Studies on the apoptosis of regulatory T cells *in vitro* and *in vivo*

Dissertation

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Mitten in der Schwierigkeit liegt die Gelegenheit

(Albert Einstein)

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Abstract

$CD4^+CD25^{++}Foxp3^+$ regulatory T cells (Treg) actively control self-reactive conventional T cells (Tcon) and other cell types and thus maintain peripheral tolerance. Accordingly, an imbalance with regard to quantity or quality of Treg-mediated suppression can lead to various immune pathologies, *e.g.* autoimmune diseases. The elucidation of mechanisms that influence Treg numbers remains therefore a major challenge for the establishment of therapies.

Recent *in vitro* and *in vivo* data point to the death receptor CD95 (APO-1/Fas) and its ligand CD95L (CD178/APO-1L/FasL) as one potential system in the control of Treg numbers. In an *in vitro* T cell death model, freshly isolated Tcon are CD95-resistant. However, after an *in vitro* expansion of 6 days they upregulate CD95 and start producing CD95L upon T cell antigen receptor (TCR) re-stimulation. Subsequent binding of endogenous CD95L to CD95 leads to suicide/fratricide *via* a process called activation-induced cell death (AICD). In contrast to Tcon, day 0 as well as day 6 Treg are highly sensitive to CD95-induced apoptosis but do not undergo AICD upon TCR re-stimulation.

In the present work two points regarding the apoptosis of Treg were investigated. First, the molecular basis for the lack of AICD in Treg was examined. Second, the sensitivity of Treg towards CD95-mediated apoptosis was analyzed *in vivo*.

Concerning the first part, one plausible explanation for the lack of AICD in Treg is insufficient CD95L expression. Indeed, the investigation of CD95L levels in Treg upon stimulation revealed that they express, compared to Tcon, less CD95L mRNA as well as protein. This diminished CD95L expression is neither due to altered kinetics nor caused by CD95L cleavage. Low CD95L expression occurs irrespective of the cellular activation status, although the Treg population consists, in contrast to Tcon, mainly of effector/memory cells. In the second part, the sensitivity of Treg to CD95-induced apoptosis was investigated *in vivo*. CD95-deficient bone marrow chimeric mice containing $CD95^+$ T cells tolerate CD95 stimulation. In this mouse model, injection of an agonistic anti-CD95 antibody resulted in reduced Treg numbers *in vivo*.

In conclusion, the resistance of Treg to AICD *in vitro* can be attributed to low stimulation-induced CD95L expression. Furthermore, the data demonstrate that Treg are sensitive to CD95-induced apoptosis *in vivo*. This apoptosis sensitivity might be exploited by $CD95L^+$ Tcon to eliminate Treg by CD95 stimulation for the establishment of powerful immune responses. Altogether, the presented findings contribute to the understanding of the mechanisms which control Treg homeostasis.

Zusammenfassung

$CD4^+CD25^{++}Foxp3^+$ regulatorische T-Zellen (engl. Treg) kontrollieren selbstreaktive konventionelle T-Zellen (engl. Tcon) und andere Zellarten und Erhalten dadurch die periphere Toleranz. Demzufolge kann ein Ungleichgewicht hinsichtlich Quantität oder Qualitiät der Treg-vermittelten Hemmung zu verschiedenen Immunpathologien, z.B. Autoimmunität, führen. Die Aufklärung der Mechanismen, die die Treg-Anzahl beeinflussen, bleibt daher eine große Herausforderung zur Etablierung von Therapien.
Kürzlich gewonnene *in vitro* und *in vivo* Daten lassen auf den Todesrezeptor CD95 (APO-1/Fas) und seinen Liganden CD95L (CD178/APO-1L/FasL) als ein potentielles System bei der Kontrolle der Treg-Anzahl schließen. In einem *in vitro* T-Zell Todesmodell sind frisch isolierte Tcon CD95-resistent. Nach einer 6-tägigen *in vitro* Expansion erhöhen sie die CD95 Expression und beginnen nach Restimulierung des T-Zell Antigenrezeptors (engl. TCR) mit der Produktion des CD95L. Anschließendes Binden des endogenen CD95L an CD95 führt zu Suizid/Fratrizid durch den Prozess des aktivierungsinduzierten Zelltods (engl. AICD). Im Gegensatz zu Tcon sind Tag 0 und auch Tag 6 Treg hochsensitiv gegenüber CD95-induzierter Apoptose, zeigen aber keinen AICD nach TCR Restimulierung.
In der folgenden Arbeit sollen zwei Punkte hinsichtlich der Apoptose von Treg untersucht werden. Zuerst soll die molekulare Basis für das Fehlen des AICD in Treg *in vitro* betrachtet werden. Zweitens soll die Sensitivität von Treg gegenüber CD95-vermittelter Apoptose *in vivo* untersucht werden.
Für den ersten Teil ist eine ungenügende CD95L Expression eine plausible Erklärung für den fehlenden AICD bei Treg. Tatsächlich zeigte die Untersuchung der CD95L Menge in Treg nach Stimulierung, dass sie, verglichen mit Tcon, wenig CD95L mRNA und auch Protein exprimieren. Die niedrige CD95L Expression ist weder bedingt durch eine veränderte Kinetik, noch verursacht durch CD95L Spaltung. Auch spielt der zelluläre Aktivierungsstatus keine Rolle, obwohl Treg, im Gegensatz zu Tcon, hauptsächlich Effektor-/Gedächtnis-Zellen sind.
Im zweiten Teil wurde die Sensitivität von Treg gegenüber CD95-induzierter Apoptose mithilfe eines Mausmodells *in vivo* untersucht. CD95-defiziente knochenmarkchimäre Mäuse, die $CD95^+$ T-Zellen enthalten, zeigen nach CD95-Stimulierung kein Leberversagen. Injektion eines agonistischen anti-CD95 Antikörpers reduzierte die Anzahl der Treg *in vivo*.
Zusammenfassend kann die Resistenz von Treg gegenüber AICD *in vitro* einer niedrigen stimulierungsinduzierten CD95L Expression zugeschrieben werden. Des weiteren zeigen die Mausmodell-Daten, dass Treg *in vivo* sensitiv gegenüber CD95-induzierter Apoptose sind. Diese Apoptosesensitivität könnte durch $CD95L^+$ Tcon ausgenutzt werden, Treg durch CD95 Stimulierung zu eliminieren, um leistungsstarke Immunantworten zu generieren. Insgesamt tragen diese Ergebnisse zum Verständnis der Mechanismen, die die Treg Homeostase kontrollieren bei.

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1 Introduction

1.1 The immune system

The immune system (lat.: *immunis* – pure) of higher organisms is a biological defense system that recognizes and antagonizes pathogens to prevent diseases. As a complex network of organs, cell types and molecules it is able to distinguish the organism's healthy cells from a wide variety of dangerous structures (bacteria, viruses, fungi, protozoa and diseased/damaged cells) and can therefore identify and eliminate foreign microorganisms and altered body cells.

1.1.1 Innate and adaptive immunity

The immune system consists of two parts: the innate and adaptive immune system are linked to each other but differ in their components and function. While the innate immune system reacts very fast upon encounter of pathogens, the adaptive immune system adjusts over time to eliminate pathogens more efficiently and has the property of immunological memory. However, only the coordinated interplay of the various components of both parts allows for the complex immune reactions of the body.

The **innate immunity** consists of anatomical and physiological barriers, cell-mediated phagocytosis, general inflammatory reactions and the complement system. The front line barriers against infection are the epithelia (skin and gastrointestinal, respiratory and urogenital tract) which protect the body against pathogens by their compact structure and *via* the production of mucus and microbicidal substances. The recognition of pathogens by macrophages, natural killer (NK) cells and neutrophils with germ-line encoded receptors represents the next line of defense. Among their receptors are the toll-like receptors (TLR) which detect extracellular as well as intracellular pathogenic features. Invariable properties of pathogens, the so-called pathogen-associated molecular patterns (PAMP), are recognized by these receptors. For instance, some cells destroy the pathogenic agent themselves while others set the body in an alarm position by the production of messengers (interleukins) to enhance

the immune reaction. Furthermore, the complement system, a network of various plasma proteins which is distributed throughout body fluids and tissues, is activated at sites of infection by a proteolytic enzyme cascade. Complement proteins protect against infection in three ways: by opsonizing pathogens for engulfment, through chemoattractants to recruit phagocytes and *via* the creation of pores in the pathogen's membrane (Liszewski et al., 1996).

The second part of the immune system, the **adaptive immunity**, is characterized by the capability to adjust to new or modified pathogenic structures. Antigen-presenting cells (APC) like dendritic cells (DC) digest invading pathogens and present pathogen-derived peptides which are recognized by antigen (Ag) receptors of lymphocytes which then establish a directed defense mechanism. Lymphocytes are divided into bone marrow (BM)-derived (B) lymphocytes which are responsible for the humoral immunity that uses antibodies as effector molecules and thymus-derived (T) lymphocytes that enable a cell-mediated immune response and support B lymphocytes. T cells can discriminate self from non-self by the so-called major histocompatibility complex (MHC) which is displayed on each cell of the body. Diseased cells that present pathogenic peptides on MHC molecules are recognized and become a target of lymphocytes. To be highly adaptable, the Ag receptor genes of T and B cells undergo recombination of their so-called V(D)J elements and in the case of the B cell Ag receptor complete a process of somatic hypermutation. These two mechanisms allow for the generation of large amounts of different Ag receptors by a restricted gene repertoire, which are then uniquely expressed on individual lymphocytes. During an immune response, lymphocytes with the required receptor specificity expand and give rise to a large population of effector lymphocytes that clear the pathogen. After a resolved infection, memory cells and specific antibodies remain which facilitate a quick reaction upon second encounter with the same pathogen.

Disorders of the immune system can result in disease. A compromised immune system leads to immunodeficiency causing recurrent and life-threatening infections which can be either genetically inherited (*e.g.* severe combined immunodeficiency

(SCID)), triggered by pharmaceuticals (*e.g.* glucocorticoids) or induced by infection (*e.g.* acquired immune deficiency syndrome (AIDS)). In contrast, autoimmune diseases occur when the immune system is hyperactive and attacks normal tissues as if they were foreign organisms. Examples for autoimmune diseases are rheumatoid arthritis, type 1 diabetes and lupus erythematosus.

1.1.2 T lymphocyte development

While B lymphocyte maturation takes place in the BM, hematopoietic progenitor cells migrate from the BM to the thymus for the generation of the T cell pool. In mice, the thymus continues to develop for 3-4 weeks after birth, which is in contrast to humans who are born with a fully developed thymus. Upon arrival in the thymus, thymocytes go through a precise screening procedure for their T cell Ag receptor (TCR), the positive and negative selection, to ensure immunogenic competence and diversity without self-reactivity. Approximately 98% of thymocytes die during the developmental differentiation process by failing either positive or negative selection, whereas the other 2% survive to give rise to T cells.

The intrathymic developmental stages of T cell progenitors can be characterized by their temporally coordinated expression of cell surface markers, *e.g.* the cluster of differentiation (CD) 3 complex, the co-receptors CD4 and CD8 as well as CD25 and CD44 (Godfrey et al., 1993). Neither CD4 nor CD8 are expressed by thymocytes in the earliest developmental stages which are therefore classed as double negative (DN) pre-T cells (Vonboehmer, 1988). During maturation, DN thymocytes give rise to two distinct T cell lineages which express different TCR chains. The majority are α:β T cells which express TCRα:TCRβ chains, while only a small percentage of cells become TCRγ:TCRδ positive. The γ:δ T cells remain CD4/CD8 negative and will not be discussed further. The development of α:β T cells will be described in more detail.

During four DN stages which can be distinguished by the differential expression of CD25 and CD44, thymocytes rearrange the gene for the TCRβ chain which pairs with a surrogate pre-TCRα chain. Expression of the resulting pre-TCR together with the

signal transducing CD3 complex leads to cell proliferation and the expression of CD4 and CD8. Due to their expression of both CD4 and CD8, these cells are called double positive (DP) thymocytes.

DP thymocytes rearrange the TCRα chain locus and start to express the complete TCR consisting of the two TCR chains and the CD3 complex. They move deeper into the thymic cortex where they are positively selected on cortical thymic epithelial cells (TEC). In this selection process, thymocytes with TCR complexes that are able to bind to self-Ag presented on MHC molecules with adequate affinity receive a survival signal. DP cells that are positively selected on MHC class II molecules stop to express CD8 and become $CD4^+$ cells, while cells positively selected on MHC class I molecules mature into $CD8^+$ cells. Developing thymocytes that cannot bind to Ag/MHC or bind with only very low affinity die by apoptosis and are rapidly phagocytosed (feeney et al., 1994; Mizuochi et al., 1992). Thymocytes that bind Ag/MHC with too high affinity become apoptotic by negative selection in the medulla with the help of medullary TEC which ensures tolerance to self-peptides (Petrie et al., 1990; Saintruf et al., 1994; Shortman and Wu, 1996). To enable T cell tolerance to a wide variety of self-Ag, the transcription factor (TF) autoimmune regulator (AIRE) allows medullary TEC to express proteins specific for other tissues in the thymus. Thereby, autoreactive T cells against various tissue Ag can be deleted in the thymus (Derbinski et al., 2001). Mature T cells that have accomplished the stringent selection processes in the thymus are released into secondary peripheral lymphatic tissues.

1.1.3 T-cell mediated immune responses

The process of somatic recombination leads to a large TCR variablity and inevitably to a small number of T cells with a particular TCR specificity. During an immune response against a certain pathogen, T cells with the required specificity are activated to proliferate and differentiate. Specialized APC like B cells, macrophages and most importantly DC are able to activate T cells (Inaba et al., 1990). Immature DC reside in tissues where they are in contact with invading pathogens, *e.g.* in the skin, in the

respiratory tract, in the blood and in the lymphatic system (Maldonado-Lopez and Moser, 2001). Detection of PAMP *via* various receptors (*i.a*. Fc- and complement receptors or TLR) by DC stimulates them to mature and migrate to lymphatic organs (Guermonprez et al., 2002; Janeway and Medzhitov, 2002; Matzinger, 1994; Caux et al., 1994). Mature DC which express high levels of MHC and co-stimulatory B7 family members (mostly CD80 and CD86) efficiently stimulate naïve T cells by the presentation of pathogenic peptide fragments complexed with MHC molecules (Trombetta and Mellman, 2005). An intracellular signaling cascade in the T cell upon binding of the TCR to Ag/MHC leads to activation and proliferation (Gascoigne and Zal, 2004; van Leeuwen and Samelson, 1999). For naïve T cells, the TCR signal must be complemented with a co-stimulatory signal delivered by CD28 on T cells upon interaction with CD80 and CD86 on DC. In the absence of this second signal, ligation of the TCR leads to T cell inactivation and anergy (Greenwald et al., 2005; Macian et al., 2004).

T cells can be grouped into $CD8^+$ cytotoxic T cells and $CD4^+$ T cells. Depending on the type of pathogen and the site of the immune response, $CD4^+$ T cells differentiate into distinct T cell subsets. The two major categories of $CD4^+$ T helper cells, characterized by their cytokine profile, are T helper 1 (Th1) and Th2 cells. Th1 cells support $CD8^+$ T cells to eliminate infected cells while Th2 cells provide help to B cells for the production of antibodies (Abbas et al., 1996). In the intestine, pro-inflammatory Th17 cells are the major $CD4^+$ T cell subset (Bettelli et al., 2008). In addition, a small percentage of $CD4^+$ T cells form the regulatory T cell (Treg) lineage. This T cell subset will be discussed in detail in chapter 1.3.

1.2 Programmed cell death

The first observations that described cell death were made 200 AD by Galenus Galen who characterized the regression of larval and foetal structures in the course of ontogeny (Barclay et al., 1944). Several hundred years later, in the middle of the 19th century, Carl Vogt discovered during his investigations of the obstetrical toad that

cells undergo programmed death (Vogt, 1842). Then, in the 20th century, inspired by the research of the embryologist Glücksmann on dying embryonic cells, Kerr, Wyllie and Currie first formulated the concept of "programmed cell death" and coined the term apoptosis (greek: *apo* - from, *ptosis* – falling) (Glücksmann, 1965; Kerr et al., 1972). Besides apoptosis, several other types of cell death are known. Necrosis, for instance, is a type of cell death that results from acute cellular injury and is accompanied by an inflammatory immune reaction and tissue destruction. Autophagy in contrast is a slow phenomenom characterized by the massive accumulation of cytoplasmic vacuoles (Galluzzi et al., 2007). In addition, also mixed forms of cell death exist. For example, although apoptosis and necrosis are different in their mode of action, there is evidence that both processes can be connected: the blockade of apoptotic signaling cascades can lead to necrotic cell death in certain scenarios (Vercammen et al., 1998).

1.2.1 Apoptosis

Together with cell proliferation, the mechanism of apoptosis provides the basis for the maintenance of tissue homeostasis in multicellular organisms. It plays a decisive role in development, proliferation and differentiation, *e.g.* during ontogeny, for the immune system and in the control of tumors (Krammer, 2000; Los et al., 1999; Vaux and Korsmeyer, 1999). It occurs when a cell is damaged beyond repair, virally infected or undergoing stress, such as starvation.

Apoptosis is a strictly regulated cellular process, during which catabolic enzymes like proteases (*e.g.* cysteine-aspartic acid proteases (caspases), see below) and nucleases are activated. Cells undergoing apoptosis show characteristic morphological and biochemical features: during early apoptosis, cell shrinkage can be observed and the cell membrane starts to build protuberances caused by a loss of membrane integrity (a process called zeiosis). In the course of the apoptotic process, the cell membrane loses its asymmetry and lipids of the inner membrane layer, *e.g.* phosphatidylserine, are exposed on the cell surface. In parallel, chromatin condenses in the periphery of the nucleus. In most forms of apoptosis the DNA is degraded by special endonucleases in

the condensed chromosomes. This DNA degradation can be visualized by gel electrophoresis as DNA ladder, which arises from internucleosomal cleavage into single or multiple nucleosomal units of 200 base pairs (Wyllie et al., 1980). In a final step, membrane bound vesicles, so-called apoptotic bodies, which contain fragments of nucleus, cytoplasm and mitochondria, are released from the dying cell. Within a short period of time, apoptotic bodies are recognized by phagocytic cells, are taken up and degraded. This prevents the release of cytoplasmic constituents which would otherwise lead to inflammatory reactions (Savill et al., 1993).

1.2.2 CD95 and other death receptor family members

The discovery of CD95 (APO-1/Fas) as target molecule for monoclonal antibodies which induced apoptosis in distinct cell lines led to the first description of an apoptosis-inducing cell surface molecule (Yonehara et al., 1989; Trauth et al., 1989; Itoh and Nagata, 1993; Oehm et al., 1992). The death receptor CD95 is a differentially glycosylated type I transmembrane protein with a molecular mass of 42 to 52 kDa which is expressed by most mammalian tissues (Leithauser et al., 1993; Watanabefukunaga et al., 1992b). CD95 belongs to the tumor necrosis factor/nerve growth factor (TNF/NGF) receptor superfamily, which is involved in the control of biological processes like differentiation, activation, proliferation and apoptosis (Fig. 1.2.1) (Locksley et al., 2001).

Besides CD95, seven other death receptors have been described in the death receptor subfamily thus far: TNF receptor 1 (TNF-R1/CD120a), DR3 (APO-3), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1/DR4/APO-2), TRAIL-R2 (DR5), DR6, ectodysplasin A receptor (EDA-R) and nerve growth factor receptor (NGF-R) (Fig. 1.2.1) (Askenasy et al., 2005; Bodmer et al., 2002; Schulze-Osthoff et al., 1998). The death receptors possess 2-4 extracellular cysteine-rich motives, a transmembrane region and an intracellular death domain (DD) which is essential for the stimulation-dependent induction of apoptosis (Itoh and Nagata, 1993; Tartaglia et al., 1993). In addition to the death receptors, four decoy receptors (DcR) have been found: DcR3, TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and Osteoprotegrin (OPG) (Fig. 1.2.1). They cannot induce apoptosis and therefore inhibit the activation of death receptors by competitive ligand binding (Sheridan et al., 1997).

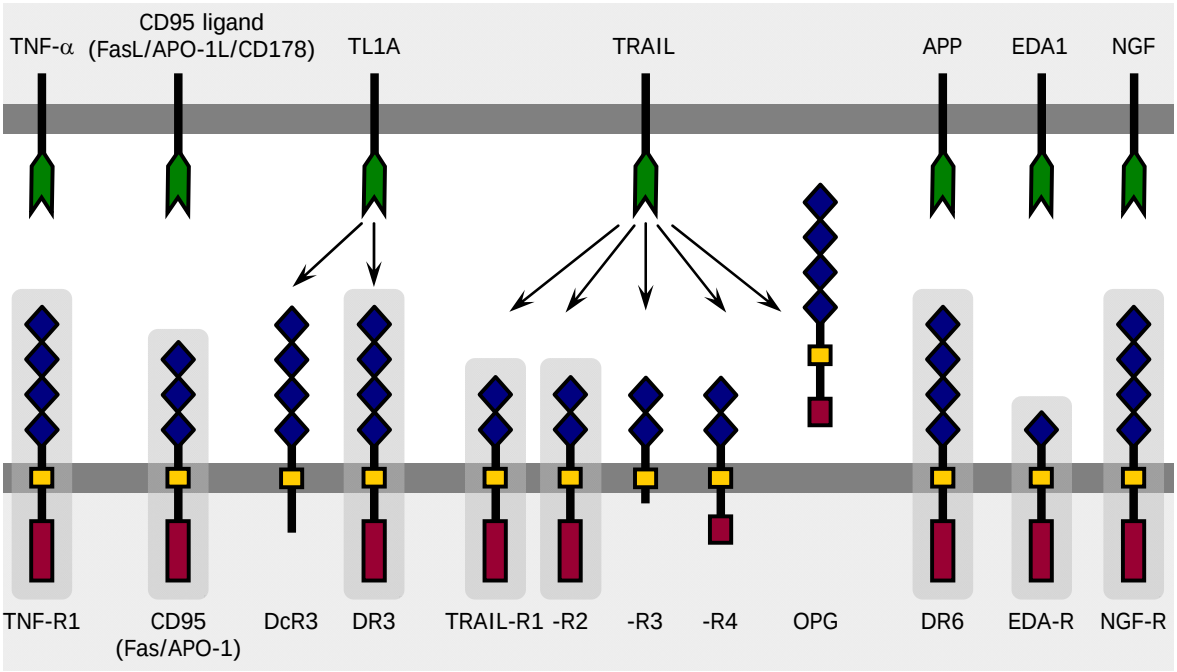

Figure 1.2.1 | Death receptors, decoy receptors and their ligands. Members of the subfamily of death receptors (highlighted by gray boxes) are characterized by 2-4 cysteine rich domains (blue), a transmembrane domain (yellow) and a cytoplasmic DD (red). Decoy receptors do not have a DD and cannot transduce the apoptotic signal but compete for ligand binding. The ligands for the death (and decoy) receptors are transmembrane proteins which mediate apoptosis by binding to their specific receptor [adapted from (Lavrik et al., 2005; Nikolaev et al., 2009)].

1.2.3 The death-inducing ligand CD95L

The ligands of the death receptors, with the exception of NGF, also belong to the TNF superfamily. The physiological ligand of the death receptor CD95, CD95 ligand (CD95L/APO-1L/FasL/CD178), is a glycosylated type II transmembrane protein with a molecular mass of 40 kDa (Nagata, 1994). Unlike CD95 which is ubiquituously expressed, the expression of CD95L is restricted to activated T cells, B cells and NK cells, as well as to some immune privileged non-lymphoid organs like the anterior chamber of the eye (Griffith et al., 1995; Kabelitz et al., 1993; Newell and Desbarats, 1999; Suda et al., 1993). Furthermore, the expression of CD95L was shown in different neoplastic cells (Hahne et al., 1996; Strand et al., 1996). Also non-lymphatic tumors can express CD95L by which they circumvent anti-tumor immune responses mounted by T cells (Green and Ware, 1997). CD95L is expressed in three different forms: (1) membrane-bound on the cell surface, (2) membrane-bound in intracellular microvesicles which are released into the extracellular space by different physiological stimuli, and (3) in a soluble form by matrix metalloprotease-mediated cleavage from the cell surface (Tanaka et al., 1998; Schulte et al., 2007; Mariani et al., 1995; Kayagaki et al., 1995; Albanese et al., 1998). Recently, it was shown that the metalloprotease a disintegrin and metalloprotease-10 (ADAM-10) plays a major role in the cleavage of CD95L (Schulte et al., 2007).

The membrane-bound form of CD95L has a high efficiency in the induction of apoptosis in mouse and man, while the soluble from has reduced apoptotic potential in humans and does not show any apoptotic function in mice (Hohlbaum et al., 2000; Schneider et al., 1998; Tanaka et al., 1995; Reilly et al., 2009). Spontaneous aggregation or association with extracellular matrix proteins is supposed to mediate the pro-apoptotic effect of soluble CD95L (sCD95L) while the ineffectiveness of sCD95L homotrimers to aggregate CD95 might result in the non-apoptotic effect (Aoki et al., 2001; Chen and Wilson, 1998; Hohlbaum et al., 2000; Schneider et al., 1998; Suda et al., 1993; Tanaka et al., 1995; Tanaka et al., 1996). In addition, a chemotactic function as

well as a function as TF of CD95L has been shown (Kirkin et al., 2007; Seino et al., 1998; Ottonello et al., 1999)

1.2.4 Induction of apoptosis

Apoptosis can be triggered *via* two fundamentally different pathways, the extrinsic (receptor-mediated) and the intrinsic (mitochondria-dependent) signaling cascade (Nagata, 1997; Green and Reed, 1998; Green, 1998). Both pathways activate the effector caspase 3 that eventually activates death substrates like caspase-activated DNase (CAD) by cleavage of the inhibitor of CAD (iCAD). CAD cleaves DNA into the characteristical 200 base pair long DNA fragments.

1.2.4.1 The extrinsic – receptor-mediated – pathway

The extrinsic signaling pathway is initiated by the activation of a death receptor through binding of its extracellular ligand or agonistic antibodies (Fig. 1.2.2) (Trauth et al., 1989; Yonehara et al., 1989; Kischkel et al., 1995; Bodmer et al., 2002). This receptor-mediated induction of apoptosis plays a major role in development, differentiation and in the homeostasis of the immune system and constitutes a part of the effector function of cytotoxic lymphocytes (Krammer, 2000). Stimulation of CD95 leads within seconds to the formation of a multiprotein complex, the death-inducing signaling complex (DISC). The CD95 DISC is composed of oligomerized (most probably trimerized) CD95 molecules, the adaptor molecule Fas-associated death domain-containing protein (FADD) and the two splice variants of the proform of caspase 8α and 8β. Caspases are proteolytic enzymes that cleave substrates at the carboxyterminal end of aspartic acid *via* a cysteine in their active center (Cohen, 1997; Villa et al., 1997; Raff, 1998; Hengartner, 2000). The CD95 DISC can also contain isoforms of cellular FLICE inhibitory protein (cFLIP) that either fulfil pro- or anti-apoptotic function (Yu and Shi, 2008). In addition, procaspase 2 and procaspase 10 can be present in the DISC (Sprick et al., 2002; Lavrik et al., 2006). Caspase 8 is activated

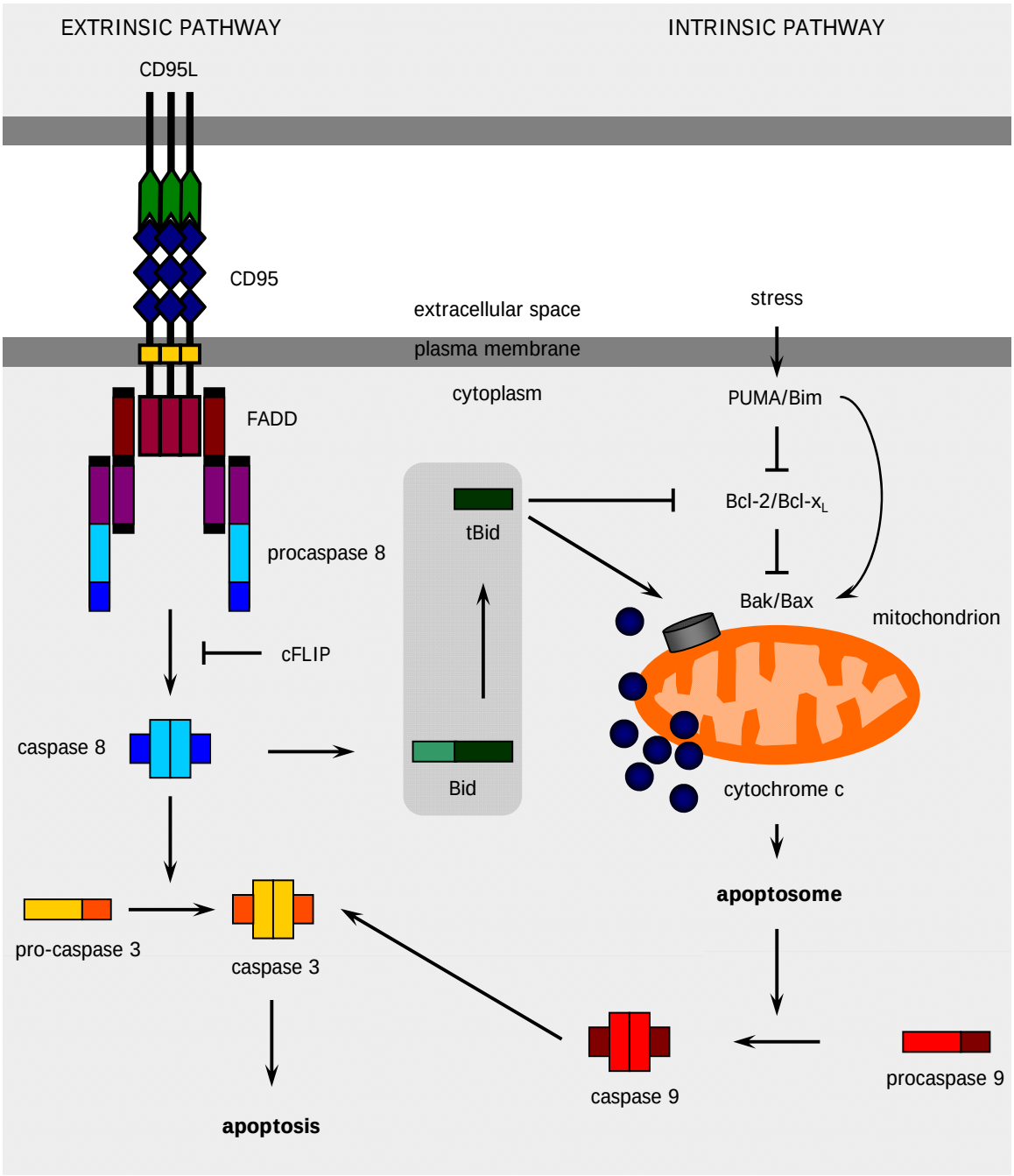

Figure 1.2.2 | The extrinsic and intrinsic apoptosis pathway. Binding of CD95L to CD95 initiates the extrinsic apoptosis pathway. CD95, FADD and caspase 8 form the DISC at which procaspase 8 is activated. Caspase 8 then activates caspase 3 by cleavage, eventually resulting in apoptosis. The intrinsic pathway triggered by stress stimuli is initiated by activation of Bim. Bim inhibits Bcl-2/Bcl-x_L which leads to the activation of Bak and Bax. These molecules trigger the release of cytochrome c from mitchondria which in turn is recruited into the apoptosome. Caspase 9 is activated in the apoptosome and finally activates caspase 3 and induces apoptosis. The generation of tBid (gray inset) by low caspase 8 activity in type II cells triggers the intrinsic pathway for the propagation of the apoptotic signal [adapted from (Bouillet and O'Reilly, 2009)].

in this multiprotein complex in an autoproteolytic fashion which triggers a caspase cascade in the cytosol (Lavrik et al., 2003). These events result in the cleavage of cellular substrates and initiate cell death (Fig. 1.2.2) (Krammer, 2000; Peter and Krammer, 2003; Yang et al., 1998).

The CD95 signal transduction differs between cell types. Based on the amount of DISC formation, cells can be divided into type I and type II (Scaffidi et al., 1998; Scaffidi et al., 1999). Due to strong DISC activity, processed caspase 8 in type I cells can directly activate effector caspases like caspase 3 to trigger the execution of apoptosis (Scaffidi et al., 1999). Type II cells have less DISC activity resulting in low caspase 8 activation. In these cells, the CD95 DISC needs an amplification loop involving mitochondria for the induction of apoptosis (see below) (Scaffidi et al., 1998; Scaffidi et al., 1999).

1.2.4.2 The intrinsic – mitochondria-dependent – pathway

The intrinsic signaling pathway is activated by intra- or extracellular stimuli – like oxidative stress, irradiation, DNA damage or cytokine deprivation – resulting in the permeabilization of mitochondria and cell death (Ferri and Kroemer, 2001; Erlacher et al., 2005; Takahashi et al., 2004; Kroemer et al., 2007). Key molecules in the permeablization of mitochondria are pro- and anti-apoptotic proteins of the B cell lymphoma 2 (Bcl-2) family. Eponym of this family is the oncogene Bcl-2 which was originally identified in follicular B cell lymphoma as gene dysregulated by chromosomal translocation (Tsujimoto et al., 1985).

Stress stimuli lead to the activation of p53-upregulated modulator of apoptosis (PUMA) or Bcl-2-interacting mediator of cell death (Bim) (Bouillet et al., 1999; Jeffers et al., 2003; Villunger et al., 2003). In contrast, in a potential cross-talk between the extrinsic and intrinsic pathway, BH3-interacting domain death agonist (Bid) is cleaved by the relatively low activity of caspase 8 in type II cells to its truncated form tBid (Fig. 1.2.2) (Yin et al., 1999). Activated PUMA, Bim and tBid inactivate pro-survival Bcl-2 family members like Bcl-2 and Bcl-x_L. This liberates the pro-apoptotic family members Bcl-2-antagonist/killer (Bak) and Bcl-2-associated X protein (Bax) resulting in

disruption of the mitochondrial outer membrane potential and the formation of pores in the membrane (Gross et al., 1999; Chao and Korsmeyer, 1998; Green and Kroemer, 2004; Kroemer et al., 1997; Petit et al., 1996). Here, cytochrome c is released from mitochondria into the cytosol where it binds to apoptotic protease-activating factor 1 (Apaf-1) and ATP. Together with procaspase 9, these molecules create a protein complex known as apoptosome (Zou, 2006; Cain et al., 2000). Procaspase 9 is cleaved into its active form at the apoptosome which in turn results in cleavage and activation of the effector caspase 3 (Fig. 1.2.2).

1.2.5 The role of CD95/CD95L in the immune system

The discovery of several mutant mouse strains with defects in the CD95/CD95L system revealed the important role of CD95 in the immune system. The mouse strain lymphoproliferation (lpr) has a recessive autoimmune phenotype which arises through insertion of a transposon into the CD95 gene (Watanabefukunaga et al., 1992a; Adachi et al., 1993; Rieux-Laucat et al., 2003). This results in decreased transcription and an almost complete lack of CD95 expression. The lpr^{cg} mutation, in contrast, is a point mutation in the DD of CD95 that abrogates signal transmission (Matsuzawa et al., 1990). A point mutation in the CD95L gene prevents the interaction of the ligand with its receptor CD95 in generalized lymphoproliferative disease (gld) mice (Roths et al., 1984). All three mouse strains develop lymphadenopathy, splenomegaly, hypergammaglobulinemia and nephritis among other autoimmune symptoms and display a characteristic accumulation of aberrant $CD3^+$ $B220^+$ $CD4^-$ $CD8^-$ T lymphocytes which might be derived from repeatedly activated T cells (Krammer, 2000; Nagata and Suda, 1995).

In 1995, a mutation in the CD95 gene in a human patient linked the autoimmune lymphoproliferative syndrome (ALPS) with a dysregulation of the CD95/CD95L system (Rieuxlaucat et al., 1995; Fisher et al., 1995). Until now, mutations of CD95 (ALPS type Ia), CD95L (type Ib) and caspase 10 (type II) which can result in ALPS have been described (Fisher et al., 1995; Rieuxlaucat et al., 1995; Jackson et al., 1999;

Lenardo et al., 1999; Straus et al., 2001; Del Rey et al., 2006; Wang et al., 1999). Yet unknown reasons are responsible for ALPS type III (Ten Bosch et al., 2001). Children suffering from ALPS show lymphadenopathy, elevated numbers of aberrant T cells and autoimmunity which resolves with age.

1.2.6 CD95/CD95L-induced apoptosis in peripheral T cells

The complex functions of the immune system regulate survival and death of T cells by finely tuned processes. Ag-specific T cells expand during an acute immune response and are removed after clearance of the pathogen except for a few cells which will give rise to Ag-specific memory T cells (Straus et al., 2001). The phenotype of lpr and gld mice points to a major role for the CD95/CD95L system and indeed CD95-mediated apoptosis represents an important mechanism in the regulation of T cell homeostasis, tolerance to self-Ag and autoimmunity (Askenasy et al., 2005).

Depending on their activation status, T cells display resistance or sensitivity towards CD95-mediated apoptosis. Naïve and resting T cells are resistant to CD95-induced apoptosis, despite small amounts of CD95 expression (Klas et al., 1993; Krueger et al., 2003b). After stimulation with Ag, naïve T cells acquire effector functions by clonal expansion, enhance the expression of CD95 but remain apoptosis resistant (Inaba et al., 1999). This resistance of activated T cells in the early stimulatory phase is caused by reduced DISC formation and a high expression of Bcl-x_L. Repeatedly activated T cells become sensitive towards apoptosis by TCR re-stimulation-induced CD95L expression. Here, CD95L triggers AICD by binding to CD95 on the surface of the cell itself (suicide) and on neighbouring cells (fratricide) (Dhein et al., 1995; Ju et al., 1995; Klas et al., 1993; Singer and Abbas, 1994; Alderson et al., 1995; Brunner et al., 1995). Memory T cells in contrast are protected from CD95-mediated apoptosis by the elevated expression of cFLIP and the anti-apoptotic molecules Bcl-2 and Bcl-x_L (Krueger et al., 2003b).

Initial studies in mice pointed to a scenario in which the pool size of activated T cell clones during immune responses is controlled and regulated by the CD95/CD95L

system. However, the induction of CD95L on T cells undergoing AICD requires TCR re-stimulation with Ag that is not present *in vivo* after the decay of the immune response, as the pathogen has been cleared. The crucial parameter for T cell apoptosis is the decline of survival signals like cytokines (*e.g.* IL-2) and growth factors which results in activated T cell autonomous death (ACAD) (Straus et al., 1999; Brenner et al., 2008; Strasser et al., 2009). It was shown that Bim plays a major role in ACAD by the activation of the intrinsic apoptosis pathway (Erlacher et al., 2005; Hildeman et al., 2002a; Sandalova et al., 2004; Willis et al., 2007). While Bim is essential for the deletion of T cells by ACAD *in vivo* after stimulation with super-Ag or challenge with Herpes Simplex Virus, CD95 is dispensable (Hildeman et al., 2002b; Strasser and O'Connor, 1998; Pellegrini et al., 2003). However, during chronic immune responses where pathogenic Ag persists to stimulate already activated T cells repeatedly, it was demonstrated that killing of activated T cells requires not only Bim but also CD95/CD95L (Hughes et al., 2008; Hutcheson et al., 2008; Strasser, 1995; Weant et al., 2008).

1.2.7 *In vitro* cell death model for T cells

For the investigation of CD95-induced apoptosis, an *in vitro* model for experiments with T cells has been established. Freshly isolated naïve (day 0) T cells are characterized by their resistance to apoptosis. They have low expression of CD95 and stimulation with a CD95L construct or plate-bound (pb) anti-CD3 monoclonal antibody (mAb) that activates the TCR does neither induce apoptosis nor AICD, respectively (Fig. 1.2.3). By expansion of day 0 T cells with anti-CD3 and anti-CD28 mAb in IL-2 containing medium for 6 days, these cells are converted into apoptosis-sensitive day 6 T cells. During culture, they upregulate the expression of CD95 and upon TCR re-stimulation, *e.g.* by pb anti-CD3 mAb they express high amounts of CD95L. Apoptosis induced by binding of endogenous CD95L to CD95 on day 6 T cells is referred to as AICD while stimulation with an artificial CD95L construct leads to CD95-induced apoptosis (Fig. 1.2.3) (Klas et al., 1993; Schmitz et al., 2003).

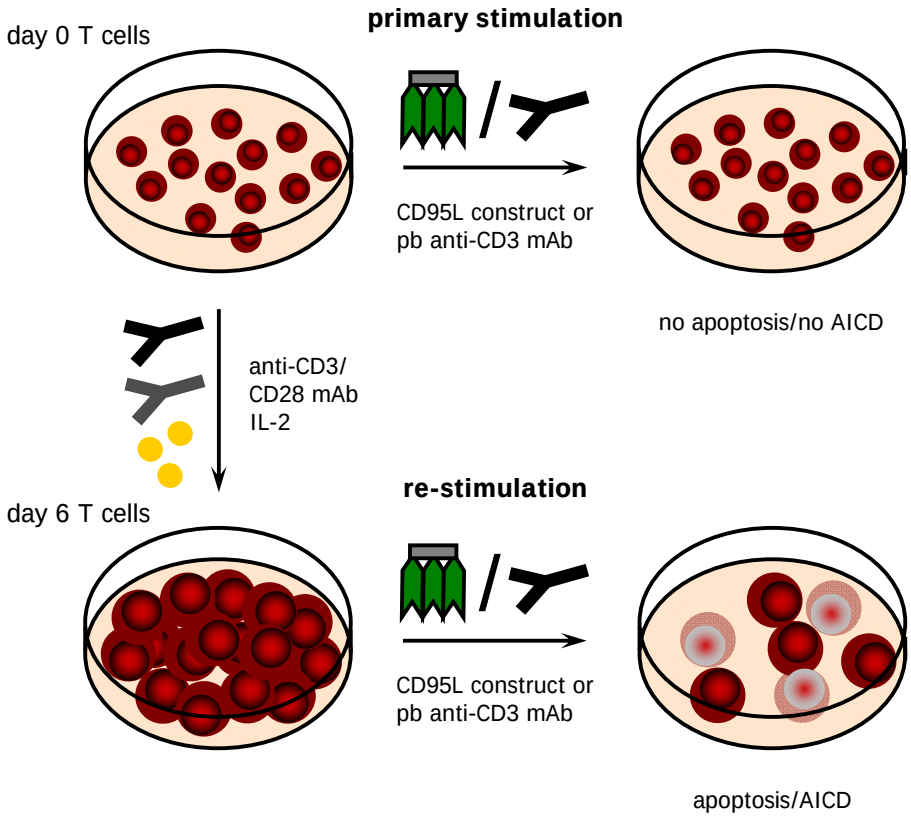

Figure 1.2.3 | Cell death model used for *in vitro* experiments with T cells. Day 0 T cells stimulated with a CD95L construct or pb anti-CD3 mAb do not become apoptotic. The expansion of day 0 T cells with anti-CD3/CD28 mAb and IL-2 results in apoptosis-sensitive day 6 T cells. Direct CD95 stimulation (with the CD95L construct) or TCR re-stimulation (with pb anti-CD3 mAb) induces apoptosis or AICD, respectively [adapted from (Strasser and Pellegrini, 2004)].

1.3 Regulatory T cells

In 1970, Gershon and Kondo reported tolerance induction to a particular Ag in mice when thymic cells were present during Ag encounter and challenge, while their presence only during challenge resulted in immunity (Gershon and Kondo, 1970). Thus, a thymus-derived cell subset seemed to mediate immune tolerance through the suppression of other cells. In 1971, two individual reports by Gershon and Droege attracted more attention to this newly defined population of suppressor T cells (Gershon and Kondo, 1971; Droege, 1971). Subsequent studies showed that mice, thymectomized 2-4 days after birth, displayed T cell-mediated lesions which could be alleviated by the transfer of thymocytes or splenocytes (Sakaguchi et al., 1982b; Sakaguchi et al., 1982a). This pointed to a population of cells generated in the mouse thymus between day 2 and 4 capable of mediating immune tolerance in a dominant, cell-extrinsic manner. However, due to skepticism caused by the lack of specific molecular markers and difficulties in isolation and culture, research on suppressor T cells was discontinued (Moller, 1988; Janeway, 1988).

Several years later, one major finding initiated the comeback of suppressor T cells. Sakaguchi and colleagues discovered that the interleukin-2 (IL-2) receptor α chain, CD25, could serve as a phenotypic marker for suppressive $CD4^+$ T cells. Transfer of CD25-depleted $CD4^+$ T cells into congenic nude mice resulted in autoimmune disease while transfer of total $CD4^+$ T cells did not (Sakaguchi et al., 1995a). A similar population of suppressor cells was also found in humans and this $CD4^+CD25^+$ T cell subset was named Treg (Taams et al., 2001a; Dieckmann et al., 2001; Jonuleit et al., 2001; Levings et al., 2001; Baecher-Allan et al., 2001).

In 2001, another important finding added to the field. Autoimmune Scurfy mice and patients suffering from immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome were found to have mutations in the gene of forkhead box P3 (Foxp3) (Wildin et al., 2001; Brunkow et al., 2001; Bennett et al., 2001). This TF was reported as the master transcriptional regulator for naturally occurring Treg (nTreg).

1.3.1 Development and homeostasis of Treg

Day 3-thymectomy and deletion of CD25-expressing cells in $CD4^+$ SP thymocytes induces autoimmunity in mice caused by the absence of peripheral Treg (Sakaguchi et al., 1995b; Sakaguchi et al., 1996). This proved that Treg develop in the thymus in a delayed fashion compared to $CD4^+CD25^-$ Tcon.

Thymic Treg differentiation (defined by Foxp3 induction) requires temporally and spatially separated signals (Fig. 1.3.1) (Josefowicz and Rudensky, 2009).

(1) Strong TCR stimulation by a high binding affinity to self-Ag/MHC on TEC or DC is needed for thymic Treg differentiation (Jordan et al., 2001; Hsieh et al., 2004; Apostolou et al., 2002). One example are recombinase activating gene 2 $(RAG2)^{+/+}$ TCR-transgenic (tg) mice in which Treg develop dependent on endogenous TCR rearrangement not using the tg TCR (Olivares-Villagomez et al., 1998). In $RAG2^{-/-}$ TCR-tg animals in contrast, Treg are generated only when the mice co-express the cognate ligand (Kawahata et al., 2002; Jordan et al., 2001).

(2) CD28 signaling triggered by CD80 and CD86 expressed on APC is indispensable for Treg development. A marked decrease in Treg frequencies is observed in CD28-deficient and CD80/CD86-deficient mice (Salomon et al., 2000; Tai et al., 2005).

(3) Common γ chain (cγ) signaling (mostly IL-2) is crucial for Treg differentiation. Mice lacking IL-2, IL-7 and IL-15 or cγ are almost completely devoid of Treg (Vang et al., 2008; Malek, 2008; Burchill et al., 2007). All together, the strong TCR signal results in the upregulation of CD25, raising the responsiveness of Treg precursor cells to IL-2 signals that result in the induction of Foxp3 (Burchill et al., 2007; Lio and Hsieh, 2008).

In the periphery, the factors controlling the homeostasis of Treg are largely unknown, certain is the dependence of Treg on IL-2 produced by effector T cells (Teff), TGF-β and CD28 costimulation (Fig. 1.3.1) (Malek, 2008).

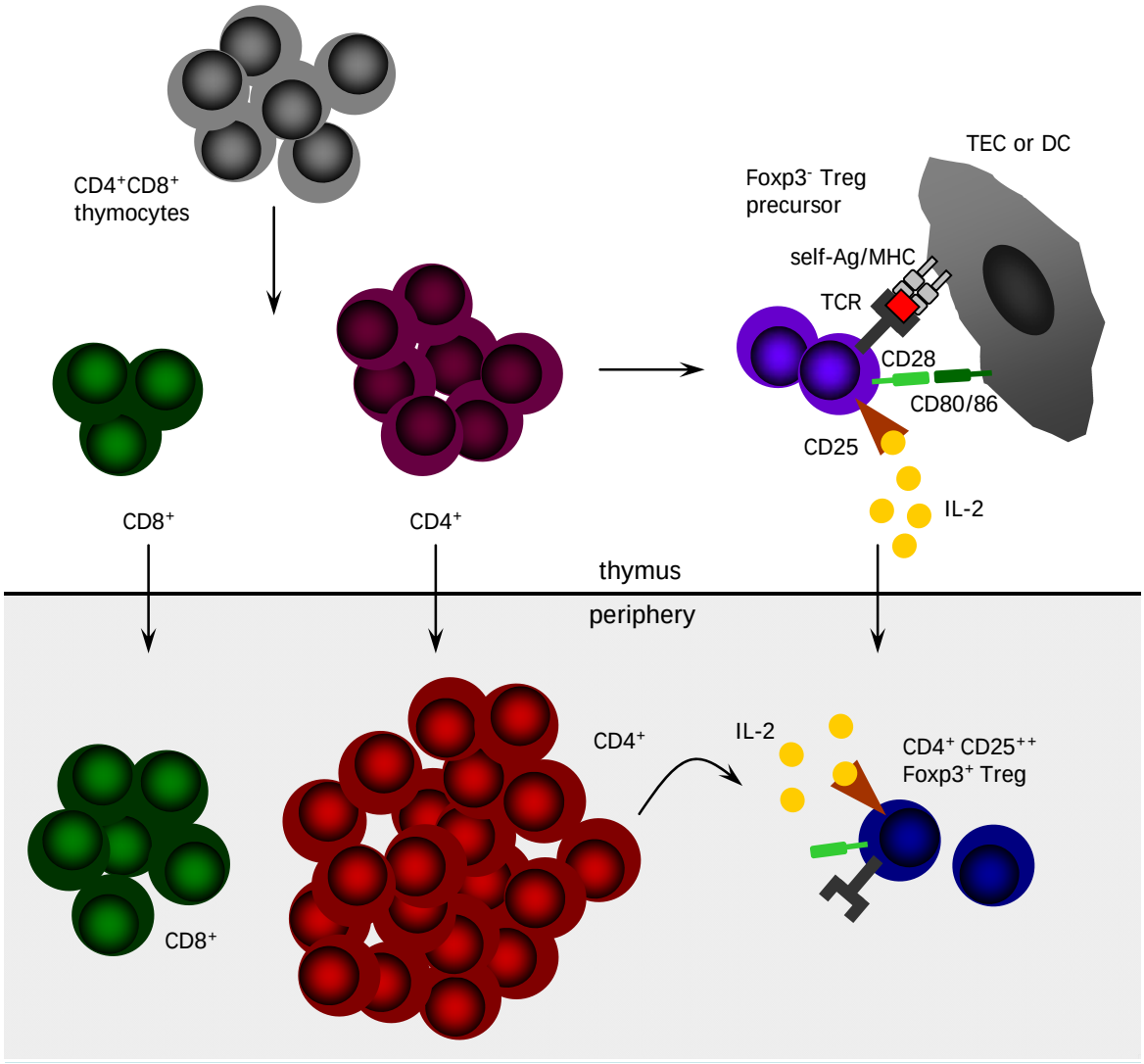

Figure 1.3.1 | Signals required for the development and maintenance of Treg. The process of Foxp3$^+$ Treg differentiation from Foxp3$^-$ CD4$^+$ thymocytes requires (1) strong TCR stimulation by self-Ag/MHC, (2) CD28 signaling triggered by CD80/86 on TEC or DC and (3) IL-2 and other γc cytokine receptor signaling. In the periphery, Treg homeostasis is mainly dependent on IL-2 produced by Teff [adapted from (Josefowicz and Rudensky, 2009)].

1.3.2 Treg lineages

In the periphery, maintenance of tolerance and prevention of autoimmunity is not only mediated by thymus-derived nTreg but also by various populations of induced Treg (iTreg) (Josefowicz and Rudensky, 2009; de Lafaille and Lafaille, 2009). iTreg can be experimentally induced or can be converted from T cells during inflammatory processes in peripheral tissues (de Lafaille and Lafaille, 2009). Different subsets of iTreg exist which can be divided into Foxp3$^+$ adaptive Treg (aTreg) and Foxp3$^-$ T regulatory 1 (Tr1) cells as well as Foxp3$^-$ T helper 3 (Th3) cells which all display suppressive capacities.

Suboptimal TCR stimulation or withdrawal of TCR signaling after short stimulation periods appear to be required for the generation of suppressive aTreg (Apostolou and von Boehmer, 2004; Sauer et al., 2008). Murine as well as human T cells can be converted into regulatory cells in the presence of TGF-β and IL-2 (Yamagiwa et al., 2001; Kretschmer et al., 2005; Chen et al., 2003).

Foxp3$^-$ suppressive Tr1 cells can be converted from naïve T cells in the presence of Ag and high amounts of IL-10 and are themselves extensive producers of IL-10 and TGF-β but produce only minimal amounts of IFN-γ and IL-5 (Shevach, 2006). The Ag-specific suppression exerted by Tr1 cells seems to be mediated by IL-10 *in vitro* as well as *in vivo* (O'Garra and Vieira, 2004). Ag-specific Foxp3$^-$ Th3 cells can be induced in the context of oral tolerance by Ag feeding *in vivo* (Weiner, 2001). *In vitro* generation of these cells is achieved by the addition of TGF-β, IL-4, IL-10 and anti-IL-12 mAb to the culture medium. Th3 cells release significant amounts of TGF-β and small amounts of IL-4 and IL-10 and can suppress Ag-specific Teff. Immunosuppressive drugs as well as immature DC can also be used for the generation of suppressive Foxp3$^-$ Tr1 or Th3 cells (Barrat et al., 2002; Jonuleit et al., 2000; Weiner, 2001). *In vivo*, Foxp3$^-$ suppressive cell types are mainly found in the intestine (Weiner, 2001).

However, thus far it is neither known to which extent iTreg contribute to peripheral tolerance nor which percentage of the peripheral Treg pool they comprise (Chen et al., 2003).

1.3.3 Characteristics of Treg

Thymus-derived nTreg comprise 1-3% of the $CD4^+$ T cell population in humans and 5-10% in mice. There is no surface marker that uniquely identifies human and murine Treg, but Treg express a distinct set of cell surface proteins compared to non-stimulated Tcon. Constitutive expression of CD25 is restricted to Treg in non-immunized mice while in humans, only cells that highly express CD25 designate Treg whereas activated Tcon acquire intermediate levels of CD25 (Sakaguchi et al., 1995b; Baecher-Allan et al., 2001). Together with CD25, cytotoxic T lymphocyte antigen-4 (CTLA-4) and glucocorticoid-induced TNF receptor (GITR) have been candidates for Treg markers as they both play a major role in the development of suppressive properties (Mchugh et al., 2002; Read et al., 2000; Shimizu et al., 2002). However, due to the fact that also activated Tcon upregulate CTLA-4 and GITR these are no longer regarded as sufficient for the explicit identification of Treg. Of the numerous other markers that have been reported to be on the surface of Treg, two recently discussed molecules should be mentioned. One is CD127, the α chain of the IL-7 receptor, and the other CD49d, the integrin α4 chain. CD127 is expressed by almost all $CD4^+$ T cells except most $CD25^+$ Treg while CD49d is present on activated T cells but not on Treg (Seddiki et al., 2006a; Liu et al., 2006; Kleinewietfeld et al., 2009; Hartigan-O'Connor et al., 2007).

1.3.4 Foxp3 – a Treg-specific transcription factor

At present, the most specific marker for the characterization of Treg is the TF Foxp3 whose discovery was achieved by the investigation of the scurfy mouse and human IPEX patients. The X chromosome-encoded Foxp3 gene belongs to the winged/helix family of TF whose members are crucial in development, metabolism, cancer and aging, and important in the immune system (Brunkow et al., 2001; Ziegler, 2006). Male scurfy mice harbor a mutation in Foxp3 which leads to severe autoimmune disease and causes death within 3-4 weeks after birth. A detailed analysis of lymphocyte populations showed that the animals suffer from hyperproliferative $CD4^+$ T cells

and from a lack of $CD4^+CD25^+$ Treg (Hori et al., 2003; Fontenot et al., 2003; Ziegler, 2006). Amelioration of the symptoms could be achieved by adoptive transfer of $CD4^+CD25^+$ T cells from wild type (wt) animals. Similar to scurfy mice, hemizygous male IPEX patients display massive inflammatory processes. The disease is treated with strong immunosuppressive drugs or even BM transplantation (Bennett et al., 2001; Wildin et al., 2001). Until now, 20 mutations in the Foxp3 gene were found to be responsible for the IPEX syndrome (Bacchetta et al., 2006).

In wt mice, up to 90% of Treg express Foxp3, while resting and activated naïve $CD4^+$ T cells do not show detectable levels. Analogous to mice, also the majority of *ex vivo* sorted human $CD4^+CD25^{++}$ Treg express Foxp3. However, while Foxp3 cannot be detected in mouse Tcon, human activated $CD4^+$ non-Treg transiently express intermediate levels of Foxp3 which can go along with suppressive function (Wang et al., 2007; Walker et al., 2003; Gavin et al., 2006).

The investigation of Foxp3-deficient Treg showed that although these cells lose their suppressive capacity *in vitro* and *in vivo* and can produce Teff cytokines, they largely retain their genetic signature and do not induce autoimmunity upon transfer into lymphopenic hosts (Gavin et al., 2007; Lin et al., 2007; Lahl et al., 2009; Kuczma et al., 2009). Interestingly, negative selection of thymocytes by a high-affinity TCR ligand is not affected by the presence or absence of Foxp3 (Hsieh et al., 2006; Chen et al., 2005). In addition, activated T cells in Foxp3-deficient mice use TCR sequences similar to Foxp3-sufficient Treg (Hsieh et al., 2006; Chen et al., 2005). These two findings indicate that T cell precursors supporting Treg differentiation are not deleted in the absence of Foxp3. Together with data showing that Foxp3-negative Treg numbers gradually decrease in $Foxp3^{+/-}$ female mice (while Foxp3-sufficient Treg numbers do not), these results imply that Foxp3 is dispensable for the development of Treg but essential for function, proliferation and survival (Lin et al., 2007; Gavin et al., 2007). Indeed, the necessity of the TF Foxp3 for suppressive capacities was demonstrated in several murine studies. Foxp3 knock-out mice develop an autoimmune-like lymphoproliferative syndrome with hyperreactive $CD4^+$ T cells and the deletion of $Foxp3^+$ T cells leads to an autoimmune disease like in scurfy mice (Lahl et al., 2007;

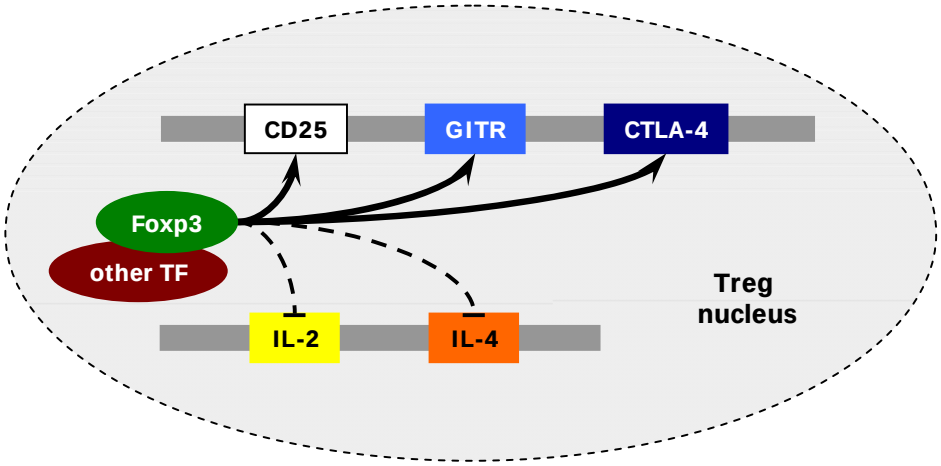

Figure 1.3.2 | Foxp3-mediated transcriptional control in Treg. Foxp3 positively regulates some genes encoding Treg-associated molecules (*e.g.* CD25, CTLA-4 and GITR) while it suppresses the transcription of others (*e.g.* effector cytokines such as IL-2 and IL-4) [adapted from (Sakaguchi, 2005)].

Kim et al., 2007; Fontenot et al., 2003). Furthermore, retroviral transduction of naïve T cells with the Foxp3 gene results in anergy, an expression profile of nTreg, and in some publications in suppressive capacity against $CD4^+$ T cells (Hori et al., 2003; Fontenot et al., 2003; Allan et al., 2005).

Association of Foxp3 with other TF, such as nuclear factor of activated T cells (NFAT), NF-κB and runt-related TF 1/acute myeloid leukaemia 1 (RUNX1/AML1) is required for its gene regulatory function (Bettelli et al., 2005; Ono et al., 2007; Wu et al., 2006). Foxp3 together with NFAT enhances CD25 and CTLA-4 transcription (Fig. 1.3.2) (Sakaguchi, 2005). In contrast, IL-2 expression which is increased by NFAT alone is repressed when this TF is complexed with Foxp3 (Fig.1.3.2) (Wu et al., 2006).

In the course of particular immune responses, Treg can express TF specific for the predominantly active T cell subset. For instance, simultaneous expression of Foxp3 and T-bet (a Th1-specific TF), interferon regulatory factor 4 (IRF4, Th2-specific) or signal transducer and activator of transcription 3 (STAT3, Th17-specific) adapts Treg to the specific environment and leads to the suppression of the corresponding T cell subsets (Chaudhry et al., 2009; Zheng et al., 2009; Koch et al., 2009). In the case of

NFAT, IRF4 and retinoid related orphan receptor (ROR)γt, a direct interaction with Foxp3 has been reported. This interaction might induce effector cell-specific suppression conditions in Treg by modified gene transcription (Wu et al., 2006; Zheng et al., 2009; Du et al., 2008). One presumable scenario is a Treg-specific expression of receptors that catch lineage defining factors from the immune response-driving T cell subsets.

Thus far, although Foxp3 is the most specific marker for Treg it remains unsuitable for the isolation of functional Treg due to its intracellular location. Hence, the discovery of a specific cell surface marker for the isolation of Treg remains a major challenge.

1.3.5 Suppression mechanisms of Treg

Recent studies have acknowledged Treg to be instrumental in the maintenance of tolerance, the prevention of autoimmune diseases, such as type 1 diabetes (Sakaguchi et al., 2001), and the limitation of chronic inflammatory diseases, such as inflammatory bowel disease (Xystrakis et al., 2006). Treg can suppress different effector functions of $CD4^+$ and $CD8^+$ T cells, B cells, NK cells and the function and maturation of DC (Piccirillo and Shevach, 2001; Thornton and Shevach, 1998; Lim et al., 2005; Ghiringhelli et al., 2005; Misra et al., 2004; Tadokoro et al., 2006). The tolerance exerted by Treg can be classified as dominant as the mere presence of Treg can inhibit autoimmunity.

Numerous mechanisms are used by Treg to suppress immune responses, *e.g.* direct cell-cell contact, the production of anti-inflammatory cytokines, and by modulating the activation state and function of APC. Another potential mechanism by which Treg mediate suppression is *via* the induction of apoptosis (this will be further discussed in paragraph 1.3.7.1). Below, some important mechanisms of suppression will be discussed briefly.

The role of TGF-β remains controversial *in vivo* whereas it does not play a role in most *in vitro* suppression assays. TGF-β-deficient Treg are suppressive, but TGF-β derived from non-Treg sources seems to be required for the protection from inflammatory

bowel disease (Fahlen et al., 2005; Mamura et al., 2004). In addition, the secretion of IL-10 by Treg *in vivo* has been shown in multiple studies to constitute an important component of their suppressive effects (Belkaid, 2007; McGeachy et al., 2005).
CTLA-4 sufficient Treg condition DC to express indoleamine 2,3-dioxygenase (IDO) which catalyzes tryptophane into proapoptotic metabolites resulting in the suppression of Tcon activation (Oderup et al., 2006; Valzasina et al., 2005; Fallarino et al., 2003) Hydrolysis of extracellular ATP (an indicator of tissue destruction with inflammatory effects on DC) to ADP or AMP by the ectoenzyme CD39 expressed by all murine Treg and about 50% of human Treg represents another anti-inflammatory Treg-mediated mechanism. Adenosine generated by CD39 in concert with the 5'-ecto-nucleoside CD73 signals *via* the A2A adenosine receptor and may inhibit the function of DC as well as act directly on activated T cells (Borsellino et al., 2007; Deaglio et al., 2007; Kobie et al., 2006). Also cAMP, which rises in $CD4^+$ T cells upon co-culture with Treg, contributes to a decrease in IL-2 production and lack of proliferation in $CD4^+$ T cells (Bopp et al., 2007).
It is not clear how and if different mechanisms are connected with each other to obtain maximal suppressive activity. The existence of multiple subpopulations of Treg leads to the hypothesis that depending on the tissue and type of immune reaction, different suppression mechanisms are important.

1.3.6 Treg in diseases

The relevance of Treg in cancer, autoimmune diseases, transplantation and virus infections has been demonstrated in clinical studies and in *in vitro* experiments (Curiel et al., 2004; Petersen et al., 2006; Kinter et al., 2007; Sakaguchi et al., 2006). The TCR repertoire of Treg is skewed towards recognizing self-Ag/MHC and Treg stimulation by their respective Ag results in the suppression of autoimmunity, hampered tumor immunity and dampened graft rejection. In that context, a diminished number of functional or normal numbers of non-functional Treg have been correlated with different autoimmune diseases (Abdulahad et al., 2007; Banica et al., 2009; Okumura

et al., 2008). In contrast, an increase of Treg can lower anti-tumor responses *via* suppression of tumor-specific effector T-cell responses (Wang and Wang, 2007). It was shown that Treg empede immunosurveillance against autologous tumor cells, because depletion of Treg before tumor challenge elicits effective immune responses to syngeneic tumors (Sutmuller et al., 2001; Onizuka et al., 1999). Furthermore, depletion of Treg and subsequent stimulation with Ag *in vitro* results in the expansion of tumor Ag-reactive T cell populations (Danke et al., 2004). Currently, different methods with beneficial outcome are being used for the manipulation of Treg in the clinic. Denileukin diftitox, for instance, a fusion protein of diphtheria toxin (DT) and IL-2 which targets CD25-expressing cells for lysis, can reduce the percentage of Treg in patients with ovarian cancer, renal carcinoma, and melanoma (Dannull et al., 2005; Rasku et al., 2008; Morse et al., 2008; Mahnke et al., 2007a; Barnett et al., 2005). Furthermore, the humanized mAb Daclizumab which binds specifically to CD25 has been given to organ transplantation patients, patients with autoimmune disorders, and cancer patients with $CD25^+$ T-cell malignancies or acute GvHD (Waldmann, 2007)

1.3.7 Treg and apoptosis

Numerous articles have been published about Treg in conjunction with apoptosis which are, however, controversial. Several laboratories show that Treg exert suppression by apoptosis while others demonstrate that Treg are killed by their target cells often involving the same molecules in both mechanisms. Discrepancies might arise from varying experimental setups and differences between the murine and human system. The most relevant data regarding apoptosis will be discussed briefly in the following two paragraphs.

1.3.7.1 Apoptosis-mediated suppression and counter-suppression

Regarding apoptosis induction in responder cells, a study proposed that murine Treg consume IL-2 and inhibit the proliferation of Tcon by Bim-dependent apoptosis (Pandiyan et al., 2007). In contrast, blockade of IL-2 binding to CD25 had no effect

on suppression of proliferation and the addition of exogenous IL-2 did not affect the suppression of IL-2 mRNA production (Tran et al., 2009; Oberle et al., 2007; Thornton et al., 2004). Furthermore, Noelle and colleagues were the first to describe that the suppressive capacity of murine Treg depends on granzyme B (GzmB), but is perforin-independent and that GzmB-deficient Treg have reduced suppressive activity *in vitro* (Cao et al., 2007; Gondek et al., 2005). In humans, Treg express GzmA and kill different cell types in a perforin-dependent manner (Grossman et al., 2004). Other studies demonstrated that a fraction of tumor-infiltrating Treg lysed NK cells and cytolytic T cells in a perforin-dependent manner (Cao et al., 2007). In addition, it has been shown that Treg can mediate lysis of B cells (Zhao et al., 2006; Janssens et al., 2003; Ludwig-Portugall et al., 2009). Furthermore, activated Treg could kill previously activated Tcon in a TRAIL-dependent and CD95-independent fashion *in vivo* (Ren et al., 2007; Baatar et al., 2007). In contrast to that, it was demonstrated in an *in vitro* model that Treg could induce apoptosis in Jurkat cells *via* CD95 (Baatar et al., 2007).
The killing of Treg by effector cells to circumvent suppression has also been investigated by several laboratories. For CD95-induced apoptosis, Reardon *et al.* demonstrated in a colitis model that Treg numbers decrease due to the presence of $CD95L^+$ cells (Reardon et al., 2008). In addition, tumor cells engineered to present CD95L were able to diminish Treg numbers in a murine tumor model (Chen et al., 2007). Furthermore, $GzmB^+$ responder cells could kill a subset of human Treg *in vitro* (Ashley and Baecher-Allan, 2009).

1.3.7.2 Treg are sensitive to CD95-induced apoptosis but lack AICD

Previous studies demonstrated that Treg behave differently from Tcon regarding apoptosis (Fritzsching et al., 2005; Taams et al., 2001b; Taylor et al., 2007; Banz et al., 2002). As described in paragraph 1.2.4, freshly isolated (day 0) Tcon express very low levels of CD95 and are resistant to apoptosis mediated by a CD95L construct as well as TCR stimulation-induced AICD (Fig. 1.3.3, day 0 Tcon). Tcon upregulate CD95 and become sensitive to apoptosis during an *in vitro* culture of 6 days with anti-

CD3/CD28 mAb and IL-2. Stimulation of day 6 Tcon with a CD95L construct leads to CD95-induced cell death, while TCR re-stimulation with pb anti-CD3 mAb results in AICD (Fig. 1.3.3, day 6 Tcon).

Day 0 and day 6 Treg express CD95 and behave similar regarding apoptosis sensitivity *in vitro*. They can easily be killed *via* cross-linking of CD95 by a CD95L construct, but they are resistant to AICD triggered by TCR re-stimulation (Fig. 1.3.3, day 0/day 6 Treg) (Taylor et al., 2007; Fritzsching et al., 2005). As the CD95-mediated death pathway is functional, the lack of Treg regarding AICD might be caused by inadequate CD95L levels (Fig. 1.3.3, day 0/day 6 Treg). It has been shown that gld mice which lack functional CD95L have increased Treg numbers whereas lpr mice were not reported to have altered Treg numbers (Mohamood et al., 2006; Zheng et al., 2007a). Therefore, a detailed analysis to elucidate the role of the CD95/CD95L system in the homeostasis of Treg is required.

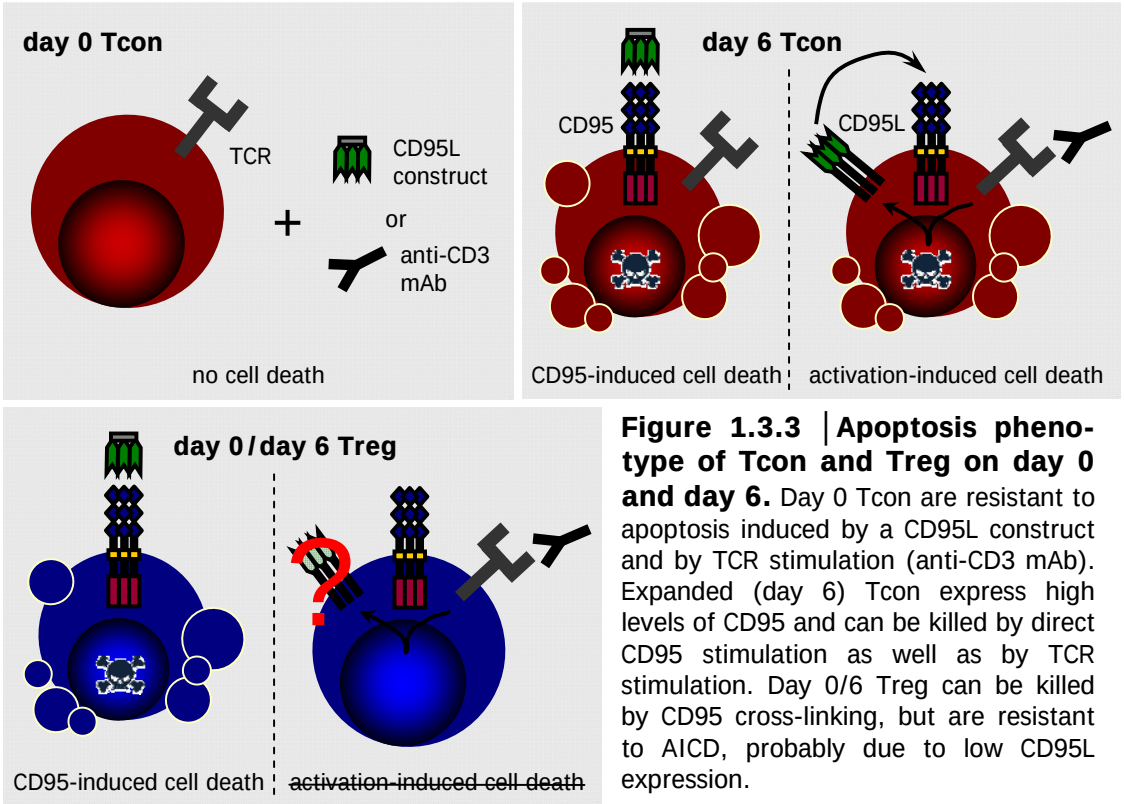

Figure 1.3.3 | Apoptosis phenotype of Tcon and Treg on day 0 and day 6. Day 0 Tcon are resistant to apoptosis induced by a CD95L construct and by TCR stimulation (anti-CD3 mAb). Expanded (day 6) Tcon express high levels of CD95 and can be killed by direct CD95 stimulation as well as by TCR stimulation. Day 0/6 Treg can be killed by CD95 cross-linking, but are resistant to AICD, probably due to low CD95L expression.

1.4 Aim of the study

Previous experiments have demonstrated that Treg are highly sensitive to CD95-induced apoptosis but simultaneously resistant to AICD *in vitro*, the latter being characterized by production and subsequent binding of CD95L to CD95. Two questions arise from these results: Firstly, do Treg have low/absent CD95L expression which makes it impossible for them to undergo AICD? This question is to be answered by the investigation of CD95L expression by Treg upon stimulation. Secondly, Treg are sensitive to CD95-induced apoptosis *in vitro* but do they also display this apoptosis phenotype *in situ* where they should receive all factors required for their survival? The involvement of CD95 in the apoptosis of Treg will be investigated *in vivo* by means of a mouse model.

2 Materials and methods

2.1 Materials

2.1.1 Laboratory materials

Consumables	Company
Reaction tubes (1.5, 2ml)	Eppendorf
Reaction tubes (5, 15, 50ml)	Becton Dickinson
Sterile filters (0.22µm, 0.45µm)	Millipore
Cell culture plates	TPP/Greiner
Cell culture flasks	TPP
Pipettes	Becton Dickinson
Pipette tips	TipOne
LS, LD, MS columns	Miltenyi Biotec

2.1.2 Equipment

Device	Company
Analysis scale AE 240	Mettler
Biofuge 15	Heraeus
Biofuge Fresco 17	Heraeus
Megafuge 3.OR	Heraeus
Omnifuge 3.ORS	Heraeus
Thermomixer compact	Eppendorf
Heating block Thermostat 5320	Eppendorf
Steril bench place HeraSafe	Heraeus
FACSCanto II	Becton Dickinson
Thermocycler DNA engine	Dyad
7500 Real Time PCR systems	Applied Biosystems

Nucleofector I	Amaxa Biosystems
IVIS 100 Imaging System	Xenogen Imaging Technologies
Magnetic stand	Miltenyi Biotec

2.1.3 Chemicals

All chemicals, if not otherwise stated, were ordered from the companies Fluka, Merck, Roth, Serva and Sigma.

2.1.4 Standard buffers

Buffer	**Reagents**
Hypotonic NaCl buffer (10x)	2% (w/v) NaCl in H_2O
PBS	137mM NaCl 8.1mM Na_2HPO_4 2.7 mM KCl 1.5mM KH_2PO_4 pH 7.4
FACS buffer	5% FCS 0.1% NaN_3 in PBS
MACS buffer (human)	0.5% HSA 2mM EDTA in PBS
MACS buffer (murine) = FASER buffer	0.5% BSA 2mM EDTA in PBS

2.1.5 Cell lines and primary cells

The erythroleukemic cell line K562 and the thereof derived CD95L$^+$ cell line KFL9 were used for CD95L control stainings.

Human and murine primary T cells were isolated from human buffy coats (Blutbank Karlsruhe) or blood donations and pooled murine lymph node (LN)/spleen cells, respectively (see paragraph 2.2.4).

BM cells were isolated from femur and tibia of mice and depleted of CD3$^+$ cells by MACS purification (see paragraph 2.2.6).

2.1.6 Media and supplements

Reagent	Company
RPMI 1640	Sigma
X-Vivo 15	Cambrex
FCS	Gibco
Glutamax	Gibco
Penicillin/Streptomycin	Sigma

2.1.7 Reagents for the isolation/depletion of T cells

Reagent	Company
Ficoll	Biochrom
Anti-human CD25 beads	Miltenyi Biotec
CD4$^+$ T cell isolation kit II, human	
CD4$^+$CD25$^+$ regulatory T cell isolation kit, mouse	
CD4$^+$ T cell isolation kit, mouse	
Anti-FITC beads	
FITC-coupled anti-mouse CD3	BD Pharmingen

2.1.8 Antibodies/reagents for the stimulation/expansion of T cells

Antibody/reagent	Company
Anti-human CD3 (OKT3)	Cytonet
Mouse IgG_{2a}	BD Pharmingen
anti-human CD28	Provided by Gerd Moldenhauer, DKFZ
Anti-mouse CD3 (2C11)	BD Pharmingen
Armenian Hamster IgG_1	BD Pharmingen
Anti-mouse CD28	BD Pharmingen
Recombinant human IL-2	Cell line LBRM-33 5A4
Phorbol 12-myristate 13-acetate (PMA)	Sigma
Ionomycin	Sigma
Dimethyl sulfoxide (DMSO)	Sigma
ADAM-10 inhibitor	Provided by Andreas Ludwig, University clinic Aachen

2.1.8 Antibodies/reagents for flow cytometry

Antibody/reagent	Company
Biotinylated anti-human CD95L (NOK1)	BD Pharmingen
Biotinylated hamster IgG_1	BD Pharmingen
FITC-coupled anti-human CD45RO	BD Pharmingen
PE-coupled anti-human CD45RA	BD Pharmingen
PE-coupled anti-human Foxp3 (259D)	Biolegend
PE-coupled mouse IgG_1	BD Pharmingen
Foxp3 staining buffer set	eBioscience
Biotinylated anti-mouse CD95L (MFL3)	BD Pharmingen
Biotinylated armenian hamster IgG_1	BD Pharmingen
PE-coupled anti-mouse CD3	BD Pharmingen

PE-Cy5-coupled anti-mouse CD4	BD Pharmingen
APC-coupled anti-mouse B220 (CD45R)	eBioscience
APC-coupled streptavidin	BD Pharmingen
APC-specific FASER kit	Miltenyi Biotec

2.1.9 RNA isolation, reverse transcription and quantitative PCR reagents

Reagent	Company
Absolutely RNA Microprep kit	Stratagene
Turbo DNA-free Kit	Ambion
Reverse Transcriptase	Applied Biosystems
RNase Inhibitor	Applied Biosystems
Power SYBR green master mix	Applied Biosystems

2.1.10 Primers

Synthetic oligodesoxyribonukleotides were designed with the program "Primer Express" (Applied Biosystems) and ordered from MWG Biotech.

Name	Sequence (5′-3′)
Primers (for the detection of human genes of interest)	
GAPDH	Forward: GCA AAT TCC ATG GCA CCG T Reverse: TCG CCC CAC TTG ATT TTG G
CD95L	Forward: TGG AAT TGT CCT GCT TTC TGG Reverse: TGT TGC AAG ATT GAC CCC G

2.1.11 siRNA reagents

Reagent	Sequence (5′-3′)/company
siRNA against Foxp3	GCA CAT TCC CAG AGT TCC T/MWG
AllStars Negative Control siRNA	Qiagen

T cell nucleofection kit	Amaxa/Lonza

2.1.12 Antibodies/reagents for *in vivo* T cell experiments

Name	Company
Anti-mouse CD95 (Jo2)	BD Pharmingen
Armenian hamster $IgG_{2\lambda1}$	BD Pharmingen
Diphtheria toxin (DT)	Sigma
D-luciferin potassium salt	Synchem OHG

2.2 Methods

2.2.1 Standard cell culture methods

Cell culture was carried out at a sterile bench. Glass ware and self-made solutions were autoclaved (30 min, 121°C). Other solutions and plastic ware was ordered sterile. Cells were cultured at 37°C and 5% CO_2.

For the culture of K562 and KFL9 cells, RPMI 1640 medium was supplemented with 10% FCS, 1% Glutamax and 1% Penicillin/Streptomycin. Medium was exchanged every 2-3 days.

Primary murine T cells were cultured in RPMI 1640 supplemented as described for K562 and KFL9 cells. 200 IU/ml IL-2 were added freshly to the culture.

For the culture of primary human T cells, X-Vivo 15 medium was supplemented with 1% Glutamax. 200 IU/ml IL-2 were added freshly to the culture.

2.2.2 Cell counting

Cells were counted and viability was measured by staining with trypan blue. 10µl of cell suspension was mixed with 10µl 1x trypan blue and counted in a Neubauer chamber. The cell number/ml was calculated according to the following formula: Counted cell number/number of counted squares x dilution factor x 10^4.

2.2.3 Freezing and thawing of cell lines

Cell lines were adjusted to 2 x 10^7/ml in RPMI containing 40% FCS and 10% DMSO and transferred into cryo tubes. Cells were frozen over night at -20°C and then stored at -80°C.
Cells were thawed quickly at 37°C and washed immediately in 50ml RPMI containing 10% FCS. The cells were resuspended in RPMI supplemented as explained in „Media and supplements“, transferred into a cell culture flask and cultured in an incubator.

2.2.4 Ficoll gradient isolation of human mononuclear cells

Primary human mononuclear cells were isolated from 500ml total blood or buffy coats according to the following protocol. Buffy coats were adjusted to 300ml with PBS. 50ml tubes filled with 15ml Ficoll were slowly overlayed with 35ml blood and centrifuged at 650 x g and 20°C for 20 min without break. Peripheral blood leukocytes (PBL) were collected from the interphase and washed once with PBS and resuspended in RPMI containing 10% FCS. PBL were transferred into cell culture flasks and cultured for 30-45 min in the incubator for the depletion of adherent cells (monocytes and macrophages). Non-adhering lymphocytes were washed from the cell culture flask with medium and stored over night at 4°C. Lymphocytes were used for the further purification of $CD4^+CD25^-$ Tcon and $CD4^+CD25^+$ Treg by magnetic sorting (see paragraph 2.2.6).

2.2.5 Isolation of murine cells from organs (LN, spleen)

Mice were killed by cervical dislocation and organs were isolated immediately. Inguinal, axillary, lateral axillary, mesenteric and sacral LN were pooled in RPMI containing 10% FCS on ice. In addition, the spleen was isolated and kept in RPMI/10% FCS on ice. Both organ types were grinded separately through a 0.45µm cell strainer to obtain single cell suspensions. Cells were pelleted by centrifugation; LN cells were resuspended in PBS and kept on ice, while splenocytes were depleted of

erythrocytes by resuspension in hypotonic NaCl solution for 30 seconds. Splenocytes were centrifuged again and resuspended in PBS. LN and spleen cells were pooled in some experiments for the isolation of either $CD4^+CD25^-$ Tcon and $CD4^+CD25^+$ Treg or total $CD4^+$ T cells by magnetic sorting (see below). In other experiments they were used separately.

2.2.6 Magnetic-activated cell separation of Tcon, Treg and $CD4^+$ T cells

The isolation of T cells was accomplished by means of magnetic-activated cell separation (MACS). Cells can be separated by MACS either by positive or negative selection. Human $CD25^{++}$ Treg were isolated by positive selection from PBL that had been isolated from buffy coats or total blood. For that, the cell suspension was incubated with a limiting amount (2µl/10^7 cells) of bead-coupled anti-CD25 mAb (Stassen et al., 2004). After a subsequent washing step, the cell suspension was loaded onto a column in a magnetic field. The magnetic beads retain the $CD25^+$ cells in the column, while the remaining cell types flow through. Outside of the magnetic field, $CD25^+$ cells were eluted from the column. The $CD25^+$ cell fraction was run a second time over a column to obtain highly purified $CD25^{++}$ Treg. After elution, the cells were stored in X-Vivo 15 until the start of experiments. The remaining cell suspension (partially depleted of $CD25^+$ cells) was used for the isolation of $CD4^+CD25^-$ Tcon with the $CD4^+$ T cell isolation kit II and an additional amount of anti-CD25 beads. During the CD4-specific negative selection, other cell types (B cells, NK cells, $CD8^+$ T cells, erythrocytes, residual $CD25^+$ cells) except the cells of interest ($CD4^+CD25^-$ Tcon) are bound by specific antibodies coupled to beads. Therefore, $CD4^+$ Tcon flow through the column in the magnetic field. After the isolation they can be used for experiments.
For the isolation of cord blood Tcon and Treg, PBL from cord blood donations were incubated with 3µl bead-coupled anti-CD25 mAb. After the positive selection which yields the $CD25^+$ cell fraction (in cord blood exclusively Treg are $CD25^+$), cells were subjected to a CD4-specific negative selection to obtain $CD4^+CD25^-$ Tcon.
Murine Tcon and Treg were isolated from organ suspension (pooled LN and spleen

cells) by means of the mouse $CD4^+CD25^+$ regulatory T cell isolation kit. In a first step $CD4^+$ T cells are isolated by negative selection. Subsequently, $CD25^+$ T cells are marked by beads and separated from the remaining $CD4^+$ T cells to yield $CD4^+CD25^-$ Tcon and $CD4^+CD25^+$ Treg.

For some experiments, total murine $CD4^+$ T cells were isolated by MACS. For that, pooled LN and spleen cells were incubated with biotinylated mAb that labels all cell types except $CD4^+$ T cells. In a subsequent step, the cell mixture was incubated with streptavidin-coupled beads. The cell suspension was run through a column resulting in non-labeled $CD4^+$ T cells in the flow through which are used for further experiments.

2.2.7 Expansion of T cells by anti-CD3 and anti-CD28 mAb

0.5-1 x 10^4 T cells were resuspended in 200µl supplemented X-Vivo 15 medium and transferred into individual wells of 96-u-well plates. Soluble anti-CD3 (0.5µg/ml) and anti-CD28 (2µg/ml) mAb as well as 200-300 IU/ml IL-2 was added to the medium. T cells were used after different *in vitro* culture periods (day 1-6).

2.2.8 Stimulation of T cells

For the investigation of CD95L expression, human and murine T cells were stimulated with 30µg/ml pb anti-human CD3 mAb (OKT3) and pb 3µg/ml anti-mouse CD3 mAb (2C11), respectively. As a control, T cells were incubated with specific pb isotype mAb. In addition, T cells were incubated with the strong stimulus PMA (10ng/ml) plus Ionomycin (1µM) or the vehicle control DMSO. For the investigation of CD95L mRNA, cells were stimulated for 2 hr, while CD95L protein expression was investigated after 20 hr of stimulation. In some experiments for the investigation of CD95L protein, cells were incubated during stimulation with 1µM of the hydroxamate based ADAM-10 inhibitor GI254023X (Hundhausen et al., 2003).

2.2.9 RNA isolation

RNA was isolated according to the manual of the absolutely RNA Microkit. Briefly, 1-6 x 10^5 cells were lysed in 200 µl lysis buffer supplemented with β-mercaptoethanol and either stored at –20°C or immediately adjusted to 400µl with 200µl 70% ethanol. The purification was achieved *via* binding to a column. Columns were washed twice with 500µl high salt buffer and 3 times with 300µl low salt buffer. The RNA was eluted with 20-40µl DEPC water (60°C) and subjected to a DNase treatment with the turbo DNA-free Kit according to the manufacturer's guidelines.

2.2.10 Reverse Transcription

RNA was reverse transcribed into complementary DNA by the use of oligo dT primers (5µM). In a 10 min step at 65°C, the RNA was stretched to allow easier attachment of the oligo dT primers. Afterwards, RNA was incubated for 45 min at 42°C in a reaction mix containing:

1x reaction buffer
5 mM $MgCl_2$
1 mM dNTP
0.5 U/µl Reverse Transcriptase
1 U/µl RNAse Inhibitor

In a final step, the reaction was stopped by incubation at 95°C for 5 min

2.2.11 SYBR green quantitative real time PCR

Expression of the gene of interest was investigated by the use of SYBR green quantitative real time PCR (qRT-PCR). This method quantifies the gene of interest by the detection of the incorporated DNA-intercalating fluorescent dye SYBR green during synthesis using the 7500 Real Time PCR systems. By accumulation of the PCR product, the detectable fluorescence was amplified by each PCR cycle. The relative expression level was determined by normalization to GAPDH with results presented

as fold expression according to the following formula (C_T is the threshold cycle value):
relative mRNA expression = $2^{-(C_T \text{ of gene of interest} - C_T \text{ of GAPDH})}$.
Reactions were carried out in a volume of 25µl in duplicates.
Reaction mix:

1x SYBR green mix (ready to use)
900 pmol forward primer
900 pmol reverse primer
H_2O
template complementary DNA

The SYBR green mix contains SYBR green, $MgCl_2$, dNTP, Hot Gold Star Taq polymerase and uracil-N-glycosylase.

2.2.12 Immunofluorescent staining for flow cytometry

For the detection of CD95L on human and murine T cells, cell pellets containing 0.5-5 x 10^5 cells were resuspended in a mixture containing 10µg/ml biotinylated anti-CD95L or isotype mAb for 60 min in FACS buffer (all steps were carried out at 4°C). After washing with FACS buffer, the cell pellet was resuspended in FACS buffer containing streptavidin-APC and incubated for 30 min Cells were washed with FACS buffer and then incubated with reagent 1 and Fc-γ receptor blocker in FASER buffer for 10 min After a wash with FASER buffer, cells were resuspended in FASER buffer containing reagent 2 and Fc-γ receptor blocker for 10 min If needed for investigation, different fluorescentlymarked antibodies for the staining of other cell surface molecules were added during this final step. Finally, cells were washed with FASER buffer and resuspended in FASER buffer for measurement.
For the immunofluorescent staining of other cell surface molecules than CD95L, 0.5-5 x 10^5 cells were pelleted, resuspended in a mix of different fluorescently marked antibodies in FACS buffer and incubated at 4°C for 20-60 min After incubation, cells were washed with FACS buffer and resuspended in FACS buffer for measurement.

The investigation of the intracellular protein Foxp3 in human T cells was carried out according to the manufacturer's protocol at 4°C. Briefly, 1-5 x 10^6 cells were washed with PBS, and incubated for 30-60 min in Fixation/Permeabilization Buffer. After a wash with PBS, cells were centrifuged twice with Permeabilization Buffer. Cell pellets were incubated in Permeablization Buffer containing normal mouse serum for 15 min (blockage of inspecific binding sites). PE-coupled anti-Foxp3 or isotype mAb was added and the mix was incubated for an additional 30 min. Subsequently, cells were washed twice with Permeabilization Buffer and resuspended in PBS for measurement at the flow cytometer.

Mean fluorescence intensity (MFI) increase in % was calculated according to the following formula: increase in MFI (%) = $[(MFI_{stimulated} - MFI_{unstimulated})/MFI_{unstimulated}] \times 100$

2.2.13 RNA interference

Human $CD4^+CD25^-$ and $CD4^+CD25^{++}$ T cells were electroporated in supplemented human T cell nucleofection solution containing 1µM control siRNA or Foxp3-specific siRNA. After 72 hr, the cells were divided to confirm the knock-down of Foxp3 by flow cytometry on the one hand and to investigate CD95L expression after stimulation on the other hand.

2.2.14 Mice

Mice (C57/BL6, lpr, Foxp3.DGL, Foxp3.DGL x lpr, $RAG2^{-/-}$, $RAG2^{-/-}$ x lpr) were housed under specific-pathogen-free conditions at the central animal facility of the German Cancer Research Center, and experiments were according to institutional guidelines and regulations. Organs of DEREG and Scurfy x DEREG mice were obtained from Tim Sparwasser, Twincore, Medical School Hanover.

2.2.15 BM chimeric mice

For BM chimeric mice, donor BM cells were T cell-depleted to prevent donor T cell-mediated graft *vs.* host disease (GvHD) which might interfere with the engraftment

process. Therefore, BM cells isolated from femur and tibia of donor mice, were subjected to MACS to obtain T cell-depleted BM. BM cells were incubated with a FITC-labeled anti-mouse CD3 mAb, washed and subsequently incubated with anti-FITC beads. $CD3^+$ T cells are retained in the column, while the remaining BM cells flow through. 3-6 x 10^6 T cell-depleted donor BM cells were injected into sublethally irradiated (4.5 Gy) 5-8 week old $RAG2^{-/-}$ x lpr mice. Donors were gender matched Foxp3.DGL and Foxp3.DGL x lpr mice.

2.2.16 *In vivo* luciferase measurement

6-10 weeks after BM reconstitution, experiments were carried out. Mice were injected i.p. with 0.25µg/g body weight anti-CD95 mAb (Jo2) and specific isotype control (Yin et al., 1999). As control, 12ng/g body weight DT was injected i.p. For the detection of luciferase activity, mice were injected i.p. with 4.5 mg luciferin in 200µl PBS. After an incubation period of at least 5 min, luminescent pictures were taken with the IVIS 100 Imaging System. Data was analyzed by means of the Igor Pro 4.09A software. The relative light unit (RLU) values were normalized to the isotype control according to the following formula: $(RLU_{treatment\ 0/24/48hr}/RLU_{treatment\ 0hr})/\ (RLU_{isotype\ 0/24/48hr}/RLU_{isotype\ 0hr})$

3 Results

3.1 *In vitro* studies

Previous *in vitro* experiments demonstrated a high sensitivity of human Treg to apoptosis induced by direct stimulation of CD95 (Aswad et al., 2005; Fritzsching et al., 2005). Nevertheless, Treg are resistant to AICD which is characterized by TCR stimulation-induced expression and subsequent binding of CD95L to CD95 (Alderson et al., 1995; Klas et al., 1993; Krueger et al., 2003a). Due to the fact that a functional CD95-triggered death pathway exists in Treg, their resistance to AICD suggests insufficient CD95L levels, which can have several reasons. For example, stimulation-induced CD95L expression could be diminished or CD95L cleavage from the cell surface could be enhanced. Therefore, the *in vitro* studies aim to investigate CD95L levels in Treg.

3.1.1 Human Treg express low CD95L mRNA compared to Tcon

CD95L protein expression upon TCR stimulation is required for the execution of AICD. Absent CD95L mRNA results in CD95L protein deficiency and might cause the resistance of Treg to AICD. Thus, CD95L mRNA expression was investigated in Treg compared to Tcon isolated from human blood. Basal CD95L mRNA expression was only slightly different in day 0 and day 6 Treg compared to day 0 and day 6 Tcon, respectively (Fig. 3.1.1). However, stimulation-induced CD95L mRNA expression revealed differences between Treg and Tcon. While day 0 Treg showed almost no increase over basal CD95L mRNA levels upon stimulation with pb anti-CD3 mAb or the strong stimulus PMA/Ionomycin (P/I), day 0 Tcon showed a stimulation-dependent increase of CD95L mRNA (Fig. 3.1.1 a). Stimulation of Tcon with pb anti-CD3 mAb marginally increased CD95L mRNA whereas P/I treatment led to substantially elevated CD95L mRNA compared to unstimulated or DMSO-treated cells (Fig. 3.1.1 a).

On day 6 of culture, induction of CD95L expression was still low in Treg irrespective

of stimulation with pb anti-CD3 mAb or P/I (Fig. 3.1.1 b). Tcon in contrast showed considerable CD95L mRNA upregulation after treatment with pb anti-CD3 mAb which was comparable to P/I stimulation-induced CD95L mRNA (Fig. 3.1.1 b). In conclusion, freshly isolated as well as expanded human Treg showed low CD95L mRNA induction upon stimulation which indicates that stimulation-dependent CD95L mRNA expression is severely blocked in human Treg.

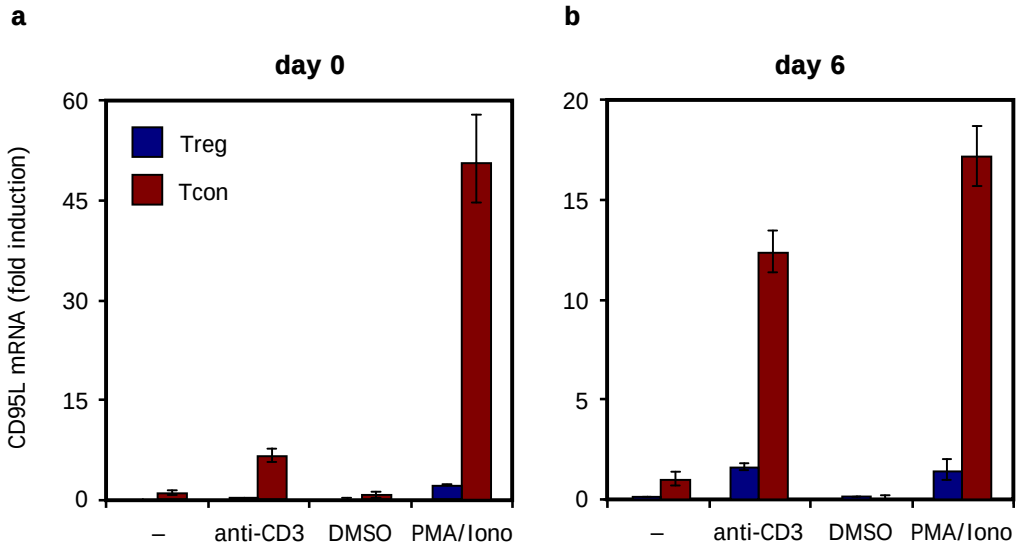

Figure 3.1.1| Human Treg have low CD95L mRNA compared to Tcon after stimulation. **a** Day 0 Treg and Tcon were left unstimulated (-) or incubated for 2 hr with 30µg/ml pb anti-CD3 mAb, DMSO or 10ng/ml PMA plus 1µM Ionomycin. RNA was isolated from cell lysates and reverse transcribed using oligo dT primers. CD95L mRNA expression was quantified by detection of incorporated SYBR green. The relative expression level was determined by normalization to GAPDH with results presented as fold expression compared to unstimulated Tcon. **b** Experiment as in **a** except with Treg and Tcon cultured for 6 days *in vitro* with soluble anti-CD3 mAb, anti-CD28 mAb and IL-2. Data are representative of 3 independent experiments.

3.1.2 Establishment and evaluation of a CD95L amplification protocol

To confirm that the low amount of CD95L mRNA in Treg cannot give rise to a substantial amount of CD95L protein in Treg, a reliable method for the detection of CD95L protein on the cell surface was needed. The commonly used flow cytometry protocol (hereafter referred to as "regular staining") makes use of a biotinylated anti-CD95L mAb (step 1) followed by an APC-coupled streptavidin (step 2) to detect CD95L on the cell surface (Fig. 3.1.2 a and b). This method inadequately visualizes CD95L on the cell surface of primary T cells and is not sensitive enough to compare CD95L expression of Tcon *vs.* Treg. To improve the existing protocol, different signal amplification methods were tested. Finally, the Fluorescence Amplification by Sequential Employment of Reagents (FASER) kit by Miltenyi Biotec proved of value for the detection of CD95L. For the new protocol, the regular staining is followed by two additional steps (step 3 and 4) of the FASER kit to amplify the CD95L surface signal (fig 3.1.2 a and c). In step 3, a biotinylated anti-APC mAb binds to the APC-coupled streptavidin. This step is followed by an additional streptavidin-APC which binds to the biotinylated anti-APC mAb (step 4).

The specificity of the established amplification protocol for the detection of CD95L surface protein was tested with the erythroleukemic cell line K562 (CD95L negative) and the K562-derived cell line KFL9 (CD95L overexpressing K562). As seen in Fig. 3.1.2 d, the signal for the CD95L protein on KFL9 cells was enhanced by the FASER method compared to the regular staining. The amplified signal was specific as the FASER method did not result in any signal on K562 cells (Fig. 3.1.2 d).

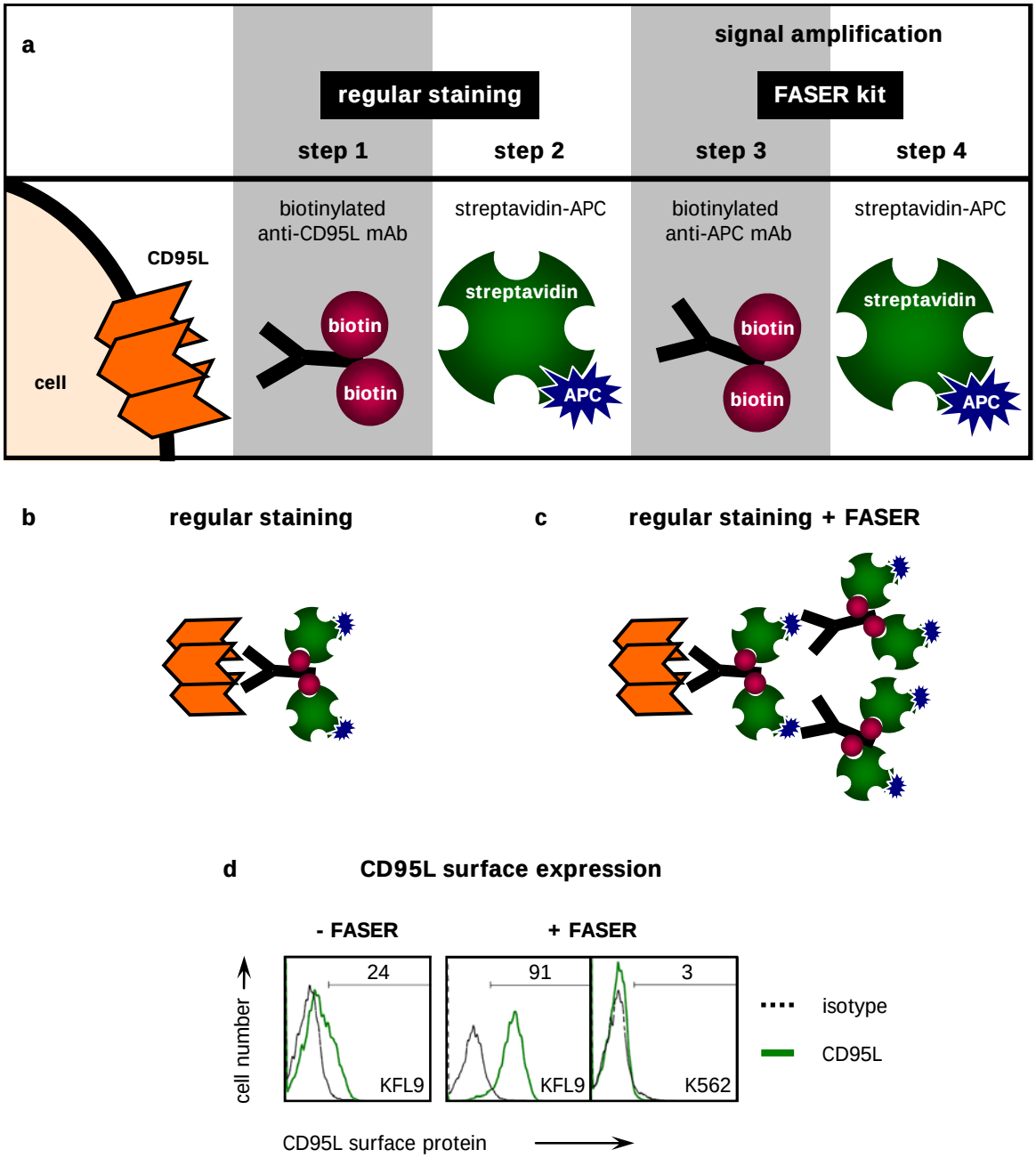

Figure 3.1.2 | CD95L amplification protocol for flow cytometry. a The regular staining detects cell surface CD95L expression by a biotinylated anti-CD95L antibody in a first step, followed by streptavidin-APC in step 2. The signal amplification by the FASER kit is achieved by two additional steps. In step 3, biotinylated anti-APC mAb binds to the APC-coupled streptavidin. This biotinylated mAb is bound by streptavidin-APC in step 4. **b** The regular staining amplifies the signal to some extent. **c** The use of the regular staining together with the FASER kit largely amplifies the signal for CD95L. **d** KFL9 (CD95L overexpressing K562) cells were stained according to the regular staining (-FASER). KFL9 or K562 (which are CD95L negative) cells were stained according to the new amplification protocol (+FASER) to evaluate the amplification potential and the specificity of the FASER kit. Data are representative of 2 independent experiments.

3.1.3 Human Treg express less CD95L protein compared to Tcon

As shown in Fig. 3.1.1, a small induction of CD95L mRNA in stimulated *vs.* not stimulated Treg was observed. This low amount of mRNA might lead to substantial CD95L protein. Hence, the CD95L surface protein level was investigated in Treg by the aforementioned FASER amplification method. 20 hr after pb anti-CD3 mAb, CD95L expression was not altered in day 0 Treg compared to isotype treated cells (Fig. 3.1.3, upper panel). Stimulation by P/I could also not increase CD95L surface expression in Treg (Fig. 3.1.3, upper panel). Contrary to Treg, day 0 Tcon showed elevated surface CD95L protein expression by P/I treatment, while no increased CD95L protein expression was observed after pb anti-CD3 mAb stimulation compared to isotype or DMSO treated cells. On day 6, only a small subpopulation of pb anti-CD3 mAb treated Treg induced CD95L protein (Fig. 3.1.3, lower panel). This subpopulation of CD95L positive Treg was slightly increased after the strong P/I stimulus compared to pb anti-CD3 mAb stimulation (Fig. 3.1.3, lower panel). Expanded Tcon could produce CD95L protein after pb anti-CD3 mAb (which is absent in freshly isolated Tcon) as well as after P/I stimulation (Fig. 3.1.3 , lower panel). Together, these results demonstrate that human Treg have very low CD95L

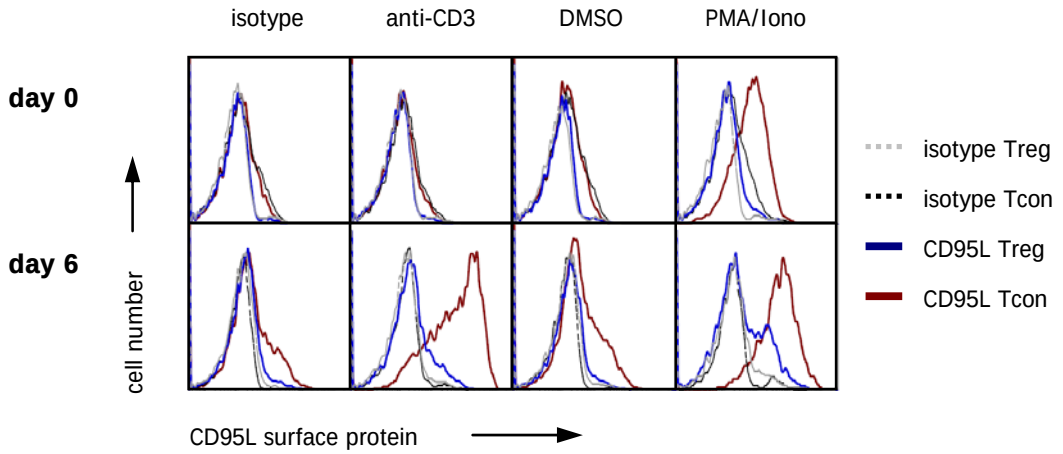

Figure 3.1.3| Treg have low CD95L surface protein compared to Tcon. Day 0 or day 6 Treg and Tcon were incubated for 20 hr with 30µg/ml pb isotype control, pb anti-CD3 mAb, DMSO or 10ng/ml PMA plus 1µM Ionomycin. CD95L surface protein expression was detected using the FASER amplification protocol. Data are representative of 3 independent experiments.

protein expression at a time point at which Tcon are already highly positive.

3.1.4 Naïve/resting human Treg express low levels of CD95L protein

CD95L expression in T cells is influenced by the cellular activation status. While activated T cells express CD95L after short stimulation periods, naïve T cells have to be stimulated repeatedly to induce CD95L expression (Strasser and Pellegrini, 2004). Whereas naïve $CD4^+$ T cells are characterized by high CD45RA and low CD45RO expression, activated/memory $CD4^+$ T cells are $CD45RA^{lo}$ and $CD45RO^{hi}$. In general, most Treg in adults have an activated/memory phenotype due to recognition of self-Ag (Fig. 3.1.4 a). Tcon from adult blood donors contain naïve ($CD45RA^{hi}$ $CD45RO^{lo}$) as well as activated cells ($CD45RA^{lo}$ $CD45RO^{hi}$) which arise from continuous contact to environmental pathogens (Fig. 3.1.4 a). Treg from cord blood, in contrast to Treg from adult blood, are almost exclusively naïve/resting which is reflected by the expression of high amounts of CD45RA and low CD45RO (Fig. 3.1.4 a). Like cord blood Treg, cord blood Tcon also have a mostly naïve phenotype (Fig. 3.1.4 a). To exclude that the activated/memory status of adult blood Treg is involved in low CD95L expression, naïve cord blood Treg were investigated regarding CD95L expression. Similar to Treg from adult blood, freshly isolated cord blood Treg produced only minimal amounts of CD95L protein upon stimulation with both pb anti-CD3 mAb and P/I (Fig. 3.1.4 b, upper panel). Day 0 Tcon from cord blood induced CD95L upon P/I treatment comparable to Tcon from adult blood (Fig. 3.1.4 b, upper panel.

On day 6 of culture, almost no cord blood Treg, like Treg in adults, showed CD95L expression upon pb anti-CD3 mAb stimulation (Fig. 3.1.4 b, lower panel). Few CD95L-positive Treg were detected after P/I stimulation (Fig. 3.1.4 b, lower panel). In contrast to Treg, day 6 Tcon from cord blood, like Tcon from adult blood, showed high CD95L expression upon pb anti-CD3 mAb and P/I treatment (Fig. 3.1.4 b, lower panel). Hence, the activation status of human Treg is not the reason for the low CD95L expression in human Treg.

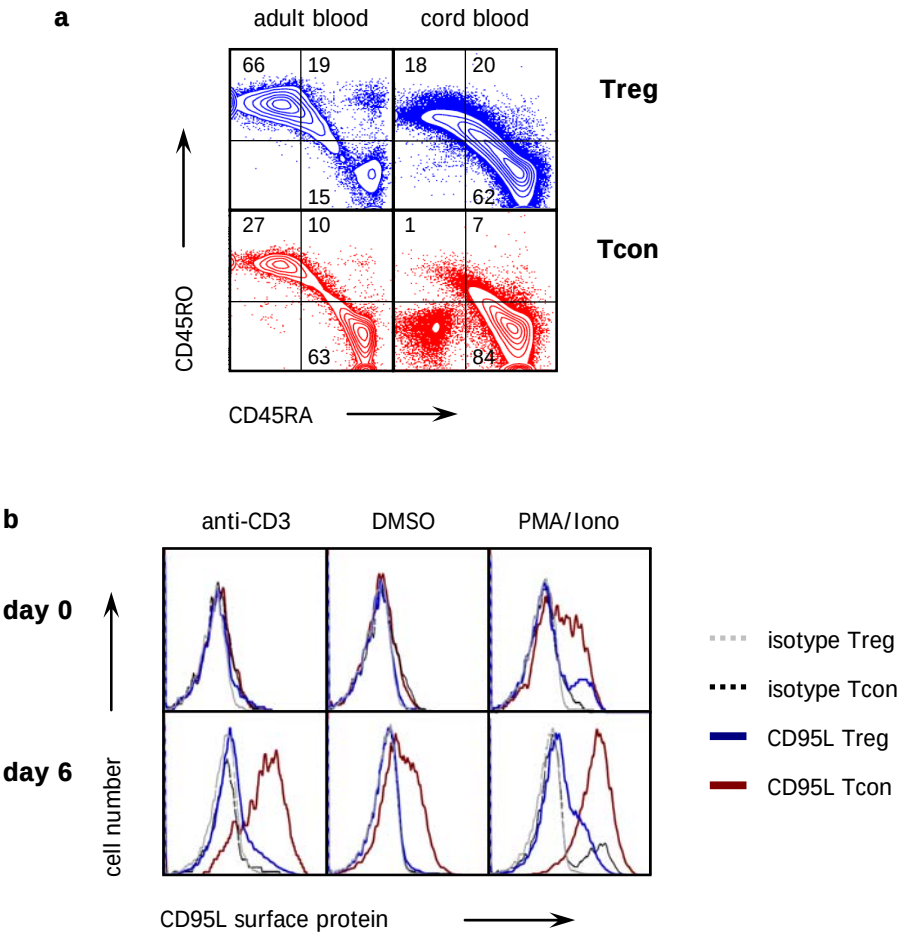

Figure 3.1.4| Cord blood Treg express low CD95L after stimulation. a Treg and Tcon from adult or cord blood were analyzed for the expression of CD45RA and CD45RO. **b** Day 0 or day 6 cord blood Treg and Tcon were incubated for 20 hr with 30µg/ml pb isotype control, pb anti-CD3 mAb, DMSO or 10ng/ml PMA plus 1µM Ionomycin. CD95L surface protein expression was detected with the help of the FASER amplification protocol. Data are representative of 2 independent experiments.

3.1.5 Kinetics of CD95L expression in human Treg

Low amounts of CD95L protein were detected on the cell surface of Treg at the same time point when Tcon showed high CD95L expression (20 hr after stimulation). Nevertheless, the possibility remains that CD95L surface protein expression follows different kinetics in Treg compared to Tcon.

Therefore, CD95L protein expression in Treg was investigated at different time points

after stimulation (Fig. 3.1.5 a). In the previously shown experiments, CD95L protein expression was investigated 20 hr after stimulation. To exclude different CD95L protein expression kinetics of Treg, further time points after stimulation (0.5, 10 and 40 hr) were examined. It has been published that surface CD95L protein increases 20-40 min after stimulation in pre-activated cells due to release from cellular granules (Schulte et al., 2007). As described in the previous paragraph, most Treg from adult blood have an activated/memory phenotype which makes the storage of CD95L protein likely and prompted the investigation of earlier stimulation time points. After 30 min of stimulation with pb anti-CD3 mAb as well as P/I which should release stored CD95L protein to the cell surface of pre-activated cells, day 0 Treg did not increase CD95L surface protein (Fig. 3.1.5 a, left). Also naïve/resting day 0 Tcon did not show a stimulation-dependent increase of CD95L expression 30 min after pb anti-CD3 mAb and P/I (Fig. 3.1.5 a, left). Furthermore, one additional early (10 hr) and one later time point (40 hr) of stimulation were tested. Stimulation of day 0 Treg for 10, 20 or 40 hr with either pb anti-CD3 mAb or P/I did not highly upregulate CD95L expression (Fig. 3.1.5 a, left). However, CD95L surface protein expression could be detected in day 0 Tcon between 10 and 40 hr after P/I stimulation with a peak of expression at 20 hr (Fig. 3.1.5 a, left). Pb anti-CD3 mAb could not induce CD95L surface protein in day 0 Tcon at any time point after stimulation (Fig. 3.1.5 a, left).

Despite the activated/memory phenotype of Treg, the *in vitro* expansion protocol might activate them even further and by that trigger CD95L expression. For that also CD95L protein expression in day 6 T cells at different time points after stimulation was investigated. Treg did not show an increase in CD95L surface protein expression at early as well as late stimulation periods with pb anti-CD3 mAb (Fig. 3.1.5 a, right). The stimulation with P/I could marginally increase CD95L surface expression in Treg at the latest tested time point (40 hr). The investigation of CD95L expression in day 6 Tcon upon pb anti-CD3 mAb revealed no detectable levels after a 30 min stimulation (Fig. 3.1.5 a, right). Nevertheless, CD95L expression increased time-dependently between 10 and 40 hr after stimulation in Tcon (Fig. 3.1.5 a, right). Tcon also produced

CD95L protein when stimulated with P/I, but this stimulus did not increase CD95L levels to the same extent as pb anti-CD3 mAb stimulation (Fig. 3.1.5 a, right).

Although there was no large difference in CD95L protein expression detected in Treg after different stimulation periods, it has to be considered that Treg could express CD95L protein at different days of *in vitro* culture compared to Tcon. On this account, Treg were stimulated at different time points (day 0 to 6) of *in vitro* culture for 20 hr and CD95L expression was analyzed. On all days of culture, pb anti-CD3 mAb could not induce substantial CD95L surface protein expression. In addition, the stimulation with P/I for 20 hr did not lead to an increase in surface CD95L expression in Treg over time (Fig. 3.1.5 b). Stimulation-dependent CD95L expression was also investigated in Tcon at each day of *in vitro* culture. At all investigated time points, Tcon produced similar CD95L protein levels upon a 20 hr P/I stimulation (Fig. 3.1.5 b). CD95L protein expression upon pb anti-CD3 mAb stimulation could not be detected from day 0 to 2 of *in vitro* culture. However, on day 3 of culture, a 20 hr stimulation with pb anti-CD3 mAb induced CD95L protein expression levels in Tcon which were comparable to P/I treatment-induced CD95L protein expression. On day 4 of culture, CD95L expression increased to approximately 3-fold over P/I treatment and reached more than 6-fold higher levels on day 5 and 6 compared to P/I stimulation (Fig. 3.1.5 b).

All together, these results show that human Treg display no altered CD95L expression kinetics and imply an impairment of human Treg to induce CD95L surface protein expression.

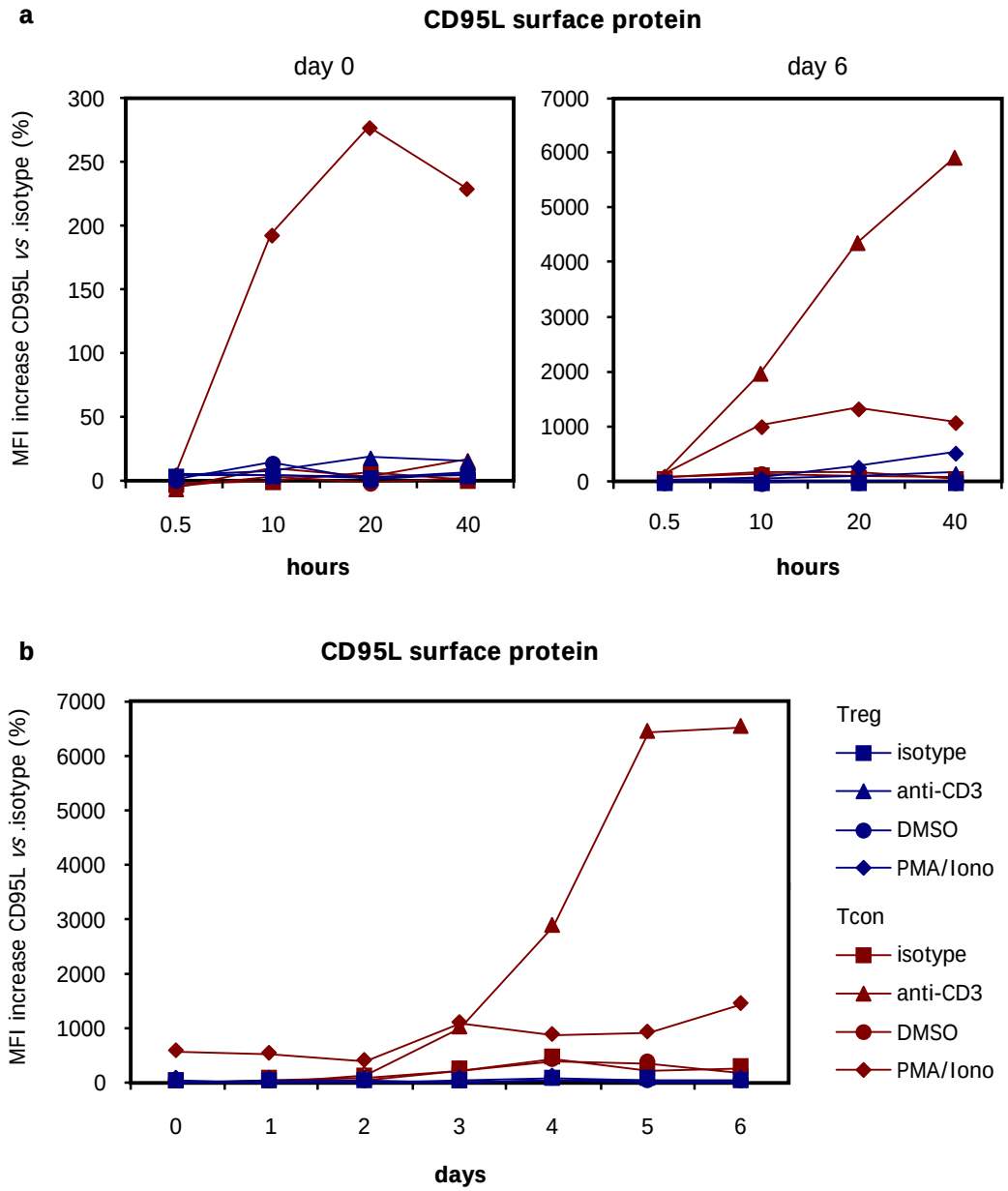

Figure 3.1.5| CD95L expression kinetics in Treg and Tcon. a Day 0 or day 6 Treg and Tcon were incubated for the indicated time points with 30µg/ml pb isotype control, pb anti-CD3 mAb, DMSO or 10ng/ml PMA plus 1µM Ionomycin. CD95L surface protein expression was detected according to the FASER amplification protocol. **b** At different days of *in vitro* culture Treg and Tcon were incubated for 20 hr with the stimuli explained in **a** and stained according to the FASER amplification protocol. Data are representative of 2 independent experiments.

3.1.5 CD95L cleavage is not causative for low CD95L expression of human Treg

Different metalloproteases can cleave CD95L from the cell surface to release a soluble form which has less apoptotic potential than the membrane bound form (Schulte et al., 2007; Schneider et al., 1998; Hohlbaum et al., 2000). Among these metalloproteases, ADAM-10 was reported to play an important role in T cells (Schulte et al., 2007). Increased cleavage of CD95L protein from the cell surface caused by higher ADAM-10 activity could be a potential cause for the low CD95L protein expression of Treg. For that reason, CD95L cleavage was investigated. Incubation with a specific ADAM-10 peptide inhibitor during stimulation had no effect on CD95L expression in day 0 Treg upon stimulation with pb anti-CD3 mAb (Fig. 3.1.6, upper panel). CD95L expression was also not altered in P/I-treated Treg incubated with an ADAM-10 inhibitor (Fig. 3.1.6, upper panel). Pb anti-CD3 mAb and P/I stimulated day 0 Tcon only marginally increased CD95L expression upon treatment with an ADAM-10 inhibitor (Fig. 3.1.6, upper panel). When day 6 Treg were incubated with an ADAM-10 inhibitor during stimulation, the level of CD95L protein was unchanged in inhibitor-treated compared to untreated cells upon stimulation (Fig. 3.1.6, lower panel). CD95L surface protein expression was similar in day 6 Tcon irrespective of incubation with an ADAM-10 inhibitor in the case of pb anti-CD3 mAb treatment (Fig. 3.1.6, lower panel). However, ADAM-10 inhibitor-treated day 6 Tcon showed a marginal increase in CD95L expression upon P/I stimulation compared to cells not treated with inhibitor (Fig. 3.1.6, lower panel).

The fact that the inhibition of the metalloprotease ADAM-10 does not increase CD95L expression in human Treg excludes CD95L cleavage as a reason for low CD95L surface protein in Treg.

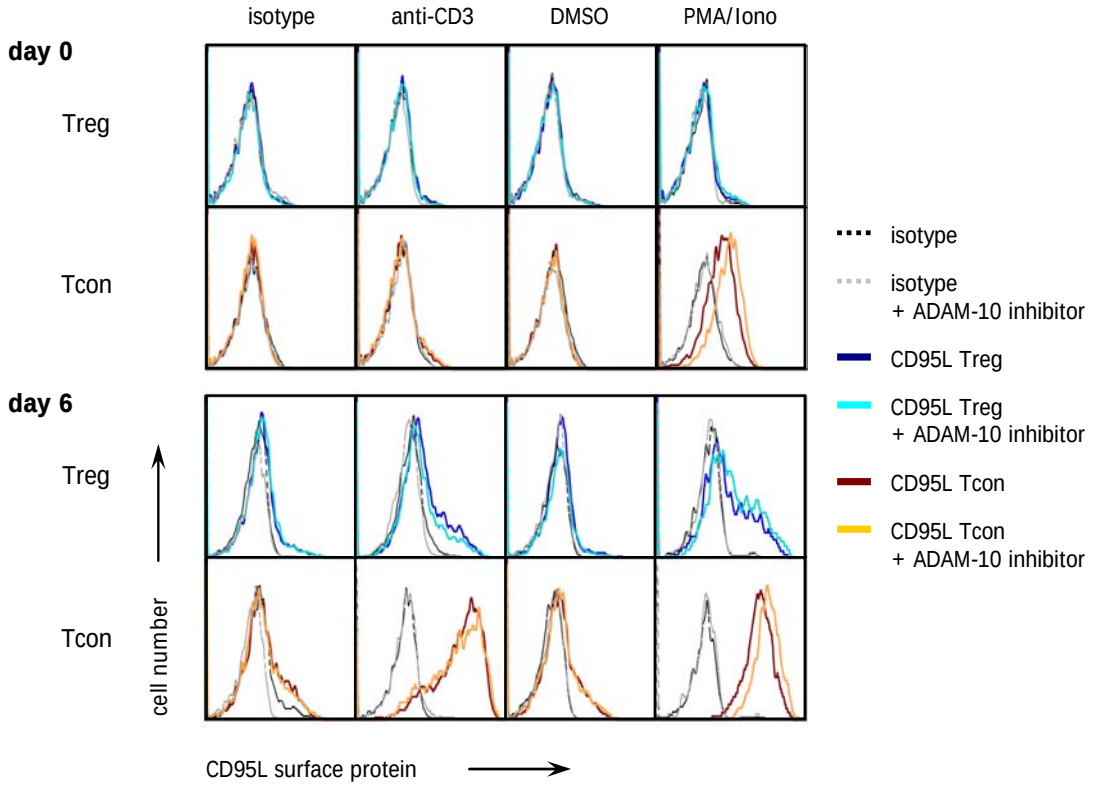

Figure 3.1.6| ADAM-10 cleavage does not cause low CD95L levels on Treg. Day 0 or day 6 Treg and Tcon were incubated for 20 hr with 30µg/ml pb isotype control, pb anti-CD3 mAb, DMSO or 10ng/ml PMA plus 1µM Ionomycin in the presence or absence of 1µM ADAM-10 inhibitor. CD95L surface protein expression was detected using the FASER amplification protocol. Data are representative of 2 independent experiments.

3.1.7 Knock-down of Foxp3 in human Treg increases CD95L expression

The data shown in the previous paragraphs demonstrate a low CD95L expression by human Treg. One factor that might be responsible for the low CD95L expression in Treg is the TF Foxp3. Foxp3 is highly expressed in Treg and can bind together with other transcriptional regulators to the promoter of cytokines like IL-2 and repress transcription (Wu et al., 2006; Fontenot et al., 2003; Chen et al., 2006; Khattri et al., 2003). In a genome-wide analysis of Foxp3 target genes, Zheng *et al.* found that Foxp3 is able to bind to the CD95L gene (Zheng et al., 2007b).

To investigate if Foxp3 negatively regulates CD95L expression in Treg, knock-down experiments were performed. Foxp3 protein expression was substantially down-regulated in Treg 72 hr after transfection with a Foxp3-specific siRNA (siFoxp3) while the scrambled control siRNA (siCtrl) could not down-regulate Foxp3 expression (Fig. 3.1.7 a and b).. To test CD95L protein expression, cells were stimulated 72 hr after Foxp3 knock-down with P/I for 20 hr. An approximately two fold increase of CD95L protein expression in siFoxp3 to control transfected Treg could be observed after stimulation with P/I while treatment of Tcon with the specific siRNA against Foxp3 did not alter their CD95L protein expression after stimulation (Fig. 3.1.7 c). Although CD95L protein expression was increased by the knock-down of Foxp3, the CD95L protein levels produced by Treg did not reach Tcon levels 72 hr after Foxp3 knock-down (Fig. 3.1.7 c). The difference between CD95L expression of Treg compared to Tcon might be due to residual Foxp3 protein after knock-down. However, this result clearly allots a role to Foxp3 in the repression of CD95L expression in human Treg.

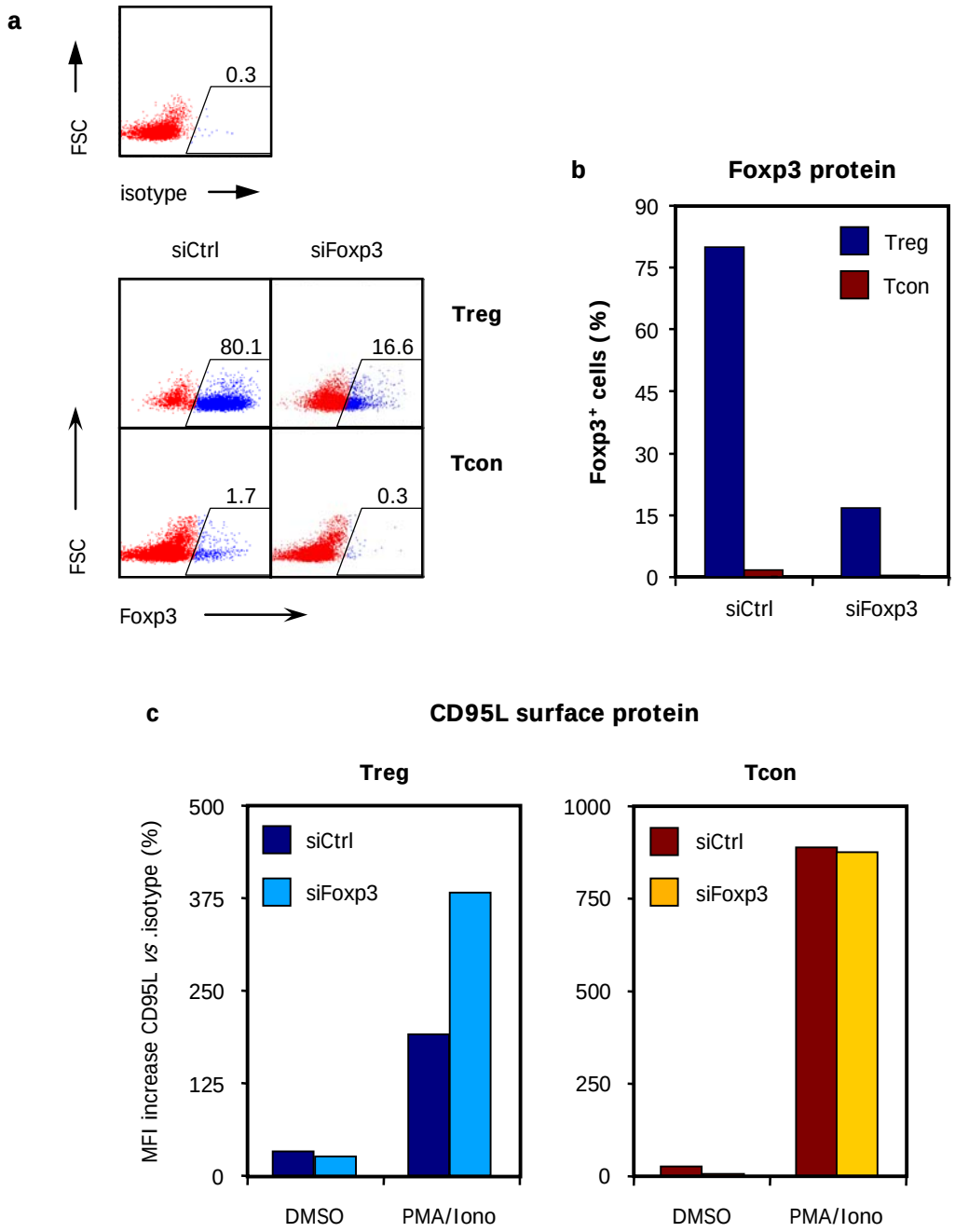

Figure 3.1.7| Foxp3 knock-down leads to increased CD95L expression in human Treg after stimulation. a Foxp3 expression levels of day 0 Treg and Tcon 72 hr after siCtrl or siFoxp3 transfection detected by intracellular flow cytometry. **b** Quantification of Foxp3 protein expression 72 hr after knock-down in day 0 Treg and Tcon. **c** 72 hr after transfection, day 0 Treg and Tcon were stimulated for 20 hr with DMSO or 10ng PMA plus 1µM Ionomycin. Surface CD95L protein expression was investigated according to the amplification protocol and calculated as MFI increase of CD95L compared to isotype stained cells. Data are representative of 3 independent experiments.

3.1.8 Murine Treg express less CD95L protein compared to Tcon

In addition to the human system, Treg and Tcon isolated from C57/Black6 (B6) wt mice were analyzed for CD95L protein expression. Freshly isolated murine Treg, like human Treg, failed to induce substantial CD95L protein upon pb anti-CD3 mAb (Fig. 3.1.8). Stimulation with P/I could also not induce CD95L protein in murine Treg which is comparable to Treg in the human system (Fig. 3.1.8). Day 0 murine Tcon could produce CD95L upon P/I treatment comparable to human Tcon (Fig. 3.1.8). However, in contrast to human day 0 Tcon which did not upregulate CD95L expression upon pb anti-CD3 mAb, murine day 0 Tcon displayed CD95L protein on the cell surface after stimulation with pb anti-CD3 mAb (compare Fig. 3.1.3 with Fig. 3.1.8). As the physiological stimulus pb anti-CD3 mAb induced CD95L expression in murine day 0 Tcon, CD95L expression was investigated exclusively in murine day 0 T cells. The obtained data regarding CD95L expression in murine Treg demonstrate that, like in the human system, Treg isolated from mouse have low stimulation-dependent CD95L protein expression.

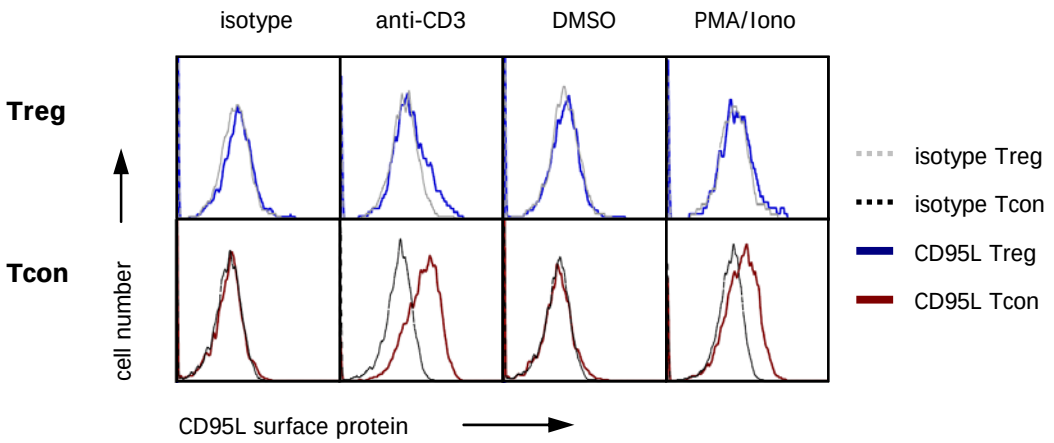

Figure 3.1.8| Murine Treg express low CD95L protein after stimulation. Freshly isolated Treg and Tcon isolated from B6 wt mice were stimulated for 20 hr with 3µg/ml pb isotype, pb anti-CD3 mAb, DMSO or 10ng PMA plus 1µM Ionomycin. CD95L surface expression was analyzed by the use of the FASER amplification method. Data are representative of 2 independent experiments.

3.1.9 Murine Foxp3-deficient Treg express CD95L

As shown before (Fig. 3.1.7), Foxp3 knock-down in human Treg led to an increased CD95L expression compared to control siRNA treated Treg after stimulation. However, CD95L expression levels in Foxp3 down-regulated Treg did not reach expression levels of Tcon which might be due to the residual Foxp3 protein expression in Treg after knock-down. To further clarify the role of Foxp3 in CD95L repression, Foxp3-deficient scurfy mice were investigated. The scurfy mouse strain harbors a spontaneous Foxp3 mutation leading to an incomplete mRNA and an unstable Foxp3 protein. As a consequence, Treg of these mice lack Foxp3 and can therefore not be isolated and investigated. To enable the analysis of Foxp3-deficient so called "would-be" Treg of scurfy mice, this strain was crossed with Depletion Of Treg (DEREG) mice. The DEREG mouse strain contains a construct in which a green fluorescent protein (GFP) - DT receptor (DTR) fusion protein is driven by an additional Foxp3 promoter. Since the Foxp3 promoter is only active in Treg, Treg from DEREG mice are GFP-DTR positive. On the basis of GFP expression, Treg can be distinguished from other $CD4^+$ cells by flow cytometry while they can be depleted by the injection of DT into living mice. Despite the mutated non-functional Foxp3 in Treg of scurfy mice the Foxp3 promoter is active. "would-be" Treg of scurfy x DEREG mice express the GFP-DTR fusion protein controlled by the additional Foxp3 promoter activity and can therefore be traced by GFP expression (Lahl et al., 2007). This makes it possible to investigate the effect of Foxp3 deficiency on CD95L expression in Treg.

$CD4^+$ T cells were isolated from spleens and pooled lymph nodes (LN) of scurfy x DEREG and control DEREG mice and stimulated for 20 hr with pb anti-CD3 mAb. As shown in Fig. 3.1.8, the stimulation of murine day 0 Tcon with pb anti-CD3 mAb induces high amounts of CD95L surface protein (in contrast to human Tcon which stay CD95L negative upon pb anti-CD3 mAb on day 0). Therefore, this more physiological stimulus and not the strong stimulus P/I was used. By gating on GFP^- (Tcon) and GFP^+ (Treg) cells, CD95L protein expression could be investigated in the two cell populations. Comparable to murine wt Treg (and human Treg), Treg from

DEREG mice failed to produce CD95L upon stimulation with pb anti-CD3 mAb (Fig. 3.1.9). Tcon from DEREG mice were able to induce CD95L expression upon pb anti-CD3 mAb stimulation (Fig. 3.1.9). Interestingly, Foxp3-deficient "would-be" Treg from scurfy x DEREG mice produced CD95L protein in amounts comparable to Tcon from scurfy x DEREG and wt DEREG mice (Fig. 3.1.9). Together with the data obtained by Foxp3 knock-down in human Treg, these results strengthen the role of Foxp3 in the negative regulation of CD95L expression in Treg.

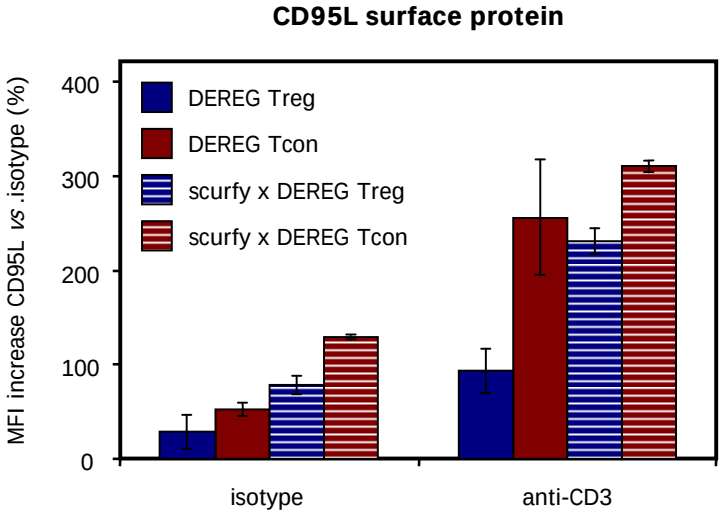

Figure 3.1.9 | CD95L expression is high in Foxp3-deficient scurfy x DEREG "would-be" Treg after stimulation.
MACS sorted $CD4^+$ T cells from DEREG and scurfy x DEREG mice were stimulated for 20 hr with 3µg/ml pb isotype control or pb anti-CD3 mAb. Cells were stained for CD95L with FASER amplification protocol. GFP^+ (Treg) and GFP^- (Tcon) were gated and analyzed for CD95L expression. Data are representative of 3

3.2 *In vivo* studies

Human Treg express the death receptor CD95 on the cell surface and are highly sensitive to apoptosis induced by exogenous CD95L *in vitro* (Fritzsching et al., 2005). Murine Treg in tumors can be depleted by CD95 stimulation and by $CD95L^+$ cells during ongoing colitis (Reardon et al., 2008; Chen et al., 2007). However, the sensitivity of Treg to CD95-induced apoptosis in healthy mice in the absence of an immune reaction remains elusive. The *in vivo* studies therefore aim to investigate, with the help of a suitable mouse model, whether Treg are sensitive to CD95-induced apoptosis *per se* and can be depleted by CD95 engagement *in vivo*.

3.2.1 Characterization of $RAG2^{-/-}$ x lpr mice

For the *in vivo* experiments in the follwing paragraphs, $RAG2^{-/-}$ x lpr mice were used. These were obtained by crossing $RAG2^{-/-}$ and lpr mice (both on the B6 background). Lpr mice have a mutation in the CD95 gene which leads to a decreased expression of CD95 on the cell surface. Among other symptoms, these mice develop lymphadenopathy and splenomegaly caused by increased numbers of aberrant $CD3^+$ $B220^+$ $CD4^-$ $CD8^-$ T lymphocytes. $RAG2^{-/-}$ mice have negligible numbers of lymphocytes due to a deficiency in RAG2, an enzyme needed for the recombination of the receptor genes of T and B lymphocytes which is reflected by a size reduction of their lymphatic organs. To investigate if the residual T and B cells in $RAG2^{-/-}$ x lpr mice can give rise to aberrant $CD3^+$ $B220^+$ T cells and lead to lymphadenopathy and splenomegaly, T and B cell markers as well as spleen and LN of $RAG2^{-/-}$ x lpr mice were compared to B6 control mice, $RAG2^{-/-}$ and lpr mice. B6 mice had normally sized spleen and LN, whereas the organs of $RAG2^{-/-}$ were reduced in size (Fig. 3.2.1 a). Lpr mice, in contrast, had enlarged spleen and LN (Fig. 3.2.1 a). The characterization of $RAG2^{-/-}$ x lpr mice showed that spleens and LN were similarly small as organs from $RAG2^{-/-}$ mice (Fig. 3.2.1 a). By flow cytometry, normal numbers of $CD3^+$ T cells and $B220^+$ B cells were detectable in spleens of B6 control mice, whereas $RAG2^{-/-}$ splenic mononuclear cells, as expected, contained reduced numbers of T and B cells. Both

mouse strains did not have elevated numbers of aberrant T lymphocytes. Lpr mice, displayed increased levels of aberrant T cells (Fig. 3.2.1 b). Interestingly, the residual T and B cells of RAG2$^{-/-}$ x lpr mice did not give rise to CD3^{+} B220^{+} T cells (Fig. 3.2.1 b). Similar results regarding CD3^{+} and B220^{+} cells were obtained with cell preparations from LN of the different mouse strains (data not shown). These results demonstrate that although RAG2$^{-/-}$ x lpr mice harbor the mutated form of CD95 the residual T and B cells do not give rise to aberrant CD3^{+} B220^{+} T cells.

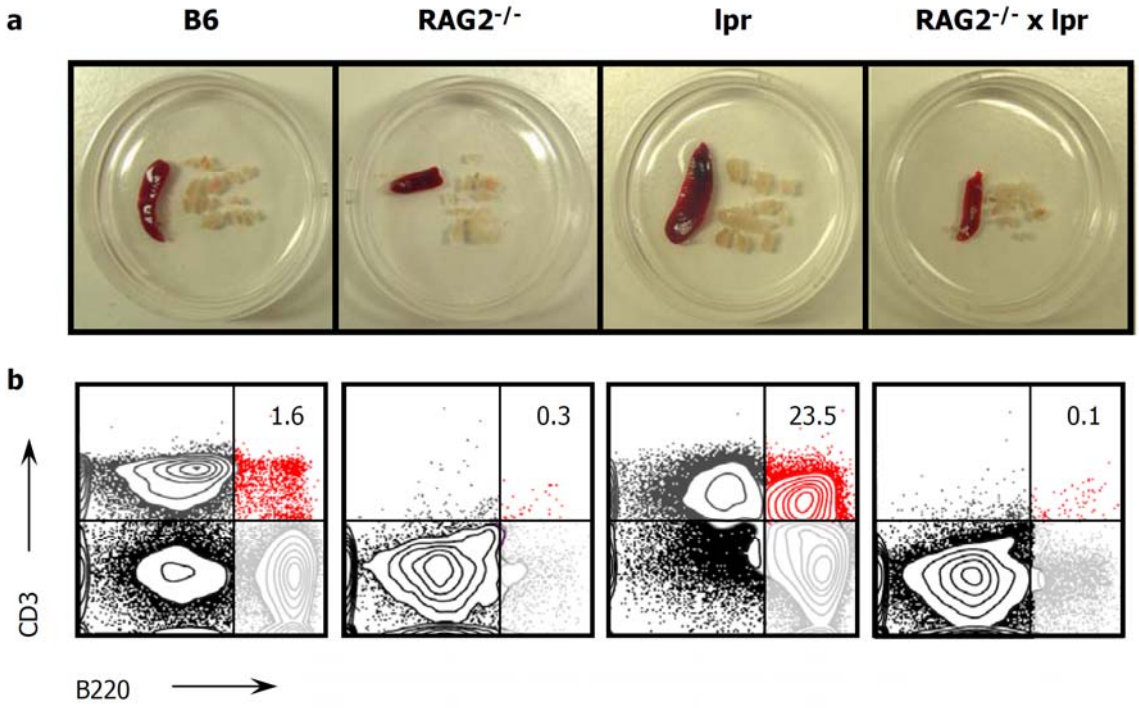

Figure 3.2.1 | Characterization of RAG2$^{-/-}$ x lpr mice. **a** LN (axillary, lateral axillary, inguinal, mesenteric, sacral) and spleens isolated from B6, RAG2$^{-/-}$, lpr and RAG2$^{-/-}$ x lpr mice. **b** Splenic mononuclear cells were stained for CD3 and B220 and analyzed by flow cytometry to detect T cells, B cells and aberrant CD3^{+} B220^{+} T cells. Data are representative of 2 independent experiments.

3.2.2 Establishment of BM chimeric RAG2$^{-/-}$ x lpr mice

To investigate the sensitivity of Treg to CD95-induced apoptosis *in vivo*, a BM chimeric mouse model was established. Upon injection of anti-CD95 mAb, CD95 wt Foxp3.DGL mice (this mouse strain will be explained below) die by liver failure while lpr mice survive owing to low CD95 expression caused by mutated CD95 (Fig. 3.2.2 a) (Kaufmann et al., 2007; Ogasawara et al., 1993). Although lpr mice survive the injection with anti-CD95 mAb, the role of CD95 cannot be investigated as all cells lack functional CD95 (Fig. 3.2.2 a). BM chimeric mice, in which the host is deficient in CD95 (lpr) and the donor lymphocytes express CD95, are suitable to answer the question if Treg are sensitive to CD95-induced apoptosis *in vivo* (Fig. 3.2.2 a). As host mice, RAG2$^{-/-}$ x lpr mice which do not die upon injection of anti-CD95 and cannot produce lymphocytes themselves were used as recipients of BM transplants (Fig. 3.2.2 a).

Sublethal irradiation partially depletes BM cells in the recipient and opens niches for the BM graft. Although the donor BM will reconstitute only a part of the BM in the RAG2$^{-/-}$ x lpr recipient mouse it will give rise to the entire lymphocyte pool as the host BM lacks RAG2.

Foxp3.DGL mice, the BM donors, contain a bacterial artificial chromosome (BAC) in which the Foxp3 promoter controls the expression of three individual genes encoding for DTR, GFP and luciferase (different from DEREG mice which express a GFP-DTR fusion protein). As the Foxp3 promoter is only active in Treg, Treg of these mice express DTR, GFP and luciferase. Treg in Foxp3.DGL mice can therefore be depleted by the injection of DT while the expression of luciferase enables the detection and quantification of Treg by *in vivo* imaging in anesthetized mice upon injection of luciferin. In addition, Treg can be traced on the basis of GFP expression by flow cytometry.

To obtain BM chimeras, irradiated RAG2$^{-/-}$ x lpr mice were intravenously injected with T cell-depleted BM from either Foxp3.DGL or Foxp3.DGL x lpr mice. The resulting BM chimeric RAG2$^{-/-}$ x lpr mice either contain T cells that are CD95

sufficient (Foxp3.DGL BM) or CD95-deficient (Foxp3.DGL x lpr BM) and can be used after a period of 6-8 weeks (donor BM engraftment period) to investigate whether Treg are sensitive to CD95-induced apoptosis in the absence of an immune response *in vivo* (Fig. 3.2.2 b).

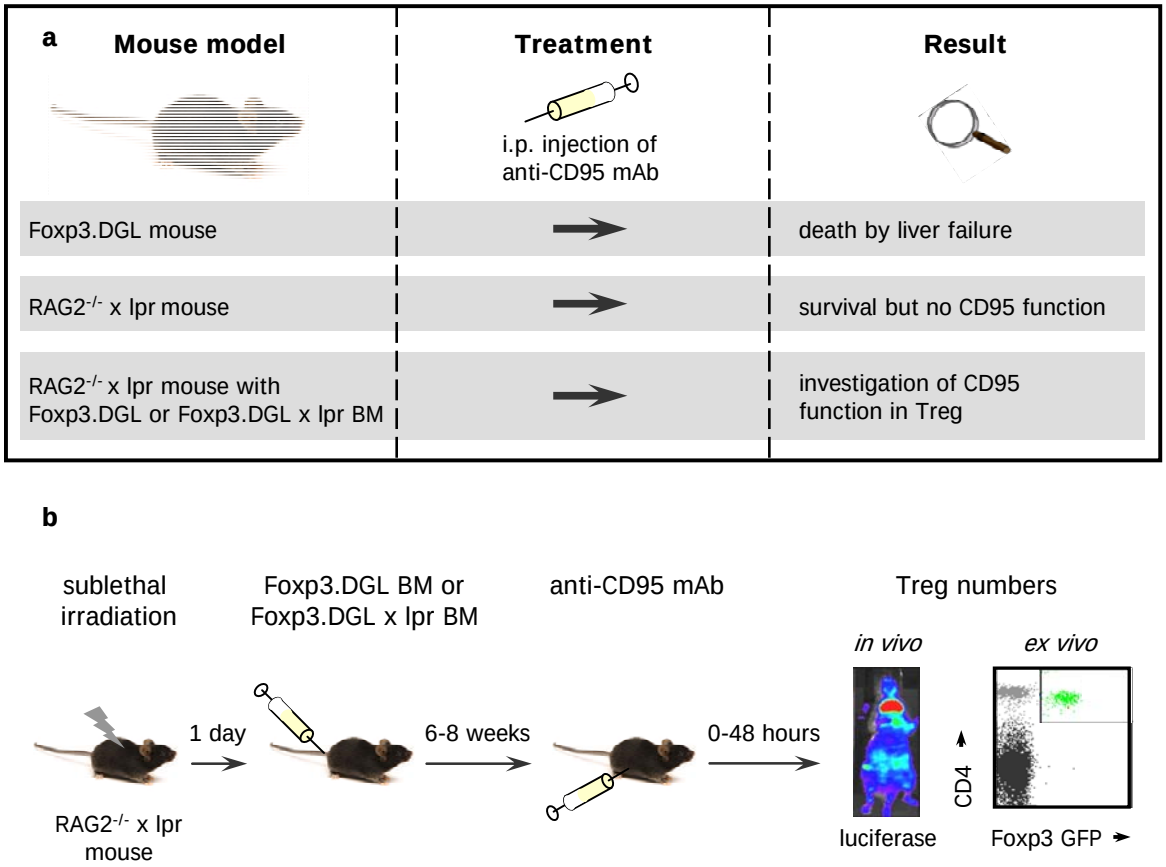

Figure 3.2.2| Protocol for BM chimeric mice. a Effect of the injection of an agonistic anti-CD95 mAb into different mouse strains. Only RAG2$^{-/-}$ x lpr mice with Foxp3.DGL BM can be used for the investigation of CD95 in Treg. **b** 5-8 week old RAG2$^{-/-}$ x lpr mice were sublethally irradiated and received one day later an i.v. injection containing 1-5 x 10^6 CD3-depleted BM cells from either Foxp3.DGL or Foxp3.DGL x lpr mice. 6-8 weeks after BM transfer, mice were i.p. injected with an isotype control or an anti-CD95 mAb. Treg numbers were monitored between 0 and 48 hr after treatment *in vivo* and *ex vivo*.

3.2.3 Depletion of Treg by CD95 stimulation

The sensitivity of Treg to CD95-induced apoptosis has not yet been investigated in healthy mice. Therefore, in the following experiments, the sensitivity of Treg to CD95-induced apoptosis *in vivo* in non-infected healthy mice was examined. BM chimeric RAG2$^{-/-}$ x lpr mice engrafted with Foxp3.DGL or Foxp3.DGL x lpr BM display luciferase activity exclusively in Treg. Mice were injected i.p. with an isotype control mAb, anti-CD95 mAb or DT (Treg depletion control). Before (0 hr) and 24 and 48 hr after treatment, luciferase activity was monitored by *in vivo* imaging. RAG2$^{-/-}$ x lpr chimeras that had received Foxp3.DGL BM (hereafter abbreviated as Foxp3.DGL → RAG2$^{-/-}$ x lpr) had lower luciferase activity levels after injection of anti-CD95 mAb compared to isotype treated mice (Fig. 3.2.3 a and b). The detected luciferase activity was lower after 24 hr of treatment with anti-CD95 mAb and started to rise again after 48 hr in one mouse whereas it remained almost identically low at both time points in the other mouse treated with anti-CD95 mAb (Fig. 3.2.3 b). The level of luciferase activity did not decrease in Foxp3.DGL x lpr → RAG2$^{-/-}$ x lpr mice, in which the T cells are CD95-deficient, independent of treatment with isotype or anti-CD95 mAb (Fig. 3.2.3 a and b). Luciferase activity even increased in Foxp3.DGL x lpr → RAG2$^{-/-}$ x lpr mice at 24 and 48 hr after treatment compared to 0 hr (Fig. 3.2.3 b). The injection of DT resulted in a decrease of luciferase activity in both Foxp3.DGL → RAG2$^{-/-}$ x lpr and Foxp3.DGL x lpr → RAG2$^{-/-}$ x lpr BM chimeras which was more pronounced after 24 hr (Fig. 3.2.3 a and b and data not shown). However, one can see that individual mice had different luciferase activity levels caused by variable degrees of BM chimerism (Fig. 3.2.3 a, *e.g.* mouse 1 and 3).

In addition, 48 hr after treatment, the amount of GFP^{+} Treg was investigated in pooled LN (axillary, inguinal, mesenteric and sacral). It revealed that substantially less GFP^{+} Treg were present in Foxp3.DGL → RAG2$^{-/-}$ x lpr mice that had received anti-CD95 mAb compared to isotype treated mice (Fig. 3.2.3 c). In contrast, GFP^{+} cell numbers remained almost constant in Foxp3.DGL x lpr → RAG2$^{-/-}$ x lpr mice that were treated with isotype or anti-CD95 mAb (Fig. 3.2.3 c). DT as positive control for the

depletion of Treg was able to diminish the numbers of GFP^+ cells in both types of BM chimeras (Fig. 3.2.3 c and data not shown). Interestingly, the numbers of $CD4^+$ GFP^- T cells (Tcon) was only slightly altered (Fig. 3.2.3 c). These results show that Treg are not only *in vitro* sensitive to CD95-induced apoptosis but also *in vivo* in the absence of an immune response.

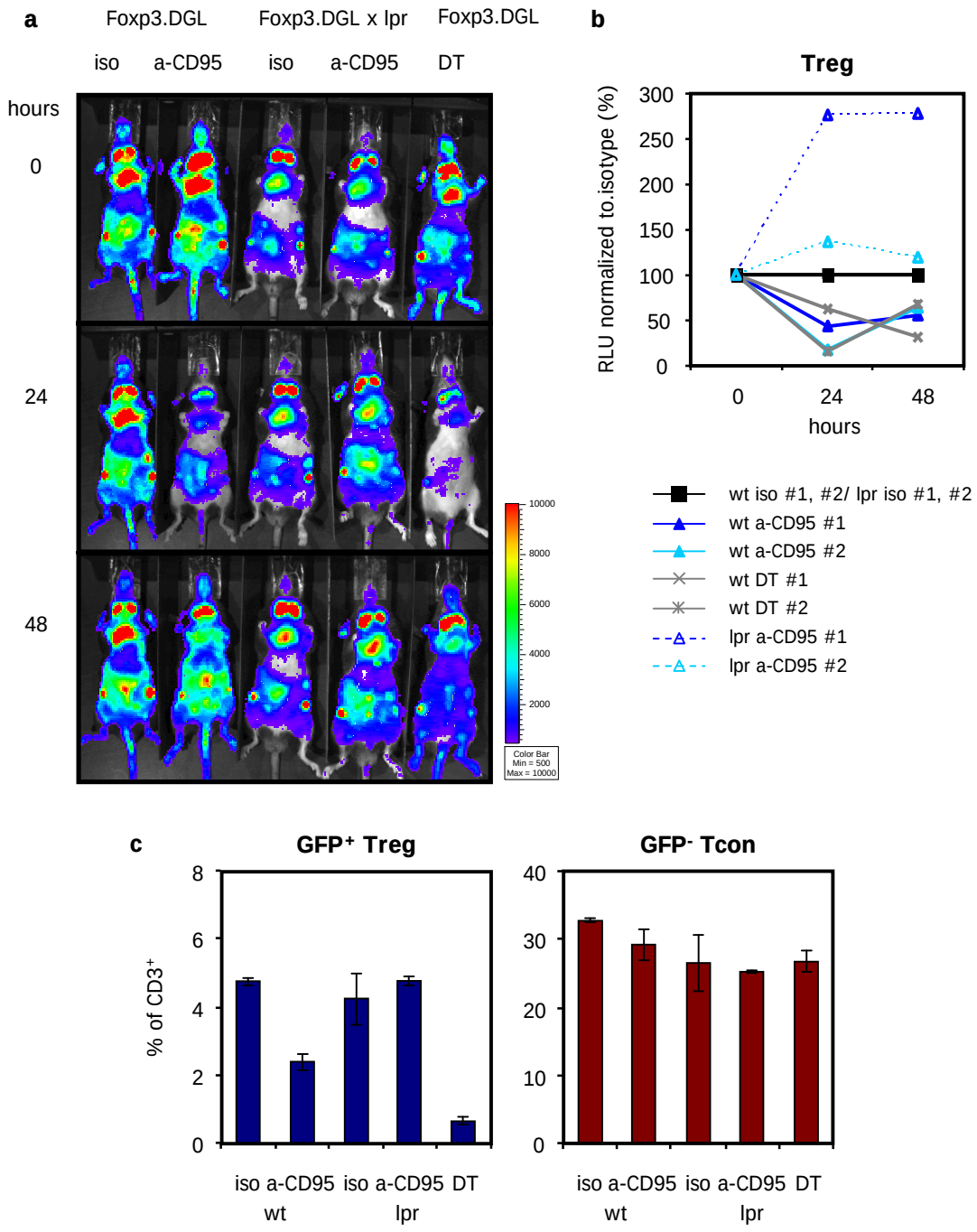

Figure 3.2.3 | Partial depletion of Treg by CD95 stimulation *in vivo*. **a** and **b** Investigation of Treg numbers by bioluminescence *in vivo*. **a** Anesthetized mice were injected with luciferin and *in vivo* bioluminescence was measured before (0 hr) and after treatment with isotype control (iso), anti-CD95 mAb (a-CD95) or DT (24 and 48 hr). One representative picture is shown. **b** Light output was quantified as relative light units (RLU) normalized to isotype control. **c** 48 hr after treatment, mice were sacrificed and pooled LN (inguinal, axillary, lateral axillary, mesenteric, sacral) were investigated for $CD4^+GFP^+$ Treg and $CD4^+GFP^-$ Tcon. Isotype n=2 (wt, lpr), anti-CD95 mAb n=2 (wt, lpr), DT, n=2 (lpr). One of three representative experiments is shown.

4 Discussion

4.1 *In vitro* experiments

Treg maintain immunological self tolerance by controlling potentially hazardous self-reactive T cells (Sakaguchi et al., 2008). Regulated proliferation and apoptosis are necessary to achieve equilibrated cell populations. In the context of apoptosis, several laboratories have demonstrated that Treg apoptosis is different in comparison to Tcon (Banz et al., 2002; Taams et al., 2001b; Fritzsching et al., 2005; Taylor et al., 2007). Effector/memory Treg, which comprise the largest fraction of Treg in adults, are characterized by elevated expression of CD95 and high sensitivity to apoptosis induced by CD95 triggering. Although Treg are sensitive to CD95-induced apoptosis, they cannot be killed by TCR stimulation which triggers AICD by CD95L production *in vitro*. In contrast, TCR re-stimulation induces CD95L expression in expanded Tcon and results in AICD (Alderson et al., 1995; Dhein et al., 1995; Klas et al., 1993; Krueger et al., 2003b). As the balance between Treg and effector cell subsets is important for initiation, course and outcome of an immune response, a detailed investigation of the mechanisms of Treg proliferation and death is fundamental to understand Treg homeostasis. The fact that Treg do not undergo AICD might be explained by diminished or altered CD95L expression. Therefore, the aim of the *in vitro* studies was to investigate the level of CD95L expression in Treg.

4.1.1 Treg have low CD95L mRNA and protein levels

The data presented in this thesis demonstrate that Treg express less CD95L mRNA upon stimulation compared to Tcon, irrespective if they were freshly isolated or *in vitro* expanded for 6 days (Fig. 3.1.1). In addition, the stimulus strength did not play a role in CD95L mRNA expression as the rather physiological stimulus pb anti-CD3 mAb as well as the strong stimulus P/I could not substantially increase CD95L mRNA in Treg (Fig. 3.1.1). The obtained data are in line with mRNA array data in which it was shown that Treg express less CD95L mRNA than Tcon (Gavin et al., 2002).

However, CD95L mRNA was not absent in Treg upon stimulation but only diminished. The low amount of CD95L mRNA could still result in CD95L protein expression. Nevertheless, investigation of CD95L surface protein expression showed that not only CD95L mRNA but also CD95L surface protein expression was reduced in Treg after stimulation (Fig. 3.1.3). This reduced CD95L expression could be observed in freshly isolated as well as *in vitro* expanded Treg. In addition, the signal strength, as for CD95L mRNA expression, did not play a crucial role in the inducibility of CD95L protein expression by Treg. In contrast, stimulation-induced expression of CD95L surface protein was highly upregulated in Tcon (Fig. 3.1.3).

Although Treg showed reduced CD95L expression in all experiments, expression levels varied between individual experiments. These differences are probably donor-dependent. However, CD95L mRNA and protein expression by Treg remained always less compared to Tcon. Moreover, on day 6 of culture, a small number of cells in the Treg population upregulated CD95L protein. These cells arise most likely from few contaminating Tcon which are present since day 0. During the culture period with anti-CD3/CD28 mAb and IL-2, these Tcon expand more vigorously than Treg and accumulate. Further cell sorting after MACS isolation to obtain even purer Treg populations reduced the amount of $CD95L^+$ cells in the Treg population on day 6, affirming that these were contaminating Tcon (data not shown).

Furthermore, CD95L protein expression of murine Tcon and Treg was investigated. Similar to the results obtained in the human system, Treg did not induce CD95L expression upon stimulation while Tcon became highly positive (Fig. 3.1.8). Together, these data imply that human as well as murine Treg are truly impaired in the expression of CD95L mRNA and protein.

So far, four different studies have investigated CD95L expression by Treg in different experimental settings using human and murine Treg. Three of them demonstrated a low CD95L expression by Treg upon stimulation (Chai et al., 2002; Singh et al., 2010; Strauss et al., 2009). In 2002, Chai and colleages investigated murine MACS-isolated Treg. Upon stimulation with anti-CD3 mAb and BM-derived APC for 2 days, Tcon displayed intracellular CD95L expression while Treg remained negative (Chai et al.,

2002). Furthermore, Strauss *et al*. investigated CD95L expression of stimulated human Treg sorted from peripheral blood. A 48 hr stimulation period with soluble anti-CD3 mAb could not induce CD95L expression in human Treg. Interestingly, Treg isolated from patients with head and neck squamous cell cancer highly upregulated CD95L protein upon TCR stimulation (Strauss et al., 2009). The finding that Treg can switch between lack of CD95L expression in healthy donors and high CD95L expression in this specific cancer type, leads to the assumption that Treg might use CD95-induced cell death as a mechanism to kill anti-tumor effector cells. In addition, the third report showed that FACS-sorted murine Treg express low amounts of CD95L protein after 3 days of stimulation with anti-CD3/CD28 mAb whereas Tcon showed high CD95L expression (Singh et al., 2010). In contrast to the results presented in this thesis and in the three described publications, a human study demonstrated that Treg can express CD95L. In this article, the authors found that $CCR4^+$ Treg, which constitute the major part of Treg from peripheral blood, produced CD95L upon culture in medium containing human serum for 16 hr (Baatar et al., 2007). Three factors are conceivable that could account for the difference between the conflicting data and the other three publications and the results presented herein. In the contradictory publication, human serum was used as stimulus while all other experimental settings included TCR stimulation to trigger CD95L expression. In addition, the isolation procedure in the conflicting publication is more stressful as the cells are sorted by three subsequent isolation steps: first, cells are isolated on the basis of CD4. This is followed by a CCR4 isolation step and finally the cells are isolated by CD25. In contrast, only 1-2 isolation steps were used in the other studies. Furthermore, it is difficult to judge the level of CD95L expression by Treg as it was not compared to Tcon. However, the authors show that CD95L-expressing Treg can kill Jurkat T cells in a CD95-dependent manner. In contrast, data obtained in this thesis show that the level of CD95L expression in stimulated Treg is insufficient to kill CD95-sensitive SKW B cells, while stimulated Tcon induce significant death (data not shown).

The low CD95L expression by Treg after stimulation *in vitro* should be considered as a prerequisite to ensure their survival, as they would constantly commit suicide or

fratricide if they expressed high levels of CD95L due to their high sensitivity to CD95-induced apoptosis. In addition, low CD95L in Treg after stimulation excludes CD95-mediated apoptosis as mechanism by which Treg suppress responder cells.

4.1.2 Low CD95L protein is not due to the activation status of Treg

Due to continuous contact to self-Ag, the Treg population in human peripheral blood isolated from adults is mainly composed of effector/memory Treg (Fritzsching et al., 2006). In contrast, Treg from cord blood contain a large proportion of naïve/resting Treg. Effector/memory and naïve/resting Treg behave differently regarding apoptosis. While effector/memory Treg are sensitive to CD95-mediated apoptosis, naïve/resting Treg are characterized by their resistance to CD95 stimulation (Fritzsching et al., 2006; Fritzsching et al., 2005). Owing to their resistance to CD95-mediated apoptosis, naïve/resting Treg could use CD95L as suppressive mechanism as they would not commit suicide or fratricide upon expression. A difference in CD95L expression between naïve/resting and effector/memory Treg was therefore possible. Nevertheless, investigation of CD95L expression by naïve/resting Treg from cord blood revealed a low CD95L expression after stimulation (Fig. 3.1.4). Thus, also naïve/resting Treg, comparable to effector/memory Treg, cannot express high amounts of CD95L upon stimulation. Although $CD45RA^+$ Treg are classified as naïve/resting while $CD45RO^+$ Treg consist of effector/memory cells, there are no major functional differences between naïve/resting and effector/memory Treg, both are immunosuppressive (Valmori et al., 2005; Seddiki et al., 2006b).

In human individuals, it has been shown that Treg have a shorter half-life compared to Tcon (Vukmanovic-Stejic et al., 2006). This might be due to the fact that effector/memory Treg are characterized by elevated expression of CD95 and high sensitivity to apoptosis induced by CD95 crosslinking. However, naïve/resting Treg are not sensitive to CD95-induced apoptosis and are spared from CD95-mediated apoptosis (Fritzsching et al., 2006). Despite their resistance to CD95, naïve/resting Treg cannot express substantial CD95L protein after stimulation. This establishes the

low CD95L expression as a characteristic of all Treg.

4.1.3 Low CD95L protein is not caused by altered expression kinetics or increased cleavage from the cell membrane

Although CD95L expression in Treg has been investigated by other laboratories, a detailed analysis has not been conducted thus far (Strauss et al., 2009; Singh et al., 2010; Chai et al., 2002; Baatar et al., 2007). CD95L mRNA and protein can be induced by Treg to some extent indicating that the signaling machinery is functional but reduced (Fig. 3.1.1 and 3.1.3). Low CD95L expression in Treg at a time point at which Tcon express substantial levels of CD95L mRNA or protein could argue for a distinct regulation of CD95L expression in Treg. Therefore, different factors that might account for the low CD95L expression in Treg were investigated.

The Treg population in human adults contains a high percentage of effector/memory cells, whereas the Tcon population comprises more naïve cells. Hence, CD95L expression in Treg might follow different expression kinetics compared to Tcon. Thus, CD95L expression at different time points after stimulation was examined in Treg. Regarding CD95L expression, it has been shown that pre-activated T cells can release CD95L from intracellular granules after very short stimulation periods (Schulte et al., 2007). Effector/memory Treg are characterized by their pre-activated status and might therefore express CD95L after very short stimulation periods. However, short-term stimulation of Treg did not induce CD95L expression which indicates that Treg, despite being continuously activated by self-Ag *in vivo*, do not store CD95L to release it upon activation (Fig. 3.1.5).

Treg did not increase CD95L after a very short stimulation period, however, CD95L expression in Treg might be present at different time points after stimulation compared to Tcon. To further investigate CD95L expression kinetics in Treg, cells were stimulated for an intermediate (10 hr) and a longer time period (40 hr). However, CD95L expression did not increase substantially in Treg at both time points while Tcon expressed high amounts of CD95L (Fig. 3.1.5). This excludes an

accelerated activation of the signaling machinery for the production of CD95L in Treg. The lack of CD95L after longer stimulation periods might be explained by a reduced or delayed stimulation-dependent signaling in Treg. A vast number of different stimulation-induced signaling molecules is required for the induction of CD95L transcription (Li-Weber and Krammer, 2003; Kavurma and Khachigian, 2003). Some of these molecules (phosphorylated Erk, $Ca2^{+}$ influx) were shown to be reduced in Treg (Tsang et al., 2006). Furthermore, activated NF-AT, which is crucial for CD95L expression, is present in reduced amount in the nucleus of Treg compared to Tcon (Hickman et al., 2006).

Not only the time point after stimulation might be decisive for CD95L in Treg, it is also possible that Treg do not express CD95L on day 0 and day 6 but on different days of *in vitro* culture. The investigation of stimulation-dependent CD95L expression on different days of culture (day 0 – day 6) demonstrated a low CD95L expression in Treg while Tcon were able to upregulate CD95L on all days of culture (Fig. 3.1.5). These results indicate that CD95L expression in Treg does not follow different *in vitro* culture kinetics.

In addition, the involvement of the metalloprotease ADAM-10 in low CD95L expression by Treg was investigated. In total T cells, ADAM-10 plays a crucial role in the cleavage of CD95L protein from the cell surface (Schulte et al., 2007). Low CD95L on Treg might be caused by increased ADAM-10-mediated CD95L cleavage due to high ADAM-10 activity. However, incubation with an inhibitor of ADAM-10 during stimulation could not increae CD95L surface expression in Treg and minimally enhanced CD95L expression by Tcon compared to cells not treated with inhibitor (Fig. 3.1.6).

Collectively, the kinetics and inhibitor data demonstrate that CD95L expression in Treg is low irrespective of the time point of stimulation or *in vitro* culture and not caused by increased CD95L cleavage from the cell membrane. It can be concluded that CD95L expression by Treg is truly impaired.

4.1.4 The absence of Foxp3 increases CD95L expression in Treg

The results obtained in this study demonstrate low CD95L expression by Treg on the mRNA as well as on the protein level. One factor that might be involved in the regulation of CD95L in Treg is the TF Foxp3 that is currently regarded as the most specific marker for the characterization of Treg (Feuerer et al., 2009). Although Foxp3 seems to be negligible for the development of Treg, it is important for their suppressive function and metabolic fitness. Foxp3 has a dual function: on some promoters it acts as transcriptional activator while it represses the activation of others. For instance, Foxp3 together with NFAT enhances the transcription of CD25, while it downregulates the transcription of IL-2 (see Fig. 1.3.2). In addition, Foxp3 can also pair with other TF besides NFAT, *e.g.* Runx/AML1 and RORγt. The presence of Foxp3 at the CD95L gene has been shown in a genome-wide Foxp3 target gene analysis (Zheng et al., 2007b). Therefore, it is possible that Foxp3 is directly involved in the repression of the CD95L promoter in Treg. To investigate the involvement of Foxp3 in CD95L expression, two experimental systems were employed. First, Foxp3 was transiently knocked down in human Treg. Second, GFP-labeled Foxp3-deficient Treg isolated from scurfy x DEREG mice were investigated. The combined use of two systems provides advantages over a single system. First, it allows the investigation of human and murine Treg. Second, although Foxp3 knock-down is not complete in human Treg, the effect of Foxp3 removal can be investigated in fully functional Treg; the murine system in contrast utilizes Foxp3$^-$ Treg and serves to investigate the complete lack of Foxp3.

The investigation of stimulated Treg treated with siRNA against Foxp3 demonstrated an approximately doubled CD95L protein expression compared to scrambled siRNA-treated Treg (Fig. 3.1.7). However, although CD95L expression was enhanced, it did not reach levels comparable to stimulated Tcon. One explanation for an elevated but still reduced CD95L protein expression in Foxp3-knocked down Treg compared to Tcon might be the residual Foxp3 protein. Despite efficient knock-down, approximately 20% of Foxp3 protein remained. This level might be enough to retain CD95L expression at a relatively low level.

In addition to the human Foxp3 knock-down studies, total $CD4^+$ T cells isolated from scurfy x DEREG mice were stimulated and CD95L expression was investigated. Similar to human siFoxp3-treated Treg, Foxp3-deficient Treg displayed elevated CD95L expression compared to Foxp3-sufficient Treg (Fig. 3.1.9). In contrast to the human system, murine Foxp3-deficient Treg showed CD95L expression levels similar to stimulated Tcon. The difference in the expression levels observed between the human and the murine system could be explained by the residual Foxp3 protein in human Treg after Foxp3 knock-down and the complete lack of Foxp3 expression in murine Treg. The residual Foxp3 protein might strongly influence the upregulation of CD95L expression in human Treg. Nevertheless, the data obtained from the human and murine experiments demonstrate that CD95L expression is affected by Foxp3 in Treg.

The obtained results do not clarify if Foxp3 is directly or indirectly involved in CD95L expression. The possibility remains that not Foxp3 itself but Foxp3-regulated factors hamper CD95L expression. These factors might still be present in Foxp3-knocked down Treg due to residual Foxp3 expression. As Foxp3 is not present at any time point in Foxp3-deficient murine Treg, Foxp3-regulated factors, *e.g.* TF like IRF6, are also absent (Zheng et al., 2007b).

To obtain insight if Foxp3 is a direct regulator for CD95L expression, further experiments have to be carried out. CD95L-promoter luciferase reporter assays in the presence or absence of Foxp3 in Jurkat T cells have so far not been conclusive (data not shown). Therefore, chromatin immunoprecipitation as well as gel shift assays would be suitable methods to clarify if Foxp3 directly binds to the CD95L promoter.

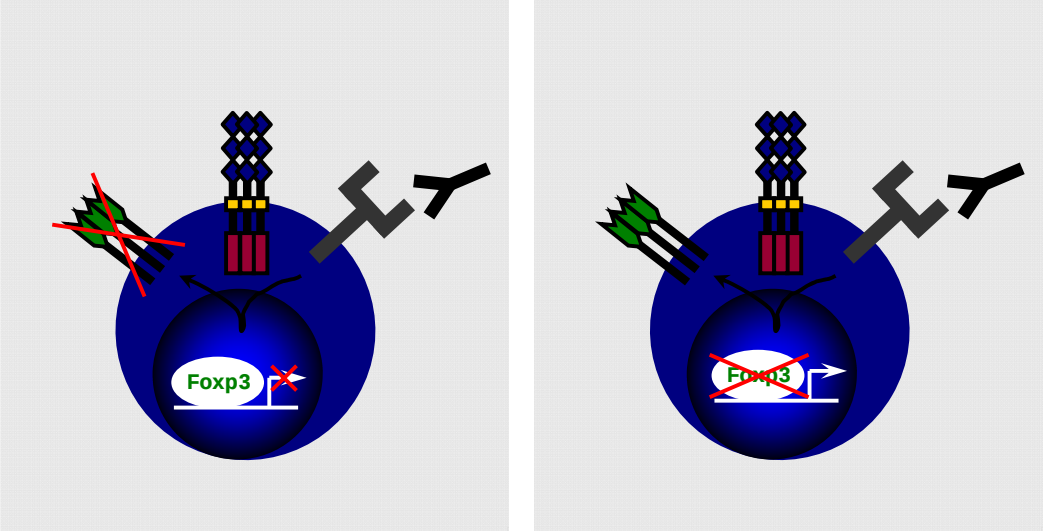

Figure 1.4.1 | The absence of Foxp3 results in stimulation-dependent CD95L expression by Treg. Foxp3-expressing Treg have low levels of CD95L surface protein whereas Foxp3 deficiency or knock-down results in elevated CD95L surface protein upon TCR stimulation.

4.2 *In vivo* experiments

During immune responses Teff are crucial for the elimination of pathogens. However, they can also participate in the development of autoimmune diseases and immune pathology. During the past years, it has become clear that every immune response involves not only the recruitment and activation of Teff but also Treg. For the proper control of the quality and magnitude of immune responses and for establishing self tolerance, the Treg-mediated maintenance of the balance of the cell populations is crucial. Consequently, the importance of Treg in this process manifests in the severe multiorgan pathology and the fatal autoimmune syndrome developed by both mice and humans lacking the Treg-specific factor Foxp3 and therefore functional Treg (Schubert et al., 2001). In addition, depletion of Treg results in the activation of self-reactive Teff and the development of autoimmune disease (Lahl et al., 2007; Kim et al., 2007).

Therefore, as Treg have to be present at the site of action in order to control Teff and other cell subsets, their homeostatic control constitutes a crucial factor. It is known that the CD95/CD95L system plays an important role in T cell homeostasis as both lpr and gld mice, harboring mutations in CD95 and CD95L, respectively, accumulate aberrant T cells. In the regulation of homeostasis of Treg, the role of the CD95/CD95L system remains controversial as gld mice have elevated Treg numbers while they do not seem to be affected in lpr mice (Mohamood et al., 2006; Zheng et al., 2007a). Therefore, a lack of data exists concerning factors and conditions that control Treg homeostasis. For this reason, the involvement of the CD95/CD95L system in the control of Treg homeostasis was investigated *in vivo*.

4.2.1 Depletion of Treg by CD95 stimulation

To investigate the involvement of the CD95/CD95L system in Treg homeostasis, a mouse model was established which enabled the examination of Treg death upon anti-CD95 mAb injection without killing the host by CD95-induced liver failure. RAG2$^{-/-}$ x lpr mice which lack functional CD95 (and tolerate anti-CD95 mAb

treatment) were reconstituted with BM isolated from either Foxp3.DGL (which give rise to CD95-sufficient T cells) or Foxp3.DGL x lpr mice (CD95-deficient T cells).
After a BM reconstitution period of 6- 8 weeks, Treg of BM chimeric RAG2$^{-/-}$ x lpr mice were investigated. Examination of luciferase activity levels (which reflect Treg numbers) 24 and 48 hr after injection of anti-CD95 mAb demonstrated that Treg numbers in Foxp3.DGL → RAG2$^{-/-}$ x lpr mice had diminished compared to the beginning of the experiment (Fig. 3.2.3). In contrast, Treg numbers of Foxp3.DGL x lpr → RAG2$^{-/-}$ x lpr mice even increased proving that the depleting effect of the anti-CD95 mAb was specific (Fig. 3.2.3). Interestingly, some Foxp3.DGL → RAG2$^{-/-}$ x lpr animals displayed stronger depletion of Treg than others. It is conceivable that the anti-CD95 mAb did not reach different tissues equally well and therefore only depleted Treg in specific organs. According to this, Treg numbers investigated by GFP measurement showed that GFP^{+} Treg decreased less in the spleen compared to LN (data not shown and Fig. 3.2.3 c).In addition, individual mice displayed a variable degree of chimerism reflected by a different amount of Treg numbers at the beginning of the experiments (Fig. 3.2.3 a, compare animal #1 with animal #3). This leads to a lower anti-CD95 mAb to Treg ratio in mice with better chimerism and could explain differences in individual mice. Furthermore, some mice had less Treg at 24 hr after injection while others had more reduced Treg numbers at 48 hr. It is possible that Treg depletion follows different kinetics in individual animals due to differential distribution of Treg or anti-CD95 mAb. Furthermore, a very restricted, but nevertheless lethal dose of anti-CD95 mAb (0.25µg/g body weight) was used. Future experiments could clarify if higher mAb doses might result in even better depletion of Treg (temporally as well as quantitatively).
Although Treg numbers decreased upon injection of anti-CD95 mAb, approximately 50% of Treg remained after anti-CD95 mAb injection. In humans, only effector/memory are sensitive to CD95-mediated apoptosis *in vitro* while resting/naïve Treg are resistant (Fritzsching et al., 2006). The Treg population in mice is heterogenous and contains effector/memory as well as resting/naïve cells. It is conceivable that only the effector/memory fraction of Treg is sensitive to CD95-

induced apoptosis *in vivo* while the resting/naïve Treg population is resistant. Resting/naïve Treg would therefore be spared from depletion by anti-CD95 engagement and explain the residual Treg population. Further experiments could clarify if the remaining Treg population is resting/naïve Treg which are less susceptible to CD95-mediated apoptosis *in vivo*.

The presented data are in line with two publications which investigated CD95-mediated apoptosis of Treg in different disease settings *in vivo* (Reardon et al., 2008; Chen et al., 2007). Chen *et al.* were interested in the effect of CD95-mediated depletion of Treg in the tumor environment. They engineered cancer cells to display CD95L on their cell surface. These cells could kill Treg at the tumor site while control cells could not. The depletion of Treg in this tumor model led to a decrease in tumor mass (Chen et al., 2007). In an additional study published in 2008, the authors investigated Treg numbers during drug-induced colitis (Reardon et al., 2008). They found that Treg were depleted from the inflamed tissue site by a yet unidentified cell type expressing CD95L at the decline of the infection. The mechanism of Treg depletion at the end of an immune response was only transient. However, it might be a short-term mechanism which allows robust pathogen-clearing immune responses following localized insult and injury.

The data obtained in this thesis demonstrate that Treg are sensitive to CD95-mediated apoptosis *in vivo*. In addition, they show for the first time that Treg can be depleted systemically in healthy non-infected mice by CD95 crosslinking. Together with the two studies mentioned above, the data demonstrate that Treg are not only sensitive to CD95-induced apoptosis during ongoing inflammation but also in non-infected animals. This suggests that CD95-mediated apoptosis is a mechanism for the homeostatic control of Treg *in vivo*. Although the $CD95L^+$ cell type remains elusive, it can be hypothesized that Teff, which express CD95L during the peak and the contraction of an immune responses, could kill CD95-sensitive Treg (Fig. 1.4.2), thereby enhancing the immune response. As explained above, this mechanism might ensure the complete elimination of pathogens that would persist if Treg-mediated suppression was already active at an earlier time point of the immune response. In

that context it was shown that CD4⁺ Tcon could kill Treg in a CD95-dependent fashion *in vitro* (Strauss et al., 2009). However, the CD95L⁺ cells will decline after clearance of the pathogen, allowing Treg to repopulate the intestine and probably help to end the immune response. In the context of autoimmune disease, however, a constant elimination of Treg by hyperactivated self-reactive Teff *via* CD95-mediated apoptosis could result in a hyperactive immune system.

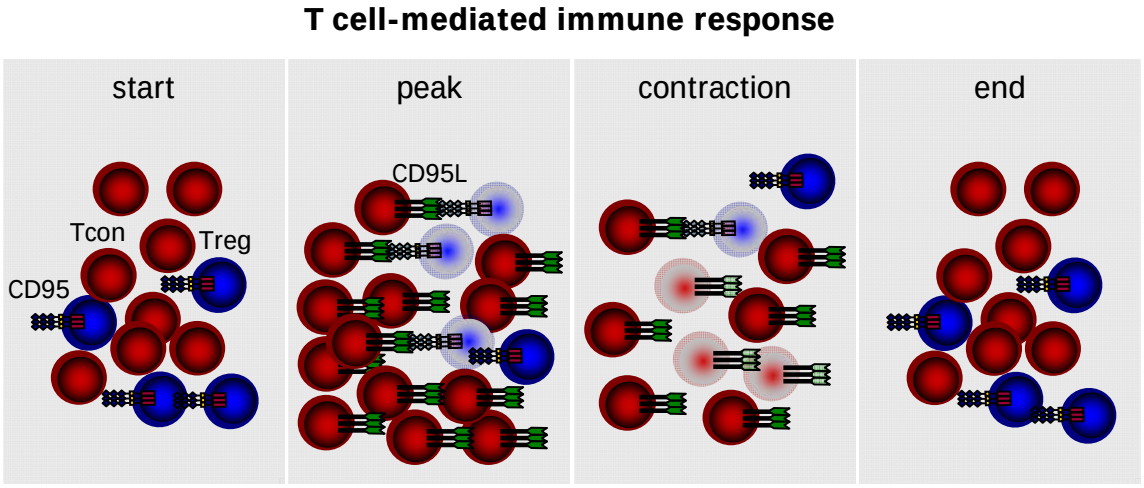

Figure 1.4.2 | Treg numbers might be regulated by CD95L-expressing Tcon during immune responses. At the beginning of an immune response, Tcon and Treg numbers are in an equilibirium. During the peak and contraction, Tcon express CD95L by which Treg and some Tcon are eliminated. This shifts the balance towards the pathogen-clearing Tcon. At the end of the immune response, the balance between both cell populations is reestablished.

4.3 Outlook

Treg depletion is being used as therapy for different diseases in which elevated Treg numbers have been reported to be disadvantageous for clinical outcome. Currently, CD25-mediated depletion of Treg is used in the clinic for the treatment of different types of cancer (Waldmann, 2007; Mahnke et al., 2007b).

The findings presented in this thesis open up the possibility to use CD95-mediated Treg depletion as novel therapeutic strategy. As the systemic use of anti-CD95 mAb would lead to liver failure in human patients, a particular therapy has to be considered. The finding of a Treg-specific surface marker would allow the application of a bispecific mAb targeting the Treg surface molecule and CD95 to specifically deplete Treg. In conclusion, the presented data might provide the basis for the development of new and powerful therapies.

5 List of abbreviations

ADAM-10	a disintegrin and metalloprotease
Ag	antigen(s)
AICD	activation-induced cell death
ALPS	autoimmune lymphoproliferative syndrome
APC	antigen presenting cell
Bcl-2	B cell lymphoma 2
BM	bone marrow
mAb	monoclonal antibody
caspase	cysteine-aspartic acid protease
CD	cluster of differentiation
CD95L	CD95 ligand
cγ	common γ chain
d	day
DC	dendritic cell(s)
DD	death domain
DEREG mice	depletion of Treg mice
DISC	death-inducing signaling complex
DMSO	dimethyl sulfoxide
DN	double negative
DP	double positive
DT	diphtheria toxin
DTR	diphtheria toxin receptor
FACS	fluorescence activated cell sorter
FASER	fluorescence amplification by sequential employment of reagents
FCS	fetal calf serum
cFLIP	cellular FLICE inhibitory protein
Foxp3	Forkhead Box P3
Foxp3.DGL mice	Foxp3.DTR-GFP-luciferase mice

GFP	green fluorescent protein
gld	generalized lymphoproliferative disease
GvHD	graft *vs.* host disease
Gzm	granzyme
hr	hour
IL	interleukin
IPEX	immune dysregulation, polyendocrinopathy, enteropathy x-linked
LN	lymph node(s)
Lpr	lymphoproliferation
MACS	magnetic activated cell sorter
MHC	major histocompatibility complex
min	minute(s)
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor κ B
NK	natural killer
PAMP	pathogen-associated molecular pattern(s)
pb	plate-bound
PBL	peripheral blood leukocyte(s)
PCR	polymerase chain reaction
PBS	phosphate buffered saline
RAG2	recombinase activating gene
rpm	revolutions per minute
RLU	relative light unit(s)
mRNA	messenger ribonucleic acid
Tcon	conventional T cell(s)
TEC	thymic epithelial cell
Teff	effector T cell(s)
TF	transcription factor
tg	transgenic
TGF-β	transfoming growth factor-β

List of abbreviations

TLR	toll like receptor
Treg	regulatory T cell(s)
nTreg	naturally occurring Treg
TCR	T cell antigen receptor
IU	International Unit
wt	wild type

6 Reference List

Abbas,A.K., Murphy,K.M., and Sher,A. (1996). Functional diversity of helper T lymphocytes. Nature *383*, 787-793.

Abdulahad,W.H., Stegeman,C.A., Van der Geld,Y.M., Doornbos-Van Der Meer,B., Limburg,P.C., and Kallenberg,C.G.M. (2007). Functional defect of circulating regulatory CD4+T cells in patients with Wegener's granulomatosis in remission. Arthritis and Rheumatism *56*, 2080-2091.

Adachi,M., Watanabefukunaga,R., and Nagata,S. (1993). Aberrant Transcription Caused by the Insertion of An Early Transposable Element in An Intron of the Fas Antigen Gene of Lpr Mice. Proceedings of the National Academy of Sciences of the United States of America *90*, 1756-1760.

Albanese,J., Meterissian,S., Kontogiannea,M., Dubreuil,C., Hand,A., Sorba,S., and Dainiak,N. (1998). Biologically active fas antigen and its cognate ligand are expressed on plasma membrane-derived extracellular vesicles. Blood *91*, 3862-3874.

Alderson,M.R., Tough,T.W., Davissmith,T., Braddy,S., Falk,B., Schooley,K.A., Goodwin,R.G., Smith,C.A., Ramsdell,F., and Lynch,D.H. (1995). Fas Ligand Mediates Activation-Induced Cell-Death in Human T-Lymphocytes. Journal of Experimental Medicine *181*, 71-77.

Allan,S.E., Passerini,L., Bacchetta,R., Crellin,N., Dai,M.Y., Orban,P.C., Ziegler,S.F., Roncarolo,M.G., and Levings,M.K. (2005). The role of 2 FOXP3 isoforms in the generation of human CD4(+) Tregs. Journal of Clinical Investigation *115*, 3276-3284.

Aoki,K., Kurooka,M., Chen,J.J., Petryniak,J., Nabel,E.G., and Nabel,G.J. (2001). Extracellular matrix interacts with soluble CD95L: Retention and enhancement of cytotoxicity. Nature Immunology *2*, 333-337.

Apostolou,I., Sarukhan,A., Klein,L., and von Boehmer,H. (2002). Origin of regulatory T cells with known specificity for antigen. Nature Immunology *3*, 756-763.

Apostolou,I. and von Boehmer,H. (2004). In vivo instruction of suppressor commitment in naive T cells. Journal of Experimental Medicine *199*, 1401-1408.

Ashley,C.W. and Baecher-Allan,C. (2009). Cutting Edge: Responder T Cells Regulate Human DR+ Effector Regulatory T Cell Activity via Granzyme B. Journal of Immunology *183*, 4843-4847.

Askenasy,N., Yolcu,E.S., Yaniv,I., and Shirwan,H. (2005). Induction of tolerance using Fas ligand: a double-edged immunomodulator. Blood *105*, 1396-1404.

Aswad,F., Kawamura,H., and Dennert,G. (2005). High sensitivity of CD4(+)CD25(+) regulatory T cells to extracellular metabolites nicotinamide adenine dinucleotide and ATP: A role for P2X(7) receptors. Journal of Immunology *175*, 3075-3083.

Baatar,D., Olkhanud,P., Sumitomo,K., Taub,D., Gress,R., and Biragyn,A. (2007). Human peripheral blood T regulatory cells (Tregs), functionally primed CCR4(+) Tregs and unprimed CCR4(-) Tregs, regulate effector T cells using FasL. Journal of Immunology *178*, 4891-4900.

Bacchetta,R., Passerini,L., Gambineri,E., Dai,M., Allan,S.E., Perroni,L., Dagna-Bricarelli,F., Sartirana,C., Matthes-Martins,S., Lawitschka,A., Azzari,C., Ziegler,S.F., Levings,M.K., and Roncarolo,M.G. (2006). Defective regulatory and effector T cell functions in patients with FOXP3 mutations. Journal of Clinical Investigation *116*, 1713-1722.

Baecher-Allan,C., Brown,J.A., Freeman,G.J., and Hafler,D.A. (2001). CD4+CD25(high) regulatory cells in human peripheral blood. Journal of Immunology *167*, 1245-1253.

Banica,L., Besliu,A., Pistol,G., Stavaru,C., Ionescu,R., Forsea,A.M., Tanaseanu,C., Dumitrache,S., Otelea,D., Tamsulea,I.,

Tanaseanu,S., Chitonu,C., Paraschiv,S., Balteanu,M., Stefanescu,M., and Matache,C. (2009). Quantification and molecular characterization of regulatory T cells in connective tissue diseases. Autoimmunity *42*, 41-49.

Banz,A., Pontoux,C., and Papiernik,M. (2002). Modulation of Fas-dependent apoptosis: A dynamic process controlling both the persistence and death of CD4 regulatory T cells and effector T cells. Journal of Immunology *169*, 750-757.

Barclay, A. E., Franklin, K. J., and Prichard, M. L. The fetal circulation and cardiovascular system and the changes they undergo at birth. 1944.
Ref Type: Generic

Barnett,B., Kryczek,I., Cheng,P., Zou,W.P., and Curiel,T.J. (2005). Regulatory T cells in ovarian cancer: Biology and therapeutic potential. American Journal of Reproductive Immunology *54*, 369-377.

Barrat,F.J., Cua,D.J., Boonstra,A., Richards,D.F., Crain,C., Savelkoul,H.F., Waal-Malefyt,R., Coffman,R.L., Hawrylowicz,C.M., and O'Garra,A. (2002). In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. Journal of Experimental Medicine *195*, 603-616.

Belkaid,Y. (2007). Regulatory T cells and infection: a dangerous necessity. Nature Reviews Immunology *7*, 875-888.

Bennett,C.L., Christie,J., Ramsdell,F., Brunkow,M.E., Ferguson,P.J., Whitesell,L., Kelly,T.E., Saulsbury,F.T., Chance,P.F., and Ochs,H.D. (2001). The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nature Genetics *27*, 20-21.

Bettelli,E., Dastrange,M., and Oukka,M. (2005). Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. Proceedings of the National Academy of Sciences of the United States of America *102*, 5138-5143.

Bettelli,E., Korn,T., Oukka,M., and Kuchroo,V.K. (2008). Induction and effector functions of T(H)17 cells. Nature *453*, 1051-1057.

Bodmer,J.L., Schneider,P., and Tschopp,J. (2002). The molecular architecture of the TNF superfamily. Trends in Biochemical Sciences *27*, 19-26.

Bopp,T., Becker,C., Klein,M., Klein-Hessling,S., Palmetshofer,A., Serfling,E., Heib,V., Becker,M., Kubach,J., Schmitt,S., Stoll,S., Schild,H., Staege,M.S., Stassen,M., Jonuleit,H., and Schmitt,E. (2007). Cyclic adenosine monophosphate is a key component of regulatory T cell mediated suppression. Journal of Experimental Medicine *204*, 1303-1310.

Borsellino,G., Kleinewietfeld,M., Di Mitri,D., Sternjak,A., Diamantini,A., Giometto,R., Hopner,S., Centonze,D., Bernardi,G., Dell'Acqua,M.L., Rossini,P.M., Battistini,L., Rotzschke,O., and Falk,K. (2007). Expression of ectonucleotidase CD39 by Foxp3(+) Treg cells: hydrolysis of extracellular ATP and immune suppression. Blood *110*, 1225-1232.

Bouillet,P., Metcalf,D., Huang,D.C.S., Tarlinton,D.M., Kay,T.W.H., Kontgen,F., Adams,J.M., and Strasser,A. (1999). Proapoptotic Bcl-2 relative bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science *286*, 1735-1738.

Bouillet,P. and O'Reilly,L.A. (2009). CD95, BIM and T cell homeostasis. Nature Reviews Immunology *9*, 514-519.

Brenner,D., Krammer,P.H., and Arnold,R. (2008). Concepts of activated T cell death. Critical Reviews in Oncology Hematology *66*, 52-64.

Brunkow,M.E., Jeffery,E.W., Hjerrild,K.A., Paeper,B., Clark,L.B., Yasayko,S.A., Wilkinson,J.E., Galas,D., Ziegler,S.F., and Ramsdell,F. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results

in the fatal lymphoproliferative disorder of the scurfy mouse. Nature Genetics *27*, 68-73.

Brunner,T., Mogil,R.J., Laface,D., Yoo,N.J., Mahboubi,A., Echeverri,F., Martin,S.J., Force,W.R., Lynch,D.H., Ware,C.F., and Green,D.R. (1995). Cell-Autonomous Fas (Cd95) Fas-Ligand Interaction Mediates Activation-Induced Apoptosis in T-Cell Hybridomas. Nature *373*, 441-444.

Burchill,M.A., Yang,J., Vang,K.B., and Farrar,M.A. (2007). Interleukin-2 receptor signaling in regulatory T cell development and homeostasis. Immunology Letters *114*, 1-8.

Cain,K., Bratton,S.B., Langlais,C., Walker,G., Brown,D.G., Sun,X.M., and Cohen,G.M. (2000). Apaf-1 oligomerizes into biologically active similar to 700-kDa and inactive similar to 1.4-MDa apoptosome complexes. Journal of Biological Chemistry *275*, 6067-6070.

Cao,X.F., Cai,S.F., Fehniger,T.A., Song,J.L., Collins,L.I., Piwnica-Worms,D.R., and Ley,T.J. (2007). Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. Immunity *27*, 635-646.

Caux,C., Massacrier,C., Vanbervliet,B., Dubois,B., Vankooten,C., Durand,I., and Banchereau,J. (1994). Activation of Human Dendritic Cells Through Cd40 Cross-Linking. Journal of Experimental Medicine *180*, 1263-1272.

Chai,J.G., Tsang,J.Y.S., Lechler,R., Simpson,E., Dyson,J., and Scott,D. (2002). CD4(+)CD25(+) T cells as immunoregulatory T cells in vitro. European Journal of Immunology *32*, 2365-2375.

Chao,D.T. and Korsmeyer,S.J. (1998). BCL-2 FAMILY: Regulators of cell death. Annual Review of Immunology *16*, 395-419.

Chaudhry,A., Rudra,D., Treuting,P., Samstein,R.M., Liang,Y.Q., Kas,A., and Rudensky,A.Y. (2009). CD4(+) Regulatory T Cells Control T(H)17 Responses in a Stat3-Dependent Manner. Science *326*, 986-991.

Chen,A.S., Liu,S.R., Park,D., Kang,Y.M., and Zheng,G.X. (2007). Depleting intratumoral CD4(+)CD25(+) regulatory T cells via FasL protein transfer enhances the therapeutic efficacy of adoptive T cell transfer. Cancer Research *67*, 1291-1298.

Chen,C., Rowell,E.A., Thomas,R.M., Hancock,W.W., and Wells,A.D. (2006). Transcriptional regulation by Foxp3 is associated with direct promoter occupancy and modulation of histone acetylation. J. Biol. Chem. *281*, 36828-36834.

Chen, W, Jin, W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, and Wahl SM. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. Journal of Experimental Medicine . 15-12-2003. Ref Type: Generic

Chen,Y.H. and Wilson,J.M. (1998). Fas ligand - a double-edged sword. Nature Biotechnology *16*, 1011-1012.

Chen,Z.B., Benoist,C., and Mathis,D. (2005). How defects in central tolerance impinge on a deficiency in regulatory T cells. Proceedings of the National Academy of Sciences of the United States of America *102*, 14735-14740.

Cohen,G.M. (1997). Caspases: the executioners of apoptosis. Biochemical Journal *326*, 1-16.

Curiel,T.J., Coukos,G., Zou,L.H., Alvarez,X., Cheng,P., Mottram,P., Evdemon-Hogan,M., Conejo-Garcia,J.R., Zhang,L., Burow,M., Zhu,Y., Wei,S., Kryczek,I., Daniel,B., Gordon,A., Myers,L., Lackner,A., Disis,M.L., Knutson,K.L., Chen,L.P., and Zou,W.P. (2004). Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nature Medicine *10*, 942-949.

Danke,N.A., Koelle,D.M., Yee,C., Beheray,S., and Kwok,W.W. (2004). Autoreactive T cells in healthy individuals. Journal of Immunology *172*, 5967-5972.

Dannull,J., Su,Z., Rizzieri,D., Yang,B.K., Coleman,D., Yancey,D., Zhang,A.J., Dahm,P., Chao,N., Gilboa,E., and Vieweg,J. (2005). Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion

of regulatory T cells. Journal of Clinical Investigation *115*, 3623-3633.

de Lafaille,M.A.C. and Lafaille,J.J. (2009). Natural and Adaptive Foxp3(+) Regulatory T Cells: More of the Same or a Division of Labor? Immunity *30*, 626-635.

Deaglio,S., Dwyer,K.M., Gao,W., Friedman,D., Usheva,A., Erat,A., Chen,J.F., Enjyoji,K., Linden,J., Oukka,M., Kuchroo,V.K., Strom,T.B., and Robson,S.C. (2007). Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. Journal of Experimental Medicine *204*, 1257-1265.

Del Rey,M., Ruiz-Contreras,J., Bosque,A., Calleja,S., Gomez-Rial,J., Roldan,E., Morales,P., Serrano,A., Anel,A., Paz-Artal,E., and Allende,L.M. (2006). A homozygous Fas ligand gene mutation in a patient causes a new type of autoirnmune lymphoproliferative syndrome. Blood *108*, 1306-1312.

Derbinski,J., Schulte,A., Kyewski,B., and Klein,L. (2001). Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. Nature Immunology *2*, 1032-1039.

Dhein,J., Walczak,H., Baumler,C., Debatin,K.M., and Krammer,P.H. (1995). Autocrine T-Cell Suicide Mediated by Apo-1/(Fas/Cd95). Nature *373*, 438-441.

Dieckmann,D., Plottner,H., Berchtold,S., Berger,T., and Schuler,G. (2001). Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. Journal of Experimental Medicine *193*, 1303-1310.

Droege,W. (1971). Amplifying and Suppressive Effect of Thymus Cells. Nature *234*, 549-&.

Du,J.G., Huang,C.J., Zhou,B.H., and Ziegler,S.F. (2008). Isoform-specific inhibition of ROR alpha-mediated transcriptional activation by human FOXP3. Journal of Immunology *180*, 4785-4792.

Erlacher,M., Michalak,E.M., Kelly,P.N., Labi,V., Niederegger,H., Coultas,L., Adams,J.M., Strasser,A., and Villunger,A. (2005). BH3-only proteins Puma and Bim are rate-limiting for gamma-radiation- and glucocorticoid-induced apoptosis of lymphoid cells in vivo. Blood *106*, 4131-4138.

Fahlen,L., Read,S., Gorelik,L., Hurst,S.D., Coffman,R.L., Flavell,R.A., and Powrie,F. (2005). T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. Journal of Experimental Medicine *201*, 737-746.

Fallarino,F., Grohmann,U., Hwang,K.W., Orabona,C., Vacca,C., Bianchi,R., Belladonna,M.L., Fioretti,M.C., Alegre,M.L., and Puccetti,P. (2003). Modulation of tryptophan catabolism by regulatory T cells. Nature Immunology *4*, 1206-1212.

feeney, A. J., Victor, K. D., Vu, K., Nadel, B., and Chukvuocha, R. U. Influence of the V(D)J recombination mechanism on the formation of the primary T and B cell repertoires. Influence of the V(D)J recombination mechanism on the formation of the primary T and B cell repertoires. 1994.
Ref Type: Generic

Ferri,K.F. and Kroemer,G. (2001). Organelle-specific initiation of cell death pathways. Nature Cell Biology *3*, E255-E263.

Feuerer,M., Hill,J.A., Mathis,D., and Benoist,C. (2009). Foxp3(+) regulatory T cells: differentiation, specification, subphenotypes. Nature Immunology *10*, 689-695.

Fisher,G.H., Rosenberg,F.J., Straus,S.E., Dale,J.K., Middelton,L.A., Lin,A.Y., Strober,W., Lenardo,M.J., and Puck,J.M. (1995). Dominant Interfering Fas Gene-Mutations Impair Apoptosis in A Human Autoimmune Lymphoproliferative Syndrome. Cell *81*, 935-946.

Fontenot,J.D., Gavin,M.A., and Rudensky,A.Y. (2003). Foxp3 programs the development and function of CD4(+)CD25(+) regulatory T cells. Nature Immunology *4*, 330-336.

Fritzsching,B., Oberle,N., Eberhardt,N., Quick,S., Haas,J., Wildemann,B., Krammer,P.H., and Suri-Payer,E. (2005).

Cutting edge: In contrast to effector T cells, CD4(+)CD25(+) FoxP3(+) regulatory T cells are highly susceptible to CD95 ligand- but not to TCR-mediated cell death. Journal of Immunology *175*, 32-36.

Fritzsching,B., Oberle,N., Pauly,E., Geffers,R., Buer,J., Poschl,J., Krammer,P., Linderkamp,O., and Suri-Payer,E. (2006). Naive regulatory T cells: a novel subpopulation defined by resistance toward CD95L-mediated cell death. Blood *108*, 3371-3378.

Galluzzi,L., Maiuri,M.C., Vitale,I., Zischka,H., Castedo,M., Zitvogel,L., and Kroemer,G. (2007). Cell death modalities: classification and pathophysiological implications. Cell Death and Differentiation *14*, 1237-1243.

Gascoigne,N.R.J. and Zal,T. (2004). Molecular interactions at the T cell-antigen-presenting cell interface. Current Opinion in Immunology *16*, 114-119.

Gavin,M.A., Clarke,S.R., Negrou,E., Gallegos,A., and Rudensky,A. (2002). Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo. Nature Immunology *3*, 33-41.

Gavin,M.A., Rasmussen,J.P., Fontenot,J.D., Vasta,V., Manganiello,V.C., Beavo,J.A., and Rudensky,A.Y. (2007). Foxp3-dependent programme of regulatory T-cell differentiation. Nature *445*, 771-775.

Gavin,M.A., Torgerson,T.R., Houston,E., deRoos,P., Ho,W.Y., Stray-Pedersen,A., Ocheltree,E.L., Greenberg,P.D., Ochs,H.D., and Rudensky,A.Y. (2006). Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. Proceedings of the National Academy of Sciences of the United States of America *103*, 6659-6664.

Gershon,R.K. and Kondo,K. (1970). Cell Interactions in Induction of Tolerance - Role of Thymic Lymphocytes. Immunology *18*, 723-&.

Gershon,R.K. and Kondo,K. (1971). Infectious Immunological Tolerance. Immunology *21*, 903-&.

Ghiringhelli,F., Menard,C., Terme,M., Flament,C., Taieb,J., Chaput,N., Puig,P.E., Novault,S., Escudier,B., Vivier,E., Lecesne,A., Robert,C., Blay,J.Y., Bernard,J., Caillat-Zucman,S., Freitas,A., Tursz,T., Wagner-Ballon,O., Capron,C., Vainchencker,W., Martin,F., and Zitvogel,L. (2005). CD4(+) CD25(+) regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. Journal of Experimental Medicine *202*, 1075-1085.

Glücksmann, A. Cell death in normal development. 1965.
Ref Type: Generic

Godfrey,D.I., Kennedy,J., Suda,T., and Zlotnik,A. (1993). A Developmental Pathway Involving 4 Phenotypically and Functionally Distinct Subsets of Cd3-Cd4-Cd8- Triple-Negative Adult-Mouse Thymocytes Defined by Cd44 and Cd25 Expression. Journal of Immunology *150*, 4244-4252.

Gondek,D.C., Lu,L.F., Quezada,S.A., Sakaguchi,S., and Noelle,R.J. (2005). Cutting edge: Contact-mediated suppression by CD4(+)-CD25(+) regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. Journal of Immunology *174*, 1783-1786.

Green,D.R. (1998). Apoptotic pathways: The roads to ruin. Cell *94*, 695-698.

Green,D.R. and Kroemer,G. (2004). The pathophysiology of mitochondrial cell death. Science *305*, 626-629.

Green,D.R. and Reed,J.C. (1998). Mitochondria and apoptosis. Science *281*, 1309-1312.

Green,D.R. and Ware,C.F. (1997). Fas-ligand: Privilege and peril. Proceedings of the National Academy of Sciences of the United States of America *94*, 5986-5990.

Greenwald,R.J., Freeman,G.J., and Sharpe,A.H. (2005). The B7 family revisited. Annual Review of Immunology *23*, 515-548.

Griffith,T.S., Brunner,T., Fletcher,S.M., Green,D.R., and Ferguson,T.A. (1995). Fas

Ligand-Induced Apoptosis As A Mechanism of Immune Privilege. Science *270*, 1189-1192.

Gross,A., McDonnell,J.M., and Korsmeyer,S.J. (1999). BCL-2 family members and the mitochondria in apoptosis. Genes & Development *13*, 1899-1911.

Grossman,W.J., Verbsky,J.W., Barchet,W., Colonna,M., Atkinson,J.P., and Ley,T.J. (2004). Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity *21*, 589-601.

Guermonprez,P., Valladeau,J., Zitvogel,L., Thery,C., and Amigorena,S. (2002). Antigen presentation and T cell stimulation by dendritic cells. Annual Review of Immunology *20*, 621-667.

Hahne,M., Rimoldi,D., Schroter,M., Romero,P., Schreier,M., French,L.E., Schneider,P., Bornand,T., Fontana,A., Lienard,D., Cerottini,J.C., and Tschopp,J. (1996). Melanoma cell expression of Fas(Apo-1/CD95) ligand: Implications for tumor immune escape. Science *274*, 1363-1366.

Hartigan-O'Connor,D.J., Poon,C., Sinclair,E., and Mccune,J.M. (2007). Human CD4+regulatory T cells express lower levels of the IL-7 receptor alpha chain (CD127), allowing consistent identification and sorting of live cells. Journal of Immunological Methods *319*, 41-52.

Hengartner,M.O. (2000). The biochemistry of apoptosis. Nature *407*, 770-776.

Hickman,S.P., Yang,J., Thomas,R.M., Wells,A.D., and Turka,L.A. (2006). Defective activation of protein kinase C and Ras-ERK pathways limits IL-2 production and proliferation by CD4(+)CD25(+) regulatory T cells. Journal of Immunology *177*, 2186-2194.

Hildeman,D.A., Zhu,Y.N., Mitchell,T.C., Bouillet,P., Strasser,A., Kappler,J., and Marrack,P. (2002a). Activated T cell death in vivo mediated by proapoptotic Bcl-2 family member Bim. Immunity *16*, 759-767.

Hildeman,D.A., Zhu,Y.N., Mitchell,T.C., Kappler,J., and Marrack,P. (2002b). Molecular mechanisms of activated T cell death in vivo. Current Opinion in Immunology *14*, 354-359.

Hohlbaum,A.M., Moe,S., and Marshak-Rothstein,A. (2000). Opposing effects of transmembrane and soluble Fas ligand expression on inflammation and tumor cell survival. Journal of Experimental Medicine *191*, 1209-1219.

Hori,S., Nomura,T., and Sakaguchi,S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. Science *299*, 1057-1061.

Hsieh,C.S., Liang,Y.Q., Tyznik,A.J., Self,S.G., Liggitt,D., and Rudensky,A.Y. (2004). Recognition of the peripheral self by naturally arising CD25(+) CD4(+) T cell receptors. Immunity *21*, 267-277.

Hsieh,C.S., Zheng,Y., Liang,Y.Q., Fontenot,J.D., and Rudensky,A.Y. (2006). An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. Nature Immunology *7*, 401-410.

Hughes,P.D., Belz,G.T., Fortner,K.A., Budd,R.C., Strasser,A., and Bouillet,P. (2008). Apoptosis regulators Fas and Bim cooperate in shutdown of chronic immune responses and prevention of autoimmunity. Immunity *28*, 197-205.

Hundhausen,C., Misztela,D., Berkhout,T.A., Broadway,N., Saftig,P., Reiss,K., Hartmann,D., Fahrenholz,F., Postina,R., Matthews,V., Kallen,K.J., Rose-John,S., and Ludwig,A. (2003). The disintegrin-like metalloproteinase ADAM 10 is involved in coinstitutive cleavage of CX3CL1 (fractalkine) and regulates CX3CL1-mediated cell-cell adhesion. Blood *102*, 1186-1195.

Hutcheson,J., Scatizzi,J.C., Siddiqui,A.M., Haines,G.K., Wu,T., Li,Q.Z., Davis,L.S., Mohan,C., and Perlman,H. (2008). Combined deficiency of proapoptotic regulators Bim and Fas results in the early onset of systemic autoimmunity. Immunity *28*, 206-217.

Inaba, K., Hosono, M., and Inaba, M. Thymic dendritic cells and B cells: isolation and

function. International Reviews of Immunology 6, 117-126. 1990.
Ref Type: Generic

Inaba,M., Kurasawa,K., Mamura,M., Kumano,K., Saito,Y., and Iwamoto,I. (1999). Primed T cells are more resistant to Fas-mediated activation-induced cell death than naive T cells. Journal of Immunology *163*, 1315-1320.

Itoh,N. and Nagata,S. (1993). A Novel Protein Domain Required for Apoptosis - Mutational Analysis of Human Fas Antigen. Journal of Biological Chemistry *268*, 10932-10937.

Jackson,C.E., Wang,J., Fischer,R.E., Hsu,A.P., Niemela,J., Dale,J.K., Fleisher,T.A., Jaffe,E.S., Lenardo,M.J., Straus,S.E., and Puck,J.M. (1999). Autoimmune lymphoproliferative syndrome (ALPS): an inherited disorder of apoptosis with predisposition to development of diverse lymphomas. American Journal of Human Genetics *65*, A48.

Janeway,C.A. (1988). Do Suppressor T-Cells Exist - A Reply. Scandinavian Journal of Immunology *27*, 621-623.

Janeway,C.A. and Medzhitov,R. (2002). Innate immune recognition. Annual Review of Immunology *20*, 197-216.

Janssens,W., Carlier,V., Wu,B., VanderElst,L., Jacquemin,M.G., and Saint-Remy,J.M.R. (2003). CD4(+)CD25(+) T cells lyse antigen-presenting B cells by Fas-Fas ligand interaction in an epitope-specific manner. Journal of Immunology *171*, 4604-4612.

Jeffers,J.R., Parganas,E., Lee,Y., Yang,C.Y., Wang,J.L., Brennan,J., MacLean,K.H., Han,J.W., Chittenden,T., Ihle,J.N., McKinnon,P.J., Cleveland,J.L., and Zambetti,G.P. (2003). Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell *4*, 321-328.

Jonuleit,H., Schmitt,E., Schuler,G., Knop,J., and Enk,A.H. (2000). Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. Journal of Experimental Medicine *192*, 1213-1222.

Jonuleit,H., Schmitt,E., Stassen,M., Tuettenberg,A., Knop,J., and Enk,A.H. (2001). Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. Journal of Experimental Medicine *193*, 1285-1294.

Jordan,M.S., Boesteanu,A., Reed,A.J., Petrone,A.L., Holenbeck,A.E., Lerman,M.A., Naji,A., and Caton,A.J. (2001). Thymic selection of CD4(+)CD25(+) regulatory T cells induced by an agonist self-peptide. Nature Immunology *2*, 301-306.

Josefowicz,S.Z. and Rudensky,A. (2009). Control of Regulatory T Cell Lineage Commitment and Maintenance. Immunity *30*, 616-625.

Ju,S.T., Panka,D.J., Cui,H.L., Ettinger,R., Elkhatib,M., Sherr,D.H., Stanger,B.Z., and Marshakrothstein,A. (1995). Fas(Cd95) Fasl Interactions Required for Programmed Cell-Death After T-Cell Activation. Nature *373*, 444-448.

Kabelitz,D., Pohl,T., and Pechhold,K. (1993). Activation-Induced Cell-Death (Apoptosis) of Mature Peripheral T-Lymphocytes. Immunology Today *14*, 338.

Kaufmann,T., Tai,L., Ekert,P.G., Huang,D.C.S., Norris,F., Lindemann,R.K., Johnstone,R.W., Dixit,V.M., and Strasser,A. (2007). The BH3-only protein bid is dispensable for DNA darnage- and replicative stress-induced apoptosis or cell-cycle arrest. Cell *129*, 423-433.

Kavurma,M.M. and Khachigian,L.M. (2003). Signaling and transcriptional control of Fas ligand gene expression. Cell Death and Differentiation *10*, 36-44.

Kawahata,K., Misaki,Y., Yamauchi,M., Tsunekawa,S., Setoguchi,K., Miyazaki,J., and Yamamoto,K. (2002). Generation of CD4(+)CD25(+) regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR

expression. Journal of Immunology *168*, 4399-4405.

Kayagaki,N., Kawasaki,A., Ebata,T., Ohmoto,H., Ikeda,S., Inoue,S., Yoshino,K., Okumura,K., and Yagita,H. (1995). Metalloproteinase-Mediated Release of Human Fas Ligand. Journal of Experimental Medicine *182*, 1777-1783.

Kerr,J.F.R., Wyllie,A.H., and Currie,A.R. (1972). Apoptosis - Basic Biological Phenomenon with Wide-Ranging Implications in Tissue Kinetics. British Journal of Cancer *26*, 239-&.

Khattri,R., Cox,T., Yasayko,S.A., and Ramsdell,F. (2003). An essential role for Scurfin in CD4(+)CD25(+) T regulatory cells. Nature Immunology *4*, 337-342.

Kim,J.M., Rasmussen,J.P., and Rudensky,A.Y. (2007). Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. Nature Immunology *8*, 191-197.

Kinter,A., McNally,J., Riggin,L., Jackson,R., Roby,G., and Fauci,A.S. (2007). Suppression of HIV-specific T cell activity by lymph node CD25(+) regulatory T cells from HIV-infected individuals. Proceedings of the National Academy of Sciences of the United States of America *104*, 3390-3395.

Kirkin,V., Cahuzac,N., Guardiola-Serrano,F., Huault,S., Luckerath,K., Friedmann,E., Novac,N., Wels,W.S., Martoglio,B., Hueber,A.O., and Zornig,M. (2007). The Fas ligand intracellular domain is released by ADAM10 and SPPL2a cleavage in T-cells. Cell Death and Differentiation *14*, 1678-1687.

Kischkel,F.C., Hellbardt,S., Behrmann,I., Germer,M., Pawlita,M., Krammer,P.H., and Peter,M.E. (1995). Cytotoxicity-Dependent Apo-1 (Fas/Cd95)-Associated Proteins Form A Death-Inducing Signaling Complex (Disc) with the Receptor. Embo Journal *14*, 5579-5588.

Klas,C., Debatin,K.M., Jonker,R.R., and Krammer,P.H. (1993). Activation Interferes with the Apo-1 Pathway in Mature Human T-Cells. International Immunology *5*, 625-630.

Kleinewietfeld,M., Starke,M., Di Mitri,D., Borsellino,G., Battistini,L., Rotzschke,O., and Falk,K. (2009). CD49d provides access to "untouched" human Foxp3(+) Treg free of contaminating effector cells. Blood *113*, 827-836.

Kobie,J.J., Shah,P.R., Yang,L., Rebhahn,J.A., Fowell,D.J., and Mosmann,T.R. (2006). T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5 '-adenosine monophosphate to adenosine. Journal of Immunology *177*, 6780-6786.

Koch,M.A., Tucker-Heard,G., Perdue,N.R., Killebrew,J.R., Urdahl,K.B., and Campbell,D.J. (2009). The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. Nature Immunology *10*, 595-U57.

Krammer,P.H. (2000). CD95's deadly mission in the immune system. Nature *407*, 789-795.

Kretschmer,K., Apostolou,I., Hawiger,D., Khazaie,K., Nussenzweig,M.C., and von Boehmer,H. (2005). Inducing and expanding regulatory T cell populations by foreign antigen. Nature Immunology *6*, 1219-1227.

Kroemer,G., Galluzzi,L., and Brenner,C. (2007). Mitochondrial membrane permeabilization in cell death. Physiological Reviews *87*, 99-163.

Kroemer,G., Zamzami,N., and Susin,S.A. (1997). Mitochondrial control of apoptosis. Immunology Today *18*, 44-51.

Krueger,A., Fas,S.C., Baumann,S., and Krammer,P.H. (2003b). The role of CD95 in the regulation of peripheral T-cell apoptosis. Immunological Reviews *193*, 58-69.

Krueger,A., Fas,S.C., Baumann,S., and Krammer,P.H. (2003a). The role of CD95 in the regulation of peripheral T-cell apoptosis. Immunological Reviews *193*, 58-69.

Kuczma,M., Podolsky,R., Garge,N., Daniely,D., Pacholczyk,R., Ignatowicz,L., and Kraj,P. (2009). Foxp3-Deficient Regulatory T Cells Do Not Revert into Conventional Effector CD4(+) T Cells but Constitute a Unique Cell Subset.

Journal of Immunology *183*, 3731-3741.

Lahl,K., Loddenkemper,C., Drouin,C., Freyer,J., Arnason,J., Eberl,G., Hamann,A., Wagner,H., Huehn,J., and Sparwasser,T. (2007). Selective depletion of Foxp3(+) regulatory T cells induces a scurfy-like disease. Journal of Experimental Medicine *204*, 57-63.

Lahl,K., Mayer,C.T., Bopp,T., Huehn,J., Loddenkemper,C., Eberl,G., Wirnsberger,G., Dornmair,K., Geffers,R., Schmitt,E., Buer,J., and Sparwasser,T. (2009). Nonfunctional Regulatory T Cells and Defective Control of Th2 Cytokine Production in Natural Scurfy Mutant Mice. Journal of Immunology *183*, 5662-5672.

Lavrik,I., Golks,A., and Krammer,P.H. (2005). Death receptor signaling. Journal of Cell Science *118*, 265-267.

Lavrik,I., Krueger,A., Schmitz,I., Baumann,S., Weyd,H., Krammer,P.H., and Kirchhoff,S. (2003). The active caspase-8 heterotetramer is formed at the CD95 DISC. Cell Death and Differentiation *10*, 144-145.

Lavrik,I.N., Golks,A., Baumann,S., and Krammer,P.H. (2006). Caspase-2 is activated at the CD95 death-inducing signaling complex in the course of CD95-induced apoptosis. Blood *108*, 559-565.

Leithauser,F., Dhein,J., Mechtersheimer,G., Koretz,K., Bruderlein,S., Henne,C., Schmidt,A., Debatin,K.M., Krammer,P.H., and Moller,P. (1993). Constitutive and Induced Expression of Apo-1, A New Member of the Nerve Growth-Factor Tumor-Necrosis-Factor Receptor Superfamily, in Normal and Neoplastic-Cells. Laboratory Investigation *69*, 415-429.

Lenardo,M., Chan,F.K.M., Hornung,F., McFarland,H., Siegel,R., Wang,J., and Zheng,L.X. (1999). Mature T lymphocyte apoptosis - Immune regulation in a dynamic and unpredictable antigenic environment. Annual Review of Immunology *17*, 221-253.

Levings,M.K., Sangregorio,R., and Roncarolo,M.G. (2001). Human CD25(+)CD4(+) T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. Journal of Experimental Medicine *193*, 1295-1301.

Li-Weber,M. and Krammer,P.H. (2003). Function and regulation of the CD95 (APO-1/Fas) ligand in the immune system. Seminars in Immunology *15*, 145-157.

Lim,H.W., Hillsamer,P., Banham,H.H., and Kim,C.H. (2005). Cutting edge: Direct suppression of B cells by CD4(+)CD25(+) regulatory T cells. Journal of Immunology *175*, 4180-4183.

Lin,W., Haribhai,D., Relland,L.M., Truong,N., Carlson,M.R., Williams,C.B., and Chatila,T.A. (2007). Regulatory T cell development in the absence of functional Foxp3. Nature Immunology *8*, 359-368.

Lio,C.W.J.Q. and Hsieh,C.S. (2008). A two-step process for thymic regulatory T cell development. Immunity *28*, 100-111.

Liszewski,M.K., Farries,T.C., Lublin,D.M., Rooney,I.A., and Atkinson,J.P. (1996). Control of the complement system. Advances in Immunology, Vol 61 *61*, 201-283.

Liu,W.H., Putnam,A.L., Xu-Yu,Z., Szot,G.L., Lee,M.R., Zhu,S., Gottlieb,P.A., Kapranov,P., Gingeras,T.R., Fazekas de St Groth,B., Clayberger,C., Soper,D.M., Ziegler,S.F., and Bluestone,J.A. (2006). CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4(+) T reg cells. Journal of Experimental Medicine *203*, 1701-1711.

Locksley,R.M., Killeen,N., and Lenardo,M.J. (2001). The TNF and TNF receptor superfamilies: Integrating mammalian biology. Cell *104*, 487-501.

Los,M., Wesselborg,S., and Schulze-Osthoff,K. (1999). The role of caspases in development, immunity, and apoptotic signal transduction: Lessons from knockout mice. Immunity *10*, 629-639.

Ludwig-Portugall,I., Hamilton-Williams,E.E., Gotot,J., and Kurts,C. (2009). CD25(+) T-reg specifically suppress auto-Ab generation against pancreatic tissue autoantigens.

European Journal of Immunology *39*, 225-233.

Macian,F., Im,S.H., Garcia-Cozar,J., and Rao,A. (2004). T-cell anergy. Current Opinion in Immunology *16*, 209-216.

Mahnke,K., Schonfeld,K., Fondel,S., Ring,S., Karakhanova,S., Wiedemeyer,K., Bedke,T., Johnson,T.S., Storn,V., Schallenberg,S., and Enk,A.H. (2007a). Depletion of CD4(+)CD25(+) human regulatory T cells in vivo: Kinetics of Treg depletion and alterations in immune functions in vivo and in vitro. International Journal of Cancer *120*, 2723-2733.

Mahnke,K., Schonfeld,K., Fondel,S., Ring,S., Karakhanova,S., Wiedemeyer,K., Bedke,T., Johnson,T.S., Storn,V., Schallenberg,S., and Enk,A.H. (2007b). Depletion of CD4+CD25+ human regulatory T cells in vivo: kinetics of Treg depletion and alterations in immune functions in vivo and in vitro. Int. J. Cancer *120*, 2723-2733.

Maldonado-Lopez,R. and Moser,M. (2001). Dendritic cell subsets and the regulation of Th1/Th2 responses. Seminars in Immunology *13*, 275-282.

Malek, TR. The biology of Interleukin-2. Annual Review of Immunology . 2008.
Ref Type: Generic

Mamura,M., Lee,W., Sullivan,T.J., Felici,A., Sowers,A.L., Allison,J.P., and Letterio,J.J. (2004). CD28 disruption exacerbates inflammation in Tgf-beta 1(-/-) mice: in vivo suppression by CD4(+)CD25(+) regulatory T cells independent of autocrine TGF-beta 1. Blood *103*, 4594-4601.

Mariani,S.M., Matiba,B., Baumler,C., and Krammer,P.H. (1995). Regulation of Cell-Surface Apo-1/Fas (Cd95) Ligand Expression by Metalloproteases. European Journal of Immunology *25*, 2303-2307.

Matsuzawa,A., Moriyama,T., Kaneko,T., Tanaka,M., Kimura,M., Ikeda,H., and Katagiri,T. (1990). A New Allele of the Lpr Locus, Lprcg, That Complements the Gld Gene in Induction of Lymphadenopathy in the Mouse. Journal of Experimental Medicine *171*, 519-531.

Matzinger,P. (1994). Tolerance, Danger, and the Extended Family. Annual Review of Immunology *12*, 991-1045.

McGeachy,M.J., Stephens,L.A., and Anderton,S.M. (2005). Natural recovery and protection from autoimmune encephalomyelitis: Contribution of CD4(+)CD25(+) regulatory cells within the central nervous system. Journal of Immunology *175*, 3025-3032.

Mchugh,R.S., Whitters,M.J., Piccirillo,C.A., Young,D.A., Shevach,E.M., Collins,M., and Byrne,M.C. (2002). CD4(+)CD25(+) immunoregulatory T cells: Gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. Immunity *16*, 311-323.

Misra,N., Bayry,J., Lacroix-Desmazes,S., Kazatchkine,M.D., and Kaveri,S.V. (2004). Cutting edge: Human CD4(+)CD25(+) T cells restrain the maturation and antigen-presenting function of dendritic cells. Journal of Immunology *172*, 4676-4680.

Mizuochi,T., Kasai,M., Kokuho,T., Kakiuchi,T., and Hirokawa,K. (1992). Medullary But Not Cortical Thymic Epithelial-Cells Present Soluble-Antigens to Helper T-Cells. Journal of Experimental Medicine *175*, 1601-1605.

Mohamood,A.S., Trujillo,C.J., Zheng,D.F., Jie,C.F., Murillo,F.M., Schneck,J.P., and Hamad,A.R.A. (2006). Gld mutation of Fas ligand increases the frequency and up-regulates cell survival genes in CD25+CD4+T(R)cells. International Immunology *18*, 1265-1277.

Moller,G. (1988). Do Suppressor T-Cells Exist. Scandinavian Journal of Immunology *27*, 247-250.

Morse,M.A., Hobeika,A.C., Osada,T., Serra,D., Niedzwiecki,D., Lyerly,H.K., and Clay,T.M. (2008). Depletion of human regulatory T cells specifically enhances antigen-specific immune responses to cancer vaccines. Blood *112*, 610-618.

Nagata,S. (1994). Apoptosis Regulated by A Death Factor and Its Receptor - Fas Ligand

and Fas. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences *345*, 281-287.

Nagata,S. (1997). Apoptosis by death factor. Cell *88*, 355-365.

Nagata,S. and Suda,T. (1995). Fas and Fas Ligand - Lpr and Gld Mutations. Immunology Today *16*, 39-43.

Newell,M.K. and Desbarats,J. (1999). Fas ligand: receptor or ligand? Apoptosis *4*, 311-315.

Nikolaev,A., McLaughlin,T., O'Leary,D.D.M., and Tessier-Lavigne,M. (2009). APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. Nature *457*, 981-9U1.

O'Garra,A. and Vieira,P. (2004). Regulatory T cells and mechanisms of immune system control. Nature Medicine *10*, 801-805.

Oberle,N., Eberhardt,N., Falk,C.S., Krammer,P.H., and Suri-Payer,E. (2007). Rapid suppression of cytokine transcription in human CD4(+)CD25(-) T cells by CD4(+)Foxp3(+) regulatory T cells: Independence of IL-2 consumption, TGF-beta, and various inhibitors of TCR signaling. Journal of Immunology *179*, 3578-3587.

Oderup,C., Cederbom,L., Makowska,A., Cilio,C.M., and Ivars,F. (2006). Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4(+) CD25(+) regulatory T-cell-mediated suppression. Immunology *118*, 240-249.

Oehm,A., Behrmann,I., Falk,W., Pawlita,M., Maier,G., Klas,C., Liweber,M., Richards,S., Dhein,J., Trauth,B.C., Ponstingl,H., and Krammer,P.H. (1992). Purification and Molecular-Cloning of the Apo-1 Cell-Surface Antigen, A Member of the Tumor-Necrosis-Factor Nerve Growth-Factor Receptor Superfamily - Sequence Identity with the Fas Antigen. Journal of Biological Chemistry *267*, 10709-10715.

Ogasawara,J., Watanabefukunaga,R., Adachi,M., Matsuzawa,A., Kasugai,T., Kitamura,Y., Itoh,N., Suda,T., and Nagata,S. (1993). Lethal Effect of the Anti-Fas Antibody in Mice (Vol 364, Pg 806, 1993). Nature *365*, 568.

Okumura,A., Ishikawa,T., Sato,S., Yamauchi,T., Oshima,H., Ohashi,T., Sato,K., Ayada,M., Hotta,N., and Kakumu,S. (2008). Deficiency of forkhead box P3 and cytotoxic T-lymphocyte-associated antigen-4 gene expressions and impaired suppressor function of CD4(+)CD25(+) T cells in patients with autoimmune hepatitis. Hepatology Research *38*, 896-903.

Olivares-Villagomez,D., Wang,Y.J., and Lafaille,J.J. (1998). Regulatory CD4(+) T cells expressing endogenous T cell receptor chains protect myelin basic protein-specific transgenic mice from spontaneous autoimmune encephalomyelitis. Journal of Experimental Medicine *188*, 1883-1894.

Onizuka,S., Tawara,I., Shimizu,J., Sakaguchi,S., Fujita,T., and Nakayama,E. (1999). Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. Cancer Research *59*, 3128-3133.

Ono,M., Yaguchi,H., Ohkura,N., Kitabayashi,I., Nagamura,Y., Nomura,T., Miyachi,Y., Tsukada,T., and Sakaguchi,S. (2007). Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. Nature *446*, 685-689.

Ottonello,L., Tortolina,G., Amelotti,M., and Dallegri,F. (1999). Soluble Fas ligand is chemotactic for human neutrophilic polymorphonuclear leukocytes. Journal of Immunology *162*, 3601-3606.

Pandiyan,P., Zheng,L.X., Ishihara,S., Reed,J., and Lenardo,M.J. (2007). CD4(+) CD25(+) Foxp3(+) regulatory T cells induce cytokine deprivation -mediated apoptosis of effector CD4(+) T cells. Nature Immunology *8*, 1353-1362.

Pellegrini,M., Belz,G., Bouillet,P., and Strasser,A. (2003). Shutdown of an acute T cell immune response to viral infection is mediated by the proapoptotic Bcl-2 homology 3-only protein Bim. Proceedings of the National

Academy of Sciences of the United States of America *100*, 14175-14180.

Peter,M.E. and Krammer,P.H. (2003). The CD95(APO-1/Fas) DISC and beyond. Cell Death and Differentiation *10*, 26-35.

Petersen,R.P., Carnpa,M.J., Sperlazza,J., Conlon,D., Joshi,M.B., Harpole,D.H., and Patz,E.F. (2006). Tumor infiltrating FOXP3(+) regulatory T-cells are associated with recurrence in pathologic stage INSCLC patients. Cancer *107*, 2866-2872.

Petit,P.X., Susin,S.A., Zamzami,N., Mignotte,B., and Kroemer,G. (1996). Mitochondria and programmed cell death: Back to the future. Febs Letters *396*, 7-13.

Petrie,H.T., Hugo,P., Scollay,R., and Shortman,K. (1990). Lineage Relationships and Developmental Kinetics of Immature Thymocytes - Cd3, Cd4, and Cd8 Acquisition Invivo and Invitro. Journal of Experimental Medicine *172*, 1583-1588.

Piccirillo,C.A. and Shevach,E.M. (2001). Cutting edge: Control of CD8(+) T cell activation by CD4(+)CD25(+) immunoregulatory cells. Journal of Immunology *167*, 1137-1140.

Raff,M. (1998). Cell suicide for beginners. Nature *396*, 119-122.

Rasku,M.A., Clem,A.L., Telang,S., Taft,B., Gettings,K., Gragg,H., Cramer,D., Lear,S.C., McMasters,K.M., Miller,D.M., and Chesney,J. (2008). Transient T cell depletion causes regression of melanoma metastases. Journal of Translational Medicine *6*.

Read,S., Malmstrom,V., and Powrie,F. (2000). Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. Journal of Experimental Medicine *192*, 295-302.

Reardon,C., Wang,A., and Mckay,D.M. (2008). Transient local depletion of Foxp3(+) regulatory T cells during recovery from colitis via Fas/Fas ligand-induced death. Journal of Immunology *180*, 8316-8326.

Reilly,L.A.O., Tai,L., Lee,L., Kruse,E.A., Grabow,S., Fairlie,W.D., Haynes,N.M., Tarlinton,D.M., Zhang,J.G., Belz,G.T., Smyth,M.J., Bouillet,P., Robb,L., and Strasser,A. (2009). Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. Nature *461*, 659-U106.

Ren,X., Ye,F., Jiang,Z., Chu,Y., Xiong,S., and Wang,Y. (2007). Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells. Cell Death and Differentiation *14*, 2076-2084.

Rieux-Laucat,F., Le Deist,F., and Fischer,A. (2003). Autoimmune lymphoproliferative syndromes: genetic defects of apoptosis pathways. Cell Death and Differentiation *10*, 124-133.

Rieuxlaucat,F., Ledeist,F., Hivroz,C., Roberts,I.A.G., Debatin,K.M., Fischer,A., and Devillartay,J.P. (1995). Mutations in Fas Associated with Human Lymphoproliferative Syndrome and Autoimmunity. Science *268*, 1347-1349.

Roths,J.B., Murphy,E.D., and Eicher,E.M. (1984). A New Mutation, Gld, That Produces Lymphoproliferation and Autoimmunity in C3H Hej Mice. Journal of Experimental Medicine *159*, 1-20.

Saintruf,C., Ungewiss,K., Groettrup,M., Bruno,L., Fehling,H.J., and Vonboehmer,H. (1994). Analysis and Expression of A Cloned Pre-T Cell-Receptor Gene. Science *266*, 1208-1212.

Sakaguchi,S. (2005). Naturally arising Foxp3-expressing CD25(+) CD4(+) regulatory T cells in immunological tolerance to self and non-self. Nature Immunology *6*, 345-352.

Sakaguchi,S., Ono,M., Setoguchi,R., Yagi,H., Hori,S., Fehervari,Z., Shimizu,J., Takahashi,T., and Nomura,T. (2006). Foxp3(+)CD25(+)CD4(+) natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunological Reviews *212*, 8-27.

Sakaguchi,S., Sakaguchi,N., Asano,M., Itoh,M., and Toda,M. (1995a). Immunological Self-

Tolerance Maintained by Activated T-Cells Expressing Il-2 Receptor Alpha-Chains (Cd25) - Breakdown of A Single Mechanism of Self-Tolerance Causes Various Autoimmune-Diseases. Journal of Immunology *155*, 1151-1164.

Sakaguchi,S., Sakaguchi,N., Asano,M., Itoh,M., and Toda,M. (1995b). Immunological Self-Tolerance Maintained by Activated T-Cells Expressing Il-2 Receptor Alpha-Chains (Cd25) - Breakdown of A Single Mechanism of Self-Tolerance Causes Various Autoimmune-Diseases. Journal of Immunology *155*, 1151-1164.

Sakaguchi,S., Sakaguchi,N., Shimizu,J., Yamazaki,S., Sakihama,T., Itoh,M., Kuniyasu,Y., Nomura,T., Toda,M., and Takahashi,T. (2001). Immunologic tolerance maintained by CD25(+) CD4(+) regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunological Reviews *182*, 18-32.

Sakaguchi,S., Takahashi,T., and Nishizuka,Y. (1982a). Study on Cellular Events in Post-Thymectomy Autoimmune Oophoritis in Mice .2. Requirement of Lyt-1 Cells in Normal Female Mice for the Prevention of Oophoritis. Journal of Experimental Medicine *156*, 1577-1586.

Sakaguchi,S., Takahashi,T., and Nishizuka,Y. (1982b). Study on Cellular Events in Postthymectomy Autoimmune Oophoritis in Mice .1. Requirement of Lyt-1 Effector-Cells for Oocytes Damage After Adoptive Transfer. Journal of Experimental Medicine *156*, 1565-1576.

Sakaguchi,S., Toda,M., Asano,M., Itoh,M., Morse,S.S., and Sakaguchi,N. (1996). T cell-mediated maintenance of natural self-tolerance: Its breakdown as a possible cause of various autoimmune diseases. Journal of Autoimmunity *9*, 211-220.

Sakaguchi,S., Yamaguchi,T., Nomura,T., and Ono,M. (2008). Regulatory T cells and immune tolerance. Cell *133*, 775-787.

Salomon,B., Lenschow,D.J., Rhee,L., Ashourian,N., Singh,B., Sharpe,A., and Bluestone,J.A. (2000). B7/CD28 costimulation is essential for the homeostasis of the CD4(+)CD25(+) immunoregulatory T cells that control autoimmune diabetes. Immunity *12*, 431-440.

Sandalova,E., Wei,C.H., Masucci,M.G., and Levitsky,V. (2004). Regulation of expression of Bcl-2 protein family member Bim by T cell receptor triggering. Proceedings of the National Academy of Sciences of the United States of America *101*, 3011-3016.

Sauer,S., Bruno,L., Hertweck,A., Finlay,D., Leleu,M., Spivakov,M., Knight,Z.A., Cobb,B.S., Cantrell,D., O'Connor,E., Shokat,K.M., Fisher,A.G., and Merkenschlager,M. (2008). T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. Proceedings of the National Academy of Sciences of the United States of America *105*, 7797-7802.

Savill,J., Fadok,V., Henson,P., and Haslett,C. (1993). Phagocyte Recognition of Cells Undergoing Apoptosis. Immunology Today *14*, 131-136.

Scaffidi,C., Fulda,S., Srinivasan,A., Friesen,C., Li,F., Tomaselli,K.J., Debatin,K.M., Krammer,P.H., and Peter,M.E. (1998). Two CD95 (APO-1/Fas) signaling pathways. Embo Journal *17*, 1675-1687.

Scaffidi,C., Schmitz,I., Zha,J.P., Korsmeyer,S.J., Krammer,P.H., and Peter,M.E. (1999). Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. Journal of Biological Chemistry *274*, 22532-22538.

Schmitz,I., Krueger,A., Baumann,S., Schulze-Bergkamen,H., Krammer,P.H., and Kirchhoff,S. (2003). An IL-2-dependent switch between CD95 signaling pathways sensitizes primary human T cells toward CD95-mediated activation-induced cell death. Journal of Immunology *171*, 2930-2936.

Schneider,P., Holler,N., Bodmer,J.L., Hahne,M., Frei,K., Fontana,A., and Tschopp,J. (1998). Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with

downregulation of its proapoptotic activity and loss of liver toxicity. Journal of Experimental Medicine *187*, 1205-1213.

Schubert,L.A., Jeffrey,E., Zhang,Y., Ramsdell,F., and Ziegler,S.F. (2001). Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. Journal of Biological Chemistry *276*, 37672-37679.

Schulte,M., Reiss,K., Lettau,M., Maretzky,T., Ludwig,A., Hartmann,D., Strooper,B., Janssen,O., and Saftig,P. (2007). ADAM10 regulates FasL cell surface expression and modulates FasL-induced cytotoxicity and activation-induced cell death. Cell Death and Differentiation *14*, 1040-1049.

Schulze-Osthoff,K., Ferrari,D., Los,M., Wesselborg,S., and Peter,M.E. (1998). Apoptosis signaling by death receptors. European Journal of Biochemistry *254*, 439-459.

Seddiki,N., Santner-Nanan,B., Martinson,J., Zaunders,J., Sasson,S., Landay,A., Solomon,M., Selby,W., Alexander,S.I., Nanan,R., Kelleher,A., and Fazekas de St Groth,B. (2006a). Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. Journal of Experimental Medicine *203*, 1693-1700.

Seddiki,N., Santner-Nanan,B., Tangye,S.G., Alexander,S.I., Solomon,M., Lee,S., Nanan,R., and Fazekas de St Groth,B. (2006b). Persistence of naive CD45RA(+) regulatory T cells in adult life. Blood *107*, 2830-2838.

Seino,K., Iwabuchi,K., Kayagaki,N., Miyata,R., Nagaoka,I., Matsuzawa,A., Fukao,K., Yagita,H., and Okumura,K. (1998). Cutting edge: Chemotactic activity of soluble Fas ligand against phagocytes. Journal of Immunology *161*, 4484-4488.

Sheridan,J.P., Marsters,S.A., Pitti,R.M., Gurney,A., Skubatch,M., Baldwin,D., Ramakrishnan,L., Gray,C.L., Baker,K., Wood,W.I., Goddard,A.D., Godowski,P., and Ashkenazi,A. (1997). Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science *277*, 818-821.

Shevach,E.M. (2006). From vanilla to 28 flavors: Multiple varieties of T regulatory cells. Immunity *25*, 195-201.

Shimizu,J., Yamazaki,S., Takahashi,T., Ishida,Y., and Sakaguchi,S. (2002). Stimulation of CD25+CD4+regulatory T cells through GITR breaks immunological self-tolerance. Nature Immunology *3*, 135-142.

Shortman,K. and Wu,L. (1996). Early T lymphocyte progenitors. Annual Review of Immunology *14*, 29-47.

Singer,G.G. and Abbas,A.K. (1994). The Fas Antigen Is Involved in Peripheral But Not Thymic Deletion of T-Lymphocytes in T-Cell Receptor Transgenic Mice. Immunity *1*, 365-371.

Singh, N, Yamamoto, M, Takami M, Seki Y, Takezaki M, Mellor AL, and Iwashima M. CD4+CD25+ Regulatory T Cells Resist a Novel Form of CD28- and Fas-Dependent p53-Induced T Cell Apoptosis . Journal of Immunology . 4-1-2010.
Ref Type: Generic

Sprick,M.R., Rieser,E., Stahl,H., Grosse-Wilde,A., Weigand,M.A., and Walczak,H. (2002). Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. Embo Journal *21*, 4520-4530.

Stassen,M., Fondel,S., Bopp,T., Richter,C., Muller,C., Kubach,J., Becker,C., Knop,J., Enk,A.H., Schmitt,S., Schmitt,E., and Jonuleit,H. (2004). Human CD25(+) regulatory T cells: two subsets defined by the integrins alpha(4)beta(7) or alpha(4)beta(1) confer distinct suppressive properties upon CD4(+) T helper cells. European Journal of Immunology *34*, 1303-1311.

Strand,S., Hofmann,W.J., Hug,H., Muller,M., Otto,G., Strand,D., Mariani,S.M., Stremmel,W., Krammer,P.H., and Galle,P.R. (1996). Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells - A mechanism of immune evasion? Nature Medicine *2*, 1361-1366.

Strasser,A. (1995). Life and Death During Lymphocyte Development and Function - Evidence for 2 Distinct Killing Mechanisms. Current Opinion in Immunology *7*, 228-234.

Strasser,A., Jost,P.J., and Nagata,S. (2009). The Many Roles of FAS Receptor Signaling in the Immune System. Immunity *30*, 180-192.

Strasser,A. and O'Connor,L. (1998). Fas ligand - caught between Scylla and Charybdis. Nature Medicine *4*, 21-22.

Strasser,A. and Pellegrini,M. (2004). T-lymphocyte death during shutdown of an immune response. Trends in Immunology *25*, 610-615.

Straus,S.E., Jaffe,E.S., Puck,J.M., Dale,J.K., Elkon,K.B., Rosen-Wolff,A., Peters,A.M.J., Sneller,M.C., Hallahan,C.W., Wang,J., Fischer,R.E., Jackson,C.M., Lin,A.Y., Baumler,C., Siegert,E., Marx,A., Vaishnaw,A.K., Grodzicky,T., Fleisher,T.A., and Lenardo,M.J. (2001). The development of lymphomas in families with autoimmune lymphoproliferative syndrome with germline Fas mutations and defective lymphocyte apoptosis. Blood *98*, 194-200.

Straus,S.E., Sneller,M., Lenardo,M.J., Puck,J.M., and Strober,W. (1999). An inherited disorder of lymphocyte apoptosis: The autoimmune lymphoproliferative syndrome. Annals of Internal Medicine *130*, 591-601.

Strauss,L., Bergmann,C., and Whiteside,T.L. (2009). Human Circulating CD4(+)CD25(high)Foxp3(+) Regulatory T Cells Kill Autologous CD8(+) but Not CD4(+) Responder Cells by Fas-Mediated Apoptosis. Journal of Immunology *182*, 1469-1480.

Suda,T., Takahashi,T., Golstein,P., and Nagata,S. (1993). Molecular-Cloning and Expression of the Fas Ligand, A Novel Member of the Tumor-Necrosis-Factor Family. Cell *75*, 1169-1178.

Sutmuller,R.P.M., van Duivenvoorde,L.M., van Elsas,A., Schumacher,T.N.M., Wildenberg,M.E., Allison,J.P., Toes,R.E.M., Offringa,R., and Melief,C.J.M. (2001). Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. Journal of Experimental Medicine *194*, 823-832.

Taams,L.S., Smith,J., Rustin,M.H., Salmon,M., Poulter,L.W., and Akbar,A.N. (2001b). Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. European Journal of Immunology *31*, 1122-1131.

Taams,L.S., Smith,J., Rustin,M.H., Salmon,M., Poulter,L.W., and Akbar,A.N. (2001a). Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. European Journal of Immunology *31*, 1122-1131.

Tadokoro,C.E., Shakhar,G., Shen,S.Q., Ding,Y., Lino,A.C., Maraver,A., Lafaille,J.J., and Dustin,M.L. (2006). Regulatory T cells inhibit stable contacts between CD4(+) T cells and dendritic cells in vivo. Journal of Experimental Medicine *203*, 505-511.

Tai,X.G., Cowan,M., Feigenbaum,L., and Singer,A. (2005). CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. Nature Immunology *6*, 152-162.

Takahashi,A., Masuda,A., Sun,M., Centonze,V.E., and Herman,B. (2004). Oxidative stress-induced apoptosis is associated with alterations in mitochondrial caspase activity and Bcl-2-dependent alterations in mitochondrial pH (pH(m)). Brain Research Bulletin *62*, 497-504.

Tanaka,M., Itai,T., Adachi,M., and Nagata,S. (1998). Downregulation of Fas ligand by shedding. Nature Medicine *4*, 31-36.

Tanaka,M., Suda,T., Haze,K., Nakamura,N., Sato,K., Kimura,F., Motoyoshi,K., Mizuki,M., Tagawa,S., Ohga,S., Hatake,K., Drummond,A.H., and Nagata,S. (1996). Fas ligand in human serum. Nature Medicine *2*, 317-322.

Tanaka,M., Suda,T., Takahashi,T., and Nagata,S. (1995). Expression of the Functional Soluble Form of Human Fas Ligand in Activated Lymphocytes. Embo Journal *14*, 1129-1135.

Tartaglia,L.A., Ayres,T.M., Wong,G.H.W., and Goeddel,D.V. (1993). A Novel Domain Within the 55 Kd Tnf Receptor Signals Cell-Death. Cell *74*, 845-853.

Taylor,S.R.J., Alexander,D.R., Cooper,J.C., Higgins,C.F., and Elliott,J.I. (2007). Regulatory T cells are resistant to apoptosis via TCR but not P2X(7). Journal of Immunology *178*, 3474-3482.

Ten Bosch,J.V., Otten,J., and Thielemans,K. (2001). Autoimmune lymphoproliferative syndrome type III: An indefinite disorder. Leukemia & Lymphoma *41*, 55-+.

Thornton,A.M., Donovan,E.E., Piccirillo,C.A., and Shevach,E.M. (2004). Cutting edge: IL-2 is critically required for the in vitro activation of CD4(+)CD25(+) T cell suppressor function. Journal of Immunology *172*, 6519-6523.

Thornton,A.M. and Shevach,E.M. (1998). CD4(+)CD25(+) immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. Journal of Experimental Medicine *188*, 287-296.

Tran,D.Q., Class,D.D., Uzel,G., Darnell,D.A., Spalding,C., Holland,S.M., and Shevach,E.A. (2009). Analysis of Adhesion Molecules, Target Cells, and Role of IL-2 in Human FOXP3(+) Regulatory T Cell Suppressor Function. Journal of Immunology *182*, 2929-2938.

Trauth,B.C., Klas,C., Peters,A.M.J., Matzku,S., Moller,P., Falk,W., Debatin,K.M., and Krammer,P.H. (1989). Monoclonal-Antibody - Mediated Tumor-Regression by Induction of Apoptosis. Science *245*, 301-305.

Trombetta,E.S. and Mellman,I. (2005). Cell biology of antigen processing in vitro and in vivo. Annual Review of Immunology *23*, 975-1028.

Tsang,J.Y.S., Camara,N.O.S., Eren,E., Schneider,H., Rudd,C., Lombardi,G., and Lechler,R. (2006). Altered proximal T cell receptor (TCR) signaling in human CD4+CD25+ regulatory T cells. Journal of Leukocyte Biology *80*, 145-151.

Tsujimoto,Y., Cossman,J., Jaffe,E., and Croce,C.M. (1985). Involvement of the Bcl-2 Gene in Human Follicular Lymphoma. Science *228*, 1440-1443.

Valmori,D., Merlo,A., Souleimanian,N.E., Hesdorffer,C.S., and Ayyoub,M. (2005). A peripheral circulating compartment of natural naive CD4(+) Tregs. Journal of Clinical Investigation *115*, 1953-1962.

Valzasina,B., Guiducci,C., Dislich,H., Killeen,N., Weinberg,A.D., and Colombo,M.P. (2005). Triggering of OX40 (CD134) on CD4(+)CD25(+) T cells blocks their inhibitory activity: a novel regulatory role for OX40 and its comparison with GITR. Blood *105*, 2845-2851.

van Leeuwen,J.E.M. and Samelson,L.E. (1999). T cell antigen-receptor signal transduction. Current Opinion in Immunology *11*, 242-248.

Vang,K.B., Yang,J.Y., Mahmud,S.A., Burchill,M.A., Vegoe,A.L., and Farrar,M.A. (2008). IL-2,-7, and-15, but not thymic stromal lymphopoeitin, redundantly govern CD4(+)Foxp3(+) regulatory T cell development. Journal of Immunology *181*, 3285-3290.

Vaux,D.L. and Korsmeyer,S.J. (1999). Cell death in development. Cell *96*, 245-254.

Vercammen,D., Beyaert,R., Denecker,G., Goossens,V., Van Loo,G., Declercq,W., Grooten,J., Fiers,W., and Vandenabeele,P. (1998). Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. Journal of Experimental Medicine *187*, 1477-1485.

Villa,P., Kaufmann,S.H., and Earnshaw,W.C. (1997). Caspases and caspase inhibitors. Trends in Biochemical Sciences *22*, 388-393.

Villunger,A., Michalak,E.M., Coultas,L., Mullauer,F., Bock,G., Ausserlechner,M.J., Adams,J.M., and Strasser,A. (2003). p53- and drug-induced apoptotic responses mediated

by BH3-only proteins Puma and Noxa. Science *302*, 1036-1038.

Vogt, Carl. 130. 1842. Solothurn, Jent und Gassman.
Ref Type: Generic

Vonboehmer,H. (1988). The Developmental Biology of Lymphocytes-T. Annual Review of Immunology *6*, 309-326.

Vukmanovic-Stejic,M., Zhang,Y., Cook,J.E., Fletcher,J.M., McQuaid,A., Masters,J.E., Rustin,M.H.A., Taams,L.S., Beverley,P.C.L., Macallan,D.C., and Akbar,A.N. (2006). Human CD4(+)CD25(hi)Foxp3(+) regulatory T cells are derived by rapid turnover of memory populations in vivo. Journal of Clinical Investigation *116*, 2423-2433.

Waldmann,T.A. (2007). Anti-tac (daclizumab, zenapax) in the treatment of leukemia, autoimmune diseases, and in the prevention of allograft rejection: A 25-year personal odyssey. Journal of Clinical Immunology *27*, 1-18.

Walker,M.R., Kasprowicz,D.J., Gersuk,V.H., Benard,A., Van Landeghen,M., Buckner,J.H., and Ziegler,S.F. (2003). Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4(+)CD25(-) T cells. Journal of Clinical Investigation *112*, 1437-1443.

Wang,H.Y. and Wang,R.F. (2007). Regulatory T cells and cancer. Current Opinion in Immunology *19*, 217-223.

Wang,J., Ioan-Facsinay,A., van der Voort,E.I.H., Huizinga,T.W.J., and Toes,R.E.M. (2007). Transient expression of FOXP3 in human activated nonregulatory CD4(+) T cells. European Journal of Immunology *37*, 129-138.

Wang,J., Zheng,L.X., Lobito,A., Chan,F.K.M., Dale,J., Sneller,M., Yao,X., Puck,J.M., Straus,S.E., and Lenardo,M.J. (1999). Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. Cell *98*, 47-58.

Watanabefukunaga,R., Brannan,C.I., Copeland,N.G., Jenkins,N.A., and Nagata,S. (1992a). Lymphoproliferation Disorder in Mice Explained by Defects in Fas Antigen That Mediates Apoptosis. Nature *356*, 314-317.

Watanabefukunaga,R., Brannan,C.I., Itoh,N., Yonehara,S., Copeland,N.G., Jenkins,N.A., and Nagata,S. (1992b). The Cdna Structure, Expression, and Chromosomal Assignment of the Mouse Fas Antigen. Journal of Immunology *148*, 1274-1279.

Weant,A.E., Michalek,R.D., Khan,I.U., Holbrook,B.C., Willingham,M.C., and Grayson,J.M. (2008). Apoptosis regulators bim and fas function concurrently to control autoimmunity and CD8(+) T cell contraction. Immunity *28*, 218-230.

Weiner,H.L. (2001). Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. Immunological Reviews *182*, 207-214.

Wildin,R.S., Ramsdell,F., Peake,J., Faravelli,F., Casanova,J.L., Buist,N., Levy-Lahad,E., Mazzella,M., Goulet,O., Perroni,L., Bricarelli,F.D., Byrne,G., McEuen,M., Proll,S., Appleby,M., and Brunkow,M.E. (2001). X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nature Genetics *27*, 18-20.

Willis,S.N., Fletcher,J.I., Kaufmann,T., van Delft,M.F., Chen,L., Czabotar,P.E., Ierino,H., Lee,E.F., Fairlie,W.D., Bouillet,P., Strasser,A., Kluck,R.M., Adams,J.M., and Huang,D.C.S. (2007). Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science *315*, 856-859.

Wu,Y.Q., Borde,M., Heissmeyer,V., Feuerer,M., Lapan,A.D., Stroud,J.C., Bates,D.L., Guo,L., Han,A.D., Ziegler,S.F., Mathis,D., Benoist,C., Chen,L., and Rao,A. (2006). FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell *126*, 375-387.

Wyllie, A. H., Kerr, J. F., and Currie, A. R. Cell death: the significance of apoptosis. Int.Rev: Cytol. 68, 251-306. 1980.
Ref Type: Generic

Xystrakis,E., Boswell,S.E., and Hawrylowicz,C. (2006). T regulatory cells and the control of

allergic disease. Expert Opinion on Biological Therapy *6*, 121-133.

Yamagiwa,S., Gray,J.D., Hashimoto,S., and Horwitz,D.A. (2001). A role for TGF-beta in the generation and expansion of CD4(+)CD25(+) regulatory T cells from human peripheral blood. Journal of Immunology *166*, 7282-7289.

Yang,Y., Kim,D., and Fathman,C.G. (1998). Regulation of programmed cell death following T cell activation in vivo. International Immunology *10*, 175-183.

Yin,X.M., Wang,K., Gross,A., Zhao,Y.G., Zinkel,S., Klocke,B., Roth,K.A., and Korsmeyer,S.J. (1999). Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature *400*, 886-891.

Yonehara,S., Ishii,A., and Yonehara,M. (1989). A Cell-Killing Monoclonal-Antibody (Anti-Fas) to A Cell-Surface Antigen Co-Downregulated with the Receptor of Tumor Necrosis Factor. Journal of Experimental Medicine *169*, 1747-1756.

Yu,J.W. and Shi,Y. (2008). FLIP and the death effector domain family. Oncogene *27*, 6216-6227.

Zhao,D.M., Thornton,A.M., DiPaolo,R.J., and Shevach,E.M. (2006). Activated CD4(+)CD25(+) T cells selectively kill B lymphocytes. Blood *107*, 3925-3932.

Zheng,L.J., Sharma,R., Gaskin,F., Fu,S.M., and Ju,S.T. (2007a). A novel role of IL-2 in organ-specific autoimmune inflammation beyond regulatory T cell checkpoint: Both IL-2 knockout and Fas mutation prolong lifespan of Scurfy mice but by different mechanisms. Journal of Immunology *179*, 8035-8041.

Zheng,Y., Chaudhry,A., Kas,A., deRoos,P., Kim,J.M., Chu,T.T., Corcoran,L., Treuting,P., Klein,U., and Rudensky,A.Y. (2009). Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. Nature *458*, 351-U116.

Zheng,Y., Josefowicz,S.Z., Kas,A., Chu,T.T., Gavin,M.A., and Rudensky,A.Y. (2007b). Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. Nature *445*, 936-940.

Ziegler,S.F. (2006). FOXP3: Of mice and men. Annual Review of Immunology *24*, 209-226.

Zou,W.P. (2006). Regulatory T cells, tumour immunity and immunotherapy. Nature Reviews Immunology *6*, 295-307.

7 List of publications

Klemke CD, Brenner D, Weiss EM, Schmidt M, Leverkus M, Gülow K, Krammer PH. Lack of T-cell receptor-induced signaling is crucial for CD95 ligand up-regulation and protects cutaneous T-cell lymphoma cells from activation-induced cell death. Cancer Res. 2009 May 15;69(10):4175-83.

8 Declaration

I hereby declare that:

- I have conducted the present work alone.
- my submission as a whole is not substantially the same as any that I have previously made or am currently making, whether in published or unpublished form, for a degree, diploma, or similar qualification at any university or similar institution.

_____ Eva-Maria Weiß (Dipl. Biol.)