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Many checkpoints on the road to cell death: regulation of Fas-FasL interactions and Fas signaling in peripheral immune responses

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Abstract

Interactions between the TNF-family receptor Fas (CD95) and Fas Ligand (FasL, CD178) can efficiently induce apoptosis and are critical for maintenance of immunological self-tolerance. FasL is kept under strict control by transcriptional and post-translational regulation. Surface FasL can be cleaved by metalloproteases, resulting in shed extracellular domains, and FasL can also traffic to secretory lysosomes. Each form of FasL has distinct biological functions. Fas is more ubiquitously expressed, but its apoptosis-inducing function is regulated by a number of mechanisms including submembrane localization, efficiency of receptor signaling complex assembly and activation, and bcl-2 family members in some circumstances. When apoptosis is not induced, Fas-FasL interactions can also trigger a number of activating and pro-inflammatory signals. Harnessing the apoptosis-inducing potential of Fas for therapy for cancer and autoimmune disease has been actively pursued, and despite a number of unexpected side-effects that result from manipulating Fas-FasL interactions, this remains a worthy goal.

1. Introduction: Fas-Fas Ligand interactions in immune responses

The discovery in the early 1990's that antibodies to the cell surface TNF-family member receptor Fas (CD95) could mediate rapid protein-synthesis independent apoptosis of a number of transformed and non-transformed cell types set the stage for the investigation of engaging Fas and related 'death receptors' as possible targets for intervention in cancer therapy. Fas also plays a critical role in immunological self-tolerance through the deletion of a number of cell types that contribute to autoimmunity. Mutations in Fas and its TNF family ligand Fas Ligand (CD178, FasL) are responsible for the single gene autoimmune *lpr* and *gld* phenotypes in mice (Ramsdell et al., 1994b; Watanabe-Fukunaga et al., 1992a) and most cases of the strikingly similar autoimmune lymphoproliferative syndrome (ALPS) in humans, which is associated in a majority of patients (Type IA ALPS) with dominant-interfering Fas mutations (Straus et al., 1999). Not surprisingly for an interaction that can permanently eliminate cells through apoptosis, it has become clear that there are many levels of regulation of Fas-FasL interactions. Both FasL synthesis and trafficking are subject to strict control, which limit the production of biologically active ligand to a few cell types. Although most activated lymphocytes express Fas, there are many levels of regulation that control the efficiency of Fas-induced apoptosis, both at the level of assembly and activation of the Fas signaling complex, and at the level of signal integration at the mitochondria. These mechanisms cooperate to create a situation where Fas-FasL interactions can efficiently eliminate autoreactive T and B cells, while having little impact on most immune responses to pathogens.

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Fas-FasL interactions have been shown to be responsible for much of the apoptosis that occurs when activated $CD4^+$ T cells are restimulated through the T-cell receptor (TCR). (Dhein et al., 1995; Ju et al., 1995). Since this process is molecularly distinct from much of the T cell death that occurs during initial T cell activation, we refer to this process as Restimulation Induced Cell Death, or RICD. Most of the death that restimulated $CD4^+$ T cells undergo is through RICD by FasL, while FasL appears to play a subsidiary role in $CD8^+$ T cells to other proteins contained in cytotoxic T cell granules such as perforin and granzymes (Davidson et al., 2002) As we will discuss in this chapter, although most activated and memory lymphocytes express cell surface Fas, RICD only kills activated T cells under conditions of chronic T-cell restimulation, due to controls on FasL expression and processing and Fas signaling that render this pathway inactive under other circumstances. Different functional subsets of $CD4^+$ T cells may also use the Fas-FasL pathway of apoptosis to greater or lesser extents. The majority of cell death that occurs after T cell activation appears to be apoptosis caused by inadequate supply of cytokines such as IL7 and IL15 that signal through gamma-chain containing cytokine receptors and Jak/STAT proteins to increase expression and function of Bcl-2 family proteins. We term this type of cell death Post-Activation Cell Death (PACD). Experiments in which activated lymphocytes are infused into IL-7 and IL-15 deficient mice have shown that these two cytokines cooperate to allow survival of T cells after activation, and genetic or pharmacological delivery of these cytokines can prolong T cell survival (Sprent and Surh, 2002; Tan et al., 2002)

In most circumstances, the massive proliferation of activated T cells during immune responses outgrows the cytokine supply and results in a balance of pro and anti-apoptotic Bcl-2 family members that favors apoptosis. This is dramatically illustrated by mice that lack the BH-3 only pro-apoptotic family member Bim. There is accumulation of excess lymphocytes in these mice and antigen-specific T cells are impaired in their ability to undergo cell death after acute antigen stimulation, while RICD of activated T cells is not affected. Conversely, Fas deficient animals have nearly normal cell death of T cells after acute activation. Mice in which Bim and Fas have both been genetically ablated have greatly enhanced pathology and autoimmune disease compared to each mutant alone, providing genetic evidence that these two pathways are distinct (Hughes et al., 2008; Hutcheson et al., 2008; Weant et al., 2008). RICD helps to maintain peripheral tolerance by eliminating reactive T cells and reducing the chance for reactive T cells to act on target cells beyond their specific effector function. Fas-FasL interactions can alter the response of activated T cells during infections, and impairment of any of the mechanisms that promote FasL mediated apoptosis discussed below may predispose towards autoimmunity.

In this chapter, we will discuss the basic biology of Fas and Fas Ligand with emphasis on the role of Fas-FasL interactions in the immune system, pointing out a number of steps that strictly regulate the scope of cells that die via Fas-FasL interactions. In particular, we will discuss findings by our group and others showing that much of the regulation of Fas signaling lies in early steps of assembly and activation of the Fas signaling complex. We will also discuss the status of attempts to harness Fas-induced apoptosis for therapeutic use in autoimmunity, transplantation and cancer therapy.

2. Fas Ligand, a highly regulated TNF family member

Fas Ligand (FasL or CD178), the sole known TNF-family ligand for Fas, is synthesized as a 281 amino acid type II transmembrane protein. Compared with other TNF family members, FasL has a relatively long N-terminal cytoplasmic domain that has been found to contain multiple sorting motifs governing the trafficking of FasL. Some groups have reported that the cytoplasmic domain of FasL can also mediate ‘reverse signaling’ upon binding to Fas. The extracellular TNF-homology domain of FasL can also be cleaved from the membrane by metalloproteinases to become a secreted trimer. However, trimeric FasL is highly unstable and

appears to be largely inert as an apoptosis-inducing ligand. These aspects of the biology of FasL make control of trafficking and cleavage of this protein as important as regulation of FasL expression.

2.1 Regulation of FasL Gene Expression

While FasL expression on activated T cells is transient, FasL expression on non-immune cells is generally constitutive. The signaling pathways and transcription factors mediating inducible FasL expression have best been studied in T cells. The calcium-sensitive NFAT (Nuclear factor of activated T-cells) transcription factor family mediates a major part of the signal by which the TCR induces FasL. Calcineurin activation results in NFAT activation and translocation to the nucleus, and inhibitors of calcineurin such as cyclosporine A block FasL expression in activated T cells (Anel et al., 1994; Brunner et al., 1996; Dhein et al., 1995; Latinis et al., 1997a). The FasL promoter has two NFAT sites, with the more distal NFAT site on the FasL promoter more important for TCR-mediated FasL expression in $CD4^+$ T cells (Latinis et al., 1997b). The Egr family of transcription factors is induced by NFAT and may act synergistically with NFAT in inducing FasL expression in some cell types (Dzialo-Hatton et al., 2001; Mittelstadt and Ashwell, 1999; Rengarajan et al., 2000). C-myc binds to a separate site on the FasL promoter and has been shown to also be required for TCR-induced FasL expression (Brunner et al., 2000; Wang et al., 1998). TCR-induced FasL expression can also be negatively regulated by a number of mechanisms. The CIITA (MHC class II transactivator) transcription factor as well as retinoic acid can block NFAT function and inhibit FasL transcription (Gourley and Chang, 2001; Lee et al., 2002). TGF-β (Transforming Growth Factor-beta) can also inhibit TCR-induced FasL expression through downmodulating c-myc expression (Genestier et al., 1999).

In parallel with NFAT, the NF–κB transcription factors, which have separate binding sites in the FasL promoter, can also induce FasL expression. Through the action of protein kinase C theta (PKC-θ), the TCR activates NF-κB and can synergize with calcineurin-dependent signaling to induce FasL expression. (Kasibhatla et al., 1999; Villalba et al., 1999; Villunger et al., 1999). The AP-1 transcription factor complex, which is activated by the TCR through MAP kinase signaling, also activates FasL expression (Matsui et al., 2000). Interestingly, inducers of nitric oxide (NO) inhibit FasL expression through blocking AP-1 activity (Melino et al., 2000). Interferon Regulatory Factors (IRFs), a family of transcription factors that induce the transcription of interferons in response to viral infection, may also cooperate with other transcription factors to maximally induce FasL in response to TCR and other stimuli (Chow et al., 2000). Viral IRF homologs from human herpesvirus 8 (HHV8), the cause of Kaposi's Sarcoma in immunocompromised patients, interferes with IRF-1 binding and downregulates FasL transcription, which may aid in the escape of infected T cells from FasL mediated apoptosis (Kirchhoff et al., 2002). A separate set of transcription factors, govern basal and constitutive FasL expression in both lymphoid and non-lymphoid cells. The transcription factor Sp1 regulates the basal FasL expression in Jurkat T cells and constitutive expression of FasL in Sertoli cells (McClure et al., 1999). Sp1 regulates FasL expression on smooth muscle cells (SMCs) by cooperating with the transcription factor Ets-1 (Kavurma et al., 2002; Kavurma et al., 2001). Transcriptional regulation of FasL results in constitutive expression in tissues such as the eye and testis that may contribute to immune tolerance through inducing apoptosis in infiltrating lymphocytes, whereas FasL dynamically expressed on T cells can eliminate Fas-sensitive cells in tissues where FasL may not be expressed (Bellgrau et al., 1995; Bonfoco et al., 1998).

2.2 Three forms of FasL controlled by post-translational modification and subcellular trafficking

Post-translational modification of FasL results in dramatically different trafficking of FasL in cells and in its ability to induce apoptosis. FasL is initially synthesized as a type II transmembrane protein containing a TNF homology domain at the C-terminal that traffics to the plasma membrane through the golgi. FasL expressed ectopically on the plasma membrane can be a strong stimulator of apoptosis (Jodo et al., 2001; Suda et al., 1997). A portion of FasL has also been reported to partition into glycosphingolipid-enriched membrane 'rafts', which may also enhance its death-inducing function(Cahuzac et al., 2006). Extracellular FasL can be cleaved by the metalloproteinase ADAM10, resulting in shedding of a 20-26KDa free extracellular domain, and the intracellular domain can be cleaved and released into the cytosol by the signal peptidase-like protease SPPL2a (Kirkin et al., 2007; Schulte et al., 2007). Soluble FasL released in this way is generally thought to be inactive or even inhibitory for FasL-mediated apoptosis, (Jodo et al., 2001; Suda et al., 1997) so metalloproteinase-dependent cleavage could be an inactivating event for FasL function as a membrane-bound ligand. The intracellular fragment traffics to the nucleus, but the function of this fragment is not clear. In addition, surface FasL can be internalized and sorted into multivesicular bodies, which when fused with the plasma membrane allow secretion of membrane bound FasL into secretory microvesicles, also known as secretory lysosomes. FasL secreted in microvesicles can be highly biologically active, so understanding of the mechanisms that regulate FasL trafficking into this compartment is important.

Sorting of FasL into secretory lysosomes occurs only in cells that have this specialized trafficking pathway, such as lymphocytes and myeloid cell lines. In fibroblasts and epithelial cell lines, FasL predominantly traffics to the plasma membrane (Blott and Griffiths, 2002). Sorting of FasL into the secretory lysosome pathway requires post-translational modification and association of specific domains in the intracellular portion of FasL with several proteins involved in protein and organelle trafficking, cytoskeletal reorganization and formation of the immunological synapse. The proline rich domain (PRD) of FasL binds to Fgr, a Src family tyrosine kinase, and deletions of the PRD as well as mutations in Fgr result in more surface FasL and less FasL in secretory lysosomes. Interestingly, similar mutaitons also appear to inhibit 'reverse signaling' through FasL that has been reported to occur after Fas binding and function to costiulate $CD8^+$ T cell activation (Sun et al., 2007). Ubiquitination and tyrosine phosphorylation at specific residues in the N-terminal portion of FasL also control trafficking of FasL into secretory lysosomes and deletion or mutation of residues important in these processes redirect FasL to the cell surface (Jodo et al., 2005; Zuccato et al., 2007). Kinases associated with actin remodeling, such as Nck, have also been been shown to co-localize with FasL (Lettau et al., 2006) and direct it to secretory lysosomes, providing another level of regulation of FasL trafficking.

These mechanisms cooperate to generate two different waves of FasL produced by T cells acutely stimulated through the TCR. The first phase of cell surface FasL occurs within 10 minutes of T cell stimulation, and is thought to derive from fusion of FasL stored in secretory vesicles with the plasma membrane. The continued stimulation of T cells results in a second wave of surface expression of FasL derived from newly synthesized protein peaking 2–4 hours after stimulation (He and Ostergaard, 2007; Lettau et al., 2004). FasL secreted in exosomal vesicles may derive from either of these pools and several nonlymphoid cells and tissues such as dendritic cells (DC) and other myeloid cells can express FasL in these various forms. Certain DC subsets have been reported to be able to kill Fas-expressing $CD4^+$ T cells (Suss and Shortman, 1996). FasL on lymph node dendritic cells have been recently shown to regulate the magnitude of $CD8^+$ effector T cell responses in the lung in the context of influenza infection through induction of T cell apoptosis via FasL (Legge and Braciale, 2005). FasL expression

on macrophages results in both macrophage and T cell apoptosis (Kiener et al., 1997; Ma et al., 2004; Monari et al., 2005; Villena et al., 2008). Epithelial cells such as those present in tissues, most likely express constitutive surface FasL.

3. Fas: an apoptosis-inducing TNF-family receptor

Unlike its ligand, Fas is expressed in many diverse cell types, tissues and organs. The initial discovery of Fas was made by two different groups screening for apoptosis-inducing antibodies against cell surface antigens. Two such antibodies, anti-Fas (for ***F***S-7 ***a***ssociated ***s***urface antigen) (Yonehara et al., 1989) and anti-Apo1 (Trauth et al., 1989) bound the same 35-52KDa protein that was termed Fas/Apo-1 (Trauth et al., 1989). Cloning and characterization studies classified Fas as a prototype of the TNF-receptor superfamily, and further designated Fas as CD95/TNFRSF6. Early work on the human Fas antigen was done mostly in lymphoma cell lines, indicating that it was highly expressed on both T and B cell lymphomas (Oehm et al., 1992). In the mouse, Fas expression was seen in the heart, liver, ovary and the thymus (Watanabe-Fukunaga et al., 1992b). This fits with expression patterns of other members of the TNF/TNFR superfamily, where expression of ligands have a propensity to be more restricted and dynamically regulated than the receptors, whose expression is regulated between cell lineages but tends to be more constant over time.

3.1 Role of Fas in lymphocyte biology

The observation that Fas-deficient *lpr* mice and *gld* mice, which carry recessive disabling mutations in the Fas and FasL genes respectively, produce autoantibodies and have excessive accumulation of $CD4^{-}CD8^{-}$ (double negative) T cells initially pointed towards a role for Fas in thymic negative selection (Watanabe-Fukunaga et al., 1992a). However, despite expression of Fas on thymocytes and the susceptibility of most thymocytes to Fas-induced apoptosis, negative selection does not appear to depend on Fas-FasL interactions, since self-reactive T cells are deleted effectively in the thymus of both *lpr* and *gld* mice (Singer and Abbas, 1994). Rather, Fas participates in the elimination of self-reactive T cells by a process known as restimulation induced cell death (RICD), an important ‘safety net’ for maintaining self-tolerance in T cells that have escaped central thymic tolerance. Despite being constitutively expressed on most T lymphoma cell lines, naïve T cells do not have surface Fas expression and therefore are highly refractory to Fas-mediated apoptosis, whereas, memory T cells have high Fas levels. Activation of the resting naïve cells via TCR stimulation upregulates surface Fas within 24 hours after activation, with highest surface levels occurring with in 6 days of stimulation (Klas et al., 1993; Miyawaki et al., 1992). However, the regulation of Fas-induced apoptosis is a multi-layered process and receptor expression alone does not render cells sensitive to Fas-induced apoptosis. It has been observed that recently activated Fas positive cells are refractory to cell death unless cultured in IL-2 for at least 48 additional hours (Peter et al., 1997). This “propriocidal death” is due to IL-2 induced cell cycle progression, which is necessary to make T cells sensitive to TCR and Fas-induced apoptosis (Lenardo et al., 1999). The T-cell receptor provides a critical and physiologically significant signal that also sensitizes T cells to Fas-mediated apoptosis, and is the basis for antigen-specific deletion of activated T cells. As discussed above, TCR engagement of activated T cells results in FasL gene upregulation and secretion (Dhein et al., 1995; Ju et al., 1995). However, mixing experiments with T cells of different specificities showed that the FasL produced by the antigen specific T cells mediates apoptosis of only the restimulated clonotype and not other Fas-expressing bystander cells. This feature of restimulation-induced cell death ensures that only chronically stimulated T cells undergo Fas-mediated apoptosis, and likely is responsible for the restricted role of Fas in elimination of T cells specific for autoantigens and chronic pathogens. The signal mediated via the TCR that sensitizes these cells to Fas-induced apoptosis is termed the “competency to die” signal. TCR and Fas engagement synergize to induce apoptosis in a

manner unaffected by protein synthesis inhibitors (Combadiere et al., 1998; Hornung et al., 1997; Wong et al., 1997). Recent work has shown that part of the TCR-induced 'competency to die' signal induces translocation of Fas to lipid rafts, also known as glycosphingolipids enriched membrane microdomains (Muppidi and Siegel, 2004). The role of lipid raft microdomains in Fas signaling is discussed in detail below.

The role of Fas in elimination of chronically restimulated T cells is largely confined to $CD4^+$ T cells, which are thought to be the key T cells that can provide help to autoreactive B cells to allow autoantibody secretion. $CD8^+$ T cells, like their $CD4^+$ counterparts, upregulate Fas upon activation, and can be induced to undergo apoptosis through Fas (Miyawaki et al., 1992). However, restimulation of $CD8^+$ T cells from Fas-deficient mice or patients with ALPS induces normal levels of cell death indicating that Fas-independent mechanisms contribute to RICD of $CD8^+$ T. However, the granzyme/perforin cytotoxic serine protease pathway is a major player in attrition of antigen specific cytotoxic T lymphocyte (CTL) response. Regulation of granzyme B is critical, since the cytoplasmic granzyme B can cause self-directed injury to the CTL producing it. Recent work identifies the presence of a serine protease inhibitor (SPI6) that binds to cytoplasmic granzyme B to form a stable complex, thereby ensuring normal antigenic response by preventing CTL loss by suicide (Zhang et al., 2006).

3.2 Function of Fas in B cells and Dendritic Cells

Fas, also regulates B cell autoantibody production and antigen presenting cell function. As in T cells, Fas is dispensable for B cell development, but important in mediating peripheral B cell tolerance. Fas is not expressed on resting B cells, but is upregulated on activated B cells and highly expressed on germinal center B cells, some of which acquire autoreactive specificities and are eliminated through BCR ligation. Since B cells are not thought to upregulate FasL upon BCR stimulation, Fas-mediated B cell apoptosis likely depends on FasL produced by other cells. In this way T cells may indirectly regulate autoreactive B cells in the periphery. Study of antigen-specific T and B cell interactions showed that $CD4^+$ T cells can specifically eliminate autoreactive B cells in a Fas dependent manner (Rathmell et al., 1995). In autoimmune Fas-deficient mice, nephritis could still occur to some degree in animals engineered to prevent antibody secretion, showing that the antigen presenting function of B cells is important in the pathogenesis of nephritis in this model system (Shlomchik et al., 1994). *Lpr* mice lacking B cells did not develop nephritis, and interestingly, this was accompanied by a concomitant reduction in the accumulation of memory phenotype $CD4^+$ T cells normally present in *lpr* mice (Chan and Shlomchik, 1998). Thus autoreactive B cells that fail to be eliminated through Fas may sustain autoimmunity through acting as antigen presenting cells for autoantigens and further activating autoreactive T cells. An essential role for B cell expression of Fas in maintaining self-tolerance was also shown in mice in which Fas was specifically eliminated in the B cell compartment. These mice developed characteristic lymphadenopathy, splenomegaly, high autoantibody titers and also accumulation of T cells, re-emphasizing the role of Fas in maintenance of peripheral B cell tolerance (Stranges et al., 2007).

Like B cells, DC may be eliminated through Fas-FasL interactions, and this may serve to downmodulate antigen presentation. Antigen-pulsed DC injected into mice were observed to disappear after 2–3 days from the draining lymph node if antigen-specific T cells are present, suggesting that T-DC interactions may be responsible for elimination of DC (Ingulli et al., 1997). Further, accumulation of DC occurs in autoimmune diseases and animal models where apoptosis pathways are disrupted, such as in human patients harboring caspase 10 mutations and *lpr* mice (Fields et al., 2001; Wang et al., 1999). Transgenic mice in which the caspase inhibitor p35 was overexpressed in DC resulted in accumulation of DC in lymph nodes, T cell hyperplasia and development of anti-nuclear antibodies in older mice. These death-resistant

DCs also increased the rapidity of autoimmune manifestations in an autoimmune-prone mouse strain (Chen et al., 2006). Mice, in which, Fas was specifically deleted in DCs also developed autoantibodies (Stranges et al., 2007). However in both of these models, the development and titer of autoantibodies were lower than in mice with universally disrupted Fas function. Taken together, these studies indicate that DC apoptosis likely occurs physiologically during antigen presentation to T cells, and that this mechanism also contributes to peripheral self-tolerance. Remarkably, elimination of Fas expression in T cells, B cells and DC all contribute to this function. This pleiotropic role of Fas likely explains why mutations affecting Fas and FasL confer such potent susceptibility to autoimmune diseases. Although additional susceptibility genes govern the nature and severity of autoimmune disease pathology, development of autoantibody production is remarkably high in mice and humans with mutations disabling this pathway.

3.3 Fas on non-immune cells

It is important to note that other cell types can also express Fas. One of the major non-immune sites of Fas expression is in hepatocytes, which are quite sensitive to Fas-induced-apoptosis (Rouquet et al., 1996). Although Fas-deficient *lpr* mice do not develop liver hyperplasia, a small amount of Fas protein may still be produced by the *lpr* mutant Fas allele, and mice engineered to completely lack Fas protein did exhibit liver hyperplasia (Adachi et al., 1995). Though Fas is expressed at high levels, FasL is not expressed by hepatocytes. However, liver sinusoids do contain T cells which may express FasL. Administration of anti-mouse Fas antibodies results in lethal acute hepatic necrosis that is dependent on hepatocyte Fas expression and Fc-mediated crosslinking of these antibodies (Adachi et al., 1995; Ogasawara et al., 1993; Xu et al., 2003). During viral and other forms of hepatitis, FasL expressed on activated T cells may also play a role in causing hepatocyte damage (Kondo et al., 1997; Seino et al., 1997). Fas expression by target tissues of T-cell mediated autoimmune disease, such as the thyroid and pancreatic islets, may also play a role in the tissue destruction in these conditions (Signore et al., 1998; Stassi and De Maria, 2002).

4. Fas receptor signaling for apoptosis: ordered assembly of oligomeric protein complexes to activate caspase-8

Activation of the Fas signaling pathway begins by binding of FasL or other receptor agonists, resulting in recruitment of the adaptor protein FADD (Fas associated death domain) and the cysteinyl aspartic proteases, caspase-8 (and caspase-10 in humans) to form a proximal signaling platform called the Death Inducing Signaling Complex (DISC) (Kischkel et al., 1995). The DISC can be detected within seconds of receptor engagement and functions to activate caspase-8/10, an essential step in initiation of programmed cell death. The recruitment and signaling specificities are maintained by alpha-helical modular domains that interact with each other and in some cases also self-associate. The death domains in the intracytoplasmic region of Fas and the C-terminal of FADD interact to recruit FADD to the receptor. FADD also contains an amino-terminal death-effector domain (DED), structurally related to the DD but with affinity for other DED modules. FADD DED binds to DEDs in the prodomain of caspase-8/10, bringing them into the DISC. Aggregation and complex formation are necessary in the DISC to catalyze caspase-8 cleavage and downstream cleavage and activation of effector caspase-3, which then culminates in apoptosis. The apoptotic machinery is irrevocable once effector caspases are activated, and many mechanisms have evolved to safeguard against wanton activation of cell death via Fas. Although post-translational modifications such as phosphorylation or ubiquitination are not required for assembly and activation of the DISC, these steps are regulated at different stages, beginning with surface receptor clustering, ligand binding and efficiency of cytoplasmic complex formation at stages downstream of the DISC which will be discussed in turn below. Fas-induced apoptosis in cell lines was originally divided

into ‘Type I’ or ‘Type II’ pathways depending on the ability of Fas-induced apoptosis to be blocked by overexpression of anti-apoptotic bcl-2 family members (Scaffidi et al., 1998). More recent data has shown that these two signaling pathways also reflect differences between more proximal events in receptor signaling, such as the localization of receptors to lipid raft microdomains which in turn regulates the preassociation of receptors prior to ligand binding. In primary T cells, as we will discuss below, there is a spectrum of sensitivity to Fas-induced apoptosis that can be regulated by cytoskeletal remodeling through the Rac family of small GTPases and likely other mechanisms as well.

Fas receptor, a 45kDa type-I transmembrane glycoprotein, is a prototypic TNFRSF death receptor with a cytoplasmic 80 amino acid death domain (DD) and 3 cysteine rich domains (CRD) in the extracytoplasmic region. Mutational analysis studies indicated that ligand binding for optimal signaling required the presence of all three CRDs (Orlinick et al., 1997). From the crystal structures of TNF ligands bound to their receptors, it was found that TNF ligands exist in trimers. The stoichiometry of ligand-receptor complexes was 3:3, and trimeric structures have also been found for adaptor molecules downstream of the receptor (Bodmer et al., 2002). This supported a model of signaling where there is cooperativity and dissemination of signaling via formation of ligand:receptor:adaptor hetero-complexes. It was therefore not surprising that in patients with Type1A ALPS, where heterozygous mutations of the Fas receptor are localized mostly to the DD, lymphocytes were highly resistant to Fas-mediated apoptosis, even though they have equal gene dosage of wild-type and mutant Fas genes. Expression of Fas constructs containing ALPS-associated mutations dominantly interfered with Fas-induced apoptosis even in the presence of wild type Fas, supporting the theory of cooperativity in signaling where inclusion of mutant Fas disrupts formation of large complexes of ligand-receptor molecules. This results in inefficient DISC formation and caspase-8 activation (Fisher et al., 1995; Martin et al., 1998; Martin et al., 1999; Vaishnaw et al., 1999).

The above model posits that FasL binding initiates receptor trimerization, and downstream cytoplasmic events. However, in some patients with type 1A ALPS, Fas mutations occurred in the extra-cellular domain that disrupted FasL binding, but had intact intracellular signaling domains. Surprisingly, it was found that these mutants could also interfere with Fas-induced apoptosis. This finding gave rise to a new hypothesis that receptors could form preassociated complexes, without necessarily requiring ligand crosslinking. A ‘pre-ligand receptor association domain’ (PLAD) in the N-terminal CRD1 portion of the receptor, distinct from the ligand binding site was subsequently identified in Fas, TNFR1, CD40, TACI, and TRAIL-receptors, and is probably a general feature of TNF receptors. (Chan et al., 2000; Clancy et al., 2005; Garibyan et al., 2007; Siegel et al., 2000). Dominant interference with receptor signaling by mutations in Fas were found to be dependent on the PLAD, favoring the hypothesis that receptor preassociation is required for and precedes ligand binding. An intact PLAD is also required for ligand binding, since ligand binding Fas mutants preassociate with wildtype receptors, but do not signal, indicating that receptor preassociation also aligns the receptors to maximally bind ligand. Interestingly, all the dominant interfering mutations identified so far in ALPS patients have an intact CRD1/PLAD region, even though some of them have mutations in the ligand binding region of CRD2, again emphasizing the role of receptor preassociation for signaling (Siegel et al., 2000). These findings make the PLAD a plausible therapeutic target to regulate TNFSF responses, and bacterially synthesized or synthetic peptides comprising of the PLAD were effective in blocking TNFR1 signaling in vitro and in a TNF-dependent model of inflammatory arthritis (Deng et al., 2005).

Engagement of Fas by FasL or agonistic anti-Fas antibodies, induces a series of events, most of which begin with receptor clustering, formation of surface microaggregates and microclusters, receptor capping and internalization. The final outcome of these receptor-aggregated superclusters at the surface is efficient DISC assembly, thereby ensuring caspase-8

activation. Formation of SDS-stable Fas microaggregates occur simultaneously with formation of the DISC, and microscopically, surface receptor aggregates can be visualized in the same time frame. These structures were termed SPOTS, for Signaling Protein Oligomerization Transduction Structures. FADD recruitment was seen to be a necessary step in formation of these large Fas surface clusters, since the SPOTS formation was drastically reduced in cells deficient for FADD or expressing Fas mutations unable to bind FADD. However, activation of caspase-8 was not necessary for formation of SPOTS (Siegel et al., 2004). Formation of receptor microclusters is further stabilized and maintained via FADD homotypic associations. In addition to its function as an adaptor protein bridging Fas and caspase -8, FADD has an innate ability to oligomerize and form lateral interactions with itself, resulting in a characteristic filamentous structure termed 'death effector filaments'. This self-association resides in the DED and is independent of Fas and caspase-8 interactions. Mutations that disrupt FADD self-association resulted in dominant interfering mutants, indicating that caspase-8 recruitment is dependent on FADD self-association (Muppidi et al., 2006; Sandu et al., 2006).

As outlined above, early events in Fas signaling proceed in a stepwise manner, beginning with receptor preassociation, formation of microaggregates upon receptor binding and finally formation of large lateral signaling platforms, SPOTS. Until this step, there is no feedback regulation of downstream molecules in the DISC on the receptor aggregation. However, after formation of SPOTS, the Fas signaling complex forms polar aggregates on one side of the cell, referred to as "capping", which are then internalized (Cremesti et al., 2001). Internalization itself may amplify Fas-induced apoptosis through further concentration of DISC components and activation of caspase-8. Receptor internalization is both actin and caspase dependent, indicating a feed -forward mechanism by which caspase-8 activity can enhance its own cleavage. The internalized receptor is then targeted to an endosomal pathway, since it colocalizes with the transferrin receptor (Algeciras-Schimnich et al., 2002). Internalization and capping occur more rapidly and prominently in Type I cells which correlates with more rapid DISC activation (Algeciras-Schimnich and E. Peter, 2003; Eramo et al., 2004; Siegel et al., 2004). Receptor endocytosis can occur either through the clathrin or caveolin mediated pathway. Fas endocytosis is exclusively dependent on the clathrin coated pits, specialized membrane vesicles formed with the help of AP-2 (adaptor proteins) and surface dynamin resulting in cytoplasmic clathrin coated vesicles. These are targeted to the endosomes and give rise to the early endosome. RNAi mediated-knockdown of endogenous clathrin heavy chain or adaptor protein complex molecules resulted in accumulation of receptor clusters on the surface, and inhibited recruitment of FADD and caspase-8 cleavage in the DISC, resulting in abrogation of apoptosis by crosslinked soluble Fas ligand. Similar observations were seen also in primary human activated T cells, indicating that endocytosis via clathrin is required for Fas-mediated apoptosis. Interestingly, blocking endocytosis and DISC formation resulted in activation of the MAP kinase (mitogen activated protein kinase) as well as NF-κB pathway, indicating that endocytosis mediates either a pro-apoptotic or proliferative outcome downstream of Fas signaling (Lee et al., 2006). A feed-forward loop in which caspase-8 activates endocytosis, which in turn promotes further receptor aggregation and caspase-8 cleavage can explain how caspases appear to be both 'upstream' and 'downstream' of receptor endocytosis.

4.1 Lipid raft microdomains as platforms for efficient Fas signaling

Lipid rafts are highly dynamic microdomains rich in sphingolipids and cholesterol, which facilitate the formation of many membrane-bound receptor signaling complexes. Lipid rafts are characterized by their insolubility in low ionic detergents, such as Triton X-100 or Brij98 (Munro, 2003; Simons and Toomre, 2000). They are less fluid than the traditional plasma membrane bilayer due to their lipid composition, allowing for isolation by low-density gradient centrifugation. Lipid raft distribution across the plasma membrane varies with cell type. For

instance, polarized epithelial cells preferentially have lipid rafts localized to their apical surface. In lymphocytes, rafts tend to distribute over the cell surface without any defined polarity (Garcia et al., 2003). Although the size of individual rafts also varies, the consensus estimates range from 20–100 nm in diameter, making them essentially submicroscopic in non-polarized cells when visualized by conventional microscopy. However, lipid rafts can coalesce and form much larger structures during signaling, as seen in the immunological synapse (Patra, 2008; Simons and Toomre, 2000).

Many recent studies have shown that lipid rafts play a critical role in immune cell signaling through the organization of signaling proteins, adaptor molecules and surface receptors at focal points on the cell membrane. It has been shown that lipid rafts act as signaling platforms for FcεRI (IgE) receptors, as well as the T cell and B cell receptor complexes, allowing the receptor to localize within close proximity to adaptor signaling components constitutively found in lipid microdomains (Cherukuri et al., 2001; Dykstra et al., 2003). Rafts are dynamic rather than static structures, allowing membrane proteins to flow in and out of them, thus changing the properties of the local protein milieu. This is the case with the FcεRI receptor on mast cells and basophils, where crosslinking of the FcεRI leads to translocation into rafts, where Lyn is a constituitive resident, and subsequent recruitment of Syk and PLCγ1(Dykstra et al., 2003). In the case of T cell receptor (TCR) activation, rafts concentrate the co-receptors CD4 and CD8 (Resh, 2006), as well as Src-family kinase Lck and many of the adaptor components needed for signaling, such as LAT (Kabouridis, 2006). Upon TCR engagement, many additional components of the signaling cascade are recruited to lipid rafts, such as PKCθ and ZAP-70, among others (Bi and Altman, 2001; Bi et al., 2001; Viola et al., 1999). CD4 partitions to lipid rafts via its interaction with Lck as well as its preferential S-palmitoylation (Resh, 2006). CD4 stimulation enhances signaling by the TCR by inducing aggregation of lipid rafts and formation of molecular assemblies at the site of the immunological synapse. During TCR activation, many of the cytoplasmic signaling proteins become detergent-insoluble, likely because of association with lipid rafts (Kabouridis, 2006; Viola et al., 1999). Studies have shown monomeric TCR complexes have weak raft affinity compared to receptor crosslinking, which increases raft-associated TCR molecules and the amount of TCR found in detergent-insoluble raft domains. Treatment of cells with methyl-β-cyclodextrin (MβCD) can dissociate these proteins from rafts and inactivates the signaling cascade (Janes et al., 1999; Montixi et al., 1998; Simons and Toomre, 2000). The cascade of interactions occurring at the site of TCR stimulation builds up the immunological synapse, with lipid raft microdomains critical to the stability and function of this complex and dynamic signaling assembly.

Membrane-anchored signaling kinases do not participate in TNF receptor family signal transduction, but the local membrane microenvironment can be just as important for efficiency of signaling. Work performed by many labs has shown very distinct functional outcomes for both TNFR1 and Fas signaling in regards to lipid rafts. TNFR1 translocated to lipid rafts very quickly after TNF treatment in HT1080 cells. Subsequent recruitment of TNFR signaling molecules RIP, TRADD and TRAF2 occurs very quickly: within 2 minutes of treatment, the TNF-induced signaling complex can be identified in lipid rafts, initiating NF-κB signaling through phophorylation of IκBα (Legler et al., 2003). Cholesterol chelation (and subsequent disruption of lipid rafts) via cyclodextrin treatment inhibited IκB phosphorylation and induced apoptosis. Similarly, blockade of signaling molecule recruitment to the lipid rafts via dipalmitoyl-phosphatidylethanolamine (DPPE) (Legler et al., 2001) also impaired IκBα phosphorylation and increased apoptosis (Legler et al., 2003).

Lipid rafts have recently emerged as important regulators of Fas-induced apoptosis through regulating the efficiency of early events in Fas signaling. We have found that in type I cells, which make a stronger DISC, a fraction of Fas resides in lipid raft constitutively, while in Type II cells the receptor seems to be excluded from rafts during the early signaling events (Muppidi

and Siegel, 2004). This pre-association of Fas with lipid rafts in Type I cells allows them to undergo apoptosis even in the presence of low-valency Fas stimuli, while Type II cells cannot. Disruption of the lipid rafts through cholesterol restores a requirement for Fas-crosslinking in Type I cells, while having no effect on Type II cells (Muppidi and Siegel, 2004). In mouse thymocytes, Fas recruitment and localization in the lipid rafts was critical for efficient DISC formation and subsequent cell death (Hueber et al., 2002). Formation of crosslinkable pre-associated receptor complexes through the N-terminal pre-ligand assembly domain (PLAD), is more efficient in cells in which Fas partitions into lipid rafts(Muppidi and Siegel, 2004; Siegel et al., 2000). Taken together, these studies suggest that lipid raft microdomains are necessary for efficient Fas signaling. Primary human $CD4^+$ T cell cultures generally respond to Fas stimuli in a Type II manner. Upon TCR engagement, however, Fas redistributes to lipid rafts and renders these cells sensitive to non-crosslinked anti-Fas antibodies or natively synthesized FasL(Muppidi and Siegel, 2004). As we will discuss in other sections, Rac-1 dependent cytoskeletal remodeling is required for this to occur.

Post-translational modification of Fas may also play a critical role in its function in lipid rafts. Modification of proteins with saturated acyl groups can result in lipid raft localization, such as Srk-family kinases (Resh, 2006). Also, proteins linked to saturated acyl chains, such as those directly acylated with two or more palmitate or a palmitate and myristate chain, can also be targeted to rafts. In fact, many membrane proteins localized to rafts carry post-translational acyl modifications, such as N-myristoylation and/or S-palmitoylation. S-palmitoylation is a reversible modification involving addition of a 16-carbon palmitate moiety to a cysteine residue via a thioester linkage, and can be readily cleaved by palmitoyl thioesterases. An interesting feature of S-palmitoylation is its dynamic nature: cycles of palmitoylation and depalmitoylation occur in a regulated fashion for many proteins, allowing for translocation in and out of lipid raft microdomains(Resh, 2006). Fas is palmitoylated at cysteine 199, just proximal to the cytoplasmic juxamembrane region (Chakrabandhu et al., 2007; Feig et al., 2007). Disruption of palmitoylation using competitive inhibitors or cleaving the thioester bond between palmitate and Fas receptor blocked Fas translocation to lipid rafts and inhibited formation of SDS-stable $CD95^{hi}$ aggregates associated with DISC formation (Feig et al., 2007). Mutation of cysteine 199 to prevent palmitoylation impaired DISC formation and inhibited Fas-induced cell death (Chakrabandhu et al., 2007; Feig et al., 2007). Palmitoylation of Fas is essential for raft association, and the apparent difference in raft-associated Fas observed in Type I-like vs. Type II-like cells could be due to differential ability of the receptor to be palmitoylated. Interestingly, the death domain of TNFR1 is required for targeting to rafts, as deletion of the domain prevented the receptor from targeting to lipid rafts and resulted in more uniform distribution across the plasma membrane (corCottin et al., 2002). It is quite evident that post-translational palmitoylation of Fas, allowing translocation to the lipid raft microdomains, is important for effective signaling. Whether there is a difference in palmitoylation states in Type I-like cells compared to Type II-like cells remains to be seen. The restricted lateral diffusion of membrane proteins found in the lipid microdomains, compared to the fluid plasma membrane, would favor oligimerization and formation of supra-clustering signaling complexes. Therefore, receptor pre-clustering via lipid raft targeting would act as a pre-signaling complex, whereby a Type I-like cell would respond to a low-level Fas stimulus more efficiently that a Type II-like cell. Fas is sequestered away from the rafts in Type II-like cells and less likely to oligimerize into larger signaling complexes from a low-level signal. They require a stronger stimulus, probably involving crosslinking of multiple Fas receptors, to bring them into closer proximity and subsequent DISC formation and signaling.

The divergent outcomes of receptor signaling of both Fas and TNFR1 reflect the role of lipid rafts in receptor signaling efficiency in the primary signaling complexes. The apparent contradiction in outcomes of lipid raft dissociation between Fas and TNFR1 become clearer when you take their respective signaling into account. TNFR1 signals through NF-κB via

receptor-associated complex I, while the death signal is transduced through complex II, which is not associated with the receptor (Micheau and Tschopp, 2003; Muppidi et al., 2004). In contrast, Fas signals through the receptor-associated DISC to trigger cell death. In one instance, cell death is promoted through dissolution of receptor-associated signaling (TNFR1), while in another apoptosis is abrogated due to inefficient formation of a signaling complex (Fas). As a result, lipid raft associated signaling has two very distinct outcomes in TNFR1 and Fas. Fas signaling should be seen as more of a dynamic process that involves more than just ligand binding to receptor, but also takes into account local microenvironment, subcellular localization, secondary modifications of Fas and global cell signaling pathways.

4.2 Cytoskeletal reorganization as a regulator of Fas-induced apoptosis

TCR induced lipid raft translocation of Fas is essential for the occurrence of Fas-mediated RICD in T cells. Our recent finding that Rac GTPases are critical for the death-inducing function of the TCR (Ramaswamy et al., 2007) implicates actin-based cytoskeletal remodeling in this process. Cytoskeletal remodeling was already known to play an important role in mediating sustained signaling as well as for strengthening and stabilizing T cell-APC contacts during initial T cell activation in the ‘immune synapse’ (IS). The IS an area of dynamic contact between a T cell and its activating APC and its formation requires cytoskeletal rearrangements that occur as a consequence of initial signaling events. The IS results in sequestration of many signaling complexes with in lipid raft moieties of the membrane with rearrangement of the actin cytoskeleton is critical for this event. Actin cytoskeletal rearrangement is dependent on Arp2/3 proteins, which are responsible for nucleating the F-actin and mediating T cell shape change to facilitate IS formation. Cytoskeletal remodeling also plays a role in negatively regulating T cell activation by terminating IS formation by the end of 6–8 hours (Dustin and Chan, 2000; Huang and Wange, 2004).

Rac GTPases are one of a number of subfamilies of small G proteins that function in cytoskeletal remodeling, and act in parallel with Rho and CDC42 GTPases to alter dynamics in T cells. We have found a unique role for Rac GTPases in TCR signaling to sensitize cells to die via Fas (Ramaswamy et al., 2007). Upon TCR ligation, the guanine-nucleotide exchange factor (GEF) Vav-1, activates Rac to mediate actin-based cytoskeletal changes (Tybulewicz, 2005). Vav1 mediated Rac activation also leads to sustained lipid raft clustering in T cells (Villalba et al., 2001). In cell lines as well as primary human cells, Rac protein knockdown blocks TCR induced Fas apoptosis. Unlike the more widespread effects of Vav1, Rac1-mediated sensitization to Fas is independent of early TCR signaling events, but appears linked to Rac1 dependent cytoskeletal reorganization, likely through dephosphorylation of The Ezrin Radixin Moesin (ERM) proteins (Ramaswamy et al., 2007). ERM family of proteins link membrane proteins to the underlying cytoskeleton and modulate various biological functions such as adhesion, motility, polarization and signaling in lymphocytes. Dephosphorylation of ERM proteins induces a conformational change that dissociates the N-terminal ERM domain from the cytoplasmic tails of membrane proteins with which they interact (Bretscher et al., 2002). Receptor triggered ERM dephosphorylation, in many instances, is a Rac1 dependent process (Nijhara et al., 2004) and during T cell activation by antigen presenting cells, Rac1 mediated ERM dephosphorylation results in a generalized increase in T cell deformability. This allows for more efficient T cell-APC conjugation and potentiates T cell activation (Faure et al., 2004). Since Fas has been found linked to the cytoskeleton through ezrin (Parlato et al., 2000), ERM dephosphorylation may allow Fas to dissociate from the cytoskeleton and migrate to lipid raft microdomains where Fas-induced apoptosis signaling is potentiated (Muppidi and Siegel, 2004). Rac1 has also been shown to promote repositioning of the centrosome toward the area of contact with target cells, which is important for efficient CTL and NK-cell mediated cytotoxicity (Billadeau et al., 1998; Gomez et al., 2007; Stinchcombe et al., 2006). Whether

or not this form of cytoskeletal rearrangement could also be important in Fas-FasL mediated apoptosis is not known.

Since Rac GTPases mediate signaling by many receptors on activated T cells, other receptors alter the threshold for Fas-induced apoptosis through this mechanism. CD44 is one such surface molecule that also activates Rac1, and enhances Fas-induced apoptosis. T cells from CD44-deficient mice are resistant to RICD and have increased pathology in a ConA-induced hepatitis model, known to be controlled by Fas elimination of activated T cells (Chen et al., 2001). Thus, CD44 acts as an endogenous sensitizer of Fas. Deliberate activation of receptors such as CD44 that activate Rac1 or low-level TCR stimulation may form a basis for curtailing autoimmune effector T cells by sensitizing them to Fas-induced apoptosis (Ramaswamy et al., 2007).

4.3 Intracellular Regulation of the Fas-induced death signal

In Type II cells, DISC formation is inefficient and activation of caspase-8 is delayed, resulting in insufficient initiator caspases to trigger apoptosis without amplification through the mitochondrial death pathway. Activation of the mitochondrial pathway is induced by intrinsic DNA damage signals or growth factor deprivation that promotes release of cytochrome C present in the intermembrane space of the outer mitochondrial membrane. The loss of mitochondrial outer membrane potential (MOMP) and cytochrome C release potentiates formation of the apoptosome complex, which is equivalent to the DISC and consists of cytochrome C complexed to APAF-1 (apoptotic protease activating factor-1). SMAC/Diablo, another molecule released from mitochondria, inactivates inhibitory proteins bound to caspases (Verhagen and Vaux, 2002). Activated APAF-1, cytochrome-c and caspase -9 form a complex (the ‘apoptosome’) that very efficiently activates the effector caspases, caspase 3 and 7 (Riedl and Salvesen, 2007). Apoptosis-inducing receptors such as Fas can activate the intrinsic cell death pathway via a member of the Bcl-2 protein family called Bid. Bid, a BH3 (Bcl-2 homology domain 3) only pro-apoptotic BCL-2 family member, is a substrate of active caspase-8, which cleaves it into a truncated form, designated tBid (Li et al., 1998; Luo et al., 1998). tBid translocates to mitochondria and allows multimerization of pro-apoptotic Bcl2 family proteins, BAK/BAX that induce loss of MOMP and cytochrome C release (Youle and Strasser, 2008). The limited amounts of caspase-8 generated in the Type II DISC is sufficient to cleave Bid, but not effector caspase-3. Hence, Type II cells are very dependent on the mitochondrial amplification via Bid and are susceptible to Bcl-2 inhibition when stimulated with Fas. Bid knockout mice have no developmental phenotype, but exhibit a dramatic resistance to the hepatic necrosis induced by anti-Fas antibody treatment, most likely due to hepatocyte resistance to Fas-induced apoptosis. However, resistance to Fas-induced apoptosis was not seen in lymphocytes, possibly because they are mostly Type I and do not require a mitochondrial amplification loop to undergo cell death (Yin et al., 1999).

In T lymphocytes the intrinsic pathway of apoptosis acts as a negative regulator in cells undergoing various forms of stress, including lack of growth factors or cytokines. Bcl-2 blocks oligomerization of Bak/Bax, thus, preventing loss of MOMP. Mice lacking Bcl-2 have severe immune defects, especially in T cell activation, and also polycystic kidney disease, whereas mice with transgenic over expression of Bcl-2 have lymphadenopathy and abnormal survival of peripheral T cells. Bim, a BH3 only Bcl-2 family member is a cytosolic protein that acts on other Bcl-2 family members to induce MOMP. Bim knock-out mice show severe lymphadenopathy and kidney disease due to autoimmune attack (Bouillet et al., 1999). In Bcl-2 deficient mice, parallel knock out of even one allele of Bim rescues many of the proliferation defects as well as the severe kidney disease, indicating that most of the Bim pro-apoptotic function is counteracted by Bcl-2. Bim knockout mice also have severe defects in thymic negative selection. T cells expressing a Bcl-2 transgene or lacking Bim, but not Fas-deficient T cells, had severe defects in undergoing apoptosis when exposed to cytokine withdrawal and

acute antigenic stimulation. However, in a more chronic stimulation model, Fas-deficient T cells were apoptosis resistant, indicating that repeated TCR stimulation as occurs in chronic infections as well as with endogenous antigens elicits the Fas pathway (Hildeman et al., 2002; Van Parijs et al., 1998). This notion of separate contributions of the intrinsic and extrinsic cell death pathways to immune homeostasis and self tolerance was born out by studies of mice doubly deficient in Bim and Fas(Hughes et al., 2008; Hutcheson et al., 2008; Weant et al., 2008). These animals develop massive splenomegaly, and lymphadenopathy at due to increases in not only T cell, but also B cell and DC numbers. Acute viral infection with HSV-1 resulted in accumulation of virus specific T cells only in Bim deficient mice, with no synergistic increase in the Bim/Fas double deficient. Interestingly, in a model of chronic infection, there is cooperativity between Fas and Bim, with synergistic accumulation virus-specific T cells (Hughes et al., 2008; Hutcheson et al., 2008; Weant et al., 2008). These findings indicate that Fas death receptor signaling intersects with the intrinsic cell death machinery at two levels, one at the membrane proximal signaling through Bid and secondly in a more indirect manner with Bim. Different models of chronic infection and autoimmunity likely involve more or less cross-talk between these two pathways.

5. Non-apoptotic signaling through Fas

Members of the TNF-receptor superfamily induce apoptosis, inflammation and proliferation. Unlike TNFR1, which induces either apoptosis or inflammation, Fas is considered a prototypic death receptor, since signaling induces apoptosis in most circumstances. However, Fas can also induce non-apoptotic signaling resulting in cell proliferation. Anti-Fas antibodies are known to costimulate T cell proliferation and secretion of cytokines such as IL-2 and IFN–γ, in the presence of minimal TCR stimulation (Alderson et al., 1993). This effect, blocked by Fas antagonists and synergistically increased by addition of exogenous FasL, appears to be caspase dependent, since caspase inhibitors block T cell proliferation costimulated through Fas (Kennedy et al., 1999). Costimulation through Fas has most often been elicited in vitro with anti-Fas antibodies or recombinant FasL. In vivo, however, T cell activation defects or lymphopenia was not observed in Fas or FasL deficient mice nor in ALPS patients, suggesting that physiologically, Fas-FasL functions mostly as an inducer of apoptosis.

Another nonapoptotic axis of the Fas signaling is mediated via the NF-κB pathway. This pathway is dependent on FADD/caspase-8 and independent of mitochondrial activation, since caspase-8 inhibitor, cFLIP, but not Bcl-2 overexpression in Type II cells, inhibits NF-κB activation (Imamura et al., 2004; Miwa et al., 1998). Interestingly, varying signaling thresholds for the apoptotic pathway appears to determine activation of alternate Fas signaling pathways. In ALPS patients with DD mutations, NF-κB signaling is activated in spite of the dominant interference caused by mutant receptors (Legembre et al., 2004). Biochemically, internalization of the Fas receptor induces the apoptotic pathway, however non-internalized receptors results in activation of NF-κB (Lee et al., 2006). Furthermore, in Fas resistant tumor cells, induction of NF-κB as well as activation of MAPK pathways increases motility and invasiveness specifically in response to Fas crosslinking, but not to TNF-α or TRAIL treatment (Barnhart et al., 2004). Therefore, Fas, similar to the dual signaler TNFR1, induces inflammatory outcomes resulting in tumor formation, dependent on different physiological conditions as well as receptor and ligand signaling thresholds. However, these alternate functions of Fas, dependent on the presence of intact downstream DISC components, may reflect nonapoptotic functions of FADD or caspase-8, two important mediators of developmental regulation as well as lymphoproliferation.

Remarkably, FADD, caspase-8 and c-FLIP, essential components of the Fas apoptosis-inducing complex, all appear to be required for embryonic development and early events in T cell signaling that lead to NF-κB activation. FADD is essential for embryonic development,

since its deletion results in embryonic lethality. This is in contrast to a lack of lethality in mice lacking receptors such as Fas or TRAIL-R that use FADD as an adapter. In order to study the role of FADD in immune system development, $FADD^{-/-}$ embryonic stem cells were reconstituted into $RAG1^{-/-}$ mice. B cell development and T cell activation required FADD function, since these mice had no B cells and very few peripheral T cells. The peripheral $FADD^{-/-}$ T cells however were totally resistant to Fas-induced apoptosis, indicating that proliferative function of FADD in the thymus is separate from the apoptosis/adaptor function in peripheral T cells (Zhang et al., 1998). However, conditional knockout studies indicate that FADD is not essential for thymic development, but is important in peripheral T cell activation and proliferation as well as in Fas-induced apoptosis (Zhang et al., 2005). The proliferative function of FADD depends on the phosphorylation of a serine residue, which is essential for cell cycle progression but not apoptosis induction (Park et al., 2005). Deficiency of caspase-8 as well as another DISC component cFLIP, results in embryonic lethality, and similar to the FADD deficient mice, T cell-specific ablation of c-FLIP or caspase-8 result in similar defects in T cell activation, proliferation and post-activation survival. (Siegel, 2006). More recently, caspase-8 was found to activate TCR induced NF-κB activation mediated through the BCM complex (Bcl-10/CARMA1/MALT1) by targeting IKK to the complex and stabilizing NF-κB activation (Su et al., 2005). A Fas-mediated caspase-8 independent pathway of apoptosis or necrosis also occurs in some primary human as well as murine T cells, where, presence of caspase inhibitor, zVAD did not prevent cell death. However, this caspase independent death was found to be dependent on FADD. It was also found that the necrotic cell death was dependent on the receptor interacting protein (RIP), which interacted with Fas in a FADD dependent manner(Kataoka et al., 2000) .

6. Differential function of Fas in $CD4^+$ T cell subsets

Upon encountering antigen, a naïve CD4 T cell undergoes a series of events beginning with activation by the antigen presenting APC followed by an enormous clonal expansion and then attrition of the response by cytokine withdrawal and finally, generation of memory cells. Among the different checkpoints that exist in controlling these events, Fas-mediated apoptosis occurs usually in cells that overcome the clonal contraction phase. However, not all T cell subset homeostasis is governed by Fas-induced apoptosis and recent studies indicate that memory T cells have intrinsic sensitivity to Fas apoptosis. Memory T cells, formed from the repertoire of antigen specific cells, are long-lived and characterized by quick induction and secretion of effector cytokines. Differential expression of the chemokine receptor CCR7 and CD27, a TNFRSF member, delineates functional memory subtypes into central and effector memory cells (Sallusto et al., 2000), with central memory cells retaining the ability to recirculate to lymph node, and effector cells residing predominantly in target tissues and more efficiently producing cytokines. There have been a number of studies recently indicating that deregulated memory T cells may be important in driving autoimmune pathology. In Fas-deficient *lpr* mice, memory T cells accumulate abnormally and a similar phenomenon has been observed in patients with systemic lupus erythematosus (SLE), a polygenic autoimmune disease (Fritsch et al., 2006). Furthemore, T cells from SLE patients were found to be resistant to TCR and Fas-mediated apoptosis (Kovacs et al., 1996). In normal donors, the effector, but not central memory cells are intrinsically sensitive to apoptosis and have upregulated levels of FasL and Bim. One mechanism of enhanced survival of central memory cells was ascribed to activation effects of the AKT kinase signaling pathway, which leads to inactivation of FOXO3a, a fork-head family transcription factor that upregulates both FasL and Bim expression (Riou et al., 2007). Since effector memory cells are potent effector cytokine producers with tissue homing abilities and a high turnover rate, any dysregulation in their numbers could lead to pathological consequences. Regulation of Fas-induced apoptosis, thereby, serves to counterbalance autoreactive T cell escape of these terminally differentiated cells.

Cytokine secretion patterns define lineages of peripheral $CD4^+$ T cells. Classically, $CD4^+$ T cells were divided into two lineages, Th1 or Th2, depending on upregulation of specific transcription factors, which induced lineage specific cytokines. Due to the diverse cytokine profiles, the lineages differ in their functionality, with Th1 effecting cell-mediated immunity and Th2 cells providing humoral defense (Abbas et al., 1996). Differential Fas sensitivity was seen in human T cell clones, where, Th1, but not Th2 clones were susceptible to TCR induced RICD, mostly regulated at the transcriptional level of FasL (Ramsdell et al., 1994a).

Interferon-γ (IFN-γ), the principal cytokine secreted by Th1 T cells, also regulates the sensitivity to Fas-mediated apoptosis, since both inhibition of IFN-γ secretion by blocking antibodies or $IFN\gamma^{-/-}$ T cells are resistant to RICD (Liu and Janeway, 1990; Refaeli et al., 2002). Another important CD4 T cell subset that is regulated by Fas pathway is the regulatory T cell lineage (Treg). This subset is necessary for maintaining peripheral tolerance through immune suppression of other effector cell types. It has been found that Tregs exhibit an activated effector phenotype, being CD25 high and CD45RO positive. Tregs are susceptible to Fas-induced apotposis ex-vivo (Fritzsching et al., 2005). Recently, however, a small subset of Fas resistant $CD45RA^+$ 'naïve' Treg were identified in cord blood (Fritzsching et al., 2006). The susceptibility of Treg to Fas-induced apoptosis may have more to do with their memory-like phenotype when commonly isolated from adults. Recent research has identified new $CD4^+$ subsets secreting IL-17, IL-21 and IL-22 (Laurence and O'Shea, 2007; Laurence et al., 2008; Tato et al., 2006). The susceptibility of these subsets to Fas- mediated apoptosis is currently being investigated.

7. Therapeutic use of Fas-FasL interactions in cancer and immunological diseases

The potential of Fas to permanently alter cell fate and eliminate cells through apoptosis has sparked efforts to harness Fas to eliminate pathogenic T and B cells or Fas-positive tumor cells in a number of clinical settings. Although anti-Fas antibodies induced acute hepatic necrosis, systemic dosing of FasL or antibodies not cross linked by Fc receptors results in reduced hepatotoxicity, and it may be possible to use this approach to selectively eliminate Fas-sensitive tumors or autoreactive lymphocytes. This approach is limited by the fact that in many cell types, both from tumors and normal tissues, Fas expression does not correlate well with sensitivity to Fas-induced apoptosis. The well-known potential of tumor cell lines to become resistant to Fas-induced apoptosis after prolonged culture due to loss of Fas or components in the Fas signaling pathway has been helpful in isolating mutants lacking Fas signaling components, but also raises concerns about the development of resistance to anti-Fas therapy if it were attempted in cancers. Stimulation of Fas on tumor cell lines resistant to Fas-induced apoptosis induces characteristics of motility and invasiveness (Barnhart et al., 2004), raising further concerns about unexpected effects of Fas stimulation in cancer.

Since endogenous FasL expression on selected tissues can induce immune tolerance and elimination of reactive T cells (Stuart et al., 1997), genetic delivery of FasL to grafts in a transplantation setting was also hoped to prolong graft survival through elimination of alloreactive lymphocytes. Despite some early reports of success with this approach (Lau et al., 1996), the vast majority of experiments in which FasL is ectopically expressed on graft tissue by retroviral transduction or transgenes under strong promoters results in hyperacute rejection with a predominantly neutrophilic infiltrate in the graft (Kang et al., 1997; Takeuchi et al., 1999). A direct chemotactic effect of soluble FasL on neutrophils has been reported (Ottonello et al., 1999), but in vivo, membrane-bound or vesicular FasL induces rapid apoptosis of macrophages, which then secrete pro-inflammatory cytokines and chemotactic factors, of which IL-1 and IL-17 appear to be important in neutrophil recruitment (Hohlbaum et al., 2001; Umemura et al., 2004). Whether or not this pro-inflammatory effect of ectopic expression

of FasL can be overcome to deliver a purely apoptotic signal to infiltrating lymphocytes in the setting of graft rejection is not known. More promising are studies in which FasL has been implicated as being more potent in mediating graft-vs. host disease than beneficial graft-vs. leukemia effects (Jiang et al., 2001; Schmaltz et al., 2001). These studies suggest that blocking Fas-FasL interactions could potentially ameliorate graft-vs. host disease in bone marrow transplants while leaving intact the beneficial graft-vs. leukemia activity by donor cells. Interestingly, as discussed above, effector memory T cells, which are very susceptible to TCR and Fas-induced apoptosis, have the ability in vivo to selectively mediate graft-vs. leukemia effects (Zheng et al., 2008). This raises the interesting possibility that alloantigen-induced Fas-mediated apoptosis in effector memory T cells may cull the immune repertoire of pathogenic graft-vs. host disease causing T cells while leaving intact graft-vs. leukemia effects, which are predominantly mediated by mechanisms other than Fas-FasL interactions.

Although the barriers to using direct ligation of Fas in immunotherapy in autoimmune disease remain high, interventions to eliminate autoreactive lymphocytes by enhancing apoptosis via Fas-FasL interactions may still prove effective. In diseases in which autoantigens are known, chronic stimulation of the TCR with antigen has been shown to mediate Fas-induced apoptosis and ameliorate animal models of antigen-specific autoimmune diseases (Critchfield et al., 1994). As understanding of the pathways that regulate Fas-induced apoptosis grows, enhancing sensitivity to Fas-mediated apoptosis through stimulation of receptors that activate the ‘competancy to die’ signal or otherwise enhance FasL sensitivity remains a worthy therapeutic goal.

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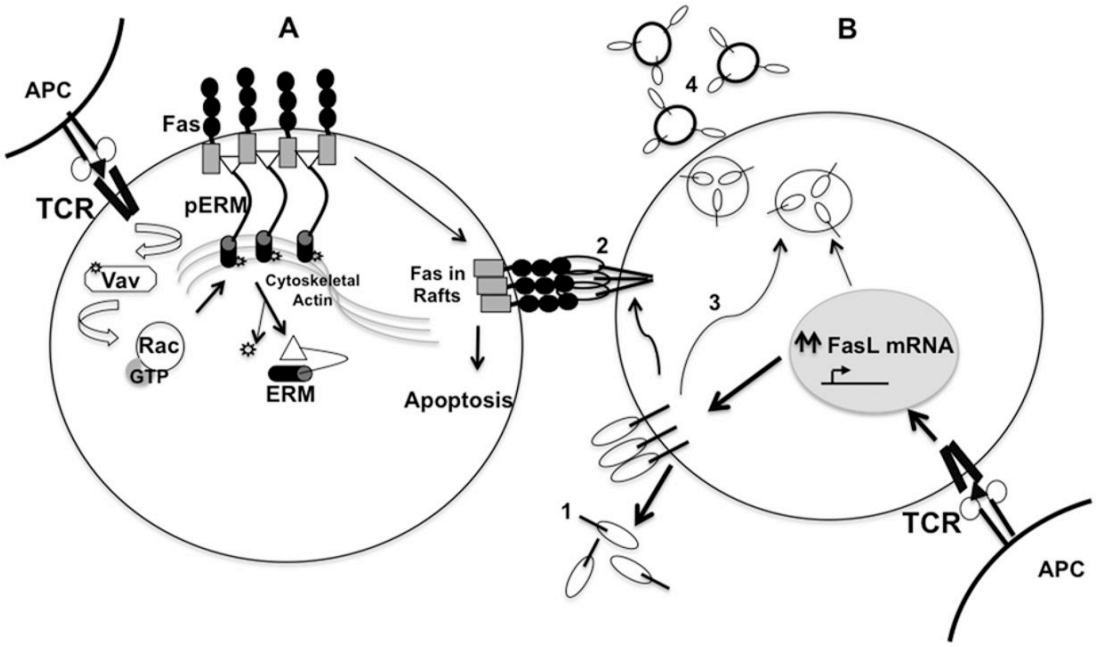

Figure 1. Schematic of regulatory checkpoints and trafficking of Fas and FasL

A. Fas Receptor- In most activated T cells, surface Fas is not associated with lipid rafts or preassociated, but is linked in monomeric form to the cytoskletal actin by phosphorylated Ezrin-Radixin-Moesin linker proteins (pERM). TCR stimulation by antigen/MHC complexes on APC activates Vav GEF and Rac GTPases, which in turn activate phosphatases. Phosphatase activity may inactivate pERM, releasing Fas to translocate to lipid rafts, where apoptosis signaling is more efficient.

B. FasL regulation- TCR stimulation in T cells also induces transcription of FasL in the nucleus, resulting in upregulation of the protein on the surface. Surface expressed FasL is either cleaved by the ADAM10 proteases to release non-apoptosis inducing soluble FasL (1) or forms a membrane bound stable trimer (2) that binds to Fas expressing cells to induce effective signaling. Some can also be released from the cells in microvesicles (4), after endocytosis (3) from the plasma membrane or from newly synthesized FasL.