have been shown in animal models that are

dependent on the presence of T cells (Uehara, Chase et al. 2005) and donor specific antibodies (Hirohashi, Chase et al. 2012).

Macrophages express pattern recognition receptors that are likely activated by alarmins released by tissue that has been injured by the transplantation procedure. Macrophages activated in this manner secrete mitogens that promote SMC proliferation and also activate allo-reactive T cells by presenting allo-antigens and expressing foreign MHC. Additionally, macrophages express Fc receptors that can bind to donor-specific antibodies, this stimulates secretion of tissue damaging cytokines and nitric oxide by the macrophages (Pober, Jane-wit et al. 2014). In mouse models, it has been shown that depletion of macrophages reduces TA (Kitchens, Chase et al. 2007).

Adaptive Immunity

B Cells

B cells are effector cells of the adaptive immune response. In response to foreign antigen and inflammation, naïve B cells differentiate into antibody-producing plasma cells that secrete large amounts of antibody, and also into APCs that activate T cells directly (Ron and Sprent 1987). B cells also contribute to immune responses by secreting cytokines, are often found as infiltrate in chronically rejected allografts, and ectopic germinal centers can be found within transplanted tissues (Kerjaschki, Regele et al. 2004; Baddoura, Nasr et al. 2005; Thaunat, Field et al. 2005). Biopsies from transplant patients with acute and chronic rejection often show evidence of B and memory T cell infiltrates (Ibrahim, Dawson et al. 1993; Ibrahim, Dawson et al. 1995; Zarkhin, Kambham et al. 2008). B cells likely present graft-derived peptides and co-stimulation to CD4 T cells *via* the indirect pathway of allo-recognition *in situ*. The activated T cells can then in turn activate B cells to proliferate and produce antibodies including donor-specific antibodies (Motallebzadeh, Rehakova et al. 2012).

Patients who develop donor-specific antibodies, which typically recognize HLA molecules expressed by graft ECs, have a higher incidence and severity of TA (Ticehurst, Molina et al. 2011). These antibodies can activate complement *via* the classical pathway resulting in the deposition of C4d on the EC lining of allograft vasculature (Frank, Molina et al. 2013). In experimental models, anti-donor antibodies

are sufficient to induce vascular lesions even in the absence of T cells (Russell, Chase et al. 1994). In heart transplant patients, the presence of B cell infiltrates is associated with recurrent rejection episodes (Sorrentino, Scarinci et al. 2006) and Rituximab, an anti-CD20 monoclonal antibody that depletes B cells, has been shown to reverse vascular rejection (Garrett, Duvall-Seaman et al. 2005).

T Cells

As shown in Figure 2, the activation of naïve T cells by cognate antigen (T cell priming) requires three different signals. Signal 1 consists of the interaction between the T cell receptor **(TCR)** and a specific peptide:MHC complex on an APC (Janeway and Bottomly 1994). Signal 2 consists of signals produced by co-stimulatory molecules on the surface of APCs that bind their ligands on T cells. The most commonly studied co-stimulatory molecules are CD80 and CD86, which bind to CD28 on T cells (Gonzalo, Delaney et al. 2001; Bour-Jordan and Blueston 2002). Signal 3 consists of inflammatory cytokines that act directly on T cells to define the type of effector response that will be generated (Curtsinger, Schmidt et al. 1999). This "three-signal" paradigm controls the activation of T cells in response to pathogen infections and also in transplantation

CD4 T cells have an essential role in allograft rejection as depicted by many studies showing that long-term allograft survival can occur in CD4-deficient recipients or by pre-treating the recipients with CD4-depleting antibodies (Shizuru, Seydel et al. 1990; Pearson, Darby et al. 1992; Pearson, Hamano et al. 1993). In addition to being essential, CD4 T cells are also sufficient to induce graft rejection as demonstrated by studies in which allogeneic cardiac transplants are performed on *rag1*$^{-/-}$ mouse recipients (which lack T and B cells); graft rejection occurs when these mice are reconstituted with only CD4 T cells (Pietra, Wiseman et al. 2000). In addition, Krieger and colleagues showed that $CD4^{+}$, but not $CD8^{+}$ T cells are required for allograft rejection (Krieger, Yin et al. 1996). Similarly, the elimination of CD4 T cells prevents TA in experimental models (Szeto, Krasinskas et al. 2002).

The role of CD8 T cells as a significant immunological cause of TA has been controversial. Clarke-Forbes showed that depletion of CD8 T cells with an anti-CD8 depleting antibody plus thymectomy, did not affect the severity of TA in a complete MHC

mismatch rat cardiac transplant model. However, in their study, CD8 depletion did not appear to be complete as CD8 T cells were detected in the spleen and lymph nodes (Forbes, Zheng et al. 1994). Using CD8-deficient and CD4-deficient mice in a complete MHC-mismatched carotid-loop model of TA, Shi *et al.* reported that CD4 T cells were critical for the development of TA, but did not observe a role for CD8 T cells (Shi, Lee et al. 1996). Using a MHC II mismatched, heterotopic heart transplant model, Fischbein *et al*. reported that both CD4 and CD8 localize to the perivascular area before TA development, and showed that while CD4 T cells are required for the development of TA, CD8 T cells also contribute to lesion progression (Fischbein, Yun et al. 2001). In addition, Allan *et al*. showed a critical role for CD8 T cells in the development of TA in a pig transplant model in which they observed significantly reduced intimal thickening after prolonged treatment with an anti-CD8 antibody (Allan, Choo et al. 1997). Later, Delfs *et al.* definitively showed that effector CD8 T cells can cause chronic rejection in the absence of CD4 T cell help (Delfs, Furukawa et al. 2001).

CD8 effector functions can be divided in to two types: direct cytotoxicity and cytokine secretion. Cytotoxicity is mediated by the release of granules containing cytotoxic proteins such as granzymes and perforin, or by engaging death receptors such as Fas with death ligands such as FasL (Kagi, Vignaux et al. 1994). Activated CD8 T cells are known to produce IFNɣ, which plays a critical role in the development of TA (Nagano, Libby et al. 1998; Eid, Rao et al. 2009).

In addition to examining CD8 T cells by eliminating this population in graft recipients, studies have also determined a role for cytotoxic granule proteins utilized by CD8 T cells in the development of TA. Choy and colleagues showed that granzyme B and perforin play a pivotal role in EC damage and the development of TA (Choy, McDonald et al. 2003; Choy, Kerjner et al. 2004). Furthermore, immunohistochemical analysis of TA lesions shows that T cells localized to the EC layer, express perforin-containing granules that are polarized toward dying ECs (Fox, Hameed et al. 1993). Additionally, mRNA encoding granzyme B, and the protein itself, are detectable in leukocytes that have infiltrated the intima and adventitia of TA-afflicted arteries. Granzyme B is also detected in association with intimal cells undergoing cell death in advanced TA lesions (Choy, McDonald et al. 2003). Regarding cytokine secretion, Stinn

et al. showed that cytotoxic T cells contribute significantly to the inflammatory cytokine environment in a transplant model (Stinn, Taylor et al. 1998), and another group showed that in the absence of CD4 lymphocytes, CD8 T cells treated with a CD40-activating antibody could drive the development of TA *via* IFNɣ production (Schnickel, Whiting et al. 2004). Lastly, memory CD8 T cells have been established as a major barrier toward achieving transplantation tolerance (Salama, Womer et al. 2007). Memory CD8 T cells are considered the main cell type responsible for the failure of co-stimulation blockade-induced tolerance in the clinic (Trambley, Bingaman et al. 1999), and depletion of memory CD8 T cells has been shown to enable tolerance in a non-human primate model of concurrent bone marrow and kidney transplantation (Koyama, Nadazdin et al. 2007).

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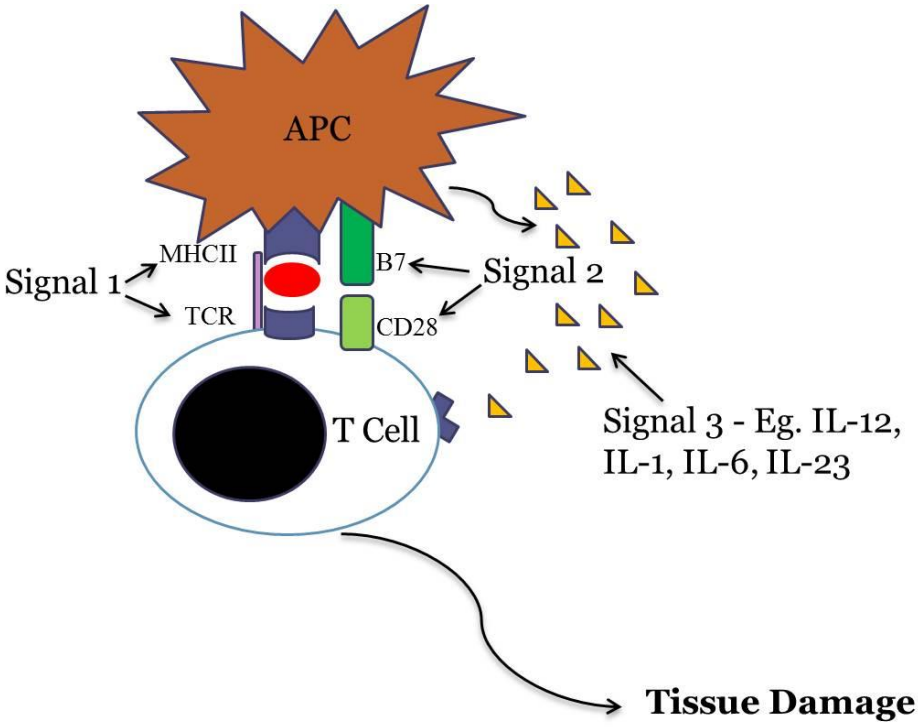


Figure 2. Three kinds of signal are required for activation of naïve T cells by antigen-presenting cells.

The binding of a TCR on a naïve T cell to the peptide-MHC on an APC delivers Signal 1 to the T cell resulting in activation. Co-stimulatory molecules on the APC, in this case B7 (CD80 or CD86), interacts with CD28 on the T cell and delivers Signal 2, which results in survival and proliferation. The cytokines produced by the APC deliver Signal 3 which results in differentiation of the naïve T cell in to an effector T cell with a specific role depending on the specificity of Signal 3.

1.3.2. Effector CD4 T Cell Responses in Transplantation

In order to understand the mechanisms by which alloimmune responses are generated, and in the hope of specifically preventing them, one should consider the basic mechanisms by which effector T cells are activated in response to antigen stimulation. In response to the three signals described above, CD4 T cells differentiate in to subsets of functionally distinct effector cells that are defined mainly by the type of cytokines they secrete. The best characterized subsets of CD4 T cells that migrate in to peripheral tissues are Th1 cells (that produce IFNɣ), Th2 cells (that produce IL-4, 5, and/or 13), and Th17 cells (that produce IL-17A, IL-17F, IL-21 and IL-22). Th1 cells activate macrophages to kill intracellular bacteria and induce class switching in activated B cells. Th2 cells also aid in class switching but particularly to IgE, a class of antibody involved in mast cell activation and allergy. The more recently characterized Th17 subset is involved in recruiting neutrophils to fight extracellular bacteria (Abbas, Murphy et al. 1996)

Allogeneic responses were traditionally thought to be mainly Th1 mediated and to be opposed by Th2 responses. In fact, a pilot human study showed that T cells obtained from patients with stable grafts produced Th2 cytokines, whereas T cells derived from patients with chronic graft rejection secreted Th1 cytokines (Waaga, Gasser et al. 2001), suggesting that Th2 conditions may be protective in an allogeneic setting. However, more recent studies have suggested that Th17 conditions can also mediate acute transplant rejection. Specifically, when a Th1 response is prevented, an aggressive inflammatory response against the graft occurs that is mediated mainly by IL-17 producing T cells (i.e., Th17 cells) (Yuan, Paez-Cortez et al. 2008). Clinically, the Th17 response has also been shown to be associated with rejection (Burlingham, Love et al. 2007), but IL-17 has also been associated with vascular features that prevent arterial occlusion in TA (Liu, Lee et al. 2012).

Th1

Vast data in the literature indicate that a Th1 immune response is the main component behind transplant rejection (Nickerson, Steurer et al. 1994; Dallman 1995). Antigen stimulation of naïve T cells, in coordination with the secretion of IL-12 from

APCs, drives the development of Th1 cells that produce IFNɣ (Bogdan and Schleicher 2006). In the absence of cognate antigen, IL-12 and IL-18 can induce secretion of high amounts of IFNɣ by artery-infiltrating memory T cells (Ranjbaran, Sokol et al. 2007). Allogeneic responses are composed primarily of Th1 cells (Jiang, Herrera et al. 2004). IFNɣ, IFNɣ-inducible chemokines, and markers of IFNɣ secretion have been found to be elevated in graft tissue from TA patients (Pattison, Nelson et al. 1996; Kao, Kobashigawa et al. 2003; van Loosdregt, van Oosterhout et al. 2006). CD4 T cell clones isolated from allograft kidneys undergoing rejection produce a high amount of IFNɣ (D'Elios, Josien et al. 1997), and T-bet, the "master-regulator" transcription factor mediating the Th1 cell phenotypes, is up-regulated in allograft kidneys undergoing rejection, but not in non-rejecting kidneys (Hoffmann, Hale et al. 2005). IFNɣ is also required for development of TA in experimental settings (Saleem, Konieczny et al. 1996; Nagano, Mitchell et al. 1997). Mouse models deficient in IFNɣ (Nagano, Mitchell et al. 1997; Nagano, Libby et al. 1998; Raisanen-Sokolowski, Glysing-Jensen et al. 1998) or in which IFNɣ has been serologically neutralized (Wang, Burns et al. 2004), show a significant reduction in intimal thickening following allogeneic cardiac transplantation. Also, the common immunosuppressants cyclosporine and sirolimus inhibit development of TA, and this is correlated with a reduction in IFNɣ production (Yi, Cuchara et al. 2006).

IFNɣ activates STAT4 in target cells, leading to cellular changes that drive tissue damage and dysfunction. IFNɣ induces ECs to produce chemokines implicated in T cell recruitment (Stanford and Issekutz 2003) up-regulates MHC class I and II expression (Tellides, Tereb et al. 2000). Tellides *et al.* showed, in a human-mouse chimeric model of arteriosclerosis, that IFNɣ was sufficient to cause intimal thickening. Mechanistically, this involved the promotion of vascular SMC mitogenesis (Tellides, Tereb et al. 2000). More recently, IFNɣ-stimulated SMC proliferation was shown to be mediated by PI3K activation of mTOR and attenuated by ASK1-interacting protein 1, which is a Ras GTPase-activating protein family member that antagonizes JAK-STAT signalling (Wang, Bai et al. 2007; Yu, Qin et al. 2011).

Th2

Th2 responses are characterized by the production of IL-4 and IL-5, and were originally associated with transplant tolerance in a number of experimental models

(Chen and Field 1995). However, it is now known that an allo-specific Th2 response is not benign or tolerizing as previously thought, and in some cases can even result in accelerated rejection (Piccotti, Chan et al. 1996; VanBuskirk, Wakely et al. 1996; Piccotti, Chan et al. 1997). IL-4 and IL-5 are chemoattractants and activators of eosinophils, which can readily cause tissue damage. Additionally, Th2 cytokines can prime allo-specific CD8 T cells to become cytotoxic (Takatsu, Kikuchi et al. 1987; Horvat, Loukides et al. 1991; Noble, Macary et al. 1995). In support of this, IFNɣ-deficient mice reject allografts at an accelerated rate, although the histological features of rejection are very different from what is typically observed in the clinic (Saleem, Konieczny et al. 1996). Furthermore, direct attempts at deviating an allo-immune response from Th1 to Th2 have failed to induce tolerance (Piccotti, Chan et al. 1996; VanBuskirk, Wakely et al. 1996; Piccotti, Chan et al. 1997).

Clinically, Th2 cytokine production has been associated with kidney rejection (Krams, Falco et al. 1992) and liver rejection (Martinez, Krams et al. 1992). In these cases IL-5, rather than IL-2 or IFNɣ, was the primary pathogenic cytokine. Furthermore, extensive evidence suggests that many different cytokines may be expressed within a rejecting allograft (Dallman 1993; Nickerson, Steurer et al. 1994). Interestingly, Chan and colleagues showed that transplant rejection in mice that are depleted of CD8 T cells, proceeds *via* a Th2 response, suggesting that CD8 T cells support Th1-dominant rejection by inhibiting the Th2 responses *in vivo* (Chan, DeBruyne et al. 1995). Although experimental models show that Th2 cells can reject organ grafts, histological features of Th2-mediated rejection (such as eosinophilia) are rarely observed in clinical cases. Therefore, a focus on Th1 rather than Th2 responses will likely provide more valuable insight in to the mechanisms regulating vascular rejection and TA.

Th17

The earliest study implicating a role for IL-17 in transplant rejection was by van Kooten *et al.* in 1998 who demonstrated the presence of IL-17 in renal transplant biopsies from six patients undergoing rejection, but not in control biopsies (Van Kooten, Boonstra et al. 1998). However, the novel subset of CD4 T cells that secretes IL-17, namely Th17 cells, was not officially recognized until 2005 (Harrington, Hatton et al. 2005; Park, Li et al. 2005). During inflammation, the differentiation of naïve CD4 T cells

to Th17 cells requires signals from an APC plus specific cytokine signals. A combination of TGF-β, IL-6, IL-1, and IL-23 (plus Signal 1 and 2) induces the development of Th17 cells from naïve CD4 T cells by a process that depends on the transcription factor RORɣt (Ivanov, McKenzie et al. 2006; Korn, Bettelli et al. 2009). The transplantation procedure as a whole induces the production of many pro-inflammatory cytokines including IL-1, IL-6, IL-21 and IL-23. TGFβ is also abundant due to its production by platelets and graft parenchymal cells (Rao and Pober 2008). The presence of these cytokines suggests Th17 cells could be induced in response to an allograft.

Experimental models have shown that IL-17 and Th17 responses can mediate transplant rejection. Th17-mediated rejection is characterized by the recruitment of neutrophils. Consistent with this, Gorbacheva and colleagues showed that IL-17 knockout recipients show delayed neutrophil infiltration after cardiac transplantation. However, these animals undergo allograft rejection with similar kinetics as wild-type recipients (Gorbacheva, Fan et al. 2010). In the same study, IL-17 knockout recipients, when depleted of CD8 T cells, showed delayed rejection relative to CD8-depleted wild-type recipients. In this model, CD4 T cell activation was not affected, however, infiltration of effector cells was inhibited in IL-17 knockout recipients suggesting that IL-17 promotes rejection by promoting T cell recruitment to the graft (Gorbacheva, Fan et al. 2010). Burrell and colleagues reported that IL-17-producing CD8 T cells mediate co-stimulatory blockade-resistant rejection in T-bet knockout recipients (which cannot mount a Th1 response) (Burrell, Csencsits et al. 2008). Using a different model, the same group showed that, unlike IFNɣ knockout recipients that are protected from TA, T-bet knockout recipients show accelerated allograft rejection characterized by a deficiency in Th1 type responses and increased production of Th2 (IL-4, IL-5, IL-10, and IL-13) and Th17 (IL-17 and IL-6) type responses. Neutralization of IL-17 inhibited this type of rejection, and depletion of CD4, but not CD8, T cells protected against TA and promoted long-term tolerance (Yuan, Paez-Cortez et al. 2008). Another group showed that blocking IL-17 with an IL-17 receptor (R):Fc fusion protein inhibits T-cell proliferative responses to alloantigens and prevents acute, but not chronic, vascular rejection (Tang, Subbotin et al. 2001). Therefore, insight into Th17 responses may provide additional information regarding the regulation of TA.

1.3.3. Inflammatory Cytokines in Transplantation

As evidenced by the sections above, immune responses involved in transplant rejection are extremely complex, and numerous cytokines are involved in transplant rejection and TA. One unavoidable feature of transplantation is inflammation that results from cellular damage as a result of ischemia and/or surgical trauma. As such, the role of inflammatory cytokines (which serve as Signal 3 during T cell activation), is important in determining the outcome of alloantigen-driven T cell responses and rejection. To this end, IL-12, IL-1 and IL-6 are inflammatory cytokines that are up-regulated after organ transplantation and that define the development of Th1 and Th17 cells. However, the mechanisms by which they affect allogeneic T cell responses are unclear.

Interleukin-1

IL-1 is a pro-inflammatory cytokine known to be involved in chronic inflammation (Oppenheim 2001). IL-1α and IL-1β are produced by two distinct genes, however, they both bind the same IL-1 receptor and induce identical signaling responses (Dinarello 1996). IL-1α is produced by many cell types of the immune system (Oppenheim 2001), as well as ECs and vascular SMCs, whereas monocytes/macrophages and dendritic cells are the predominant source of IL-1β. Injured ECs produce IL-1α that can trigger the release of IL-1β from monocytes, and both isoforms go on to stimulate inflammatory T cell responses (Rao and Pober 2008).

IL-1 has been well documented to have an essential role in Th17 differentiation (Acosta-Rodriguez, Napolitani et al. 2007; Wilson, Boniface et al. 2007). Targeting IL-1 in transplant models has been shown to be beneficial. For instance, in a rat heart transplant model, administration of low dose cyclosporine with an IL-1 receptor antagonist resulted in significantly increased transplant survival, characterized histologically by a reduction in graft infiltrating leukocytes (Shiraishi, Csete et al. 1995). In a rat cardiac transplant model, injection of a soluble IL-1 receptor prolonged allograft survival and eliminated lymph node hyperplasia (Shiraishi, Csete et al. 1995). IL-1 also promotes the development of TA and the development of artery-reactive human memory T cells that secrete IL-17 (Rao, Tracey et al. 2007).

Interleukin-6

IL-6 is secreted by APCs and some non-hematopoietic cells such as ECs and SMCs (Schuett, Luchtefeld et al. 2009). The IL-6 receptor (IL-6R) consists of an α-chain that binds IL-6. The IL-6/IL-6R complex then associates with a signal transducing receptor subunit, gp130, to activate signalling pathways within target cells (Hirano 1998). Gp130 is expressed on most cell types, whereas IL-6R expression is limited to hepatocytes and leukocytes (Saito, Yoshida et al. 1992). However, a soluble form of IL-6 receptor exists, which is capable of binding IL-6 and activating gp130 on cells lacking the IL-6 receptor, a process known as IL-6 *trans*-signalling (Montero-Julian 2001). IL-6 can therefore affect a great diversity of cells and tissues

IL-6 regulates T cell responses in a number of ways. Reports show that IL-6 promotes Th17 responses (Joseph, Miner et al. 1998; Yamamoto, Yoshizaki et al. 2000; Bettelli, Carrier et al. 2006), and neutralization of IL-6 prevents the development of Th17 responses that prevent co-stimulation blockade-induced tolerance in T-bet$^{-/-}$ allograft recipients (Burrell, Csencsits et al. 2008). Additionally, several studies have found that IL-6 inhibits the induction of regulatory T cells **(Tregs)**, which are a subpopulation of T cells that down-regulate immune responses and maintain self-tolerance (Dominitzki, Fantini et al. 2007; Fogal, Yi et al. 2011), see discussion below. Moreover, the presence of IL-6 has been shown to render activated T cells resistant to suppression by Tregs (Pasare and Medzhitov 2003)

In transplantation, high levels of IL-6 are correlated with vascular rejection in primates (Matsumiya, Gundry et al. 1997), and with poor outcome after heart transplantation in humans (Deng, Plenz et al. 2002; Plenz, Eschert et al. 2002). Experimentally, studies have shown that production of this cytokine by graft cells is important in driving transplant rejection (Liang, Christopher et al. 2007), and might augment T cell responses by opposing the development of Tregs, and by inhibiting T cell death (Fogal, Yi et al. 2011; von Rossum, Krall et al. 2011). In addition to regulating T cell responses, IL-6 has several vascular effects. For instance, inflammatory activation of the endothelium by this cytokine increases T cell and monocyte adhesion and recruitment into tissues (Watson, Whittaker et al. 1996; Romano, Sironi et al. 1997; Kishimoto 2005; Tieu, Lee et al. 2009). At the cellular level, IL-6 increases the

expression of cell adhesion molecules (ICAM and VCAM) and the secretion of chemokines (IL-8 and MCP-1) (Watson, Whittaker et al. 1996; Romano, Sironi et al. 1997). In addition to regulating the inflammatory activation of the endothelium, IL-6 also increases the proliferation and migration of circulating endothelial progenitor cells, which could increase endothelial regeneration (Fan, Ye et al. 2008).

1.3.4. Tolerance

Immunological tolerance is defined as a state in which the immune system does not mount a pathogenic response to specific antigens. In transplantation, this would be reflected in the specific unresponsiveness of immune cells toward the graft, thereby permitting acceptance, while immunological responses to other antigens, such as those associated with pathogens, would be maintained (Li, Wells et al. 2000; Lechler, Sykes et al. 2005). Transplantation tolerance, like rejection, is a T cell mediated affair (Li 2004). In principle, the nature of inflammatory cytokine stimulation during T cell activation can determine the induction of tolerance *versus* immune activation. For instance, IL-6 blockade has been shown to result in increased differentiation of Tregs in rheumatoid arthritis, which could be tolerogenic.

Tregs

Tregs generally characterized by $CD4^{+}CD25^{high}FoxP3^{+}$ are specialized T cells with specific immunosuppressive activity. Many research efforts have gone in to trying to harness their regulatory function as a novel approach to induce tolerance in transplantation (Hippen, Riley et al. 2011; Lombardi, Sagoo et al. 2011). Tregs modulate immune responses via a variety of mechanisms including secretion of immunosuppressive cytokines, direct suppression of CD4 and CD8 effector T cells, and modulation of APC and EC function (Shevach 2009).

Studies have revealed that kidney transplant recipients who are hyporesponsive toward their donor antigens have elevated numbers of $CD4^{+}FoxP3^{+}$ in graft infiltrate as compared to recipients who remain reactive to donor antigens (Bestard, Cruzado et al. 2007). Higher FoxP3 mRNA levels have also been observed in biopsies from tolerant combined kidney and bone marrow transplant recipients (Kawai, Cosimi et al. 2008), and

higher numbers of FoxP3^{+} T cells are detected in biopsies from tolerant liver recipients (Li, Zhao et al. 2008). Additionally, liver transplant recipients undergoing immunosuppression withdrawal protocols with establishment of tolerance have expanded numbers of circulating Tregs (Nafady-Hego, Li et al. 2010). Shan *et al*. in a meta-analysis showed that localization of Tregs to the allograft, whether detected by elevated numbers of FoxP3+ cells or by increased mRNA expression, generally correlate with better graft survival (Shan, Guo et al. 2011).

Several challenges remain in the translation of Treg therapy to the clinic. Safe protocols for the selection and expansion of donor allo-specific Tregs must be developed that include Tregs with indirect allospecificity (Jiang, Tsang et al. 2006; Peters, Hilbrands et al. 2008). Also, there is the potential of the transferred alloantigen-specific Tregs to convert to proinflammatory effector T cells (Chadha, Heidt et al. 2011). Finally, memory T cell responses are known to be a barrier toward allograft tolerance and Tregs have shown limited ability to suppress memory T cells (Yang, Brook et al. 2007; Afzali, Mitchell et al. 2011).

Induction of Tolerance

There are two temporally and mechanistically distinct phases of allograft tolerance, induction and maintenance, which operate via different mechanisms and are differentially susceptible to induction and blockade (Chiffoleau, Walsh et al. 2003). Several mechanisms have been proposed as the inducers of transplantation tolerance, and these can be classified broadly into two categories: deletional mechanisms in which alloreactive cells are eliminated, and immunoregulatory mechanisms in which the alloreactive cell processes have been altered such that they no longer respond to the alloantigens. These mechanisms are not mutually exclusive and likely are complementary (Li, Wells et al. 2000). An example of immunoregulatory mechanisms is described above in the discussion on Tregs. The focus of my studies is on the regulation of deletional mechanisms, which is discussed below.

Li *et al.* proposed that reduction of the extraordinarily large alloreactive T cell pool by deletional mechanisms must occur in order for allograft acceptance to be achieved, and that the few remaining alloreactive clones may then be controlled through

immunoregulatory processes (Li, Wells et al. 2000). Thus, for induction of tolerance, it is important to decrease the size of the cytopathogenic T cell compartment. This is further supported by numerous studies showing that animal models in which activated T cells are resistant to cell death, are also resistant to tolerance induction (Li, Li et al. 1999; Wells, Li et al. 1999; Wekerle, Kurtz et al. 2001). Moreover, T cell deletion is evident in spontaneously accepted MHC-mismatched liver allografts in mice (Qian, Lu et al. 1997). There are two main types of T cell deletion: central, which occurs in the thymus and eliminates autoreactive T cells during maturation, and peripheral, which eliminates immunopathologic T cells in peripheral tissues (Stockinger 1999).

1.4. T Cell Elimination

1.4.1. Central Deletion

During maturation in the thymus, highly autoreactive T cell precursors are deleted, and thus inhibited from reaching the periphery where they could cause tissue damage. This process results in what is known as self-tolerance. Self-tolerance and allograft tolerance are alike in that the goal is tolerance to a massive amount of antigens (Li, Wells et al. 2000). Estimates claim that 90-99% of T cells undergoing maturation in the thymus are eliminated (Suchin, Langmuir et al. 2001). This is comparable to the magnitude of alloreactive T cells that would have to be eliminated in an allograft scenario. The story linking central tolerance and transplantation tolerance goes back to over 60 years ago when Medawar and colleagues at the University College of London performed ground-breaking studies on acquired immunological tolerance (Billingham, Brent et al. 1953). The work was based on an observation by Ray Owens at Cal Tech, that a pair of dizygotic bovine twins had two sets of red blood cells, one set that derived from themselves and one set derived from their twin (Owen 1945). Owen concluded that the twins must have exchanged blood cells *in utero* and these cells survived into adulthood of the animal. This was the first documented account of immunological tolerance, and it led Burnet and Fenner in 1949 to hypothesize that antigen exposure early in life, induced immunological tolerance to that antigen. In order to test this hypothesis, Medawar *et al.* in 1953 used a transplantation model in which they used

strain A mice as allogeneic donors and CBA mice as recipients. In a series of experiments, they showed that adult CBA mice readily rejected skin grafts from strain A mice. However, if the CBA fetuses were injected *in utero* with strain A tissues, they accepted skin grafts from strain A mice as adults, while still rejecting skin grafts from a different allogeneic mouse strain (Billingham, Brent et al. 1953). With this work, Medawar not only defined the basis for immunological tolerance to self-antigens, but also set the stage for advances in clinical transplantation. Clonal deletion of self-reactive T cells in the thymus during development, together with regulatory mechanisms to inhibit the few self-reactive clones that made it to the periphery, are the mechanisms that prevent autoimmune diseases from developing (Kappler, Roehm et al. 1987; Sakaguchi 2000), and may underline the principles by which transplantation tolerance can be achieved. However, thymic output is highly restricted in adults; therefore, peripheral deletion of T cells may be the predominant tolerance mechanism that is operational for adult transplant tolerance.

1.4.2. Peripheral Deletion

Activated T cells that have the potential to cause immunopathologies are actively deleted through cell death mechanisms in the periphery. Activated T cell death is required for organ transplant survival in response to immunosuppressive therapy as well as for the induction of tolerance in experimental models (Wells, Li et al. 1999). This is supported by numerous studies showing that tolerance cannot be induced in animal models in which T cells are resistant to cell death (Li, Li et al. 1999; Wells, Li et al. 1999; Wekerle, Kurtz et al. 2001). The peripheral deletion of T cells relies on cellular mechanisms of regulated T cell death, of which apoptosis is the best understood.

1.4.3. Apoptosis

Because peripheral deletion of T cells relies on the induction of regulated forms of cell death, it is important to understand these basic mechanisms. Apoptosis is the most studied form of regulated cell death. The term coined by *Kerr et al.* in 1972 means “leaves falling from a tree” (Kerr, Wyllie et al. 1972), and refers to the programmed elimination of cells that are no longer useful to the organism. Apoptosis is a highly

conserved essential process occurring in all multicellular organisms, and is a prominent attribute of the immune system. Immune cell homeostasis is maintained by this process, eliminating effector cells that are no longer needed and whose persistence may result in immunopathologies. Several years of experimental data now suggest that in the setting of organ transplantation, T cell apoptosis may be a pivotal event in the prevention of rejection, and in the induction of tolerance.

The Extrinsic Apoptotic Pathway

The extrinsic apoptotic pathway occurs *via* death receptors of the TNFR family and includes Fas (APO-1, CD95), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and TNF-R1 (Chiffoleau, Walsh et al. 2003). These receptors contain a death domain in their cytoplasmic tail by which the apoptotic signal is transduced following receptor activation (Krammer 2000). When death receptors are engaged by their ligands, it results in recruitment of adaptor proteins to the receptor complex that recruits and drives the catalytic activity of pro-caspase-8; this comprises the death-inducing signalling complex **(DISC)**. Formation of the DISC allows for pro-caspase-8 molecules to come in close proximity to each other resulting in their oligomerization, proteolytic cleavage, and activation of downstream effector pro-caspases (Krammer 2000; Chiffoleau, Walsh et al. 2003). At this point, apoptosis may proceed *via* two different pathways (Scaffidi, Fulda et al. 1998). In so called “type I” cells, there is robust caspase-8 activation at the DISC, this results in the direct cleavage and activation of pro-caspase-3, which then goes on to proteolytically dismantle the cell. In “type II” cells, however, a lower amount of activated caspase-8 is produced. This is compensated for by concurrent activation of the mitochondrial apoptotic pathway resulting in activation of pro-caspase-9, and subsequent activation of executioner caspases. In type II, but not type I cells, over expression of Bcl-2 or Bcl-x_L prevents apoptosis (Scaffidi, Fulda et al. 1998). A switch from apoptosis sensitivity to resistance appears to correlate with a switch from a type I to a type II cell (Krammer 2000).

c-FLIP

The susceptibility of cells to death receptor-mediated cell death is controlled in large part by the intrinsic expression of anti-apoptotic proteins. Cellular FLICE-inhibitory

proteins **(c-FLIP)** were first described in certain viruses (v-FLIP) as proteins that share significant homology with caspase-8 (Hu, Vincenz et al. 1997), and that inhibit cell death induced by death receptors, by preventing caspase-8 recruitment to the DISC and activation. Expression of FLIP has been shown to inhibit apoptosis induced not only by Fas but also TRAIL-R1, TNF-R1, and TRAMP/DR3 (Bertin, Armstrong et al. 1997; Hu, Vincenz et al. 1997; Thome, Schneider et al. 1997). Three different c-FLIP splice variants are expressed as proteins, the long form **(c-FLIP$_L$)** of 55kDA, the short form **(c-FLIPs)** of 26kDA and a 24kDA form c-FLIP$_R$ (Tschopp, Irmler et al. 1998; Golks, Brenner et al. 2005). All forms can competitively inhibit pro-caspase-8 recruitment to the DISC, thereby inhibiting its activation and its downstream effects (Tschopp, Irmler et al. 1998).

The Intrinsic Apoptotic Pathway

Cell death can also be initiated through intrinsic cell signals that occur in response to cellular stress, such as irradiation and nutrient deprivation, the mitochondria are essential regulators of this death pathway (Kroemer, Galluzzi et al. 2007). The proteins of the respiratory chain (respiratory complexes I-IV and cytochrome c) are found in the inner mitochondrial membrane. This membrane is impermeable to most ions including protons, which permits the generation of the proton gradient necessary for the process of oxidative phosphorylation to occur and the production of ATP (Mitchell and Moyle 1965; Mitchell and Moyle 1965). The mitochondrial apoptotic pathway is activated when the outer mitochondrial membrane undergoes permeabilization. At this point, the inner mitochondrial proteins, which are normally confined between the inner and outer mitochondrial membranes, are released into the cytosol, thus arresting the bioenergetic function of the mitochondrion (Kroemer, Galluzzi et al. 2007; Galluzzi, Blomgren et al. 2009). The process of mitochondrial outer membrane permeabilization is regulated by the Bcl-2 family proteins, some of which are pro-survival (Bcl-2, Bcl-x$_L$) and some of which are pro-apoptotic (effector molecules BAX, BAK, and BH3- only proteins BAD, BID, BIK, BIM, PUMA, Noxa) (Kroemer, Galluzzi et al. 2007). In general, upon exposure to cellular stressors such as noxious chemicals, UV radiation, or growth factor withdrawal, BAX and BAK act on the outer mitochondrial membrane to induce permeabilization that results in cytochrome c release (Ow, Green et al. 2008). Once in the cytosol, cytochrome c associates with a caspase recruitment domain **(CARD)**-containing apoptotic protease-activating factor-1 **(APAF-1)** molecule. In the presence of

dATP, these cytochrome c-APAF-1 complexes oligomerize into a wheel-like structure called the apoptosome. The fully-formed apoptosome contains CARD domains in the appropriate formation to recruit and activate pro-caspase-9 (Zou, Li et al. 1999). Activated caspase-9 is an initiator caspase that catalyses the proteolytic cleavage and activation of downstream executioner caspases. These are able to cleave many targets in the cell including other caspases, thus initiating an amplification loop that ultimately results in apoptosis (Kroemer, Galluzzi et al. 2007). In addition to caspases, mitochondria-mediated cell death can also proceed in the absence of caspase activation through generation of reactive oxygen species and the release of cytotoxic proteins such as apoptosis-inducing factor (Ruefli, Ausserlechner et al. 2001).

As reviewed by Chipuk and Green, controversy remains regarding the nature of the protein-protein interactions among Bcl-2 family members required for outer membrane permeabilization and apoptosis (Chipuk and Green 2008). The “Rheostat model” proposes that the fate of the cell is dependent on the balance between pro-apoptotic and anti-apoptotic Bcl-2 protein expression. While consistent with many observations, this model does not account for the relatively high expression of uninhibited BAX and BAK, or the fact that different Bcl-2 proteins have different functions and different targets. The more current anti-apoptotic protein neutralization model proposes that BAX and BAK must be continually bound/inhibited by anti-apoptotic proteins. Thus, these anti-apoptotic proteins must be neutralized for BAX and BAK to induce outer membrane permeabilization and cell death. The pro-apoptotic Bcl-2 family proteins BID, BIM, and PUMA can neutralize all anti-apoptotic proteins, whereas BAD and Noxa have specific targets. However, as with the Rheostat model, this model ignores the fact that BAX and BAK are expressed at high levels in the cell, and not all individual BAX and BAK proteins are bound by anti-apoptotic proteins. In a third model, BID and BIM directly activate BAX and BAK, and that all other pro-apoptotic Bcl-2 family proteins are de-repressors or increase sensitivity to BID and BIM. In this model, cell stress induces BAX/BAK activation by BID/BIM. The occasional BIM/BID that becomes inappropriately activated, is sequestered by anti-apoptotic Bcl-2 proteins. However, affinity between BID/BIM and effector molecules remains unclear (models reviewed in (Chipuk and Green 2008)).

1.4.4. Cell Death Pathways That Control Peripheral Deletion

As shown in Figure 3, the two main pathways by which activated T cells undergo cell death are *(i)* AICD, which occurs *via* the extrinsic pathway and is triggered by repeated high-affinity stimulation of the TCR (Chiffoleau, Walsh et al. 2003; Green, Droin et al. 2003), and *(ii)* cytokine deprivation-induced cell death **(CDICD)**, which occurs *via* the intrinsic pathway in response to a paucity of stimulating cytokines and growth factors (Duke and Cohen 1986; Boise, Gonzalez-Garcia et al. 1993; Boise, Minn et al. 1995). Both processes are active in the setting of transplantation.

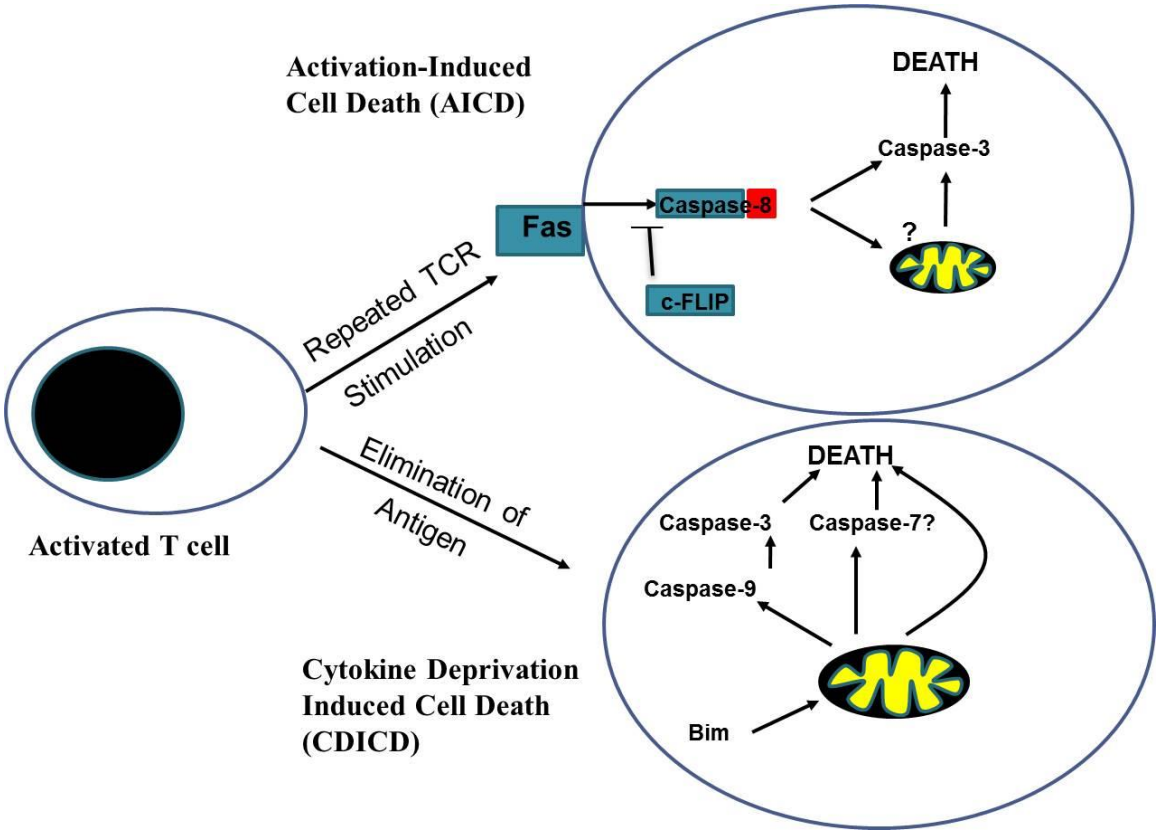


Figure 3. Activated T cells undergo cell death via two main pathways, AICD and CDICD.

AICD occurs as a result of TCR re-stimulation *via* the extrinsic cell death pathways involving death ligands and receptors such as Fas-FasL. CDICD occurs when an activated T experiences a lack of cytokines/survival signals in its environment consistent with the contraction phase of an immune response when the stimulus is no longer present. This occurs *via* the intrinsic/mitochondrial cell death pathway.

Activation Induced Cell Death

AICD is an essential tolerance mechanism that prevents immunopathologic responses (Green, Droin et al. 2003; Krammer, Arnold et al. 2007). AICD is dependent on IL-2 (Lenardo 1991; Refaeli, Van Parijs et al. 1998), can be inhibited by expression of c-FLIP (Irmler, Thome et al. 1997; Tschopp, Irmler et al. 1998), and is generally not inhibited by expression of Bcl-2 family anti-apoptotic proteins (Strasser, Harris et al. 1995). T cells from mice with a conditional deletion in c-FLIP display significantly reduced numbers of mature T lymphocytes presumably caused by augmented apoptosis *in vivo* (Zhang and He 2005). Thus, c-FLIP may be a relevant clinical target for promoting activated T cell death in the setting of allograft rejection

IL-2 is a cytokine produced by activated T cells and is essential for T cell survival, proliferation, and differentiation. However, IL-2 is also absolutely required for AICD (Lenardo 1991). Mice deficient in IL-2 do not develop immunodeficiency, but rather display autoimmunity and severe lymphoproliferation (Schorle, Holtschke et al. 1991); a similar phenotype is observed in mice deficient in the IL-2 receptor alpha- chain (Willerford, Chen et al. 1995). Furthermore, IL-2-deficient mice are resistant to induction of tolerance after receiving islet or cardiac allografts (Li, Li et al. 1999). It has been proposed that whether IL-2 sensitizes or protects T cells from AICD is dependent on the timing of IL-2 exposure; specifically, previous exposure to IL-2 increases T cell susceptibility to AICD, but this situation can be overruled by exposure to IL-2 at the time of TCR re-stimulation (Pender 1999). The type of signal transduced may also be dependent on the IL-2 concentration at the time of exposure. For example, a study using human Tregs and human CD4 responder T cells in co-culture showed that in the presence of high concentration of IL-2, the CD4 T cells are eliminated by the Tregs, whereas in the presence of low amounts of IL-2, the Tregs are eliminated by the CD4 responder cells (Czystowska, Strauss et al. 2010). Therefore, even though IL-2 may be involved in allograft rejection, it seems to be required for induction of tolerance, perhaps due to its role in sensitizing T cells to AICD.

AICD by definition is an antigen-specific process. In a transplant setting, the large majority of T cells within grafts are alloantigen-specific effector cells that could be targeted to undergo AICD. However, when donor-derived APCs are cleared there may

be a paucity in the immune response, or pockets lacking inflammatory signals may develop, where these alloantigen-specific T cells may undergo CDICD.

Cytokine Deprivation Induced Cell Death

CDICD is triggered when T cells experience the absence of cytokine and growth factor signals (Duke and Cohen 1986), consistent with the elimination of pathogen-derived antigens that precedes the contraction phase of an immune response. This type of cell death is important in the maintenance of immune homeostasis and in the prevention of autoimmunity (Bouillet, Metcalf et al. 1999). It is thought that the lack of growth factors causes a rapid down regulation of anti-apoptotic Bcl-2 family molecules (Hildeman, Zhu et al. 2002), accompanied by increased expression of the pro-apoptotic Bcl-2 family member Bim (Hildeman, Zhu et al. 2002). This seems to be the pivotal step in determining the fate of the T cell (Chiffoleau, Walsh et al. 2003). Bim is a pro-apoptotic BH3-only member of the Bcl-2 family (Bouillet, Metcalf et al. 1999) that antagonizes the survival actions of Bcl-2 and Bcl-xL, thereby triggering mitochondrial permeabilization and cell death (Hildeman, Zhu et al. 2002).

Bim regulates T cell development as well as the persistence of effector T cell responses. It was simultaneously discovered by two different groups, *(i)* Hsu and collegues who screened a yeast two-hybrid library from ovarian tissue using MCL-1 (BCL-2 family member that inhibits cell death) as bait (Hsu, Lin et al. 1998), and *(ii)* O'Connor *et al*. who screened a λ-phage expression library from a T cell lymphoma using BCL-2 as bait (O'Connor, Strasser et al. 1998). There are at least three isoforms of Bim produced by alternative splicing, Bim_{EL} (extra long), Bim_{L} (long), and Bim_{S} (short), and these all differ in their pro-apoptotic capacity (O'Connor, Strasser et al. 1998). Bim_{S} is the most potent inducer of cell death since it cannot be sequestered by the microtubular dynein-motor complex the way Bim_{EL} and Bim_{L} are (Puthalakath, Huang et al. 1999). Bim is expressed in most lymphoid and myeloid cells, with Bim_{EL} being the most highly expressed variant followed by Bim_{L}, and Bim_{S} expression is usually low (O'Reilly, Cullen et al. 2000).

During T cell development in the thymus, more than 90% of cells undergo cell death (Marsden and Strasser 2003). During a process called positive selection,

immature T cells that have failed to produce a functional TCR do not receive survival signals and will undergo cell death, ensuring that only T cells with functional TCRs end up in the periphery. Subsequently, immature T cells whose TCR gene rearrangement produced a TCR with a high affinity for self-antigen undergo cell death in a process called negative selection, this ensures that strongly self-reactive T cells are eliminated (Strasser 2005). Bim is essential for the induction of T cell death during this process, and Bim deficient mice have a striking defect in negative selection (Bouillet, Purton et al. 2002).

In the mature immune system, Bim is required for the contraction of CD8 T cell responses following both acute and chronic viral infections (Hildeman, Zhu et al. 2002; Hughes, Belz et al. 2008; Weant, Michalek et al. 2008). Bim is sufficient for contraction after acute viral infection, whereas Bim and Fas act cooperatively to shut down chronic viral infections and to preclude autoimmunity (Hughes, Belz et al. 2008). The importance of Bim-mediated T cell death in immune homeostasis is highlighted by the development of certain autoimmune-like manifestations in mice that lack this protein (Bouillet, Metcalf et al. 1999). Paradoxically, in addition to regulating T cell death, recent evidence indicates that Bim is also required for optimal T cell activation in autoimmune encephalomyelitis, diabetes, and graft-versus-host disease (Ludwinski, Sun et al. 2009; Yu, Yu et al. 2012). However, nothing is known about how the opposing effects of Bim on T cell activation and death cooperate to define the outcome of immunopathological conditions, whether Bim differentially affects CD4 and CD8 T cell responses to alloantigens, and how Bim affects immune-mediated vascular injury.

1.4.5. Therapeutic Elimination of T Cells

As discussed, removal of alloreactive T cells is likely a cardinal event in the induction of allograft tolerance. One way to effectively eliminate T cells is to deplete them using antibodies. Some of the earliest attempts at T cell depletion involved administering anti-lymphocyte globulin, anti-thymocyte globulin, or anti-lymphoid serum, which is serum that contains anti-lymphocyte antibodies (Starzl, Marchioro et al. 1967; Starzl, Marchioro et al. 1967). These treatments resulted in effective depletion of peripheral lymphocytes. Unfortunately, the use of these agents also leads to non-

specific immunosuppression and the accompanying morbidity (Steward-Tharp, Song et al. 2010). Additionally, in the absence of additional immunosuppression, neutralizing antibodies towards depleting antibodies rapidly develop (Preville, Flacher et al. 2001). Despite the associated problems, this therapy is still used in certain high-risk transplant cases (Steward-Tharp, Song et al. 2010). T cell depleting antibodies with greater specificity were subsequently developed, such as the clinically significant OKT3 mouse monoclonal antibody against CD3, a signalling component associated with the TCR (reviewed in (Chatenoud and Bluestone 2007)). This was the first monoclonal antibody approved by the U.S. Food and Drug Administration and continues to be used today. The use of this antibody however can also lead to T cell activation resulting in a septic-shock like reaction (Ferran, Sheehan et al. 1990). Less toxic, modified anti-CD3 monoclonal antibodies have since been engineered to reduce the T cell activating signal (Friend, Hale et al. 1999). These are reported to induce apoptosis and promote expansion of regulatory T cells (Tregs) (reviewed in (Chatenoud and Bluestone 2007)). Another therapeutically used monoclonal antibody is anti-CD25 (alemtuzumab / Campath-1H); this antibody is used with varying effectiveness in both solid and hematological transplants (Friend, Hale et al. 1989; Hale and Waldmann 1994; Alba, Morales et al. 2010). As for complications with this treatment, CD25 is widely expressed in immune cells; therefore it not only effectively depletes B and T cells, but can also cause pancytopenia (Steward-Tharp, Song et al. 2010). Paradoxically, it has also been reported to cause autoimmune thyroiditis (Coles, Wing et al. 1999). Also puzzling, treatment of renal transplant recipients with Campath-H1 alone or administered alongside rapamycin, has resulted in acute rejection mediated by monocytes (not lymphocytes), although these episodes were reversible, and following treatment, the patients remained rejection-free with reduced immunosuppressants despite lymphocyte levels returning to normal (Kirk, Hale et al. 2003). The use of anti-CD25 is also complicated by the fact that it depletes Tregs, which express high levels of CD25.

Depleting antibodies against CD2, a cell adhesion molecule on the surface of memory T cells and most natural killer (NK) cells, have also been developed (Branco, Barren et al. 1999; Xu, Kolber-Simonds et al. 2004). Of these, BTI-322 has been shown to prevent acute rejection in renal transplantation (Mourad, Besse et al. 1997; Squifflet, Besse et al. 1997). More recently, it was shown that the $F(ab')_2$ fragment of this

antibody failed to prevent skin allograft rejection, while the whole IgG could, indicative of a Fc-dependent cell mediated effect on CD2 expressing cells (Snanoudj, Rouleau et al. 2004). It should be noted that a subset of immuno-competent memory-like T cells appears to be resistant to antibody depletion therapy. However, these have been easily controlled with common immunosuppressants (Pearl, Parris et al. 2005).

Directed depletion of cells of the immune system can be advantageous for the prevention of transplant rejection or autoimmunity. However, more specific targets must be identified in order to prevent generalized immunosuppression and other adverse effects such as lymphocyte activation

1.5. Current Diagnosis and Treatment of TA

1.5.1. Diagnosis

There are two main methods by which TA is diagnosed. The most widely used method is coronary angiography, which is a test that uses dye and special x-rays to show the insides of coronary arteries. This method requires comparison of diseased vessels to normal vessels in order to estimate severity of the disease. However, due to the diffuse nature of TA, it often results in underestimation of the disease as there is usually no healthy segment for proper comparison (Kittleson and Kobashigawa 2014). Intravascular ultrasound (IVUS) is the other method used to detect TA, this method offers cross-sectional views of the diseased vessels and is more sensitive than coronary angiography (Kapadia, Nissen et al. 1998; Rickenbacher 1998; Kapadia, Nissen et al. 1999). However, IVUS is invasive, expensive, and can only be used on major epicardial vessels (Kittleson and Kobashigawa 2014).

1.5.2. Treatment

Immunosuppression is the main therapy for the prevention of both acute and chronic rejection. Immunosuppression therapy is separated into three phases – induction, maintenance, and anti-rejection depending on the time of the therapy and the state of the grafted organ (Costello, Mohanakumar et al. 2013). Induction therapy refers

to the administration of a high dose of immunosuppressants, usually anti-lymphocyte globulin, anti-thymocyte globulin or IL-2R, at the time of transplantation and in the early postoperative period (Lund, Edwards et al. 2014). Approximately half of adult heart transplant recipients receive induction therapy while 70% of pediatric patients receive this therapy (Kirk, Edwards et al. 2011; Stehlik, Edwards et al. 2012; Lund, Edwards et al. 2014). There is still some debate surrounding the safety of induction therapy, as it increases risk of opportunistic infections and malignancy (Kittleson and Kobashigawa 2014). Maintenance immunosuppression is the main therapy used to prevent rejection and consists of a cocktail of three different types of drugs – cell cycle inhibitors, calcineurin inhibitors **(CNIs)**, and steroids (Stehlik, Edwards et al. 2012). Most current immunosuppression regimens consist of a combination of the three (Baran, Zucker et al. 2011). Currently, Tacrolimus is the most widely used calcineurin inhibitor for heart transplants, whereas mycophenolate mofetil is the most commonly used cell-cycle inhibitor (Stehlik, Edwards et al. 2012). After the first year post-transplantation, approximately 90% of patients take steroids as part of their maintenance immunosuppression regime, but by 5 years post-transplantation 51% of patients have been weaned off of steroids (Stehlik, Edwards et al. 2012).

Rapamycin (sirolimus) and everolimus are cell activation and growth inhibitors that prevent the activation of the mammalian target of rapamycin **(mTOR)**, and both have been shown to prevent development of TA (Eisen, Tuzcu et al. 2003; Keogh, Richardson et al. 2004; Raichlin, Bae et al. 2007). Both sirolimus and everolimus interfere with T cell activation and proliferation by inhibiting progression through the cell cycle (Schuler, Sedrani et al. 1997; Abraham 1998; Sehgal 1998). These drugs bind to FK506 binding protein-12 (FKBP12), and the FKBP12-SIR or FKBP12-EVL complex binds to mTOR, causing the inhibition of activation of p70s6k, a kinase that phosphorylates ribosomal S6 protein, ultimately causing cell arrest in late G1 phase just before entering S phase (Sehgal 1998; Olyaei, de Mattos et al. 2001; Nashan 2002; Shitrit, Yussim et al. 2004). This new generation of immunosuppressants was initially used in “no nephrotoxicity” regimes that avoided use of CNIs. However, the use of these drugs resulted in a high frequency of acute rejection episodes as well as extensive side effects; therefore today these drugs are typically used in combination with low dose CNIs (Cibrik, Silva et al. 2013).

It is important to emphasize that the existing immunosuppressive strategies for organ transplantation and TA have many limitations. Steroids, CNIs, and cell cycle inhibitors cause hyperlipidaemia, so statins are often given alongside the immunosuppression regime to reduce low-density lipoprotein cholesterol. Pravastatin is the favored statin for transplant recipients, as it has a reduced chance of interacting with CNIs (Page, Miller et al. 2005). Statins have additionally been shown to act as anti-inflammatory agents and have direct immunomodulatory properties (Vaughan, Murphy et al. 1996; Aikawa, Rabkin et al. 2001; Libby and Aikawa 2002). Shimizu and colleagues. showed that cerivastatin reduces macrophage activation *in vitro* and *in vivo* (Aikawa, Rabkin et al. 2001). Atorvastatin has been reported to reduce MHC class II expression and mixed lymphocyte reactions *in vitro* (Kwak, Mulhaupt et al. 2000), and pravastatin has been shown to reduce NK cytotoxicity *in vivo,* and CTL activity *in vitro,* resulting in reduce human kidney transplant rejection (Katznelson, Wang et al. 1998).

Post-transplant therapy also worsens existing diabetes, and up to 32% of transplant recipients become diabetic 5 years post-transplant (Stehlik, Edwards et al. 2010). Vertebral compression fractures are also common since steroid use exacerbates osteoporosis; this can be quite a debilitating problem, therefore, transplant recipients are recommended Vitamin D and calcium supplements, as well as to engage in weight bearing exercises (Kittleson and Kobashigawa 2014).

Renal failure is often a problem due to the toxicity of CNIs; up to 8% of heart transplant recipients develop end-stage renal failure at just 5 years post-transplant, and that percentage goes up for every additional year post-transplant (Zietse, Balk et al. 1994; Goldstein, Zuech et al. 1997; Stehlik, Edwards et al. 2010). In these individuals, their immunosuppressant regime may be switched to a “renal sparing” one by reducing CNI dose, or substituting for rapamycin (Gustafsson and Ross 2009). Other common problems associated with the use of immunosuppression include: gout, diuretic use for renal insufficiency, malignancy that usually appears 3 – 5 years after transplantation (Vajdic and van Leeuwen 2009), as well as lymphoproliferative disorders – usually B cell lymphomas related to EBV infection (Swinnen, Costanzo-Nordin et al. 1990; Opelz and Henderson 1993).

Once advanced TA is present, percutaneous coronary intervention may be performed at specific *foci*. However, restenosis, which refers to an abnormal constriction of the vessel is, common and it is unclear whether it changes the overall disease prognosis (Sharifi, Siraj et al. 2000). Patients with multi-vessel disease may be candidates for surgical revascularization with coronary artery bypass grafting (Kittleson and Kobashigawa 2014). Finally, re-transplantation becomes the only option for end-stage graft failure (Smith, Ribakove et al. 1995). Overall, it is clear that existing therapeutic strategies for TA and transplant rejection are suboptimal. New strategies are needed to prevent these conditions without causing morbidity or mortality of graft recipients. To achieve this, a more advanced understanding of how alloimmune responses are regulated is required.

1.6. Rationale, Overall Objective, and Overarching Hypothesis

Rationale

Given that activated T cell death is a regulator of allogeneic immune responses, and that studies have shown that it is required for induction of tolerance and for graft survival in response to immunosuppression (Wells, Li et al. 1999; Wells, Li et al. 2001), I set out to study the regulation of T cell death in alloimmune responses and in vascular rejection. Since the two main pathways of activated T cell death are AICD and CDICD, my goal was to greater elucidate their regulation in the context of vascular rejection. My first specific goal was to explore the role of inflammatory cytokines present during T cell activation on Fas-mediated AICD in human CD8 T cells. Specifically, I compared the susceptibility of human CD8 T cells activated in Th17-inducing conditions (IL-1, IL-6, and IL-23) and ones activated in Th1-inducing conditions (IL-12) to Fas-mediated AICD. I focused on Th17-inducing conditions because they have been implicated, but poorly characterized, in transplant rejection and Th1-inducing conditions since this is the main type of immune response involved in transplant rejection (as discussed in Section 1.3.2). I chose to focus on CD8 T cells since these are the cells that cause direct damage to the cells of the graft. I observed that the CD8 T cells activated in IL-1/6/23 had a significantly reduced susceptibility to Fas-mediated AICD than those activated in IL-12. I

then tested the cytokines individually, and in combination, to specifically determine what cytokine was responsible for what effect. The focus of the study then shifted to IL-1 and IL-6. During this investigation, I found that IL-1 reduced FasL expression and that IL-6 caused increased expression of c-FLIPs. The results of this study are explained in Chapter 3 and have also been published (von Rossum, Krall et al. 2011).

As a continuation of my first goal, my second specific goal was to study how the effects of IL-6 on AICD affect alloimmune responses and vascular rejection *in vivo*. For this, I moved to a mouse model and analyzed the effect of IL-6 on CD4 and CD8 T cell death, FOXP3 expression, and vascular rejection. Here, I found that mice transplanted with IL-$6^{-/-}$ arteries developed less severe vascular rejection than those transplanted with IL-$6^{+/+}$ arteries – this was associated with an increase in cell death in splenic CD4 and CD8 recipient T cells. These results are described in Chapter 4.

My third specific goal was to study the role of CDICD in the regulation of alloimmune responses and vascular rejection *in vivo*. For this goal, I focused on the pro-apoptotic protein Bim that, as detailed above (Section 1.4.4), is the pivotal regulator of cell death, and is also essential for negative selection during T cell development in the thymus. I studied the effect of Bim in alloimmune-induced T cell activation, T cell death, and on vascular rejection. I found that Bim was unexpectedly required for T cell proliferation in response to alloantigen stimulation *in vitro* and that a partial reduction in Bim expression was sufficient to attenuate activation, whereas complete elimination of Bim was required to prevent T cell death – these effects resulted in significantly less vascular rejection in $Bim^{+/-}$, but not $Bim^{-/-}$, graft recipients. This study is presented in Chapter 5 and also has been published (von Rossum, Enns et al. 2014). Overall, these studies provide valuable insight into the regulation of allogeneic immune responses and may have clinical implications.

Overall Objective

To understand the role and regulation of activated T cell death pathways in alloimmune-induced vascular rejection and development of transplant arteriosclerosis.

Overarching hypothesis

The activated T cell death pathways AICD and CDICD regulate the development and severity of transplant arteriosclerosis.

Chapter 2. Inflammatory Cytokines Determine the Susceptibility of Human CD8 T Cells to Fas-Mediated Activation-Induced Cell Death Through Modulation of FasL and c-FLIPs Expression.

2.1. Preface

The content of this chapter has been published. von Rossum, A., Krall, RL., Escalante, NK., and Choy, JC. Inflammatory Cytokines Determine the Susceptibility of Human CD8 T Cells to Fas-mediated Activation-induced Cell Death through Modulation of FasL and c-FLIPS Expression. *J Biol Chem*. 2011. 286: 21137-44.

2.2. Introduction

Specific biological conditions determine the types of cytokines produced during inflammation, and the nature of these inflammatory cytokines dictates the fate of CD8 effector T cell responses. For instance, IL-12 is produced in response to several pathogens and high levels of this cytokine during CD8 T cell activation drives the development of short-lived effector cells characterized by potent effector functions, such as IFNɣ production and cytotoxic properties. In contrast, the presence of lower levels of IL-12 favours the generation of long-lived memory CD8 T cells (Joshi, Cui et al. 2007). In addition to pathogen infection, ischemic damage also triggers an inflammatory response that clearly affects immune responses in organ transplantation, cardiovascular diseases, stroke, and in response to tumours (Snoeijs, van Heurn et al. 2010). IL-1α, IL-1β, and IL-6 are produced rapidly in response to ischemic damage (Morgan, Pelletier et al. 1993), and IL-23 has been shown to be produced following ischemic damage of heart and liver allografts (Husted, Blanchard et al. 2006). The combination of IL-1, IL-6, and

IL-23 is also involved in the differentiation of CD4 T cells into IL-17-producing effector cells, termed Th17 cells, which have distinct roles in physiology and pathology (Acosta-Rodriguez, Napolitani et al. 2007; Wilson, Boniface et al. 2007). Furthermore, CD8 T cells are recruited early on into cardiac allografts associated with ischemic damage (Schenk, Nozaki et al. 2008). Because CD8 T cells are exposed to the combination of IL-1, IL-6, and IL-23 during inflammatory conditions, understanding the effects of these cytokines on CD8 T cell biology provides important insight into the regulation of adaptive immune responses.

The induction of effector T cell death during immune responses is crucial for preventing the development of immunopathology and for prolonging allograft survival in response to tolerizing regimens or immunosuppressive drugs (Wells, Li et al. 1999; Krammer, Arnold et al. 2007). However, very little is known about how the specific inflammatory cytokines present during T cell activation affect the susceptibility of resultant effector T cells to AICD.

The intrinsic susceptibility of T cells to AICD is regulated by expression of c-FLIP, (Irmler, Thome et al. 1997). c-FLIP is expressed in activated T cells where it prevents caspase-8 activation in response to FasL and other death-inducing ligands. c-FLIP$_S$ has been suggested to be the main isoform that regulates the susceptibility of activated human T cells to AICD (Kirchhoff, Muller et al. 2000). However, activation of T cells in this mentioned study was conducted by stimulation with phytohemagglutinin, which provides only TCR stimulation. Physiologically, T cell responses are influenced by the presence of TCR stimulation (Signal 1), co-stimulation (Signal 2), and cytokine signals (Signal 3).

I have examined the effects of inflammatory cytokines on the susceptibility of human CD8 T cells to post-activation cell death. I focused specifically on the response of human T cells because there are some distinctions between the mechanisms by which inflammatory cytokines affect the biology of human as compared to mouse effector T cells (Huber, Ramos et al. 2010). Activated CD8 T cells express IL-12Rβ1 and IL-12Rβ2 (Desai, Quinn et al. 1992). In addition, CD8 T cells respond to IL-1 stimulation, express IL-6R and gp130 in resting conditions, and some express IL-23R

after activation (Mizuochi, McKean et al. 1988; Hirata, Taga et al. 1989; Wang, Taga et al. 1998; Huber, Heink et al. 2009). Thus, I examined the effects of IL-12 as well as the combination of IL-1, IL-6, and IL-23 (IL-1/6/23) on post-activation cell death of human CD8 T cells. I found that human CD8 T cells activated in the presence of IL-1/6/23 are less susceptible to Fas-mediated AICD than those activated in IL-12, as a result of decreased FasL expression mediated by IL-1 and an increase in c-FLIPs expression mediated by IL-6.

2.3. Materials and Methods

2.3.1. Human Cell Isolation and Culture

Human peripheral blood cells were obtained by phlebotomy from healthy volunteers with approval of the SFU Institutional Review Board. Mononuclear cells were isolated from peripheral blood by density gradient centrifugation. CD8 T cells were purified to >98% purity by positive selection with Dynal magnetic beads (Invitrogen, Carlsbad, CA) using CD8 as the cell surface marker and including a bead removal step as described (Choy, Wang et al. 2007). In some experiments, purified T cells were stained with a PE-conjugated CD45RO antibody (BD Biosciences, Franklin Lakes, NJ) before cell sorting using a BD FACS Aria.

CD8 T cells were stimulated with plate-bound anti-CD3 (3 µg; eBioscience, San Diego, CA) and soluble anti-CD28 (1 µg/mL; eBioscience) in RPMI containing 10% FCS (v/v) as described (Choy and Pober 2009). Cells were treated with recombinant IL-12 (10 ng/mL; R&D Systems, Minneapolis, MN), or IL-1α (10 ng/mL; NCI Biological Resources Branch Preclinical Repository), IL-6 (50 ng/mL; Peprotech, Rocky Hill, NJ), and/or IL-23 (50 ng/mL; R&D Systems). A neutralizing antibody to IFNɣ (10 µg/mL; BD Biosciences) was also added in some experiments. In certain experiments, a FasL neutralizing antibody (10 µg/mL; BD Biosciences) was added, a caspase-8 inhibitor z-IETD.fmk (EMD Chemicals, Gibbstown, NJ) added on days 3 and 5 post-stimulation (25 µM on each day), or an agonistic Fas antibody (10 µM; clone CH11; Millipore, Billerica, MA) added at day 4 post-stimulation.

In co-culture experiments, CD8 T cells were activated in the presence of either IL-12 or IL-1/6/23 for four days, and then harvested and washed. CD8 T cells activated in IL-1/6/23 were CFSE (Carboxyfluorescein succinimidyl ester) labelled (0.5 μM; Invitrogen) as described (Choy, Wang et al. 2007) whereas those activated in IL-12 were not. CFSE-labelled CD8 T cells that had been activated in IL-1/6/23 were then co-cultured at a 1:1 ratio with unlabelled CD8 T cells that had been activated in IL-12. Anti-CD3 and anti-CD28 stimulation was maintained in these co-cultures.

2.3.2. Quantification of Cell Death and Viability

Cell death was measured by staining cells with propidium iodide (PI; Invitrogen) as described in (Choy and Pober 2009). We were not able to evaluate phosphatidylserine exposure in addition to membrane permeabilization in our experiments because activation of human CD8 T cells induces transient exposure of this lipid on the outer membrane that is unrelated to cell death, thereby confounding the interpretation of data obtained by this method [data not shown and (Fischer, Voelkl et al. 2006)]. Total cell viability was quantified with a MTS assay (Promega, Madison, WI) as described (Choy and Pober 2009).

AICD was induced using conditions described previously (Ramaswamy, Dumont et al. 2007; Choy and Pober 2009). Specifically, human CD8 T cells were stimulated in the presence of IL-12 or IL-1/6/23 for three days, removed from stimulation, washed extensively, and cultured in IL-2 (50 U/mL; NCI) for at least five days. T cells were then either left unstimulated or were re-stimulated with plate-bound anti-CD3 (3 μg) for 16 h and cell death measured by PI staining.

2.3.3. Flow Cytometry

Flow cytometry was performed as described previously (Choy, Wang et al. 2007). In some experiments, CD8 T cells were CFSE labeled prior to stimulation. Fas and FasL surface protein expression was examined by staining cells with a PE-conjugated antibody to Fas (BD Biosciences) or a biotin-labelled antibody to FasL (BD

Biosciences) followed by PE-conjugated streptavidin. All data was acquired on a BD FACS Aria.

2.3.4. Analysis of RNA Expression and Cytokine Secretion

Total RNA was isolated using a RNA Minikit (Qiagen, Valencia, CA) and Taqman quantitative RT-PCR (RT-qPCR) performed as described (Choy, Wang et al. 2007) using validated Taqman primer/probe sets (Applied Biosystems, Foster City, CA). Data was acquired on an ABI 7900HT iCycler. IFNɣ levels were quantified using an ELISA kit (Invitrogen).

2.3.5. Cell Lysis and Western Blotting

CD8 T cells were lysed and Western blotting performed as described (Choy and Pober 2009) using c-FLIP (Upstate, Lake Placid, NY and Abcam) and β-actin (Sigma) antibodies.

2.3.6. siRNA Knockdown

5 x 10^6 primary resting human CD8 T cells were transfected with 200 pmol of siRNA that targets all isoforms of c-FLIP (Qiagen) or a scrambled control sequence (Qiagen) by amaxa nucleofection using program U-014. Transfected CD8 T cells were rested for 6 h prior to activation.

2.3.7. Statistical Analysis

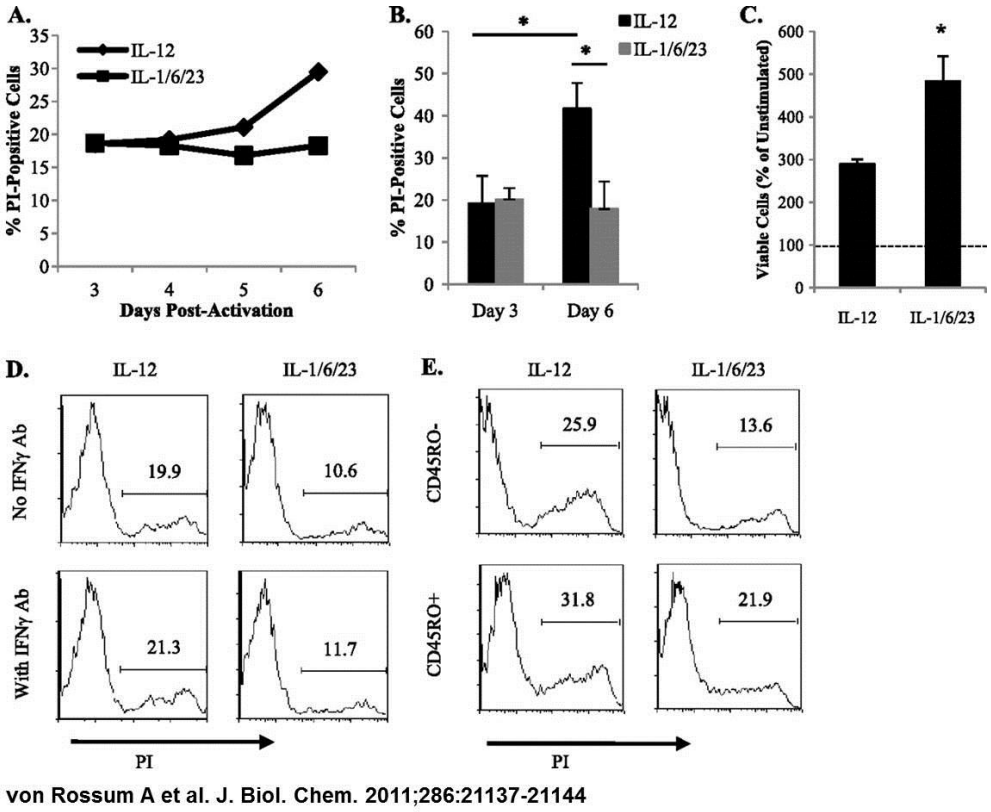
A two-tailed Student's t-test was performed to determine significant differences between groups. An α-value of <0.05 was chosen to be significant.

2.4. Results

2.4.1. Inflammatory Cytokines Differentially Affect the Susceptibility of Human CD8 T Cells to AICD

To begin examining the effects of inflammatory cytokines on CD8 T cell death, primary human CD8 T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 in the presence of IL-12 or IL-1/6/23, and cell death examined by PI staining at various time points after activation. There was no difference in the background level of cell death of CD8 T cells activated in the two conditions at early time-points up to day 3 post-stimulation. Starting after day 3, there was a progressive increase in cell death of CD8 T cells activated in IL-12 that continued until day 6. In contrast, cell death did not increase in CD8 T cells activated in the presence of IL-1/6/23 at any time point (Figure 4 A). When cell death was compared in cells isolated from five different donors in separate experiments, there was significantly less cell death in CD8 T cells activated in IL-1/6/23 as compared to IL-12 at day 6 post-stimulation (Figure 4 B; $p<0.02$). Further, consistent with the reduced percentage of CD8 T cells undergoing cell death after activation in IL-1/6/23, there was increased accumulation of viable human CD8 T cells seven days after activation in IL-1/6/23 as compared to IL-12 (Figure 4 C).

Some reports have suggested that certain forms of cell death that occur after TCR stimulation can be mediated by IFNɣ (Liu and Janeway 1990; Refaeli, Van Parijs et al. 2002). Production of IFNɣ by human CD8 T cells increased after activation in the presence of IL-12, as compared to IL-1/6/23 (data not shown). However, neutralization of IFNɣ did not affect cell death in our experiments (Figure 4 D). Finally, we also evaluated the effects of IL-12 and IL-1/6/23 on the death of human naïve and memory CD8 T cells after activation. A similar pattern of cell death was observed when purified $CD45RO^-$ or $CD45RO^+$ human CD8 T cells were activated under the two conditions (Figure 4 E). Therefore, human CD8 T cells undergo less cell death and accumulate to a greater extent after activation in IL-1/6/23 as compared to IL-12, and this process is independent of the levels of IFNɣ produced under the two conditions.



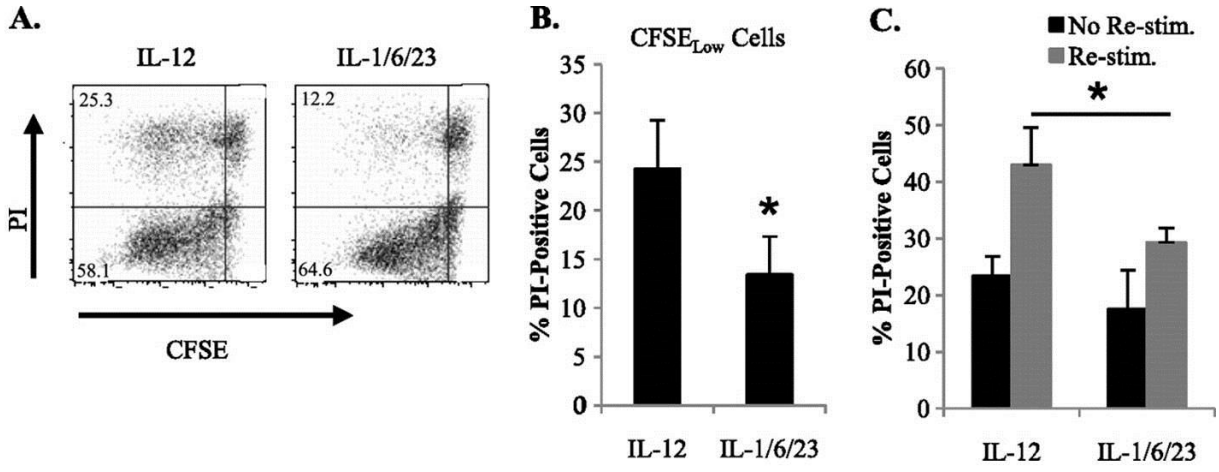
von Rossum A et al. J. Biol. Chem. 2011;286:21137-21144

Figure 4. Human CD8 T cells activated in IL-1/6/23 undergo less post-activation cell death than those activated in IL-12.

Human CD8 T cells activated in IL-1/6/23 undergo reduced post-activation cell death compared to those activated in IL-12. **A**, human CD8 T cells were activated in the presence of IL-12 or IL-1/6/23, and cell death was quantified by PI staining at days 3–6 post-stimulation. Data from one of three independent experiments are shown. **B**, cell death at day 3 and day 6 post-stimulation of human CD8 T cells activated in the presence of IL-12 or IL-1/6/23 was examined in cells from five different individuals in separate, independent experiments. Data presented are the mean ± S.D. of the % PI-positive cells. *, $p < 0.02$. **C**, human CD8 T cells were activated in the presence of IL-12 or IL-1/6/23, and the accumulation of viable cells was quantified with an MTS assay at day 7 post-stimulation. Data presented are the mean ± S.D. of triplicate measurements in one representative experiment out of three. *, $p < 0.01$. **D**, human CD8 T cells were activated in the presence of IL-12 or IL-1/6/23. An IgG isotype control or neutralizing IFNγ antibody was added to the cultures, and cell death was examined by PI staining after 6 days. Representative data from one of two independent experiments with different donors are shown. **E**, $CD45RO^-$ and $CD45RO^+$ human CD8 T cells were purified before activation in the presence of IL-12 or IL-1/6/23. Cell death was measured at day 6 post-stimulation by PI staining. Data from one of two independent experiments with different donors are shown.

Since the cell death we observed occurred in the presence of continuous TCR stimulation, I examined the effects of IL-12 and IL-1/6/23 on the susceptibility of human

CD8 T cells to AICD. Proliferation was observed in almost all T cells at day 6 after activation and there was no difference in the proportion of CD8 T cells that had undergone proliferation after activation in IL-12 as compared to IL-1/6/23 (82.8 ± 1.4% of cells were $CFSE_{Low}$ after activation in IL-12 as compared to 77.5 ± 4.9% of cells after activation in IL-1/6/23; n=4; p = NS). However, the proportion of proliferated cells that were undergoing cell death was significantly lower in CD8 T cells that had been activated in the presence of IL-1/6/23 as compared to IL-12 (Figure 5 A and B). The effect of cytokines on AICD was then examined using a well-established *in vitro* model in which the human CD8 T cells were stimulated with anti-CD3 and anti-CD28 in the presence of IL-12 or IL-1/6/23 for three days, removed from stimulation, washed extensively, and cultured in IL-2 for 5 days. T cells were then either left unstimulated or were re-stimulated with plate-bound anti-CD3 for 16 h at which point cell death was measured by PI staining (Ramaswamy, Dumont et al. 2007). CD8 T cells that were initially activated in IL-1/6/23 underwent significantly less TCR re-stimulation-induced death as compared to those activated in IL-12, indicating that they were less susceptible to AICD (Figure 5 C).

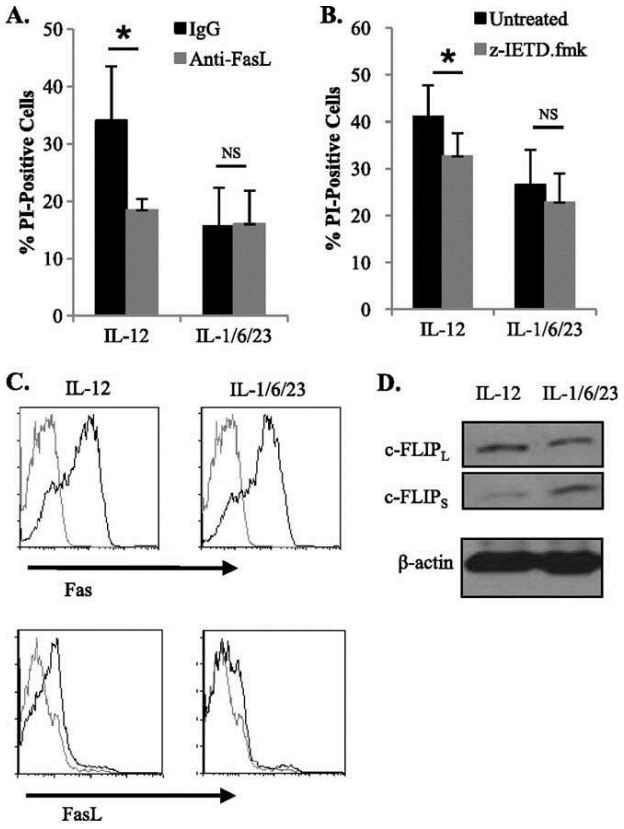


von Rossum A et al. J. Biol. Chem. 2011;286:21137-21144

Figure 5. Human CD8 T cells activated in IL-1/6/23 are less susceptible to AICD as compared with those activated in IL-12

Human CD8 T cells activated in IL-1/6/23 are less susceptible to AICD as compared with those activated in IL-12. **A**, human CD8 T cells were CFSE-labeled prior to activation in the presence of IL-12 or IL-1/6/23. Cells were harvested at day 6, and cell death was examined by PI staining. Data are representative of four independent experiments with different donors. **B**, mean ± S.D. of % PI-positive cells in the $CFSE_{Low}$ population of CD8 T cells activated in the presence of IL-12 or IL-1/6/23 over a series of four independent experiments with different donors. *, $p < 0.01$. **C**, CD8 T cells were activated in the presence of IL-12 or IL-1/6/23 for 3 days, removed from TCR stimulation and cultured in IL-2 (50 units/ml) for 5 days before being re-stimulated with plate-bound anti-CD3. Cell death was examined after 16 h by PI staining. Data presented are the mean ± S.D. of three independent experiments with separate donors. *, $p < 0.05$.

I then determined the role of the Fas pathway in cell death of CD8 T cells by inhibiting FasL activity with a neutralizing antibody or preventing caspase-8 activity with a pharmacological caspase-8 inhibitor (z-IETD.fmk). FasL neutralization significantly inhibited the death of CD8 T cells activated in IL-12, but had no effect on CD8 T cells activated in IL-1/6/23 (Figure 6 A). Similar findings were obtained when caspase-8 was inhibited (Figure 6 B), although greater inhibition was observed with FasL neutralization, which is likely due to reduced stability of the caspase-8 inhibitor in aqueous solution over days 3-6 of the experiments. Analysis of Fas, FasL, and c-FLIP expression indicated that human CD8 T cells activated in IL-1/6/23 expressed similar levels of Fas, lower levels of FasL and higher levels of c-FLIPs as compared to cells activated in IL-12 (Figure 6 C-D).



von Rossum A et al. J. Biol. Chem. 2011;286:21137-21144

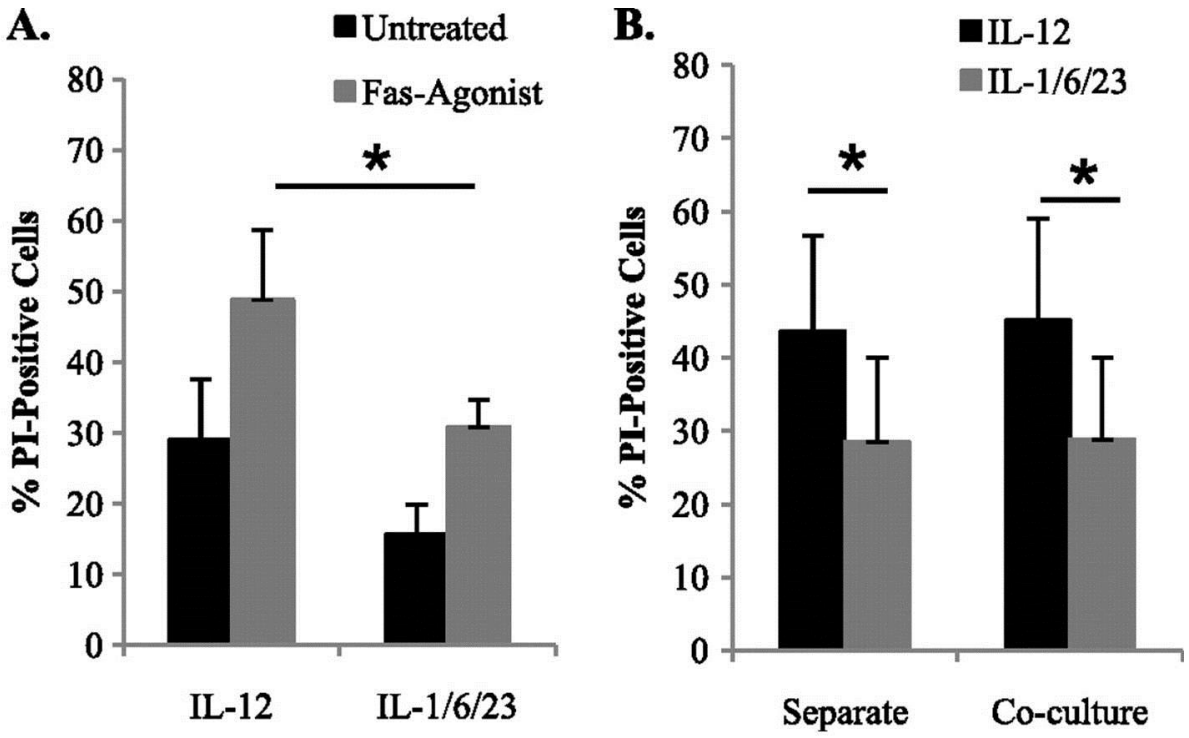
Figure 6. Human CD8 T cells activated in the presence of IL-1/6/23 do not undergo Fas-mediated cell death and differentially express FasL and c-FLIPs as compared with cells activated in IL-12

Human CD8 T cells activated in the presence of IL-1/6/23 do not undergo Fas-mediated cell death and differentially express FasL and c-FLIPS as compared with cells activated in IL-12. Human CD8 T cells were activated in the presence of IL-12 or IL-1/6/23. **A**, a control IgG or FasL-neutralizing antibody was added, and cell death was measured by PI staining at day 6 post-stimulation. Data presented are the mean ± S.D. of three independent experiments with different donors. *, $p < 0.05$, NS = not significant. **B**, cells were untreated or treated with a pharmacological inhibitor of caspase-8 (z-IETD.fmk). Cell death was measured by PI staining at day 6 post-stimulation. Data presented are the mean ± S.D. of three independent experiments with different donors. *, $p < 0.05$, NS = not significant. **C**, Fas and FasL expression was examined by flow cytometry at day 3 post-stimulation. Gray lines: IgG staining control; black lines: Fas or FasL staining. Representative data from one of three independent experiments are shown. **D**, at day 3 post-stimulation, T cells were lysed and c-FLIP expression was examined by Western blot. Detection of β-actin was used as a loading control. Representative data from one of three independent experiments are shown.

The findings above indicate that, as compared to IL-12, IL-1/6/23 attenuates Fas-mediated AICD in human CD8 T cells and that this is potentially due to a lower level of FasL expression and/or a higher level of c-FLIPs expression. To examine this, I

determined the intrinsic susceptibility of human CD8 T cells activated in IL-12 or IL-1/6/23 to Fas-induced cell death by adding an agonistic Fas antibody. This bypasses the requirement for endogenous FasL expression to induce cell death, and is expected to induce the same level of cell death in both conditions if reduced FasL expression was solely responsible for the attenuated induction of AICD observed in the presence of IL-1/6/23. The concentration of Fas-agonistic antibody used was 10-fold higher than that needed to induce maximal cell death in Jurkat cells, indicating that the cells were treated with a maximal concentration of this death stimulus. Fas-induced cell death of CD8 T cells activated in IL-1/6/23 was significantly lower than that of cells activated in IL-12 (Figure 7 A). Therefore, although CD8 T cells activated in IL-1/6/23 express less FasL than those activated in IL-12, replacement of saturating levels of Fas stimulation still resulted in a lower level of cell death. This suggests that CD8 T cells activated in the presence of IL-1/6/23 are intrinsically resistant to this form of cell death.

To further determine whether CD8 T cells activated in IL-1/6/23 were intrinsically less sensitive to Fas-mediated cell death as compared to those activated in IL-12, the nature of the extrinsic signals was normalized by co-culturing T cells that had been activated in IL-12 with those that had been activated in IL-1/6/23. In these experiments, T cells were activated in IL-12 or IL-1/6/23 for four days. Cells were then harvested and those activated in the presence of IL-1/6/23 were labelled with CFSE prior to being co-cultured at a 1:1 ratio with unlabelled CD8 T cells that had been activated in IL-12. Cell death in the CFSE-positive cells (*e.g.,* those originally activated in IL-1/6/23) and CFSE-negative cells (*e.g.,* those originally activated in IL-12) was determined after two days in co-culture. CD8 T cells originally activated in IL-1/6/23 underwent significantly less cell death in these co-cultures as compared to those originally activated in IL-12 (Figure 7 B), thereby establishing that CD8 T cells activated in IL-1/6/23 are intrinsically resistant to Fas-induced AICD.



von Rossum A et al. J. Biol. Chem. 2011;286:21137-21144

Figure 7. Human CD8 T cells activated in IL-1/6/23 are intrinsically less susceptible to Fas-mediated cell death than those activated in IL-12

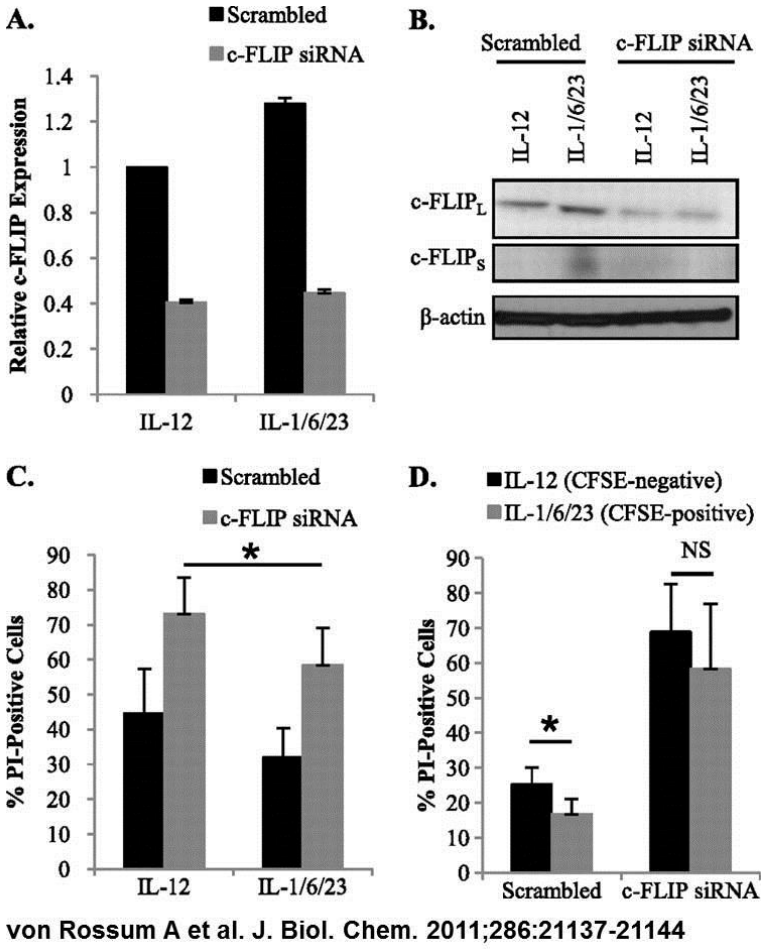
Human CD8 T cells activated in IL-1/6/23 are intrinsically less susceptible to Fas-mediated cell death than those activated in IL-12. **A**, human CD8 T cells were activated in the presence of IL-12 or IL-1/6/23. At day 4 post-stimulation, cells were either left untreated or treated with an agonistic Fas antibody (10 μm). Cell death was measured at day 6 post-stimulation by PI staining. Data presented are the mean ± S.D. of three independent experiments with different donors. *, $p < 0.05$. **B**, CD8 T cells were activated in the presence of either IL-12 or IL-1/6/23 for 4 days. Cells were then harvested, and those activated in IL-1/6/23 were labeled with CFSE. CD8 T cells that had been activated in IL-12 (unlabeled) or IL-1/6/23 (CFSE-labelled) were then cultured separately or co-cultured together at a 1:1 ratio. Cell death was quantified in each population after 2 days. Data represent the mean ± S.D. of four independent experiments with different donors. *, $p < 0.04$.

2.4.2. IL-1/6/23 Inhibits AICD Through Up-regulation of cFLIPs

The role of c-FLIP expression in regulating post-activation cell death of human CD8 T cells activated in IL-12 and in IL-1/6/23 was then examined by inhibiting c-FLIP expression using siRNA gene silencing. In CD8 T cells transfected with non-specific control RNA, total c-FLIP mRNA levels and c-$FLIP_S$ protein levels were higher after activation in IL-1/6/23 as compared to activation in IL-12 for five days. Transfection of CD8 T cells with c-FLIP siRNA consistently inhibited expression of this anti-apoptotic

molecule to similar levels in both cytokine conditions (Figure 8 A and B). Inhibition of c-FLIP expression increased cell death of CD8 T cells activated in both cytokine conditions. However, the amount of cell death in CD8 T cells activated in IL-1/6/23 after inhibition of c-FLIP expression, remained slightly lower than those activated in IL-12 that also received c-FLIP siRNA (Figure 8 C).

Because there is differential expression of FasL on the surface of CD8 T cells activated in IL-12 as compared to IL-1/6/23, we determined whether inhibition of c-FLIP expression normalized the intrinsic susceptibility of CD8 T cells activated in these cytokine conditions to cell death by using the co-culture system described in Figure 7 B. CD8 T cells were transfected with control or c-FLIP siRNA prior to being activated in the presence of IL-12 or IL-1/6/23 for four days. The cells that were activated in the presence of IL-1/6/23 were then CFSE-labelled and co-cultured with corresponding control or c-FLIP siRNA transfected CD8 T cells that had been activated in the presence of IL-12 and were not CFSE-labelled. Cell death in these experiments was measured after one day in co-culture, because the induction of cell death was accelerated after inhibition of c-FLIP expression; in addition, the advanced stage of cell death in cells receiving c-FLIP siRNA after 48 h in these co-cultures resulted in technical difficulties in accurately quantifying PI-positivity in the CFSE-labelled population. In co-cultures of CD8 T cells receiving control RNA, cells originally activated in IL-1/6/23 underwent significantly less cell death than those originally activated in the presence of IL-12. However, in co-cultures of CD8 T cells receiving c-FLIP siRNA, there was no difference in death of cells that had been activated in IL-1/6/23 as compared to IL-12 (Figure 8 D). Taken together, these findings indicate that the higher level of c-FLIP$_S$ expression in human CD8 T cells activated in IL-1/6/23 accounts for the intrinsic resistance of these cells to Fas-mediated AICD but that lower levels of FasL expression dictated by IL-1/6/23 can result in less induction of Fas-mediated cell death



von Rossum A et al. J. Biol. Chem. 2011;286:21137-21144

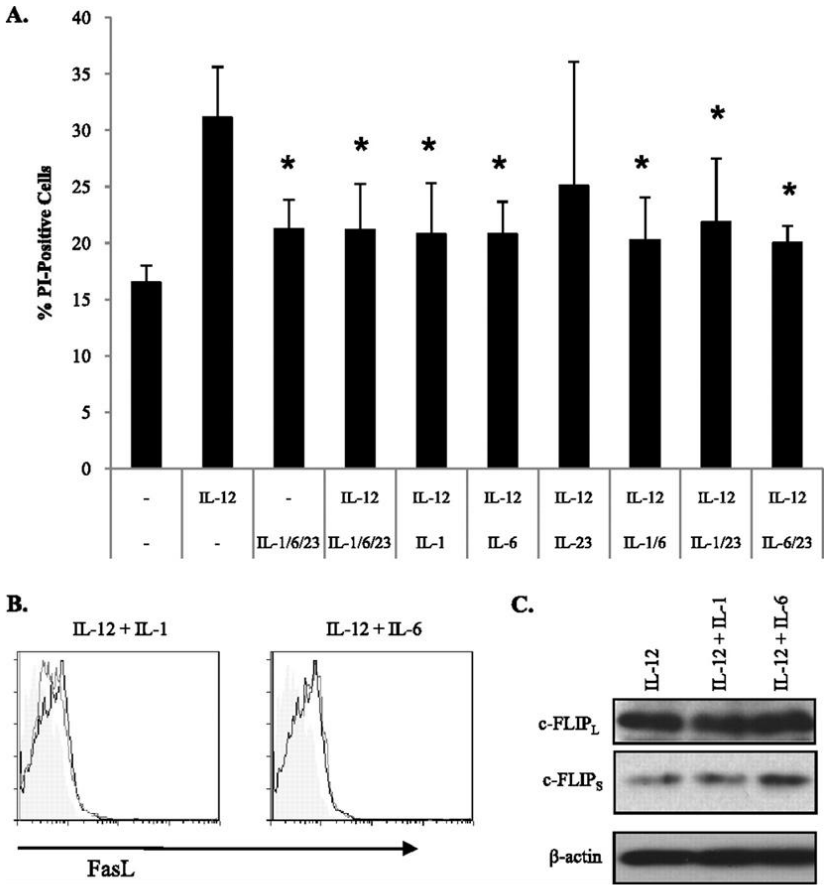
Figure 8. Increased expression of c-FLIP protects CD8 T cells activated in the presence of IL-1/6/23 from AICD

Increased expression of c-FLIP protects against CD8 T cells activated in the presence of IL-1/6/23 from AICD. **A** and **B**, human CD8 T cells were transfected with nonspecific "scrambled" control RNA or siRNA targeting c-FLIP by Amaxa nucleofection prior to stimulation in IL-12 or IL-1/6/23. **A**, RNA was isolated at day 5 after activation, and c-FLIP expression was examined by RT-qPCR. Data presented are the mean ± S.D. of triplicate measurements in one representative experiment out of three. **B**, cells were lysed at day 5 after activation, and c-FLIP expression was examined by Western blot. Representative data from one of three independent experiments are shown. **C**, after nucleofection with scrambled control or c-FLIP siRNA, CD8 T cells were activated in the presence of IL-12 or IL-1/6/23, and cell death was examined at day 6 post-stimulation. Data presented are the mean ± S.D. of three independent experiments. *, $p < 0.05$. **D**, after nucleofection with scrambled control or c-FLIP siRNA, CD8 T cells were activated in either IL-12 or IL-1/6/23 for 4 days. Cells that were transfected with control or c-FLIP siRNA and activated in IL-1/6/23 were then CFSE-labeled prior to being co-cultured with unlabeled cells that had been transfected with control or c-FLIP siRNA and activated in IL-12. Cell death in the CFSE-negative and CFSE-positive populations was quantified by PI staining after 1 day in co-culture. Data are the mean ± S.D. of three independent experiments with different donors. *, $p < 0.02$; NS = not significant, $p > 0.10$.

2.4.3. IL-1 or IL-6 Is Sufficient to Inhibit AICD, but Does So through Different Mechanisms

I then examined the specific cytokines that determine the susceptibility of human CD8 T cells to post-activation cell death. The lower level of death in CD8 T cells activated in the presence of IL-1/6/23 could result from the absence of IL-12, which appears to direct human CD8 T cells to die late after activation, or could involve active inhibition of cell death. In order to determine whether IL-1/6/23 actively inhibits post-activation cell death, we examined the effects of IL-12 plus IL-1/6/23 on human CD8 T cell death. CD8 T cells activated in the presence of IL-12 underwent more cell death than those activated in the absence of exogenous cytokines, confirming that IL-12 increases the susceptibility of human CD8 T cells to AICD. Human CD8 T cells activated in IL-12 plus IL-1/6/23 underwent significantly less cell death than those activated in IL-12 alone, and the amount of cell death was quantitatively similar to that of CD8 T cells activated in IL-1/6/23, indicating that IL-1/6/23 actively inhibits AICD (Figure 9 A).

The effects were then examined of IL-1, IL-6, or IL-23 alone, on the death of human CD8 T cells activated in the presence of IL-12. IL-1 or IL-6 alone was capable of significantly inhibiting the death of CD8 T cells that occurs in the presence of IL-12. Although the death of CD8 T cells activated in IL-12 plus IL-23 was decreased on average as compared to IL-12 alone, the difference did not reach statistical significance; this may be due to a large amount of donor variability. Combinations of any two cytokines together did not inhibit cell death to a greater extent than IL-1 or IL-6 alone (Figure 9 A). Finally, I examined the effects of IL-1 and IL-6 on FasL and c-FLIP expression. IL-1 inhibited the expression of FasL on the surface of CD8 T cells that had been activated in the presence of IL-12, but IL-6 did not (Figure 9 B). In contrast, IL-6 alone increased the expression of c-FLIPs in CD8 T cells that had been activated in the presence of IL-12, but IL-1 did not (Figure 9 C). These findings indicate that IL-1 and IL-6 attenuate Fas-mediated AICD through distinct mechanisms, namely the attenuation of FasL expression and induction of c-FLIPs expression, respectively.



von Rossum A et al. J. Biol. Chem. 2011;286:21137-21144

Figure 9. IL-1 or IL-6 alone is able to inhibit cell death of human CD8 T cells activated in IL-12.

IL-1 or IL-6 alone is able to inhibit cell death of human CD8 T cells activated in IL-12. **A**, human CD8 T cells were activated in the presence of the indicated cytokines, and cell death was quantified at day 6 post-stimulation by PI staining. Data presented is the mean ± S.D. of four experiments with different donors. *, $p < 0.05$ as compared with CD8 T cells activated in the presence of IL-12. **B** and **C**, CD8 T cells were activated in the presence of IL-12, IL-12 plus IL-1, or IL-12 plus IL-6 for 3 days. **B**, FasL expression was examined by flow cytometry. Shaded area: isotype staining control; black line: IL-12; gray line: IL-12 + IL-1 or IL-12 + IL-6. Representative data from one of three independent experiments are shown. **C**, c-FLIP expression was examined by Western blot. Representative data from one of three independent experiments are shown.

2.5. Discussion

I have shown that the presence of IL-12 during human CD8 T cell activation increases the susceptibility of resultant effector T cells to Fas-mediated AICD, whereas the presence of IL-1/6/23 attenuates the induction of this cell death pathway.

Mechanistically, this difference results from differential expression of FasL and c-FLIP$_S$. It is important to point out that even a moderate reduction in activated T cell death resulted in significant effects in overall T cell viability (Figure 4 C). This may be a consequence of the continual accumulation of cell death over time. These findings have implications for our understanding of the physiological and pathological properties of CD8 T cell responses.

Variations in cytokine conditions differentially affect the susceptibility of effector T cells to post-activation cell death. For instance, IFNα augments the susceptibility of human T cells to AICD (Kaser, Nagata et al. 1999). Lee and colleagues (Lee, Park et al. 2003) showed that IL-12 inhibits cell death of murine CD8 T cells in response to primary stimulation with anti-CD3 alone, and that this is associated with the attenuation of FasL expression by IL-12. However, IL-12 induces high levels of FasL expression in CD8 T cells that are stimulated by cognate antigen in the presence of co-stimulation (Kilinc, Rowswell-Turner et al. 2009), thereby suggesting that the effects of IL-12 in T cells may depend on the absence or presence of co-stimulatory signals. Also, IL-12 drives the differentiation of CD8 T cells into short-lived effector cells characterized by high levels of effector molecules, whereas lower levels of IL-12 during CD8 T cell activation favours the development of long-lived memory T cells (Joshi, Cui et al. 2007).

Following on this, I have observed that IL-12 increases the susceptibility of human CD8 effector T cells (generated in the presence of TCR activation and CD28-mediated co-stimulation) to AICD *in vitro,* and that there is less AICD CD8 T cells activated in IL-1/6/23. This is due to lower levels of FasL expression and higher levels of c-FLIPs in CD8 T cells activated in IL-1/6/23 as compared to those activated in IL-12. These findings also indicate that IL-1 and IL-6 act in a dominant manner through inhibition of FasL expression and induction of c-FLIPs expression, respectively, to inhibit the programming of AICD by IL-12. Interestingly, mouse CD4 T cells that are differentiated in IL-1 and IL-6 (as well as TGFβ) were recently shown to be resistant to AICD due, in part, to higher levels of c-FLIP expression. This contributes to their persistence and pathological potential in an experimental setting of ocular autoimmunity (Zhang, Xu et al. 2008; Shi, Ramaswamy et al. 2009; Yu, Iclozan et al. 2009).

In the experiments presented here, I have mostly evaluated the effects of cytokine combinations on CD8 T cell death, because T cells are likely to be exposed to multiple cytokines during inflammation. This is exemplified by the concurrent expression of IL-1 and IL-6 observed in response to ischemic damage soon after organ transplantation (Morgan, Pelletier et al. 1993). However, IL-1 or IL-6 alone was sufficient to reduce the susceptibility of IL-12-treated human CD8 T cells to AICD, indicating that both cytokines may act in an overlapping manner to control this aspect of CD8 T cell responses. Recent findings showing that cytokines act on all lymphocytes within reactive lymph nodes during active immune responses indicates that inflammatory cytokines produced by APCs are likely to affect the function of all reactive T cells (Perona-Wright, Mohrs et al.). I performed some experiments with isolated naïve and memory CD8 T cells, as determined by CD45RO expression, and determined that IL-12 and IL-1/6/23 similarly affect the susceptibility of $CD45RO^-$ and $CD45RO^+$ human CD8 T cells to post-activation cell death.

The expression of c-FLIP may dictate the pathological nature of human T cells as its expression is increased in T cells from patients with multiple sclerosis and Crohn's disease (Semra, Seidi et al. 2001; Monteleone, Monteleone et al. 2006). Further, the effects of IL-1 and IL-6 on the resistance of human CD8 T cells to Fas-mediated AICD may augment the pathological nature of this cell type in autoimmune diseases and organ transplantation (Tajima, Wakita et al. 2008; Goverman 2009). Indeed, polymorphisms in IL-1RA are associated with susceptibility to multiple sclerosis (Crusius, Pena et al. 1995) and IL-1 blockade prolongs allograft survival in mice (Shiraishi, Csete et al. 1995). IL-6 is increased in plasma of patients with multiple sclerosis and its deficiency ameliorates disease in mouse models of this disease (Frei, Fredrikson et al. 1991; Samoilova, Horton et al. 1998). While reduced susceptibility to AICD may be pathological in long-term conditions such as autoimmunity and chronic infections, it may increase the protective potential of CD8 T cells during acute infection and in response to malignancies. In mouse models, CD8 T cells activated in the presence of IL-1β, IL-6, IL-23, IL-21, and TGFβ mediate protective immunity against influenza infection and some tumours (Hamada, Garcia-Hernandez Mde et al. 2009; Hinrichs, Kaiser et al. 2009).

Inflammatory conditions are known to direct the differentiation of naïve CD4 T cells into effector cells that produce distinct cytokines, such as IFNɣ, IL-4, and IL-17. Although CD8 T cells have been reported to produce these effector cytokines, the large majority of human CD8 T cells produce IFNɣ after TCR activation, and only a very small minority can be induced to express IL-4 or IL-17 (Fong and Mosmann 1990; Vukmanovic-Stejic, Vyas et al. 2000; Kondo, Takata et al. 2009). I examined the production of IL-17 from human CD8 T cells activated in IL-1/6/23 because these cytokines facilitate the differentiation of IL-17-producing CD4 T cells (although TGFβ is also necessary in mouse T cells and perhaps in human T cells) (Acosta-Rodriguez, Napolitani et al. 2007; Wilson, Boniface et al. 2007; Manel, Unutmaz et al. 2008). Consistent with previous reports (Kondo, Takata et al. 2009), I observed IL-17 production in less than 0.5% of peripheral blood human CD8 T cells after by IL-1/6/23. It is likely that these IL-17-producing cells are derived from a small subset of $CD161^{positive}$ precursors (Cosmi, De Palma et al. 2008; Billerbeck, Kang et al.). Thus findings appear to be unrelated to the very limited differentiation of total human CD8 T cells into IL-17-expressing effectors.

In summary, I have shown that the nature of inflammatory signals received by human CD8 T cells during TCR stimulation dictates their susceptibility to AICD. Specifically, CD8 T cells activated in the presence of IL-1/6/23 undergo less Fas-mediated AICD than those activated in the presence of IL-12. This is related to lower expression of FasL and is regulated by the expression of c-FLIP. These findings provide important insight into the biology of human effector T cell responses, and have implications for understanding the regulation of CD8 effector T cells. Importantly, this study contributes to our understanding of mechanisms regulating alloimmune responses during TA. Human CD8 T cells are an important effector T cell in TA, and their expansion is regulated by a balance of activation and cell death. As such, a better understanding of how inflammatory cytokines affect the susceptibility of effector T cells to AICD, and the proteins are involved in this process, are essential for the identification of novel therapeutic targets that can result in more specific and less toxic immunosuppression.

Chapter 3. IL-6 Inhibits T Cell Death during Vascular Rejection and Contributes to Alloimmune-Mediated Vascular Injury.

3.1. Introduction

In Chapter 2, I showed that IL-6 and IL-1 inhibit AICD of human CD8 T cells in vitro by up-regulating c-FLIPs and down-regulating FasL expression, respectively. Since AICD is a regulator of alloimmune responses, I set out to study the effect of inflammatory cytokines on the development of vascular rejection and T cell death in a murine model. I focused on the role of IL-6 because this cytokine controls the intrinsic susceptibility of T cells to AICD as opposed to IL-1, which controls the expression of FasL that is likely to be presented by non-T cells in a physiological context.

In transplantation, it has been shown that increased levels of IL-6 correlate with increased severity of vascular injury (Matsumiya, Gundry et al. 1997) and with poor transplant outcome in humans (Boratynska, Klinger et al. 2001; Deng, Plenz et al. 2002; Plenz, Eschert et al. 2002). Experimentally, reports show that IL-6 promotes or augments Th1 and Th17 responses (Joseph, Miner et al. 1998; Yamamoto, Yoshizaki et al. 2000; Bettelli, Carrier et al. 2006), and that neutralizing IL-6 ablates the Th17 response and reverses co-stimulation blockade-resistant graft rejection (Burrell, Csencsits et al. 2008). Furthermore, IL-6 may inhibit induction and function of some types of regulatory T cells; Pober and colleagues showed that neutralizing human IL-6 reduced vascular rejection in a humanized mouse model of TA (Fogal, Yi et al. 2011). They further showed IL-6 blockade increases the expansion of a unique type of FOXP3-expressing Tregs that is defined by the expression of CD161. Another study found that IL-6 trans-signaling in T cells results in the activation of CD4 $CD25^-$ T cells that are resistant to the induction of FOXP3 expression; this inhibited induction of tolerance in a

mouse model of colitis (Dominitzki, Fantini et al. 2007). Mechanistically, IL-6 may alter FOXP3 function at the posttranslational level by inducing covalent modifications of FOXP3 and reducing FOXP3-chromatin binding in human CD4 $CD25^{+}$ Treg cells (Samanta, Li et al. 2008).

Both vascular EC and SMC have been shown to produce IL-6 *in vitro* (Loppnow and Libby 1989; Loppnow and Libby 1989), and Liang and colleagues showed that $IL6^{-/-}$ allografts transplanted in to wild type recipients survive approximately 3 times longer than wild type allografts. Transplanting a wild type allograft into an $IL\text{-}6^{-/-}$ recipient does not seem to offer protective effects, indicating that the graft tissue is an important source of IL-6 (Liang, Christopher et al. 2007). In this study, by using a CD25 antibody to deplete Tregs, they showed a role for Tregs in reducing rejection that occurs in the absence of IL-6. However, the direct effect of IL-6 on Treg expansion was not addressed. An additional source of IL-6 comes from donor APCs that are carried within the graft into the recipient. These APCs migrate to the recipient's draining lymph nodes and spleen, where they can allogeneically activate T cells and induce rejection.

In addition to the effects of IL-6 on Tregs, I have shown that IL-6 augments the development of effector CD8 T cell responses by preventing cell death (von Rossum, Krall et al. 2011). Despite the varied mechanisms by which IL-6 contributes to the development of effector T cell responses, the specific effects of this cytokine on Tregs and T cell death during rejection has remained poorly understood. I have studied these processes in a mouse model of alloimmune-induced vascular injury. In this model, alloimmune targeting of transplanted aortic segments triggers the development of intimal thickening, thus providing a measure of the extent of immune-mediated damage and TA.

3.1.1. Murine Aortic Interposition Model of Arterial Rejection and TA

Until the late 1980s there was no experimental model for the study of TA, which limited studies on the immunology and biochemistry of the disease. In 1991, Mennander *et al.* developed an aortic interposition model in rats in which aortic segments are grafted into complete MHC-mismatched recipients. The grafted artery segments develop histopathological and morphological lesions comparable to those observed in TA,

including the intimal thickening resulting from deposition and proliferation of SMCs. In this model, an acute rejection episode appears to be a significant initiator of TA followed by adventitial inflammation that eventually culminates in intimal hyperplasia (Mennander, Tiisala et al. 1991).

Aortic interposition grafting across a complete MHC barrier permits a comprehensive evaluation of T cell responses that drive development of TA. This includes direct CD4 and CD8 T cell recognition and targeting of foreign peptide-MHC presented by graft-derived cells, indirect CD4 (and possibly CD8) T cell recognition and targeting of graft-derived alloantigens presented by recipient APCs, and CD4 T cell-dependent antibody-mediated recognition of foreign MHC molecules on vascular cell surfaces. All of these processes are reflected in a complete MHC mismatched model, but not in other antigen mismatched models that lead to reduced immunological damage (Stevenson, Shaffer et al. 1996; Derhaag, Duijvestijn et al. 2000; Choy 2010). I have used this model to examine the mechanisms of immune-mediated vascular damage and rejection that lead to TA. However, it should be noted that there are limitations on the types of conclusions that can be drawn regarding vascular cell responses in aortic interposition models that involve a complete MHC mismatch. The intima of artery segments transplanted across a complete MHC barrier is composed of SMC derived mainly from the recipient (Johnson, Carpenter et al. 2002). A striking difference with TA observed in human transplants in which almost all intimal SMCs are of donor origin (Atkinson, Horsley et al. 2004).

3.2. Materials and Methods

3.2.1. Animals

C57Bl/6, BALB/c and BALB/c IL-$6^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Mice were bred in house by breeding an IL-$6^{+/-}$ female with an IL-$6^{-/-}$ male, and used for experimentation at 8 – 12 weeks of age. Genotyping was done on ear punches using REDExtract-N-Amp™ Tissue PCR Kit (Sigma Aldrich XNAT-100RXN) as per the manufacturer's instructions using the following primers: TTC CAT

CCA GTT GCC TTC TTG G – Common, TTC TCA TTT CCA CGA TTT CCC AG – Wild type reverse, CCG GAG AAC CTG CGT GCA ATC C – Mutant reverse (Figure 10). All the protocols used in this study were reviewed and approved by the Simon Fraser University animal ethics review board.

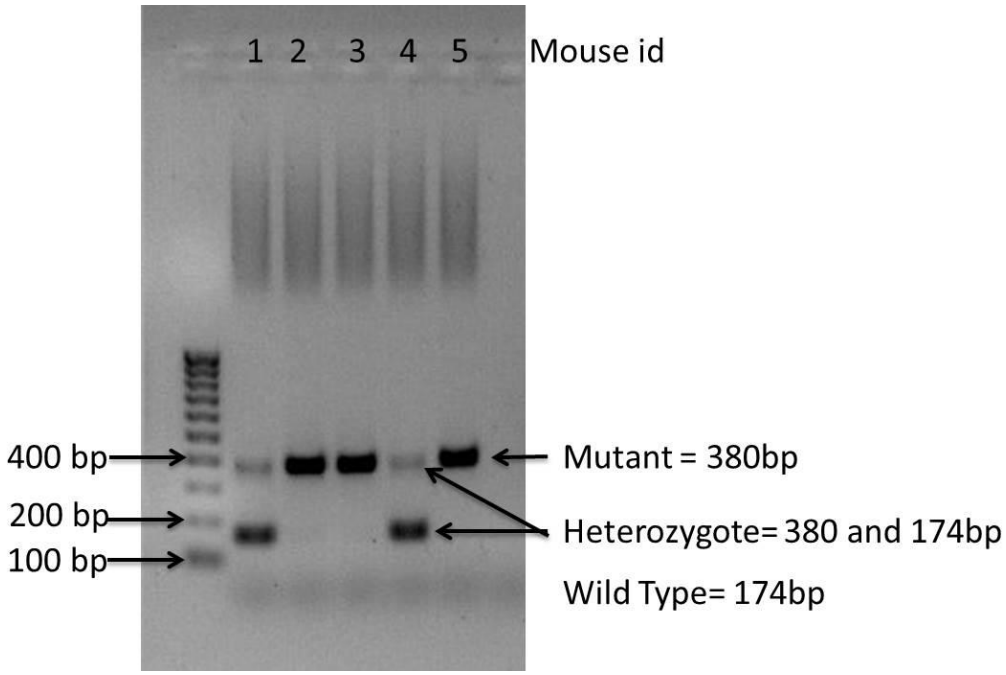


Figure 10. IL-6 genotyping gel data
Mouse DNA was obtained from ear punches and amplified using REDExtract-N-Amp™ Tissue PCR Kit as per manufacturer's instructions. PCR products were run on 0.5% agarose gel for 30 min at 100 V.

3.2.2. Cell Isolation and Cell Culture

For isolation of T cells, spleens were collected from 8-12 week old C57Bl/6 mice, and splenocytes isolated by sifting through 70 µm cell strainers. CD4 and CD8 T cells were purified from splenocytes by negative selection using CD4 and CD8 EasySep T cell Enrichment kits (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. The isolated CD4 and CD8 T cells were labeled with CFSE and resuspended in RPMI containing 10% FBS (Invitrogen, Carlsbad, CA). Macrophages were isolated as described (Hamilton, Antignano et al. 2010). Briefly, the peritoneum of BALB/c IL-$6^{+/+}$ and BALB/c IL-$6^{-/-}$ mice were lavaged 3 times with 5 mL Hank's buffered saline containing 2% FBS. The collected macrophages were resuspended in RPMI containing 10% FBS and 50,000 cells/well plated in flat-bottom 96

well plates. Cells were allowed to adhere for 3 h before washing 3x to remove non-adherent cells. Macrophage preparations were routinely greater than 95% $CD11b^+$ and $F4/80^+$.

3.2.3. Analysis of Alloantigen-induced T Cell Death *in Vitro*

CFSE-labeled CD4 and CD8 T cells were cultured with isolated allogeneic IL-$6^{+/+}$ and IL-$6^{-/-}$peritoneal macrophages at a 5:1 ratio. On Day 4, a Fas-agonist antibody (clone Jo2, BD Pharmingen) or IgG control was added. After 6 days, T cells were collected and stained with fluorescently-labeled antibodies to CD4 (BD Pharmingen,), CD8 (BD Pharmingen), and with PI (Invitrogen, Burlington, ON) prior to flow cytometric analysis on a BD FACSJazz I. Data were analyzed using FlowJo Software (TreeStar Inc).

3.2.4. Analysis of Cytokine Secretion

Supernatants from cell cultures were collected and IL-6 levels quantified using a Ready-SET-Go! cytokine ELISA kit as per the manufacturer's instructions (eBioscience, San Diego, CA).

3.2.5. Murine Aortic Interposition Grafting

Murine aortic interposition grafting was performed by Ms. Winnie Enns as described (Choy, Wang et al. 2007). Briefly, segments of abdominal aorta from Balb/c ($H2^d$) IL-$6^{+/+}$ or IL-$6^{-/-}$ donor mice were interposed into the resected infrarenal aorta of C57BL/6 ($H2^b$) mice. Arteries from C57Bl/6 donors into C57Bl/6 recipients served as syngeneic controls. Total ischemic time was less than 30 min for each surgery.

3.2.6. Morphological analysis

At day 30 post-transplantation, grafted artery segments were perfusion-fixed with 4% (v/v) paraformaldehyde, and then excised and frozen in optimum cutting temperature (OCT) medium. Ten µm sections were prepared and stained by Ms. Winnie Enns using

an elastic van Giesen protocol to visualize the elastic laminae. Percent luminal narrowing was quantified on ImageJ using the following formula:

%Luminal Narrowing = [(area within the internal elastic lamina – area within the endothelial cell layer) / area with the internal elastic lamina]x100

3.2.7. Immunohistochemistry

Cross-sections of arteries were stained by immunohistochemistry using a rabbit polyclonal antibody to CD4 (10 mg/ml; Abnova, Neihu District. Taipei City, Taiwan), a rabbit polyclonal antibody to CD8 (1:50, Abnova), a rabbit polyclonal antibody to smooth muscle cell (SMC) α-actin (1:100 Abnova), a rabbit polyclonal antibody to CD31 (1:100 Abnova), and a rat monoclonal antibody to Mac-3 (2.5ng/ul BD Pharmingen). Primary antibody incubations were followed by incubation of sections with biotin conjugated secondary antibodies followed by horseradish peroxidase-conjugated avidin (DakoCytomation, Denmark). Staining was visualized using AEC substrate-chromagen (Vector Laboratories, Burlingame, CA), which results in red staining, and sections were counterstained with hematoxylin to visualize cell nuclei. The accumulation of T cells, macrophages and dendritic cells was quantified by manual counting of positive cells, then dividing the respective values by the total number of intimal cells to calculate the % positive cells in the intima. EC lining was quantified by measuring the length of CD31 staining bordering the luminal side of the intima and then dividing that value by the total circumference of the lumen. SMC accumulation in the intima was quantified by measuring the percentage of intima area that was positive for SMC α-actin staining using *Image J*.

3.2.8. *Ex Vivo* Analysis of T Cell Proliferation, Death and FOXP3

Spleens were collected from mice on day 7 after interposition grafting of aortic segments. Splenocytes were stained with fluorescently conjugated antibodies to CD4 (clone RM4-5, BD Pharmingen), CD8 (clone 53-6.7, BD Pharmingen), and CD44 (clone IM7, BD Pharmingen) prior to being fixed and permeabilized using a BD Cytofix/Cytoperm kit (BD Pharmingen). Cells were subsequently stained with

fluorescently-conjugated antibodies to Ki67 (eBioscience), active caspase-3 (BD Pharmingen) and FOXP3 (clone MF23 BD Pharmingen) to determine cell proliferation, death and Tregs respectively. Data were acquired on a BD LSRII and analyzed using FlowJo Software. All gates defining positive and negative staining were set using isotype staining controls to normalize values in separate runs.

3.2.9. Statistical Analysis

Differences between two groups were determined using a Student's t-test. Significant differences are defined as having a $p < 0.05$.

3.3. Results

3.3.1. IL-6 Is Produced Mainly by APCs

To characterize the production of IL-6 in alloimmune reactions, CD4 and CD8 T cells were isolated from C57Bl/6 mice, and then stimulated with either IL-$6^{+/+}$ or IL-$6^{-/-}$ allogeneic macrophages. Co-culture of T cells with C57Bl/6 macrophages served as syngeneic controls. IL-6 levels were quantified by ELISA after 6 days (Figure 11 A and B). IL-6 was produced almost exclusively by the macrophages as evidenced by the near complete absence of this cytokine in cultures containing IL-$6^{-/-}$ macrophages. IL-6 production was up-regulated by alloantigen activated CD4 and CD8 T cells.

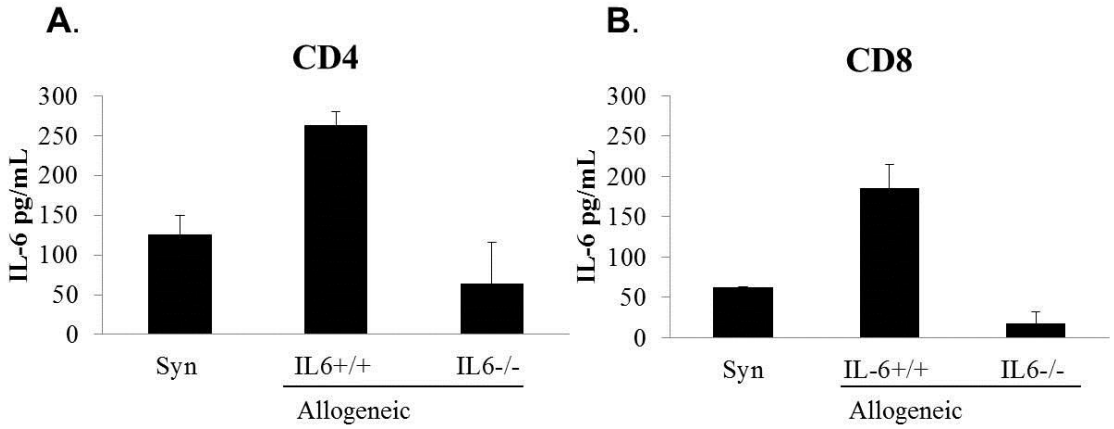


Figure 11. IL-6 is produced by antigen presenting cells

C57Bl/6 CD4 and CD8 T cells were co-cultured with BALB/c IL-$6^{+/+}$ or IL-$6^{-/-}$ macrophages. C57Bl/6 macrophages were used as syngeneic controls. Supernatants were collected at 6 days and IL-6 cytokine secretion quantified by ELISA**. A**, Representative histogram showing average ± SE of IL-6 in pg/mL in the co-culture wells containing macrophages and CD4 T cells. **B**, Representative histogram showing average ± SE of IL-6 in pg/mL in co-culture wells containing macrophages and CD8 T cells.

3.3.2. IL-6 Is Needed for Rejection of Aortic Interposition Grafts

The role of IL-6 in regulating T cell-mediated allograft vascular damage and rejection was then examined by interposition grafting of aortic segments from complete MHC-mismatched IL-$6^{-/-}$ or IL-$6^{+/+}$ Balb/c donors in to C57Bl/6 recipients. Immune-mediated vascular injury in this model initiates vascular reparative responses that culminate in luminal narrowing (Mennander, Tiisala et al. 1991; Shi, Lee et al. 1996; Johnson, Carpenter et al. 2002). The grafted arteries were harvested at day 30 post-transplantation for analysis of percent luminal narrowing, which serves as a readout for the severity of vascular injury. IL-$6^{-/-}$ transplanted artery segments had reduced luminal narrowing compared to IL-$6^{+/+}$ transplanted arteries (Figure 12 A). The effect of IL-6 on vascular cells within the grafts was also determined. EC vessel lining and the accumulation of intimal SMCs were studied by staining sections of the allograft arteries for CD31 and smooth muscle α-actin, respectively at 30 days post-transplantation. No significant difference was observed in luminal EC lining (Figure 12B) or in the proportion of SMCs among intimal cells (Figure 12 C), of IL-$6^{-/-}$ or IL-$6^{+/+}$ transplanted arteries. The effect of IL-6 on the accumulation of mononuclear cells in allograft arteries was then examined by quantifying the number of CD4 and CD8 T cells, and macrophages within

allograft arteries 30 days post-transplantation. CD4 (Figure 12 D) and CD8 (Figure 12 E) T cell accumulation was significantly reduced in IL-$6^{-/-}$ allograft arteries as compared to IL-$6^{+/+}$ allograft arteries with no difference in macrophage accumulation (Figure 12 F).

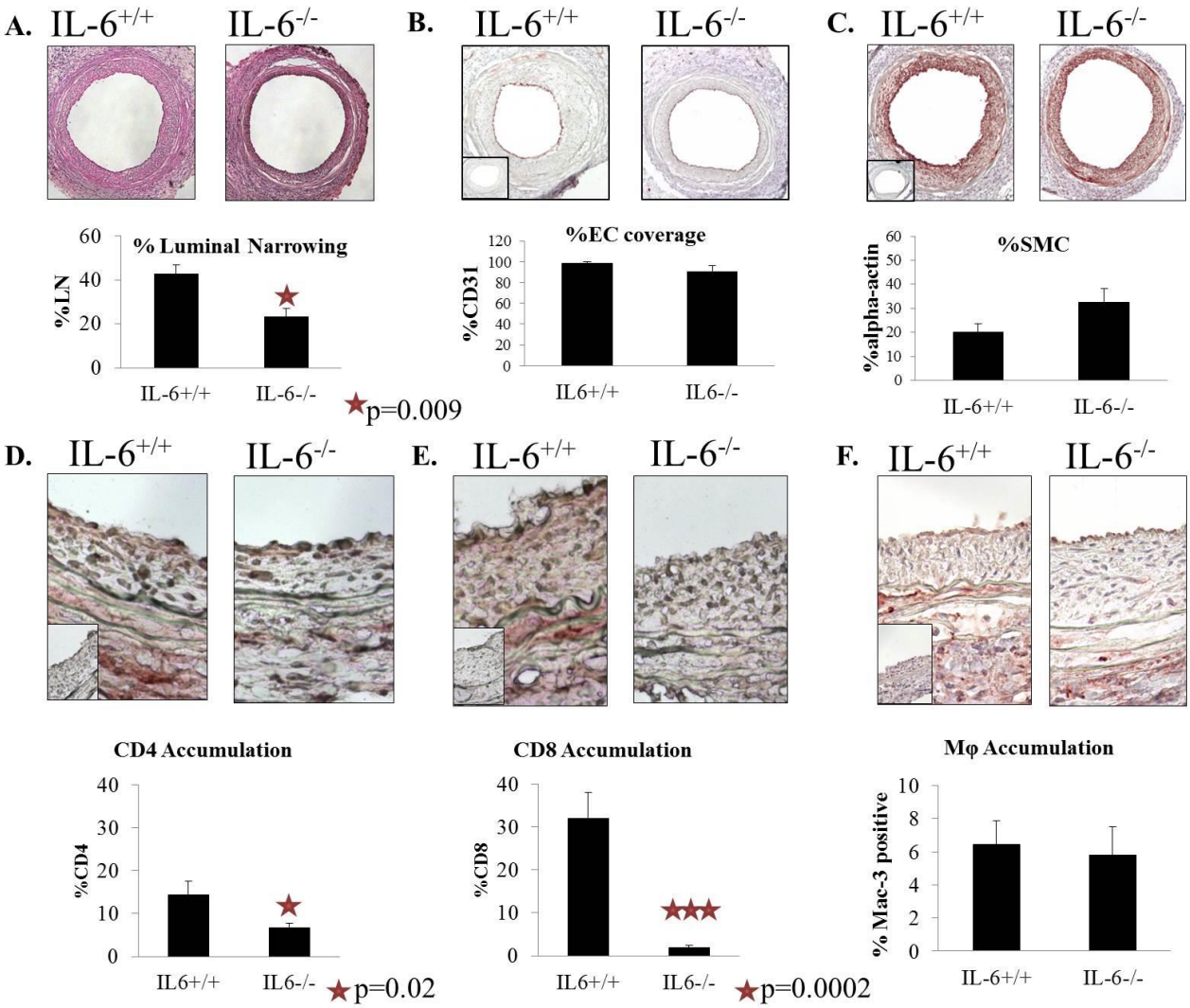


Figure 12. IL-6 augments vascular rejection and the accumulation of effector T cells in allograft arteries.

Segments of abdominal aorta from IL-$6^{+/+}$ or IL-$6^{-/-}$ BALB/c mice were interposed into the resected abdominal aorta of C57Bl/6 mice. The grafted arteries were harvested at day 30 post transplant**. A,** Representative photomicrographs of elastic van Giesen stained IL-$6^{+/+}$ and IL-$6^{-/-}$ grafted arteries. Magnification ×100. The graph depicts the %luminal narrowing in IL-$6^{+/+}$ (n=6) and IL-$6^{-/-}$ (n=4) allograft arteries.p=0.009. **B** & **C**, Representative photomicrographs of IL-$6^{+/+}$ and IL-$6^{-/-}$ allograft arteries immunohistochemically stained for **B**, CD31 and **C**, smooth muscle α-actin. Quantification of **B**, CD31 positivity and **C,** smooth muscle α-actin positivity was performed. **F**, Representative photomicrographs of IL-$6^{+/+}$ and IL-$6^{-/-}$ allograft arteries immunohistochemically stained for **D**, CD4, **E**, CD8, and **F**, Mac-3. The percentage +/- SE of $CD4^{positive}$, $CD8^{positive}$, and Mac-$3^{positive}$ cells within the intima of allograft arteries was quantified by manual counting.*p= 0.02, ****p= 0.0002

3.3.3. IL-6 Prevents T Cell Death *In vivo,* but Has No Apparent Effect on T Cell Proliferation or FOXP3 Expression

Because the absence of IL-6 expression reduced luminal narrowing and attenuated the accumulation of CD4 and CD8 effector T cells in allograft arteries, T cell proliferation and death in response to allograft arteries was examined. Segments of IL-$6^{+/+}$ and IL-$6^{-/-}$ abdominal aortas were transplanted into recipient mice, and splenocytes isolated at day 7 post-transplant. Flow cytometric analysis was performed for CD4, CD8, CD44, Ki67, active caspase-3, and FOXP3 to quantify CD4 and CD8 T cell activation, proliferation, death, and Tregs, respectively. There was a substantial and significant increase in cell death of both CD4 and CD8 T cells in mice that received IL-$6^{-/-}$ allograft arteries as compared to mice that received wild type arteries (Figure 13 A and B). However, there was no significant difference in the quantity of Ki67-positive CD4 and CD8 T cells in response to IL-$6^{-/-}$ allograft arteries as compared to in response to IL-$6^{+/+}$ ones, although there was a trend toward a reduction (Figure 13 B and C). Finally, no difference in the frequency of FOXP3 positive T cells was observed in the spleens of mice that received IL-$6^{-/-}$ allograft arteries as compared to those that received IL-$6^{+/+}$ ones, suggesting that IL-6 does not affect expansion or differentiation of Tregs (Figure 13 E).

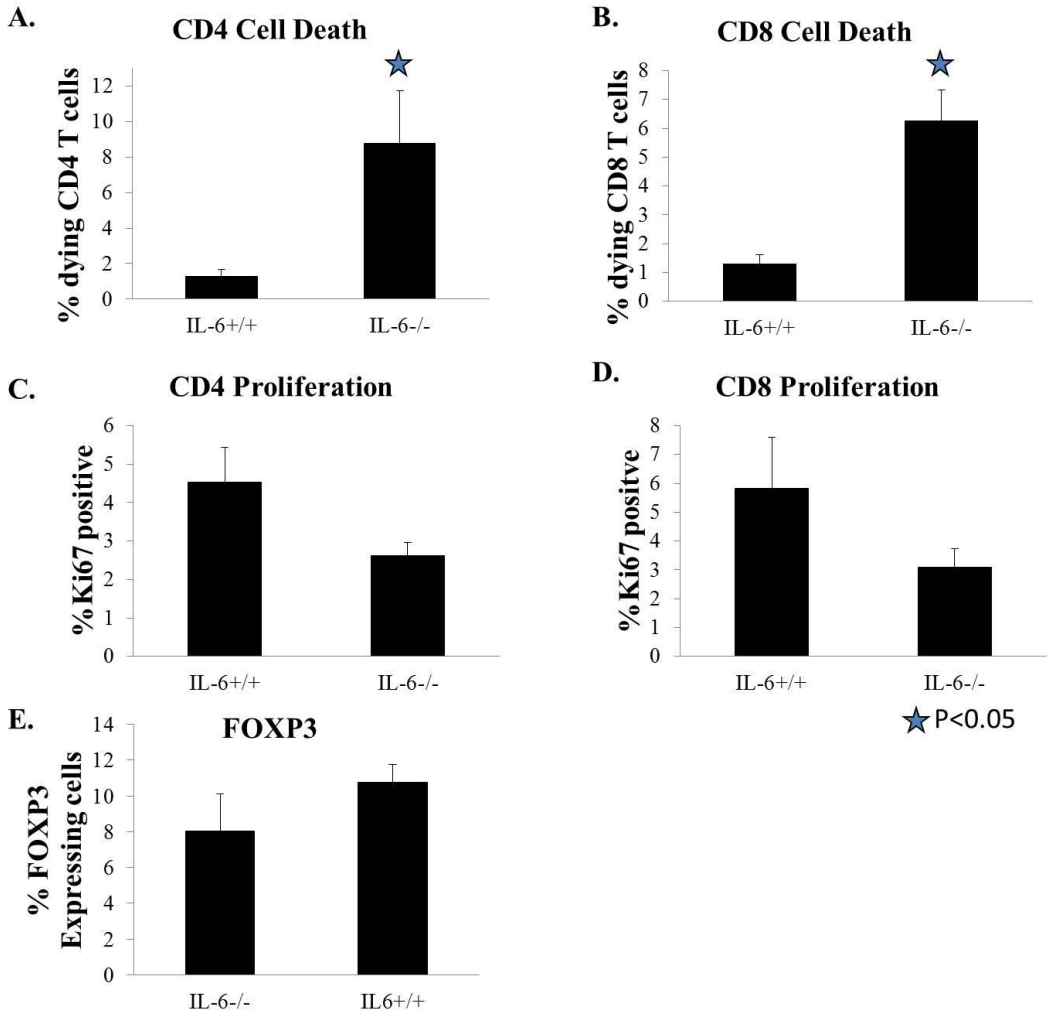


Figure 13. IL-6 Prevents T Cell Death but does Not Affect Tregs

Segments of abdominal aorta from either an IL-$6^{+/+}$ or an IL-$6^{-/-}$ BALB/c mouse were interposed into the resected abdominal aortas of a C57Bl/6 mouse. Syngrafts (syn) were performed as controls. Splenocytes were isolated at day 7 post transplantation and examined by flow cytometry**. A** & **B**, Average ± SE of the percentage of **A**, $CD4^{+}CD44^{+}$ and **B**, $CD8^{+}CD44^{+}$ effector T cells that are active caspase-3 positive in IL-$6^{+/+}$ (n=4) and IL-$6^{-/-}$ (n=4) allograft artery recipients. **p* <0.05. **C** & **D**, Average ± SE of the percentage of **C**, CD4 and **D**, CD8 T cells that are Ki67 positive in IL-$6^{+/+}$ (n=7) or IL-$6^{-/-}$ (n=5) allograft artery recipients *p*=NS.**E**, Average ± SE of the percentage of $CD4^{+}CD44^{+}$ effector T cells that are FOXP3 positive in IL-$6^{+/+}$ (n=3) and IL-$6^{-/-}$ (n=3) artery recipients. p=NS.

3.3.4. IL-6 Inhibits Fas-Mediated T Cell Death After Alloantigen Stimulation *In Vitro*

Because the lack of IL-6 increased the frequency of activated T cells undergoing cell death after artery transplantation, the effect of IL-6 was studied on Fas-mediated T

cell death after alloantigen stimulation *in vitro*. Isolated CD4 and CD8 T cells were CFSE labelled and stimulated with allogeneic macrophages, and T cell death was examined after 6 days by staining cells for CD4 and CD8 plus PI, and then quantifying the frequency of $CFSE_{low}$ cells (*i.e.,* those that had been activated by alloantigen stimulation and proliferated) that were PI positive. To study Fas-mediated T cell death, a Fas-agonistic antibody was added to the cultures at day 4. Stimulation of T cells with this antibody did not increase death of T cells that had been stimulated with IL-$6^{+/+}$ macrophages, indicating that CD4 and CD8 T cells activated by IL-$6^{+/+}$ allogeneic macrophages were resistant to Fas-induced cell death. However, there was a significant increase in Fas-induced cell death of CD4 and CD8 T cells after activation with IL-$6^{-/-}$ allogeneic macrophages (Figure 14). This suggests that the production of IL-6 by APCs reduces the susceptibility of alloantigen-induced effector CD4 and CD8 T cells to Fas-induced death.

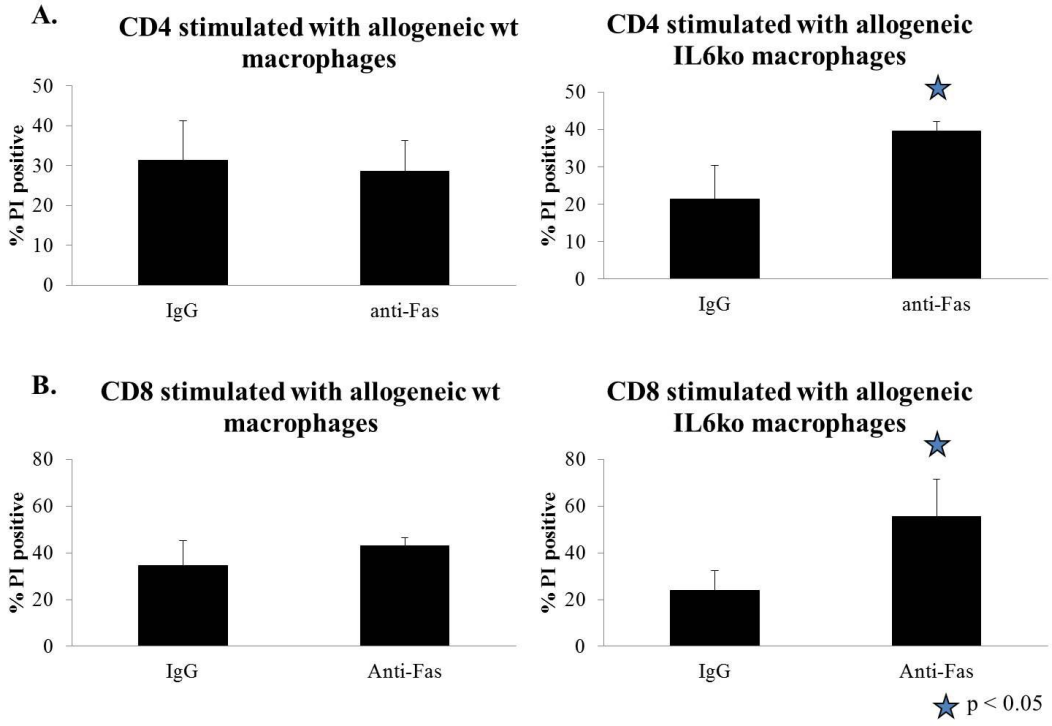


Figure 14. IL-6 protects against Fas-mediated cell death of alloantigen-stimulated T cells *in vitro.*

CFSE labelled C57Bl/6 **A**, CD4 and **B**, CD8 T cells from C57Bl6 mice were co-cultured with BALB/c IL-$6^{+/+}$ or IL-$6^{-/-}$ macrophages. A Fas-agonistic antibody or IgG isotype control was added on day 4, and cell death measured on day 6 in the $CFSE^{low}$ populations by PI exclusion. $p < 0.05$.

3.4. Discussion

In these experiments, IL-6 deficient allografts developed less severe vascular injury than wild type allografts, indicating that graft derived IL-6 is an essential component of the initiation of alloimmunity. Also, the reduction in luminal narrowing observed in the IL-$6^{-/-}$ arteries was accompanied by a significant reduction in CD4 T and CD8 T cell accumulation in allograft arteries. This reduction in T cell accumulation is likely to result from increased T cell death since there was a substantial increase in dying T cells in response to grafts that lacked IL-6. The findings here support the findings in Chapter 2 where I showed that human CD8 T cells activated in the presence of IL-6 are resistant to activation induced cell death.

IL-6 augments T cell responses by preventing expansion/differentiation of Tregs, reducing the susceptibility of effector T cells to Treg-mediated suppression, and/or by inhibiting AICD. Although several studies have found that the presence of IL-6 renders some T cells resistant to FOXP3 induction and that activating T cells in the absence of IL-6 induces immunosuppressive T cells, these studied focused on specific subtypes such as $CD161^{+}$ CD4 T cells (Dominitzki, Fantini et al. 2007; Fogal, Yi et al. 2011). However, no difference in the frequency of splenic Tregs was observed in response to IL-$6^{+/+}$ arteries compared to IL-$6^{-/-}$ ones, suggesting Treg induction may not be the main mechanism by which IL-6 acts in vascular rejection. However, although the spleen is a main site of alloantigen presentation, the accumulation of Tregs in artery grafts should be assessed in future studies. An additional drawback in this study was the inability to directly measure the sensitivity of alloreactive effector T cells to Treg-mediated suppression. Nevertheless, the combined demonstration of *(i)* a substantial increase in effector T cell death in the absence of graft-derived IL-6 *in vivo,* and *(ii)* an increased susceptibility of alloantigen-induced effector T cells to Fas-mediated cell death *in vitro,* suggests a pathogenic role for IL-6 in mediating T cell survival.

To my knowledge, this is the first time that graft-derived IL-6 deficiency has been shown to reduce severity of vascular injury in relation to a reduced susceptibility of activated T cells to AICD. In addition to its effects on cells of the immune system, it is also possible that IL-6 contributes to luminal narrowing through a direct effect on

vascular cells. IL-6 affects the migration of SMCs (Wang, Castresana et al. 2001; Wang, Liu et al. 2007) and can stimulate the proliferation of SMCs in both PDGF-dependent as well as PDGF-independent manners (Nabata, Morimoto et al. 1990; Ikeda, Ikeda et al. 1991; Klouche, Rose-John et al. 2000). It is therefore possible that the observed reduction in % luminal narrowing is not entirely caused by a decrease in effector T cell activity owing to increased cell death, but rather by a lack of mitogen production from the graft itself. Based on this, I anticipated, yet did not observe, a difference in SMC accumulation in the intima of grafts that lack IL-6 or any effects on the integrity of the endothelium. Thus, the activation status of the endothelium should be studied in future work.

IL-6 is likely to affect vascular rejection through its actions on CD4 T cells as it has been shown that intimal thickening in complete MHC-mismatched aortic interposition grafts depends mainly on this T cell subset (Shi, Lee et al. 1996). I have evaluated the effect of IL-6 on alloantigen-driven immune responses and associated allograft vascular injury using aortic interposition grafting across a complete MHC barrier. This is an antigen mismatch situation reflects almost all heart transplants and permits a comprehensive evaluation of T cell responses that are known to be involved in organ transplant rejection (Choy 2010). However, immune-mediated arterial damage is severe in this model, and results in the rapid population of the intima by SMCs derived from circulating recipient stem cells (Johnson, Carpenter et al. 2002). This feature of the murine model differs from clinical observations in that almost all of the intimal SMCs are of donor origin in humans (Minami, Laflamme et al. 2005). As such, the analysis presented here contributes to the basic immune mechanisms behind allograft vascular injury and rejection, but the specific vascular cell responses that contribute to intimal thickening in our model must be distinct from those driving similar changes in the clinical setting. (Stevenson, Shaffer et al. 1996; Choy 2010)

The results of this study have clinical implications, because IL-6 targeting therapeutics are currently used in the clinic for other inflammatory diseases. The anti-IL-6R monoclonal antibody tociluzimab, is used in some patients with rheumatoid arthritis (Navarro-Millan, Singh et al. 2012), and is also approved for the treatment of Castleman's disease, and both polyarticular and systemic juvenile idiopathic arthritides

(O'Shea, Kanno et al. 2014). Further, a small study was conducted in eight patients and showed that tociluzimab may be effective in treating corticosteroid-refractory graft-*versus*-host disease, but several of the patients developed infections (Drobyski, Pasquini et al. 2011). Currently, a Phase II trial is underway studying the effects of tociluzimab in patients with glucocorticoid-refractory acute graft-*versus*-host disease (NLM identifier NCT01757197), as well as a Phase I/II clinical trial investigating the effectiveness of tociluzimab in kidney transplantation (NLM identifier NCT01594424). In preliminary work, Tociluzimab has been shown to be effective in the treatment of giant cell arteritis, an arterial disease with many similarities to TA (Unizony, Arias-Urdaneta et al. 2012), and is currently in Phase III clinical trials for this disease (NLM identifier NCT01791153). Additionally, sirukumab, a human anti-IL-6 monoclonal antibody, and sarilumab, a human anti-IL-6-receptor monoclonal antibody, are currently in Phase III trials for rheumatoid arthritis, (NLM identifiers NCT01606761 and NCT01061736 respectively) (O'Shea, Kanno et al. 2014)

In summary, I have shown that IL-6 is an important regulator of activated T cell death and is involved in the development of TA. This reveals an important role for graft derived IL-6 in TA and suggests IL-6 as a potential therapeutic target for the prevention of acute and chronic rejection.

Chapter 4. Regulation of Alloimmune Responses by the Cell Death Regulatory Protein Bim

4.1. Preface

The content of this chapter has been published. von Rossum,A. Enns, W. Shi, Y.P. MacEwan, G.E. Malekesmaeli, M. Brinkman, R. Choy, J.C. Bim Regulates Alloimmune-Mediated Vascular Injury Through Effects on T-Cell Activation and Death. Arterioscler *Thromb Vasc Biol. 2014*. 34:1290-97 Copyright 2014 American Heart Association, Inc. Some of the experiments in this chapter were conducted with the technical assistance of honours undergraduate student Patrick Shi and undergraduate summer student Grace E. MacEwan. All surgeries were performed by Ms. Winnie Enns, and all animal work was done according to the guidelines of the Canadian Council on Animal Care.

4.2. Introduction

Chapters 2 and 3 above describe my study of the extrinsic signals that control the susceptibility of T cells to post-activation cell death, with the majority of work focusing on IL-6. As detailed in Chapter 1, T cell intrinsic processes are also important in defining the outcome of pathological T cell responses. Thus, in this chapter, I describe the role of the intrinsic cell death pathway that induces CDICD in T cells, in the development of vascular rejection and TA.

Bcl-2 proteins are known to control T cell death after transplantation. By using mice in which T cells overexpress the anti-apoptotic Bcl-2 protein Bcl-xL, Wells *et al*. (Wells, Li et al. 1999) showed that Bcl-xL increases T cell persistence after heart transplantation, and by virtue of this, augments acute and chronic heart transplant rejection. Bim is a pro-apoptotic BH3-only member of the Bcl-2 family, and is a pivotal

initiator of T cell death triggered by cytokine deprivation (Bouillet, Metcalf et al. 1999). Bim antagonizes the survival actions of Bcl-2 and Bcl-xL, thereby triggering mitochondrial permeabilization and resultant cell death (Hildeman, Zhu et al. 2002). The importance of Bim-mediated T cell death in immune homeostasis is highlighted by the development of autoimmune-like manifestations in mice that lack this protein (Bouillet, Metcalf et al. 1999). Paradoxically, in addition to regulating T cell death, recent evidence indicates that Bim is also required for optimal T cell activation in autoimmune encephalomyelitis, diabetes, and graft-*versus*-host disease (Ludwinski, Sun et al. 2009; Yu, Yu et al. 2012).

Using a combination of *in vitro* experiments and a complete MHC-mismatch model of aortic interposition grafting, I show in this chapter that Bim is required for the optimal activation of both CD4 and CD8 T cells in response to allogeneic stimulation. This unexpected role of Bim is independent of its effect in driving T cell death. I further identify a quantitative difference in the reliance of T cells on Bim for activation, as opposed to cell death, with a partial reduction in Bim expression preventing T cell proliferation but not death, and complete elimination of Bim expression preventing both proliferation and death. Aortic interposition grafting of artery segments into $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ recipients showed that reduction of Bim expression (in $Bim^{+/-}$ mice), but not complete elimination (in $Bim^{-/-}$ mice), reduces immune-mediated vascular injury and rejection. This coincided with attenuated T cell proliferation, but not cell death, in $Bim^{+/-}$ graft recipients. Both T cell proliferation and death were attenuated in $Bim^{-/-}$ graft recipients, likely resulting in offsetting effects on immune activation and inactivation in this setting. Altogether, these findings provide important insight into the control of allogeneic T cell responses, and show that the effect of Bim on alloantigen-induced T cell responses is complex and regulated by its opposing effects on T cell proliferation and death. This has implications for understanding alloimmune-mediated vascular damage that contributes to organ transplant failure.

4.3. Materials and Methods

4.3.1. Animals

C57Bl/6 $Bim^{+/+}$, C57Bl/6 $Bim^{+/-}$, C57Bl/6 $Bim^{-/-}$, and BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, Maine), bred continually in-house, and used for experimentation at 8 – 12 weeks of age. All mice were maintained on identical diets in the same room. The breeding was performed as suggested by Jackson Laboratories. Specifically, female $Bim^{+/-}$ mice were bred with male $Bim^{-/-}$ mice to generate $Bim^{+/-}$ and $Bim^{-/-}$ offspring, and these littermates were used for experimentation. Genotyping was performed using REDExtract-N-Amp™ Tissue PCR Kit (Sigma Aldrich) as per the manufacturer's instructions using the following primers: CAT TCT CGT AAG TCC GAG TCT – Common, GTG CTA ACT GAA ACC AGA TTA G– Wild type reverse, CTC AGT CCA TTC ATC AAC AG – mutant reverse (Figure 15). All the protocols used in this study were reviewed and approved by the Simon Fraser University animal ethics review board.

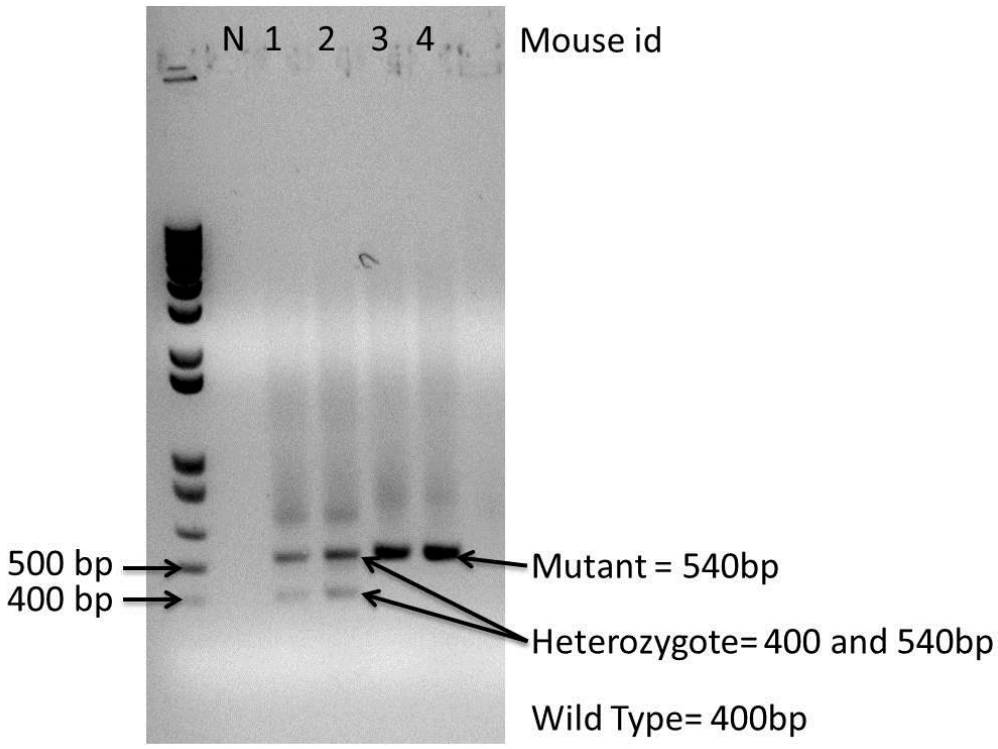


Figure 15. Bim genotyping

Mouse DNA was obtained from ear punches and amplified using REDExtract-N-Amp™ Tissue PCR Kit as per manufacturer's instructions. PCR products were run on 0.5% agarose gel for 30 min at 100 V.

4.3.2. Cell Isolation and Cell Culture

For isolation of T cells, spleens were collected from 8-12 week old $Bim^{+/+}$, $Bim^{+/-}$ and $Bim^{-/-}$ mice, and splenocytes isolated by sifting through 50 μm cell strainers. CD4 and CD8 T cells were purified from splenocytes by negative selection using CD4 and CD8 EasySep T cell Enrichment kits (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. The isolated CD4 and CD8 T cells were resuspended and cultured in RPMI containing 10% FBS (Invitrogen, Carlsbad, CA).

Macrophages were isolated as described (Hamilton, Antignano et al. 2010). Briefly, the peritoneum of BALB/c or C57Bl/6 mice were lavaged 3 times with 5 mL of Hank's buffered saline containing 2% FBS. The collected macrophages were resuspended in RPMI containing 10% FBS and 50,000 cells/well plated in flat bottom 96 well plates. Cells were allowed to adhere for 3 h before washing 3x to remove non-

adherent cells. Macrophage preparations were routinely greater than 95% CD11b and F4/80-positive.

4.3.3. Analysis of Alloantigen-induced T Cell Proliferation and Death *in Vitro*

T cells were labelled with CFSE as described previously (Choy, Wang et al. 2007; von Rossum, Krall et al. 2011). 2 x 10^5 CFSE-labeled CD4 and CD8 T cells were cultured with isolated macrophages. After 6 days, T cells were collected and stained with fluorescently-labeled antibodies to CD4 (clone RM4-5, BD Pharmingen, Franklin Lakes, NJ), CD8 (clone 53-67, BD Pharmingen), and with PI (Invitrogen, Burlington, ON) prior to flow cytometric analysis on a BD Aria. Data was analyzed using FlowJo Software (TreeStar Inc).

4.3.4. Analysis of Cytokine Deprivation-induced Cell Death

For examination of CDICD of freshly isolated T cells, CD4 and CD8 T cells from $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ mice were cultured in RPMI containing 10% FBS (1x10^6 cells/mL) in the absence of stimulation or growth factors. Cell survival was then measured by quantifying PI exclusion at 24, 48, and 72 h.

For examination of cytokine deprivation-induced cell death of activated T cells, isolated CD4 and CD8 T cells from $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ mice, were stimulated with plate-bound anti-CD3 (2.5 µg, eBioscience) and soluble anti-CD28 (2 µg/mL, eBioscience). T cells were removed from stimulation after 72 h, washed extensively, and cultured in the absence of stimulation or growth factors and in the presence of an IL-2 neutralizing antibody (10 µg/mL, eBioscience). Cell survival was analyzed by quantifying PI exclusion. All flow cytometric data was acquired on a BD FACS Aria or a BD FACSJazz. Data was analyzed using FlowJo Software (TreeStar Inc).

4.3.5. Analysis of Cytokine Secretion

Supernatants from cell cultures were collected and IL-2 levels quantified using a cytokine ELISA kit as per the manufacturer's instructions (eBioscience, San Diego, CA).

4.3.6. Murine Aortic Interposition Grafting

Murine aortic interposition grafting was performed by Ms. Winnie Enns as described (Choy, Wang et al. 2007). Segments of abdominal aorta from BALB/c donor mice were interposed into the resected infrarenal aorta of C57Bl/6 $Bim^{+/+}$, $Bim^{+/-}$, or $Bim^{-/-}$ mice. Arteries from C57Bl/6 donors into C57Bl/6 recipients served as syngeneic controls. Total ischemic time was less than 30 min for each surgery.

4.3.7. Morphological Analysis

At day 30 post-transplantation, grafted artery segments were perfusion-fixed with 4% paraformaldehyde and then excised and frozen in optimum cutting temperature (OCT) medium. Ten μm sections were prepared and stained using an elastic van Giesen protocol by Ms. Winnie Enns to visualize the elastic laminae. Intimal thickening was quantified as described (Choy, Kerjner et al. 2004; Choy, Cruz et al. 2005).

4.3.8. Immunohistochemistry

Cross-sections of arteries were stained by immunohistochemistry using a rabbit polyclonal antibody to CD4 (10 mg/ml; Abnova, Neihu District, Taipei City, Taiwan), a rabbit polyclonal antibody to CD8 (1:50, Abnova), a rabbit polyclonal antibody to smooth muscle (SM) α-actin (1:100, Abnova), a rabbit polyclonal antibody to CD31 (1:100, Abnova), and a rat monoclonal antibody to Mac-3 (1:50, BD Pharmingen). Primary antibody incubations were followed by incubation of sections with biotin conjugated secondary antibodies followed by horseradish peroxidase-conjugated avidin (DakoCytomation, Denmark). Staining was visualized using AEC substrate-chromagen (Vector Laboratories, Burlingame, CA), which results in red staining, and sections were counterstained with hematoxylin to visualize cell nuclei.

The accumulation of T cells, macrophages and dendritic cells was quantified by manual counting of positive cells and then dividing the respective values by the total number of intimal cells (as determined by counting of nuclei in the intima) to calculate the % positive cells in the intima. EC lining was quantified by measuring the length of CD31 staining bordering the luminal side of the intima and then dividing that value by the

total luminal circumference. SMC accumulation in the intima was quantified by measuring the percentage of intima area that was positive for SM α-actin staining using *Image J*.

4.3.9. *Ex Vivo* Analysis of T Cell Proliferation and Death

Spleens were collected from mice on day 7 after interposition grafting of aortic segments. Splenocytes were stained with fluorescently conjugated antibodies to CD4 (BD Pharmingen), CD8 (BD Pharmingen), and CD44 (clone IM7, BD Pharmingen) prior to being fixed and permeabilized using a BD Cytofix/Cytoperm kit (BD Pharmingen). Cells were subsequently stained with fluorescently-labeled antibodies to Ki67 (eBioscience) and active caspase-3 (BD Pharmingen) to determine cell proliferation and death, respectively. Data were acquired on a BD FACS Aria and analyzed using FlowJo. All gates defining positive and negative staining were set using isotype control staining controls.

4.3.10. Statistical Analysis

Differences between two groups were determined using a Student's t-test and between multiple groups using an ANOVA followed by a Tukey's post-hoc test. Significant differences were defined as having a $p \leq 0.05$.

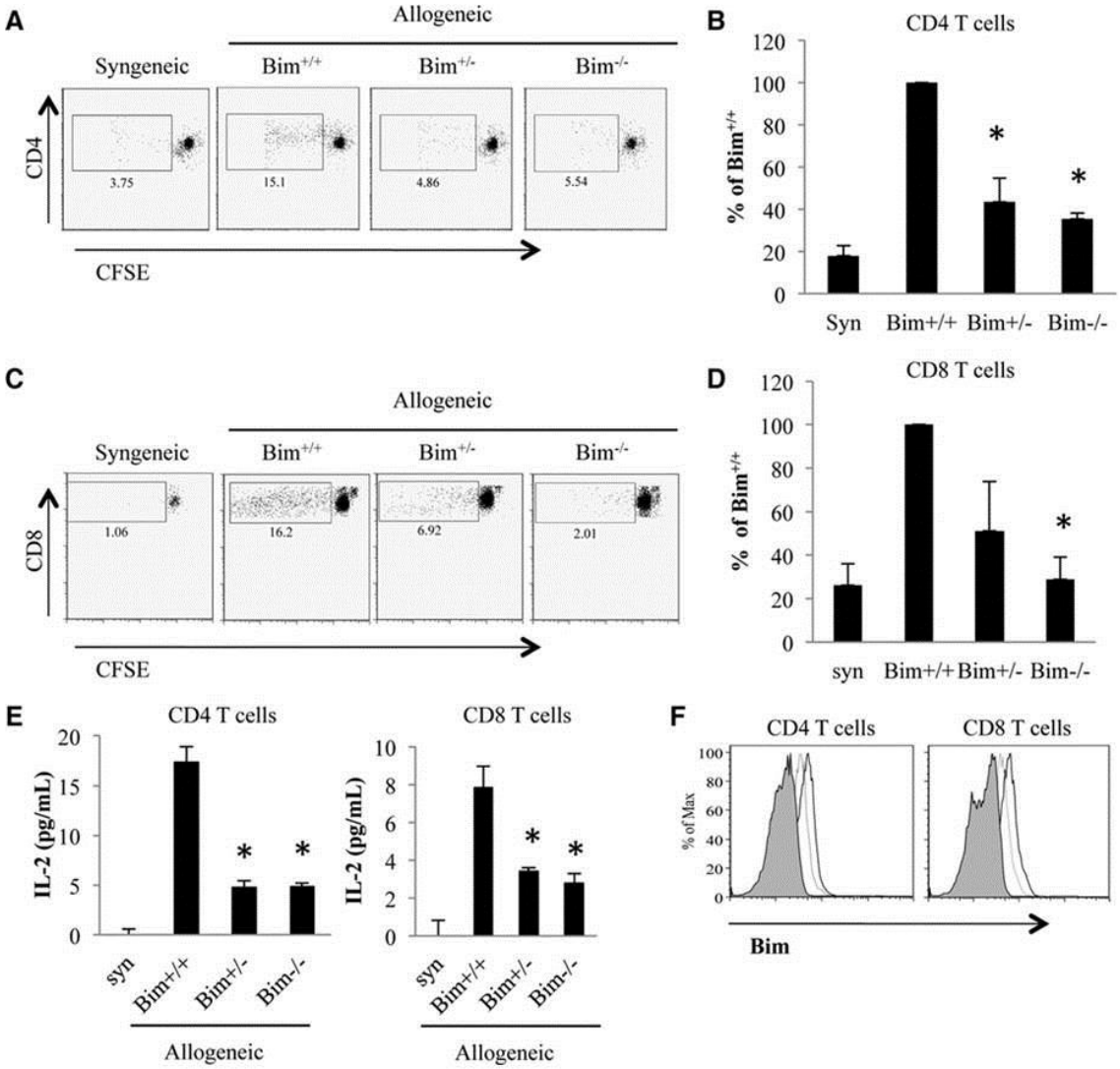
4.4. Results

4.4.1. Bim Is Required for Alloantigen-Induced Activation of T Cells

To begin examining the role of Bim in controlling the alloantigen-induced activation of T cells, CD4 and CD8 T cells were isolated from $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ mice, labelled with CFSE, and then stimulated with allogeneic macrophages. Proliferation was examined by measuring CFSE dilution after 6 days. There was no proliferation in syngeneic controls, but there was robust proliferation of $Bim^{+/+}$ T cells in response to alloantigen stimulation. Both $Bim^{+/-}$ and $Bim^{-/-}$ CD4 T cell proliferation was significantly reduced compared to $Bim^{+/+}$ CD4 T cells, and the defect in proliferation was

comparable between $Bim^{+/-}$ and $Bim^{-/-}$ CD4 T cells (Figure 16 A and B). When alloantigen-induced proliferation of CD8 T cells was examined, proliferation of $Bim^{-/-}$ CD8 T cells was significantly reduced as compared to their $Bim^{+/+}$ counterparts. There also appeared to be reduced proliferation of $Bim^{+/-}$ CD8 T cells compared to $Bim^{+/+}$ cells, but this outcome did not reach statistical significance (Figure 16 C and D, $p = 0.15$).

IL-2 secretion from CD4 and CD8 T cells was then evaluated. IL-2 was absent in T cells cultured with syngeneic macrophages, yet there was substantial IL-2 secretion by $Bim^{+/+}$ T cells in response to allogeneic stimulation. There was a significant reduction in IL-2 production by both CD4 and CD8 T cells isolated from $Bim^{+/-}$ and $Bim^{-/-}$ mice in response to allogeneic stimulation as compared to cells isolated from $Bim^{+/+}$ mice (Figure 16 E). Thus, this Bcl-2 protein is required for the optimal activation of CD4 and CD8 T cells. Moreover, profiling of Bim expression by isolated T cells from $Bim^{+/-}$ mice revealed expression levels at ~50% that of $Bim^{+/+}$ T cells (Figure 16 F), indicating that a partial reduction in Bim expression is sufficient to attenuate alloantigen-induced T cell activation.



von Rossum A et al. Arterioscler Thromb Vasc Biol. 2014;34:1290-1297

Figure 16. Bim is required for alloantigen-driven activation of T cells

CD4 and CD8 T cells from C57Bl/6 $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ mice were labelled with CFSE and cultured with peritoneal macrophages from BALB/c mice. T cells cultured with macrophages from C57Bl/6 $Bim^{+/+}$ mice served as syngeneic controls. **A**, Representative dot plots showing proliferation of CD4 T cells in response to allogeneic macrophages. **B**, Mean ± SE of relative CD4 T cell proliferation for a series of 3 separate experiments. *p<0.05 compared with $Bim^{+/+}$. **C**, Representative dot plots showing CD8 T cell proliferation in response to allogeneic macrophages. **D**, Mean ± SE of relative CD8 T cell proliferation for a series of 3 separate experiments. *p<0.05 compared with $Bim^{+/+}$. **E**, Supernatants from allogeneic co-cultures were harvested at 24 hours post stimulation and IL-2 levels measured by ELISA. *p<0.01 as compared with $Bim^{+/+}$ cells. **F**, Representative histograms of Bim expression in CD4 and CD8 T cells isolated from $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ mice. Black line, $Bim^{+/+}$ cells; gray line, $Bim^{+/-}$ cells; and shaded area, $Bim^{-/-}$ cells.

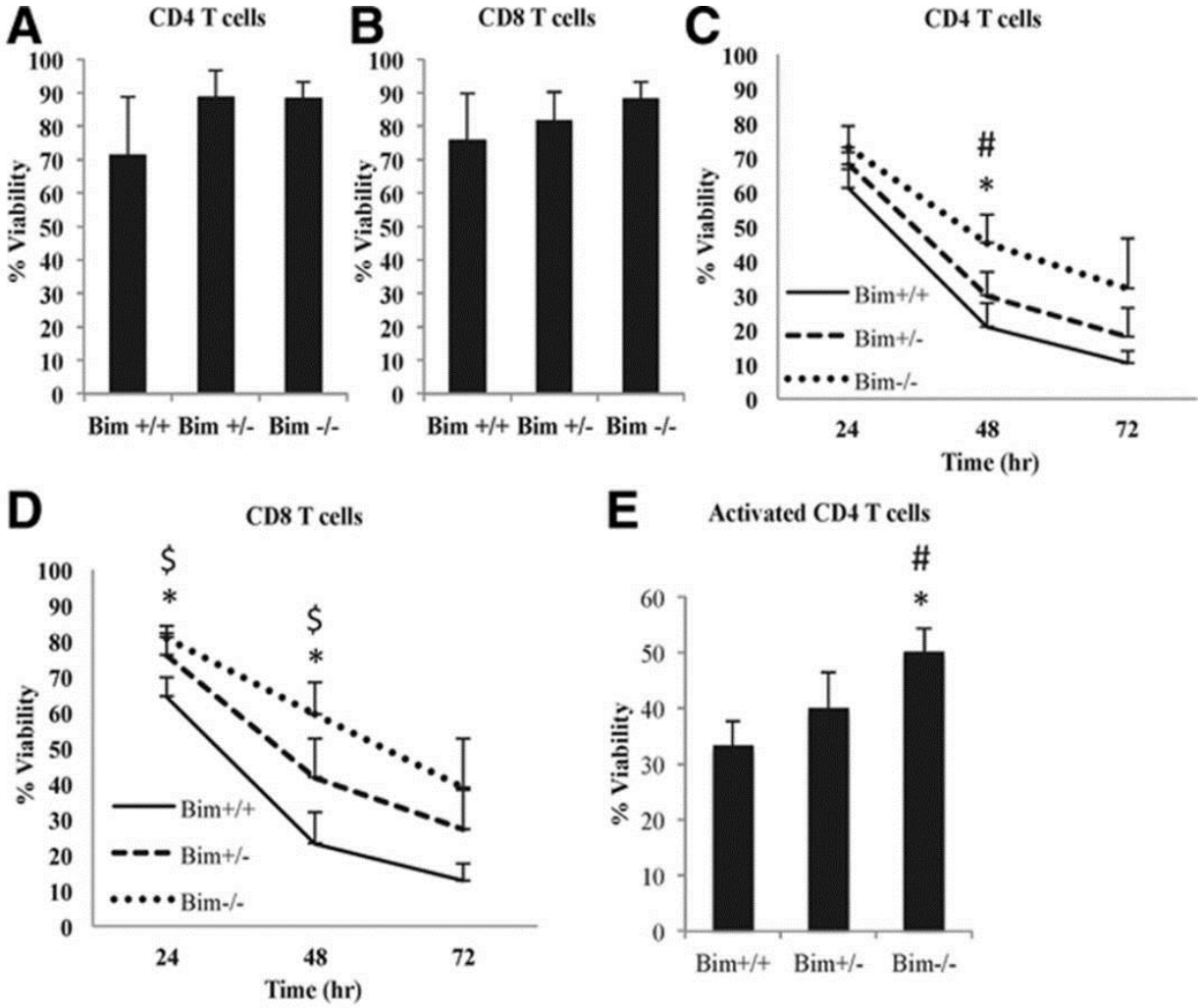
4.4.2. Complete Elimination of Bim Expression Prevents Cytokine Deprivation-Induced T Cell Death

Two types of T cell death regulate allogeneic responses: activation AICD that is caused by continuous exposure of T cells to antigen stimulation, and cytokine CDICD (Wells, Li et al. 1999). T cell viability was therefore analyzed in $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ T cells that were continually stimulated with allogeneic macrophages. There was no difference in the survival of CD4 or CD8 T cells in any of the groups (Figure 17 A and B), indicating that Bim does not affect T cell death in this setting. These findings also support a role for Bim in regulating T cell activation that is independent of its role in controlling cell death.

In addition to AICD, T cell responses are also regulated by CDICD in transplantation. This is demonstrated by experiments showing that transplant outcome is affected by the expression of Bcl-xL, a key regulator of cell death in response to cytokine deprivation, in T cells (Wells, Li et al. 1999). This form of cell death is likely to be triggered in the setting of transplantation by local regions of reduced cytokine availability, down-regulation of cytokine receptors from the surface of activated T cells, or the elimination of donor-derived APCs that directly present alloantigens to T cells in lymphoid tissues and the graft. As such, I characterized the role of Bim in regulating CDICD of CD4 and CD8 T cells using an established *ex vivo* protocol that involves culturing freshly isolated T cells in the absence of TCR or cytokine stimulation (Bouillet, Metcalf et al. 1999; Villunger, Michalak et al. 2003). $Bim^{+/+}$ CD4 T cells died at a steady rate with less than 10% survival after 72 h. As expected, $Bim^{-/-}$ CD4 T cells were significantly protected from CDICD. Interestingly, $Bim^{+/-}$ CD4 T cells underwent cell death at an identical rate as $Bim^{+/+}$ CD4 T cells, indicating that a partial reduction in Bim levels is not sufficient to attenuate CDICD in CD4 T cells (Figure 17 C). Importantly, $Bim^{+/-}$ CD4 T cells were also significantly more susceptible to CDICD than $Bim^{-/-}$ T cells. With regard to CD8 T cells, $Bim^{+/+}$ CD8 T cells underwent a steady rate of death in response to cytokine deprivation and, as expected, $Bim^{-/-}$ CD8 T cells were resistant to this form of cell death. However, unlike CD4 T cells, $Bim^{+/-}$ CD8 T cells were partially protected from cell death, undergoing levels that were intermediate between $Bim^{+/+}$ and $Bim^{-/-}$ cells (Figure 17 D). These results show that complete elimination of Bim expression protects both CD4 and CD8 T cells from CDICD, but that a partial reduction

in Bim levels does not affect this cell death process in CD4 T cells and partially reduces cell death of CD8 T cells.

We also examined CDICD of TCR activated T cells *in vitro*. For this, isolated CD4 and CD8 T cells were stimulated with anti-CD3 plus anti-CD28, removed from stimulation, and further cultured in the presence of an IL-2 neutralizing antibody for 72 h. IL-2 was neutralized because short-term stimulation of T cells with anti-CD3 and anti-CD28 *in vitro* is known to support ongoing proliferation and cytokine secretion for several days after the cells are removed from stimulation (Kaech and Ahmed 2001; Raue, Beadling et al. 2013). There was no difference in cell death between any of the groups prior to 72 h after removal from stimulation. At 72 h, activated CD4 T cells from $Bim^{-/-}$ mice were significantly protected from cell death but cells from $Bim^{+/-}$ mice were not (Figure 17E). Unexpectedly, Bim did not affect CD8 T cell death in these *in vitro* experiments. This *in vitro* model may not reflect the *in vivo* response of CD8 T cells because $Bim^{-/-}$ CD8 T cells are firmly established as being resistant to cell death caused by cytokine deprivation *in vivo* (Hildeman, Zhu et al. 2002; Strasser and Pellegrini 2004; Hughes, Belz et al. 2008).



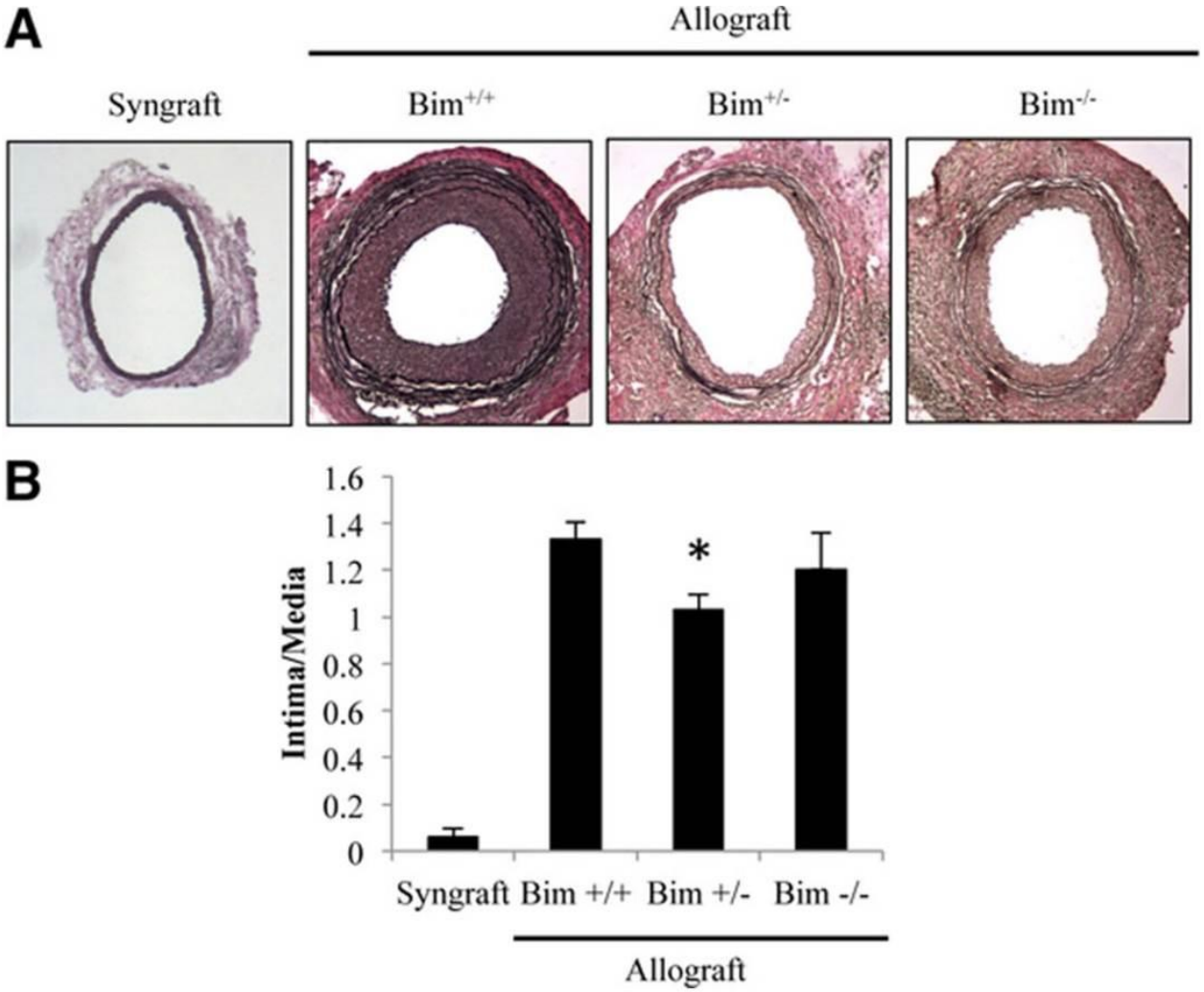
von Rossum A et al. Arterioscler Thromb Vasc Biol. 2014;34:1290-1297

Figure 17. Bim does not affect cell death of T cells continually exposed to alloantigen, but attenuates CDICD.

A, CD4 and **B**, CD8 T cells from C57bl/6 $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ mice were cultured with peritoneal macrophages from BALB/c mice. After 6 days, cell viability was analyzed by quantifying PI exclusion by flow cytometry. Data presented are the mean±SE of cell viability for a series of 3 separate experiments. **C**, CD4 and **D**, CD8 T cells from $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ mice were cultured in the absence of cytokines or growth factors. Cell viability was analyzed by PI exclusion. Data presented are the mean ± SE of cell viability for a series of 4 independent experiments. *p<0.05 $Bim^{-/-}$ compared with $Bim^{+/+}$ cells; # p<0.05 $Bim^{+/-}$ compared with $Bim^{-/-}$ cells; $ p<0.05 $Bim^{+/-}$ compared with $Bim^{+/+}$. **E,** CD4 T cells from $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ mice were stimulated with anti-CD3 plus anti-CD28 for 72 h, removed from stimulation and further cultured in the absence of stimulation for 72 h. An interleukin-2 neutralizing antibody was added to T cells after their removal from stimulation. Cell viability was determined by quantifying PI exclusion. Data presented are the mean ± SE of cell viability for a series of 3 independent experiments. *p≤0.05 as compared with $Bim^{+/+}$ cells; #p≤0.05 as compared with $Bim^{+/-}$ cells.

4.4.3. Partial Reduction, but Not Complete Elimination, of Bim Attenuates Immune-Mediated Vascular Rejection

The role of Bim in regulating T cell-mediated allograft vascular damage and rejection was then examined by interposition grafting of aortic segments from complete MHC-mismatched donors into $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ recipients. Immune-mediated vascular injury in this model initiates vascular reparative responses that culminate in rapid intimal thickening (Mennander, Tiisala et al. 1991; Shi, Lee et al. 1996; Johnson, Carpenter et al. 2002). The grafted arteries were harvested at day 30 post-transplantation for analysis of intimal thickening. Artery segments transplanted into $Bim^{+/-}$, but not $Bim^{-/-}$ mice, developed significantly less intimal thickening as compared to $Bim^{+/+}$ recipients (Figure 18).



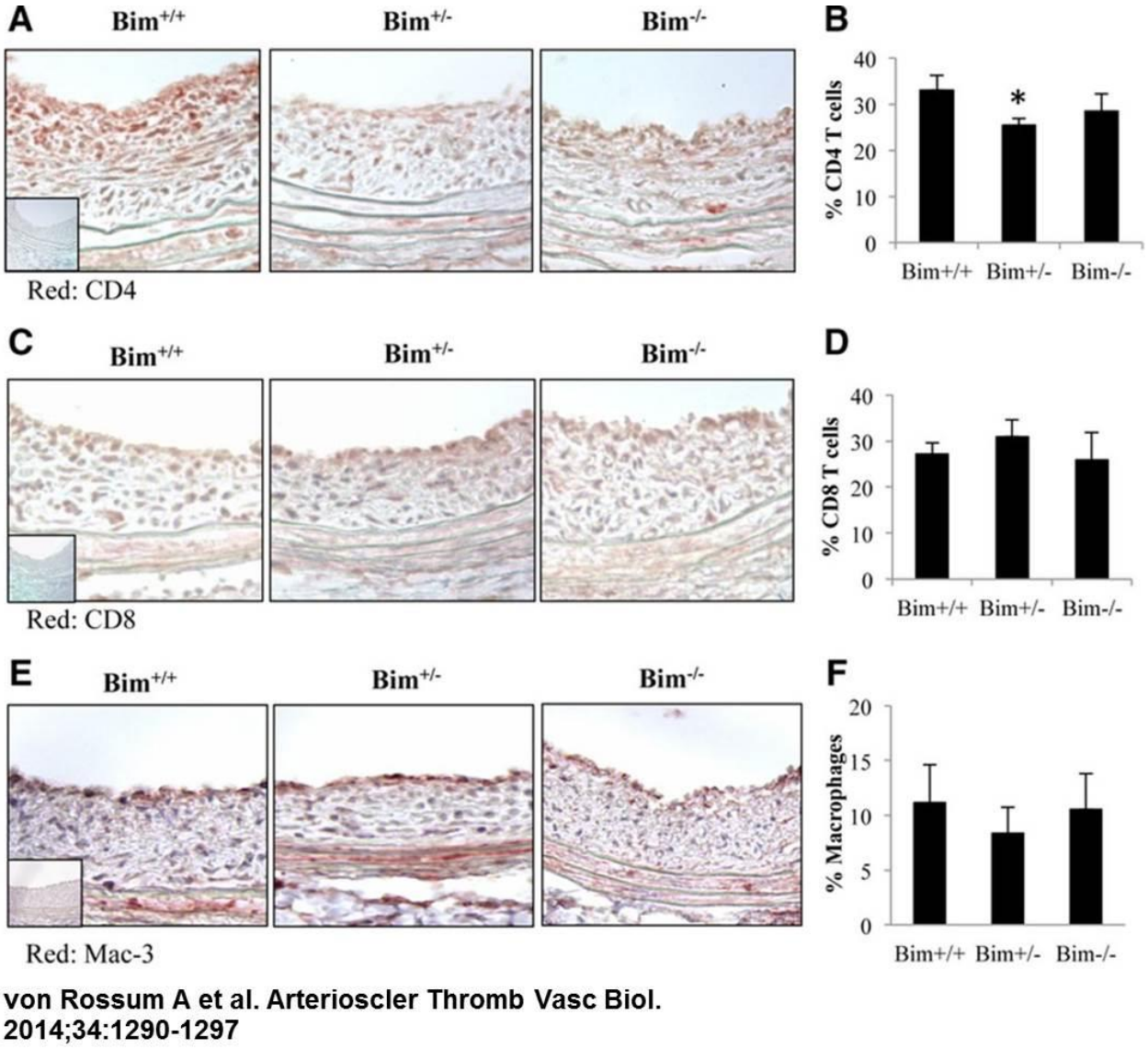
von Rossum A et al. Arterioscler Thromb Vasc Biol. 2014;34:1290-1297

Figure 18. Partial reduction, but not complete elimination, of Bim in graft recipients attenuates intimal thickening of allograft arteries

Segments of abdominal aorta from BALB/c mice were interposed into the resected abdominal aortas of C57Bl/6 $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ mice. The grafted arteries were harvested at day 30 post transplant. **A**, Representative photomicrographs of elastic van Giesen stained arteries from $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ graft recipients. Magnification ×100. **B**, Quantification of intimal thickening in allograft arteries from $Bim^{+/+}$ (n=14), $Bim^{+/-}$ (n=10), and $Bim^{-/-}$ (n=6) mice. *$p<0.05$ compared with $Bim^{+/+}$.

The effect of Bim on the accumulation of leukocytes in allograft arteries was then examined by quantifying the number of CD4 T cells, CD8 T cells, and macrophages within allograft arteries. There was a significantly reduced CD4 T cell accumulation in

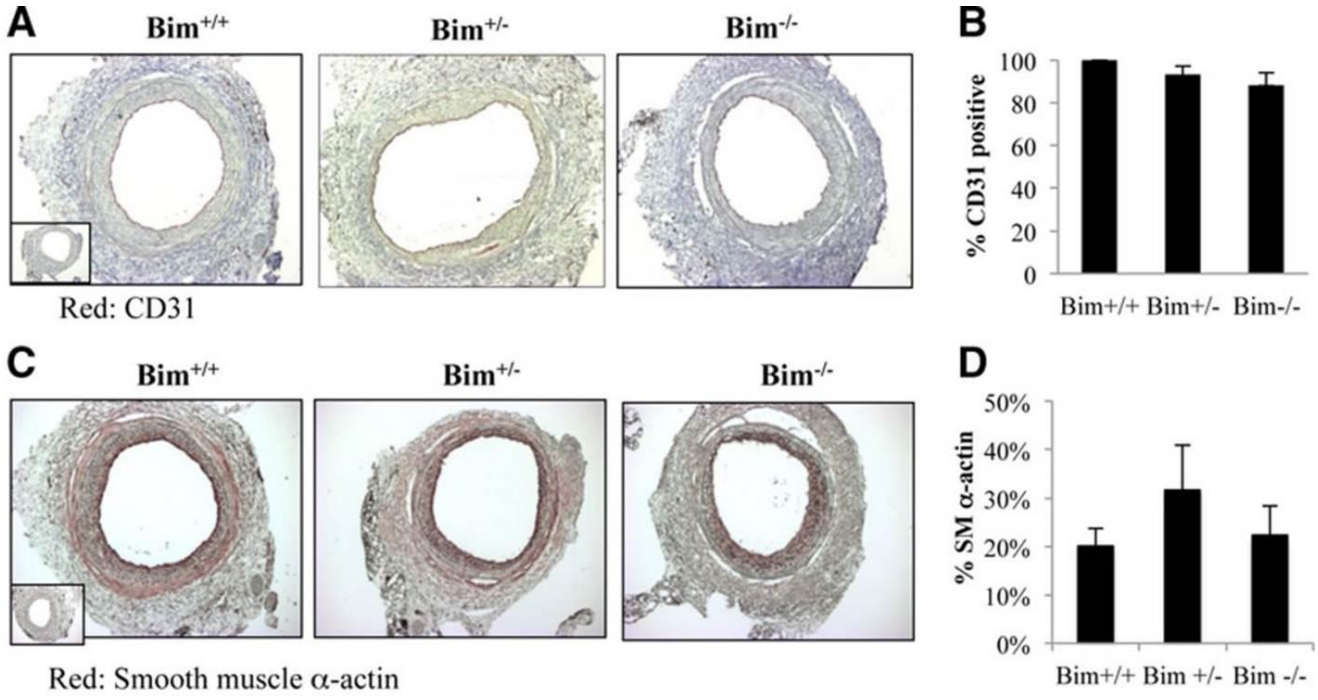
allograft arteries transplanted into $Bim^{+/-}$ mice, as compared to the $Bim^{+/+}$ counterparts, but CD4 T cell accumulation did not differ between arteries harvested from $Bim^{-/-}$ and $Bim^{+/+}$ mice (Figure 19 A and B). There was no significant difference in the accumulation of CD8 T cells (Figure 19 C and D) or macrophages (Figure 19 E and F) in allograft arteries from $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ mice. Staining for dendritic cells revealed too few cells in the intima to conclusively determine differences between the groups. The effect of Bim on vascular cells within the grafts was also determined. EC vessel lining and the accumulation of intimal SMCs were studied by staining sections of the allograft arteries for CD31 and smooth muscle α-actin, respectively. No significant difference was observed in EC lining of the lumen (Figure 20 A and B) or in the percentage of SMCs in the intima of allograft arteries in any of the groups (Figure 20 C and D).



von Rossum A et al. Arterioscler Thromb Vasc Biol. 2014;34:1290-1297

Figure 19. Partial reduction, but not complete elimination, of Bim reduces CD4 T-cell accumulation in allograft arteries, but does not affect CD8 T-cell or macrophage accumulation

Representative photomicrographs of allograft arteries from $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ graft recipients immunohistochemically stained for **A**, CD4, **C**, CD8, and **E**, Mac-3. The percentage of **B**, CD4-positive, **D**, CD8-positive, and **F**, Mac-3–positive cells within the intima of allograft arteries was quantified by manual counting. Data presented in **B**, **D**, and **E** are the mean ± SE of the percentage of all intimal cells that are CD4-, CD8-, and Mac-3–positive in arteries from $Bim^{+/+}$ (n=14), $Bim^{+/-}$ (n=10), and $Bim^{-/-}$ (n=6) mice. *$p<0.05$ compared with $Bim^{+/+}$. The inset photomicrographs depict isotype staining controls.



von Rossum A et al. Arterioscler Thromb Vasc Biol. 2014;34:1290-1297

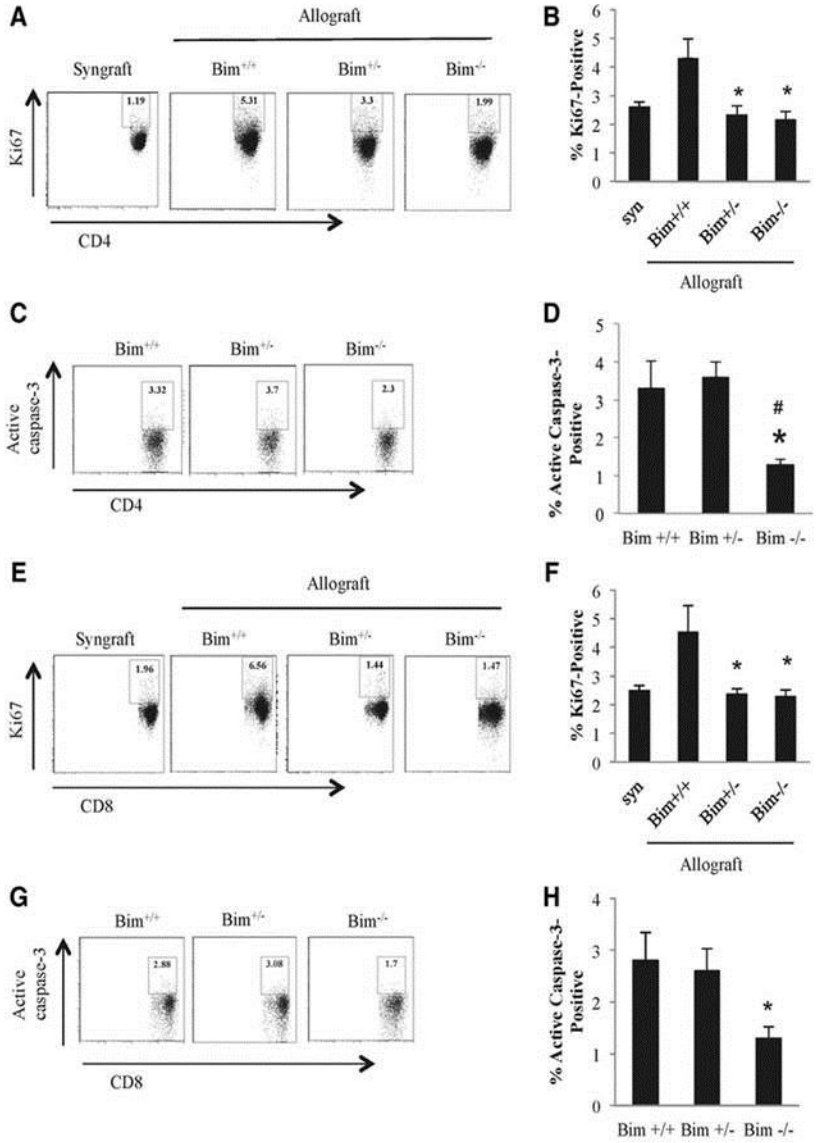
Figure 20. Bim does not affect endothelial lining or smooth muscle cell accumulation in the intima of allograft arteries.

Representative photomicrographs of allograft arteries from $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ graft recipients immunohistochemically stained for **A**, CD31 and **C**, smooth muscle α-actin. Quantification of **B**, CD31 positivity and **D**, smooth muscle α-actin positivity was performed. The data presented in **B** and **D** are the mean ± SE of the percentage luminal lining that is $CD31^{+}$ or the percentage smooth muscle α-actin positive area in the intima.

4.4.4. Partial Reduction of Bim Levels Attenuates T Cell Proliferation, but Not Death, in Response to Allograft Arteries

Since a decrease in Bim expression levels reduced intimal thickening and attenuated the accumulation of CD4 effector T cells in allograft arteries, T cell proliferation and death in response to allograft arteries was examined. Segments of abdominal aorta were transplanted into $Bim^{+/+}$, $Bim^{+/-}$, or $Bim^{-/-}$ mice, and splenocytes isolated at day 7 post-transplantation. Flow cytometric analysis was performed for CD4, CD8, CD44, Ki67, and active caspase-3 to quantify CD4 and CD8 T cell proliferation and death, respectively. Significantly fewer Ki67-positive CD4 T cells were observed in both $Bim^{+/-}$ and $Bim^{-/-}$ mice in response to allograft arteries as compared to $Bim^{+/+}$

counterparts (Figure 21 A and B). Also, there was no difference in cell death of CD4 T cells in $Bim^{+/-}$ as compared to $Bim^{+/+}$ artery graft recipients, but T cell death was significantly reduced in $Bim^{-/-}$ mice as compared to either $Bim^{+/+}$ or $Bim^{+/-}$ counterparts (Figure 21 C and D). Proliferation of CD8 T cells was also reduced in both $Bim^{+/-}$ and $Bim^{-/-}$ mice in response to allograft arteries (Figure. 21 E and F). Cell death of CD8 T cells was significantly reduced only in $Bim^{-/-}$ as compared to $Bim^{+/+}$ graft recipients (Figure 21 G and H).



von Rossum A et al. Arterioscler Thromb Vasc Biol. 2014;34:1290-1297

Figure 21. Partial reduction in Bim levels is sufficient to attenuate T cell proliferation in response to allograft arteries, but complete elimination of Bim is needed to prevent T cell death.

Segments of abdominal aorta from BALB/c mice were interposed into the resected abdominal aortas of C57Bl/6 $Bim^{+/+}$, $Bim^{+/-}$, or $Bim^{-/-}$ mice. Syngrafts (syn) were performed as controls. Splenocytes were isolated at day 7 post transplant and examined by flow cytometry. **A**, Representative dot plots showing the frequency of CD4 T cells that are Ki67 positive in $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ graft recipients. **B**, Mean ± SE of the percentage of CD4 T cells that are Ki67 positive in $Bim^{+/+}$ (n=9), $Bim^{+/-}$ (n=8), and $Bim^{-/-}$ (n=8) graft recipients. *p<0.05 compared with Bim+/+ mice. **C**, Representative dot plots showing the frequency of $CD4^+CD44^+$ effector T cells that are active caspase-3 positive in $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ graft recipients**. D**, Mean ± SE of the percentage of $CD4^+CD44^+$ effector T cells that are active caspase-3 positive in $Bim^{+/+}$ (n=9), $Bim^{+/-}$ (n=9), and $Bim^{-/-}$ (n=9) graft recipients. *p<0.05 compared with $Bim^{+/+}$ mice, #p<0.05 compared with $Bim^{+/-}$ mice. **E**, Representative dot plots showing the frequency of CD8 T cells that are Ki67 positive in $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ graft recipients. **F**, Mean ± SE of the percentage of CD8 T cells that are Ki67 positive in $Bim^{+/+}$ (n=9), $Bim^{+/-}$ (n=7), and $Bim^{-/-}$ (n=8) graft recipients. *p≤0.05 compared with $Bim^{+/+}$ mice. **G**, Representative dot plots showing the frequency of $CD8^+CD44^+$ effector T cells that are active caspase-3 positive in $Bim_{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ graft recipients. **H**, Mean ± SE of the percentage of $CD8^+CD44^+$ effector T cells that are active caspase-3 positive in $Bim^{+/+}$ (n=9), $Bim^{+/-}$ (n=7), and $Bim^{-/-}$ (n=9) graft recipients. *p<0.05 as compared with $Bim^{+/+}$.

4.5. Discussion

In this study, I have shown that Bim is required for the activation of T cells in response to stimulation by alloantigen and that this process is involved in immune-mediated vascular damage and rejection that causes TA. Furthermore, the effect of Bim on T cell activation and death can be separated by differential reductions in Bim levels. Specifically, a partial reduction in Bim expression, as seen in $Bim^{+/-}$ T cells, is sufficient to attenuate T cell activation, and complete elimination of Bim expression is required to prevent CD4 T cell death.

In response to allogeneic stimulation, both $Bim^{+/-}$ and $Bim^{-/-}$ CD4 and CD8 T cells display reduced proliferation as compared to $Bim^{+/+}$ cells. Interestingly, a half reduction in Bim levels, as occurs in $Bim^{+/-}$ T cells, is sufficient to produce this effect. Cell death was measured in these cultures and no difference was observed between any of the groups, indicating that the observed effect on the accumulation of proliferated cells was not due to altered cell survival. Since T cells in this setting are exposed to continuous antigen, cell death in these experiments was likely a result of AICD, Bim has been reported to be involved in the induction of AICD (Snow, Oliveira et al. 2008), although its role in driving T cell death in response to cytokine deprivation is more extensively understood (Bouillet, Metcalf et al. 1999; Hughes, Belz et al. 2008). I also characterized the role of Bim in CDICD and confirmed that both CD4 and CD8 T cells that lack Bim are

protected from this form of cell death. Interestingly, a partial reduction in Bim expression, as occurs in $Bim^{+/-}$ mice, did not inhibit CD4 T cell death indicative of Bim haplosufficiency. CD8 T cells from $Bim^{+/-}$ mice underwent cell death at an intermediate level between the $Bim^{-/-}$ and the $Bim^{+/+}$ CD8 T cells. This cell death pathway may regulate T cell death in organ transplantation, because graft-derived professional APCs that directly present alloantigens to T cells are eliminated as allogeneic immune responses progress over time, and this may result in localized regions in which graft-reactive T cells experience a paucity of survival signals (Pietra, Wiseman et al. 2000; Game and Lechler 2002). Also, down-regulation of certain cytokine receptors, such as CD25 and CD127, by effector T cells may render them unable to respond to survival signals even in settings of persistent antigen (Kalia, Sarkar et al. 2010).

An unexpected role for Bim in regulating T cell activation has recently been reported in animal models of autoimmune encephalomyelitis, diabetes, and graft-*versus*-host disease (Ludwinski, Sun et al. 2009; Yu, Yu et al. 2012). However, the interplay between the regulation of T cell activation and death by Bim was not examined in these studies. My findings extend existing knowledge by establishing *(i)* a role for Bim in controlling both the activation and death of T cells in response to alloantigen stimulation, *(ii)* that a partial reduction of Bim expression inhibits T cell proliferation without affecting CD4 T cell death, for which complete elimination of Bim is necessary, and *(iii)* that both processes control immune responses that lead to allograft vascular injury and rejection. It is important to note that, although Bim is required for certain types of clonal deletion in the thymus (Suen and Baldwin 2012), we did not observe splenomegaly in $Bim^{+/-}$ mice in this study. Also, systemic elimination of Bim has been reported to result in an increase in dysfunctional Foxp3-expressing T regs (Zhan, Zhang et al. 2011); however, I did not observe an increase in Foxp3-expressing T cells in $Bim^{+/-}$ or $Bim^{-/-}$ graft recipients (data not shown).

I observed a very early defect in T cell activation in the absence or reduced levels of Bim. Although I examined IL-2 secretion as a surrogate indicator of effector cytokine production *in vitro*, other cytokines are clearly involved in supporting the expansion of effector T cells in the setting of transplantation *in vivo* (Gaston 1994). Bim likely affects the acquisition of a large array of T cell effector functions since it has been

thought to act very proximally in TCR signalling pathways (Ludwinski, Sun et al. 2009). Ludwinski *et al.* (Ludwinski, Sun et al. 2009) reported that $Bim^{-/-}$ T cells activated with agonistic CD3 antibody had significantly reduced levels of calcium influx as compared to $Bim^{+/+}$ T cells, and that this resulted in reduced activation of NFAT. Bcl-2 and Bcl-xL are known to localize to the endoplasmic reticulum and interact with the inositol 1,4,5 triphosphate receptor (IP3-R) to either inhibit or potentiate calcium release (Chen, Valencia et al. 2004; Oakes, Scorrano et al. 2005). Given this, antagonism of Bcl-2 effects by Bim may serve to augment TCR-mediated calcium signalling in T cells. Further studies are needed to define these processes in T cells.

My data establish that the reduction, but not the elimination, of Bim expression in graft recipients attenuates vascular injury and rejection. Partial reduction in Bim levels also reduced accumulation of effector CD4 T cells in the intima of allograft arteries, and reduced proliferation of alloreactive T cells but did not affect CD4 T cell death. On the other hand, both T cell activation and death were attenuated when Bim expression was completely absent in graft recipients. When assessed in combination with the *in vitro* findings, which show that Bim expression in T cells regulates T cell activation and death in a similar manner, these data indicate that in the setting of $Bim^{+/-}$ graft recipients, CD4 T cell activation is compromised, but death is not, with this resulting in reduced allograft damage. However, when Bim expression is completely absent in $Bim^{-/-}$ recipients, attenuated T cell activation is offset by increased survival of activated T cells and this results in no net effect on rejection. Interestingly, partial reduction of Bim levels in $Bim^{+/-}$ mice did not appear to affect CD8 T cell accumulation in allograft arteries in our studies. Instead, Bim is likely to affect vascular rejection through its actions in CD4 T cells, because intimal thickening in complete MHC-mismatched aortic interposition grafts depends mainly on this T cell subset (Shi, Lee et al. 1996). Finally, a limitation in this study is that Bim is systemically absent in $Bim^{-/-}$ mice. This means that potentially, the effects we observe in this model *in vivo*, may also be reflecting the effect of Bim deficiency on other cells of the immune system. In the future, this limitation could be overcome by using T cell specific Bim knockout and heterozygous transgenic mice, or by grafting artery segments in to $RAG^{-/-}$ mice and reconstituting the recipients with $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ T cells.

In summary, I have shown that reduction, but not elimination, of Bim in T cells inhibits T cell activation in response to alloantigen stimulation and attenuates allograft vascular injury. The regulation of allogeneic immune responses by Bim is complex and involves the control of both T cell activation and death, and these processes are differentially susceptible to reductions in Bim expression. These findings have implications for understanding the regulation of immune responses in transplantation, and may have clinical implications given the investigation of BH3 mimetics for therapeutic applications (Labi, Grespi et al. 2008).

Chapter 5. Summary and Future Directions

It is well established that T cell death is an important regulator of transplant rejection and TA. However, the exact contribution of specific cell death pathways to the development of TA remains to be fully understood. In this dissertation I have elucidated two novel mechanisms regulating main forms of activated T cell death. Additionally, I provide evidence that these processes play a role in the immune-mediated vascular rejection that causes TA.

In an immune response, there is vast interplay between cytokines, growth factors, chemokines and other effector molecules and processes that shape the outcome a specific immune response. These include, but are not limited to, the nature of the peptide antigen being presented, the type of MHC it is presented on, the type of co-stimulation, soluble factors in the immediate environment, as well as the presence of inhibitory and regulatory mechanisms. Additionally, there is a great deal of redundancy in processes that control allogeneic reactions, as evidenced by the numerous studies in which specific aspects of immune responses were actively inhibited with the goal of attenuating the immune system, only to observe an equally aggressive or sometimes even more severe response and rejection (Piccotti, Chan et al. 1996; VanBuskirk, Wakely et al. 1996; Piccotti, Chan et al. 1997; Yuan, Paez-Cortez et al. 2008). I have shown that the nature of inflammatory signals received by human CD8 T cells during TCR stimulation dictates their susceptibility to AICD. Specifically, CD8 T cells activated in the presence of IL-6 are protected from Fas-mediated AICD. This is mediated by increased expression of c-FLIPs. In order to establish the significance of this finding *in vivo,* I used IL-$6^{-/-}$ mice and a transplant model to show that IL-6 drives vascular rejection, the accumulation of T cells in allografts, and inhibits Fas-mediated T cell death. This reveals an important role for graft-derived IL-6 and suggests that IL-6 produced by donor-derived APCs is a potential therapeutic target for the prevention of acute and chronic rejection, since defects in T cell survival were observed in the spleen

anatomical location in which graft APCs interact with T cells. However, graft SMCs and ECs also secrete and respond to IL-6. IL-6 is normally produced by graft cells in response to injury, it is a known mitogen and migration mediator of circulating endothelial progenitor cells (Fan, Ye et al. 2008), and is involved in the migration and proliferation of SMCs (Nabata, Morimoto et al. 1990; Ikeda, Ikeda et al. 1991; Klouche, Rose-John et al. 2000; Wang, Castresana et al. 2001; Wang, Liu et al. 2007). Future studies should establish the relative contribution of IL-6 deficiency in donor APCs *versus* IL-6 deficiency in donor graft cells toward the development of vascular disease. This could be done by transplanting IL-$6^{-/-}$ arteries that are depleted of APCs in to wild type recipients. Systemic dendritic cell and macrophage depletion can be achieved using Macrophage-Fas-induced apoptosis (MAFIA) mice, in which administration of AP20187 dimerizing ligand results in apoptosis in colony-stimulating factor-1 receptor–positive [CSF-$1R^{+}$] cells as described previously (Burnett, Kershen et al. 2004). Additionally, the role of cFLIPs in activated T cells should be established in this model, which may provide a better indication of whether this protein could be a potential therapeutic target. This could be done directly by forcing expression of c-FLIPs in T cells in the absence of IL-6 signaling during activation or indirectly by studying the importance of the Fas-FasL pathway *in vivo*. Ideally, this would involve transplanting arteries from $Fas^{-/-}$ and $IL6^{-/-}$ double-knockout donors into allogeneic recipients and comparing their vascular disease to that of allogeneic donors receiving $Fas^{-/-}$ $IL6^{+/+}$ transplants.

Cell death pathways exhibit an extraordinary amount of complexity and redundancy. There are clear roles for not only the mitochondria, but also the plasma membrane, nucleus, endoplasmic reticulum, and lysosomes. Usually these pathways converge at the mitochondrion, where a common cell death pathway takes over *via* activation of caspases, although caspase-independent cell death can also occur (Lemasters 2005). I have shown that the pro-apoptotic protein Bim is required for CDICD and for proper T cell activation. Perhaps the most novel and interesting part of this study is the finding that a partial reduction in Bim levels (as occurs in $Bim^{+/-}$ mice) leads only to a defect in T cell activation but not CDICD in CD4 T cells. The exact mechanism by which Bim governs activation pathways has not been established although there are several clues in the literature. Ludwinski and colleagues had reported that Bim knockout T cells, when activated *via* the TCR with anti-CD3 and anti-CD28, displayed a global

defect in cytokine production, including IL-2, IL-4, IL-6, IL-10, and IL-17, not only at the protein level, but also at the message level (Ludwinski, Sun et al. 2009). However, when they activated T cells with phorbol myristate acetate and ionomycin, which directly stimulate protein kinase C and calcium release from the endoplasmic reticulum, cytokines were produced at levels observed in wild type T cells. This suggests that the Bim defect in T cells lies proximal to the TCR. When they studied the signalling pathways that were affected by Bim deficiency, they found that upon TCR stimulation, NFATc2 proteins were nearly all dephosphorylated in wild type cells, but remained phosphorylated in $Bim^{-/-}$ cells. NFATc2 dephosphorylation is mediated by calcineurin, a serine phosphatase that is in turn activated by calcium/calmodulin (Hogan, Chen et al. 2003). Stimulation of the TCR triggers phospholipase C (PLC)–mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate to produce inositol-1,4, 5-trisphosphate (IP3) and polyunsaturated diacylglycerol (DAG). IP3 then induces calcium release from the endoplasmic reticulum. A sustained increase in calcium elevation is essential during T cell activation, specifically for the production of effector cytokines (Kane, Lin et al. 2000; Winslow, Neilson et al. 2003). While calcium release does occur in $Bim^{-/-}$ T cells, its peak value was reported to be significantly reduced as compared to $Bim^{+/+}$ T cells (Ludwinski, Sun et al. 2009). It is not clear how Bim affects calcium release. It may alter the control of calcium signalling by Bcl-2 which is known to bind and modulate activity of IP3R, or it may be that Bim affects calcium release directly as it has been shown to be associated with the endoplasmic reticulum membrane (Morishima, Nakanishi et al. 2004). These findings have implications for understanding the regulation of immune responses in transplantation, and may have clinical implications given the investigation of BH3 mimetics for therapeutic applications (Labi, Grespi et al. 2008). Future studies should focus on targeting calcium signalling specifically in T cells, since toxicity is a well-documented effect of global inhibition of calcium signalling such as in the case of CNI therapies.

Data in the literature suggest that the elimination of alloreactive T cells is key in the prevention of rejection and in the induction of tolerance. While some success has been accomplished *via* depletion by antibodies, induction of activated T cell death is a more attractive therapeutic option since it has been shown to also promote immunoregulatory pathways, and there is a strong correlation between

immunoregulation and tolerance (Chiffoleau, Walsh et al. 2003). It is important to note that a small to moderate reduction in activated T cell death in the early stages of an alloimmune response, can lead to large effects long term. Given the complexity of human immunology and the heterogeneity among individuals, it is unlikely that there will be a single “magic bullet” agent for induction of allograft tolerance. It is important to keep in mind that immunosuppression and tolerance are fundamentally different concepts, and immunosuppression may hinder the development and maintenance of tolerance in the clinical setting. Nonetheless, over the past 50 years there has been tremendous improvement in prevention of acute rejection (85-95% survival >1 year) (Lechler, Sykes et al. 2005), and patient quality of life. As for the prevention of chronic or long-term rejection, induction of alloreactive T cell death in combination with immunoregulation may be a promising approach.

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