Fas (CD95/Apo-1) ligand regulation in T cell homeostasis, cell-mediated cytotoxicity and immune pathology

Thomas Brunner*, Christoph Wasem, Ralph Torgler,
Igor Cima, Sabine Jakob, Nadia Corazza

*Division of Immunopathology, Institute of Pathology, University of Bern,
Murtenstrasse 31, P.O. Box 62, 3010 Bern, Switzerland*

Abstract

Members of the tumor necrosis factor (TNF) superfamily are crucially involved in the regulation of T cell activation, homeostasis and cytotoxicity. In particular, Fas ligand (FasL), expressed by activated T lymphocytes, induces cell-mediated cytotoxicity and may also be responsible for apoptotic suicide. Tight regulation of this death-inducing ligand is a prerequisite for proper immune defense and homeostasis. In this review, we will discuss various aspects of FasL regulation in cell-mediated cytotoxicity, immune homeostasis and the immunopathology of diseases.

Keywords: Apoptosis; Death receptors; Disease; Lymphocyte; Transcription

1. Introduction

In complex multicellular organisms, cell growth and cell death has to be tightly regulated in order to avoid serious damage due to uncontrolled growth or loss of vital cellular functions. Similarly, it is fundamental that cell death can be induced in single cells to eliminate individual cells with a defined function or defect, and to avoid extensive death of bystander cells. Thus, induction of cell death must be an active and well-controlled process. Cell death induction via *apoptosis* appears to fulfill all of these criteria. Apoptosis can be targeted to individual cells and proceeds in a cell-autonomous manner. Apoptosis-inducing signals activate the cell's own suicide machinery, which executes the cell's demise in an active and energy-dependent fashion. Most importantly since energy production is maintained until late stages of apoptotic cell death, plasma membranes remain intact, and thus, the release of pro-inflammatory cytoplasmic components, as it occurs during accidental cell death like necrosis, is prevented. In addition, changes in the plasma membrane, e.g. phosphatidylserine flip, target the apoptotic cell to rapid and efficient removal by phagocytic cells. Therefore, under normal circumstances, i.e. physiological rates of cell death, apoptosis

occurs in a silent non-inflammatory manner (reviewed in [1,2]).

The cell's apoptosis machinery is initiated by a variety of stimuli. At least in vitro, at certain concentrations most chemicals can cause sufficient cellular stress to initiate apoptotic cell death, whereas a further increase of the dose often results in necrosis. While individual triggers may lead to the same result, i.e. apoptotic death of the cell, the pathways initiated by these triggers and leading to the cell's demise are often distinct. Most physical and biochemical triggers are sensed within the cell, and thus initiate the apoptosis signaling cascade directly inside the cell. Other stimuli use cell surface receptors for apoptosis induction. Thus, apoptotic events that are initiated inside the cell proceed via the so-called *intrinsic pathway*, whereas the so-called *extrinsic pathway* is activated by extracellular signals via specific cell surface receptors (reviewed in [1]). For example, UV-irradiation, DNA damage, or steroids usually activate the intrinsic pathway, whereas death receptors of the tumor necrosis factor (TNF) family, as will be further discussed below, cause death via the extrinsic pathway. In general, a given stimulus initiates either one or the other pathway, however, a certain cross-talk between these pathways has also been observed, i.e. that apoptosis signals initiated within the cell may also cause signaling via the extrinsic pathway. Undoubtedly, both signaling pathways are of major importance for the control of immune cells by apoptotic cell death. In this review, however, we will limit our

* Corresponding author.

E-mail address: tbrunner@pathology.unibe.ch (T. Brunner).

discussion on the extrinsic pathway initiated by Fas ligand (FasL).

2. Fas ligand, a death factor

Years before the cloning of FasL, it was noticed that activated CD4⁺ T cells exert a cytolytic activity, which is Ca²⁺-independent and cannot be attributed to perforin and granzymes. Further studies by Golstein and co-workers demonstrated that this cytolytic activity is initiated by the ligand of the recently cloned apoptosis-inducing receptor Fas (also called Apo-1 or CD95), since activated CD4⁺ T cells killed only Fas-expressing target cells but not Fas-negative cells [3]. Soon after, Nagata and co-workers cloned the rat, mouse and human gene for FasL and demonstrated that FasL induces apoptosis via interaction with the Fas receptor [4–6].

FasL belongs to the large family of TNF-like molecules, which includes other apoptosis-inducing ligands like TNF α and TRAIL (TNF-related apoptosis-inducing ligand), as well as non-apoptosis-inducing molecules, such as the ligands for CD27, CD30, CD40, etc. (reviewed in [7]). FasL is expressed as a 40 kDa type II transmembrane molecule, with strong sequence homology to other TNF family members in the C-terminal extracellular receptor-binding domains. Binding of FasL to its receptor causes the rapid assembly of a multimeric protein complex (the DISC, death-inducing signaling complex) crucial for the activation of the apoptosis signaling cascade [8]. While most TNF receptor-like molecules require trimerization for the recruitment of signaling molecules and activation of the signaling cascade, the Fas receptor appears to pre-exist in a trimeric (inactive) form [9]. It is thus believed that FasL binding to its receptor causes a higher-order aggregation of the receptor molecules, and thus, the recruitment of signaling molecules. Immediately after activation, the adapter molecule FADD (Fas-associated death domain) binds to the so-called death domain in the cytoplasmic tail of Fas. Binding of FADD causes the recruitment of the inactive pro-form of caspase 8, a member of the family of cysteine proteases, intimately involved in apoptosis signaling. Self-activation of caspase 8 leads then to the initiation of a caspase cascade, eventually resulting in the apoptotic death of the cell. The signaling events involved in Fas-induced apoptosis have been extensively reviewed elsewhere (reviewed in [10–12]) and will thus not be further discussed in this review.

Although FasL expression was initially thought to be restricted to activated T cells and NK cells [4,13], a plethora of publications has demonstrated FasL expression in many different cell types, including non-hematopoietic tissue cells (reviewed in [12,14]). The initial observation of FasL activity in activated T cells suggested that FasL may represent a cytotoxic T cell effector mechanism. However, its ubiquity expression under a variety of physiological and pathological conditions indicates a multifunctional role for this molecule. Consequently, FasL has been implicated in im-

mune privilege [15–17], tumor escape [18–20], inflammation [21,22], tissue cell turnover [23], tolerance induction [24,25], hematopoiesis [26], and many other events. While the importance of FasL in these processes is not always well understood, its role and regulation in T cell homeostasis and effector functions is much better characterized.

3. FasL in T cells: a story about killers and victims

3.1. The role of FasL in immune homeostasis

In recent years, gene-deficient mice have become an important research tool to investigate the role of a given molecule under physiological and disease conditions. In the case of FasL, there was no requirement to generate such a gene-deficient mouse since such a phenotype occurs naturally. Generalized lymphoproliferative disorders (*gld*) is a natural mutant mouse strain which displays strong lymphoproliferative disorders and autoimmune diseases with increased age. When FasL was cloned, sequence analysis revealed a single point mutation in the *fasL* gene of *gld* mice, causing the generation of a mutant FasL protein, which is unable to bind and trigger the Fas receptor [6,27,28]. Similarly, a natural mutant mouse strain was identified with aberrant Fas expression, the so-called lymphoproliferative disorder (*lpr*) mice, due to a retroviral transposon in the *fas* gene [27,29]. The strong autoimmune phenotype of the *gld* and *lpr* mice strongly suggested defects in central deletion (thymic negative selection) and/or peripheral tolerance induction. While the later has been supported by many different studies, the role of FasL in thymic negative selection is less clear. Thymocytes, particularly at the double-positive stage (CD4⁺CD8⁺), are sensitive to Fas ligation [30], however, *gld* mice show a normal distribution of CD4⁺ and CD8⁺ thymocyte subsets. In addition, experimental studies using T cell receptor (TCR) transgenic (tg) mice have found no differences in antigen-induced negative selection in *gld* versus wild-type mice [31]. Sprent and co-workers, however, observed normal negative selection at low antigen concentrations, but reduced negative selection when antigen was administered at high concentrations to *lpr* mice [32]. Thus, depending on antigen concentrations different effector molecules may be involved in central deletion. While this is an experimental observation, the question remains of how much self-antigen is sufficient to drive negative selection and what are physiological self-antigen concentrations.

Mature T cells from *lpr* mice are not only resistant to Fas-induced apoptosis but also to antigen-driven apoptosis induction in vitro and in vivo. In vivo activation of peripheral T cells causes clonal expansion, but also the induction of apoptotic cell death to eliminate undesired antigen-specific T cells at the end of an immune response (peripheral deletion). Similarly, in vitro restimulation of previously activated T cells induces apoptosis by *activation-induced cell death* (AICD). Both, *lpr* and *gld* T cells, show defective peripheral

deletion and AICD, indicating a major role for Fas/FasL interaction in T cell homeostasis (reviewed in [27]). In 1995, several groups have independently described the underlying mechanism of AICD in T cells [33–36]. Upon restimulation of previously activated T cells FasL expression is rapidly induced and subsequent interaction with the Fas receptor induces cell death. Surprisingly, at least in vitro this Fas/FasL-dependent antigen-driven suicide can even occur in a cell-autonomous manner [33,34]. The mechanism of how FasL can activate the Fas receptor on the same cell is currently not very clear. FasL, like most other TNF members, can be cleaved by metalloproteases releasing a soluble molecule. It is tempting to speculate that released soluble FasL may bind back to membrane-bound Fas and thereby induce apoptosis. Surprisingly, however, cleaved soluble FasL is an inefficient apoptosis trigger, presumably due to the lack of membrane retention, but may rather act as a competitor of Fas/FasL interaction [37–39]. It is therefore more likely that membrane-bound FasL may be released from the cell surface of activated T cells on microvesicles, which then engages the Fas receptor in a cell-autonomous manner [40] (Fig. 1).

Besides cell-autonomous suicide, FasL can also kill in a fratricide manner, i.e. by inducing the death of neighboring T cells. It might thus appear obvious that in vivo T cells are similarly killed in a crowd of activated FasL-expressing T cells or commit cell-autonomous suicide. Yet, this does not seem to be the case. Recent work by Green and co-workers has analyzed the requirements for T cell-expressed FasL in antigen-driven peripheral deletion using bone marrow chimeras ([41,42]; Pinkoski and Green, personal communication). Surprisingly, wild-type T cells failed to undergo peripheral deletion in *gld* recipients, whereas *gld* T cells were normally depleted in wild-type recipients. This strongly supports the idea that tissue cell-expressed FasL is responsible for the induction of apoptosis in activated T cells. Interestingly, these authors have found a strong induction of FasL expression in the intestinal epithelium, which was dependent on $\text{TNF}\alpha$ released by activated T cells [42]. These findings indicate that the intestinal mucosa may represent a tissue involved in the elimination of activated T cells, almost like an immunological graveyard. *gld* and *lpr* mice, however, do not only show defective peripheral deletion of activated T cells, but also an

Fig. 1. Multiple roles of FasL in T cell-mediated cytotoxicity and T cell homeostasis. Upon T cell receptor (TCR) activation, FasL is transcribed and expressed on the cell surface, where it can induce killing of Fas⁺ target cells or activated B cells (cytotoxicity). T cell-expressed FasL can also induce apoptosis in neighboring activated T cells (fratricide killing). Alternatively, FasL is released from the cell surface on microvesicles and can induce apoptosis in a cell-autonomous manner. Upon stimulation by inflammatory cytokines (e.g. $\text{TNF}\alpha$), FasL expression is induced on tissue cells and can cause apoptosis in activated T cells. BCR, B cell receptor; GrzB, granzyme B; MHC, major histocompatibility complex; TCR, T cell receptor.

accumulation of autoreactive B cells and high titers of autoantibodies with increasing age. Thus, T cell-expressed FasL may be still crucial in the regulation of autoreactive B cells through the induction of Fas-induced apoptosis [43] (Fig. 1).

The importance of FasL in the regulation of immune homeostasis and in the control of autoreactive T and B cells is also supported by the phenotype of human patients with mutations in FasL, Fas or Fas-associated signaling molecules like FADD. Many of these patients with autoimmune lymphoproliferative syndrome (ALPS) suffer from lymphadenopathy, splenomegaly, and various autoimmune diseases (reviewed in [44,45]). Similar to T cells from ALPS patients, T cells from FADD-deficient mice show defective Fas-induced apoptosis, further demonstrating its crucial role as an adapter molecule in the Fas-induced death signaling pathway [46]. Surprisingly, however, FADD-deficient T cells also exhibit defective proliferative responses upon TCR stimulation [47]. This rises the question whether death receptors, and in particular Fas, may have also other functions than apoptosis induction. This idea is supported by various observations that Fas ligation in resting T cells causes co-stimulation [48–51]. This activation pathway initiated by Fas ligation is thought to proceed via cFLIP, initially described as a caspase 8 homologue and Fas inhibitor (reviewed in [52]). In addition to its inhibitory activity it may divert the Fas signal towards activation of NF κ B and the MAP kinase pathway [53,54]. However, the *in vivo* relevance of Fas-mediated co-stimulation has yet to be demonstrated.

4. The role of FasL in cell-mediated cytotoxicity

Since FasL/Fas interactions are involved in induction of T cell apoptosis, it appears obvious that FasL is also used by activated T cells as a cytotoxic effector mechanism. FasL-mediated cytotoxicity represents the most important killing mechanism of CD4⁺ T cells, whereas CD8⁺ T cells may eliminate their targets by perforin/granzyme B as well as FasL. Although the mechanisms of killing are different, perforin/granzyme B- and FasL-induced cytotoxicity have many things in common. FasL initiates apoptosis induction through binding to its receptor and proximal caspase activation. In contrast, the serine protease granzyme B has to enter the target cell with the help of perforin to exert its cytotoxic activity. Nonetheless, both mechanisms kill the target cell by specific activation of caspases. Similar to Fas, granzyme B directly activates caspases (caspases 3 and 8) through proteolytic activation of the pro-enzymes. In addition, recent reports have shown that granzyme B also utilizes the mitochondrial pathway for amplification of the death signal by specific cleavage of the pro-apoptotic Bcl-2 member Bid and induction of cytochrome *c* release (reviewed in [55,56]).

Another common feature of perforin/granzyme B- and FasL-mediated cytotoxicity is the fact that primed T cells kill

more efficient than resting T cells. Resting CD8⁺ T cells do not express perforin or granzyme B, but synthesize and store these proteins in their cytolytic granules upon primary stimulation, and release its content after restimulation of these primed T cells. Similarly, stimulation of resting T cells only inefficiently induces FasL gene expression, whereas restimulation of primed T cells causes a rapid induction of FasL transcription and protein expression. Naive (CD45RA⁺) T cells express little or no FasL, whereas previously activated (CD45RO⁺) T cells express relatively high amounts of FasL [57]. This observation supports the notion that only primed T cells are thought to be effective killers. For both effector mechanisms this may prevent inappropriate target cell death or even bystander killing. Finally, while perforin/granzyme B-mediated cytotoxicity was thought to be regulated predominantly through post-translational events, i.e. the degranulation process, and FasL-mediated cytotoxicity primarily through transcriptional control, there is accumulating evidence that FasL can be similarly stored and released from cytoplasmic granules as do perforin and granzyme B [58–62]. This aspect of post-transcriptional regulation of FasL-mediated cytotoxicity will be further discussed later.

When do T cells use FasL and when perforin/granzyme B? Why do T cells use multiple cytolytic effector mechanisms at all? These are important questions with no readily available answers. Under many disease conditions, FasL and cytolytic granule proteins are co-expressed, for example, in CD8⁺ CTLs. However, experimental studies using perforin-deficient animals or *gld* mice have revealed that CTLs preferentially use one cytotoxic effector mechanism over the other. For example, infection of mice with the lymphocytic choriomeningitis virus induces a massive expansion of virus-specific CD8⁺ cytotoxic T cells, which kill virus-infected cells and control the viral expansion. Perforin appears to be the dominant cytotoxic effector mechanism in this protective immune response, since perforin-deficient animals are unable to control viral replication, whereas *gld* mice show no defects in virus elimination [63]. In contrast, hepatocyte damage, as it occurs during hepatitis B and C infection, appears to be dominated by FasL-mediated killing [64,65]. Finally, in the same diseased animal different target organs may show differential sensitivity to one or the other cytotoxic effector mechanism. Transfer of C57Bl/6-derived spleen cells or bone marrow into C57Bl/6 $\times$ DBA2 F1 recipients induces a strong MHC class I-dependent acute graft-versus-host disease (GvHD). Lack of perforin or FasL expression in donor T cells only partially rescues recipients from death, whereas inhibition of both pathways fully protects from lethality [66,67]. Thus, the lethal effects of GvHD are mediated by both pathways. However, when tissue damage is assessed in individual target organs, it becomes apparent that one or the other effector mechanism is dominant. For example, we have found that apoptosis induction of intestinal epithelial cells during GvHD is almost exclusively mediated by FasL [68]. Similarly, hepatic lesions are

improved upon neutralization of FasL [69], whereas skin lesions and lymphatic hypoplasia are unaffected [70].

Finally, we have found that even the same cell type can differentially use one or the other cytotoxic effector mechanism, depending on the mode of activation. Intraepithelial lymphocytes (IEL) of the intestinal mucosa consist mostly of CD8⁺ T cells. Freshly isolated naive IEL can kill target cells in a FasL- but not perforin-dependent manner in an 18 h assay. In contrast, in vivo primed IEL preferentially use perforin/granzyme B to eliminate target cells. However, in the absence of perforin (i.e. perforin-deficient mice), the lack of this effector mechanism is compensated by FasL [71].

Most likely, the expression levels of Fas and the relative Fas sensitivity determines whether target cells can be killed by FasL-expressing CTLs. Hepatocytes are well known to be particularly sensitive to Fas ligation, since injection of anti-Fas antibody induces rapid death due to liver failure [72]. Similarly, intestinal epithelial cells express Fas [68] and might become further sensitized to Fas-mediated apoptosis by pro-inflammatory cytokines like IFN γ [73]. In contrast, in other target cells Fas-induced apoptosis might be effectively inhibited by a variety of specific and broad-spectrum inhibitors. For example, cFLIP is abundantly expressed in heart and skeletal muscle, but only at low levels in liver and colon [74]. Upon infection with certain viruses, target cells might express virus-encoded vFLIP, which also contributes to Fas resistance [75]. On the other hand, other viruses have

developed strategies to evade perforin/granzyme B-mediated killing of the host cells. For example, the adenovirus protein L4-100K directly inhibits granzyme B [76]. In conclusion, different cytolytic effector mechanisms are needed to eliminate a broad spectrum of target cells with different resistance mechanisms.

5. Transcriptional regulation of the deadly weapon

The crucial role of FasL in the regulation of immune homeostasis and its devastating effect in some immunopathologies strongly indicates that its expression must be tightly controlled. As mentioned above, FasL expression was initially thought to be restricted to T cells and NK cells, and to be regulated predominantly at the transcriptional level. Various studies have taught us about the different cellular sources of FasL, and several post-transcriptional regulations of FasL have been described. Yet, several lines of evidence support the idea that the transcriptional control of FasL gene expression is one of the most important events in regulating FasL bioactivity (Fig. 2). Various transcription factors have been implicated in FasL expression in different cell types. To this end, large segments of the human and mouse FasL promoter have been sequenced and numerous transcription factor consensus sequences have been described. Among of the first identified have been the two binding sites for nuclear factor of activated T cells (NFAT). While both sites

Fig. 2. Transcriptional and post-transcriptional regulation of FasL. T cell receptor (TCR) ligation causes the activation of various downstream signaling molecules, which eventually results in the activation of transcription factors. Different transcription factors synergistically act on the *fasL* promoter and induce FasL transcription. *fasL* promoter activation can also be induced by stress signals or cytokines. Upon translation, FasL is directly transported to the cell surface, or can be stored in secretory vesicles until further activation of the cell. Cell surface-expressed FasL can interact with membrane-bound Fas and induce apoptosis, or be cleaved by metalloproteases to produce an antagonistic soluble FasL. CaM, calmodulin; CaN, calcineurin; DAG, diacylglycerol; ER, endoplasmic reticulum; JNK, Jun kinase; PKC, protein kinase C; PLCγ1, phospholipase Cγ1; sFasL, soluble FasL; ZAP-70, z-associated protein-70.

participate in regulating FasL promoter activity, mutational analysis revealed that the distal NFAT site has a more prominent effect on the promoter activity. Importantly, NFAT is crucial in the regulation of activation-induced promoter activity in T cells, but dispensable for constitutive FasL expression in Sertoli cells [77]. These findings by Latinis and co-workers are strongly supported by our and others results that activation-induced FasL expression is efficiently blocked by the immunosuppressive drug cyclosporin A (CsA) and FK506, which inhibit the calmodulin-dependent phosphatase calcineurin, responsible for NFAT activation [34,78–80]. Interestingly, the inhibitory effect of CsA on activation-induced FasL promoter activity may not only be mediated by inhibition of NFAT activity. CsA also inhibits the expression of the two transcription factors EGR-2 and -3, which have been shown to promote FasL transcription via a responsive element at –220 to –205 in the FasL promoter [81,82]. However, it is possible that the contribution of EGR-2 and -3 in the regulation of the FasL promoter may be cell type-specific. Activation-induced FasL transcription was found to be abrogated by the protein synthesis inhibitor cycloheximide in some cell types (suggesting the requirement for de novo synthesis, i.e. EGR-2 and -3) [81,82], whereas we have found no requirement for de novo protein synthesis in an other cell type [83].

Calcineurin and subsequently NFAT become most likely activated via a rise in intracellular Ca^{2+} , initiated by phospholipase $\gamma 1$ (PLC $\gamma 1$) activation and the hydrolysis of the membrane phospholipid phosphoinositol 4,5-bisphosphate (PIP $_2$) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP $_3$). IP $_3$ induces a rise in intracellular Ca^{2+} , whereas DAG activates protein kinase C (PKC). The requirement for IP $_3$ can be by-passed by Ca^{2+} ionophores such as ionomycin. Interestingly, however, ionomycin is not sufficient to induce FasL promoter activity, but requires the simultaneous activation of PKC by phorbol ester or DAG. Recent publications by Villunger et al. and Villalba et al. have shown that PKC θ cooperates with NFAT in the activation of the FasL promoter [84,85]. In these studies, PKC θ was found the most potent PKC isoform in activating the FasL promoter. Activation of PKC θ as well as other isoforms may cause the activation of the transcription factor NF κ B and AP-1 (Jun/Fos). Response elements for both transcription factors are found in the FasL promoter (AP-1 nt –1048, NF κ B nt –1080) and have been implicated in its activation [86–88]. Interestingly, however, whereas Villunger et al. [85] have found that the effect of PKC θ is exclusively mediated via the AP-1 binding site, suggesting cooperation between NFAT and AP-1 in FasL promoter activation, Villalba et al. observed that the NF κ B site also contributes to the effect mediated by PKC θ [84]. Even more puzzling is the observation made by Kasibhatla et al. [89], that the NF κ B site but not the AP-1 site in the promoter is required for phorbol ester and ionomycin-induced FasL promoter activation. It is thus conceivable to believe that PKC θ -mediated AP-1 activation may cooperate with NFAT

in FasL promoter activation, however, that another PKC isoform may be responsible for the NF κ B-mediated activation of the promoter upon stimulation with phorbol ester and ionomycin.

While AP-1 may not be required for activation-induced FasL promoter activity in T cells, it is certainly a crucial element in the activation of FasL transcription in response to cellular stress, e.g. DNA damage. Chemotherapeutic agents can induce Fas/FasL-dependent apoptosis in leukemic T cells [86,90]. This effect appears to be mediated via stress-induced activation of the Jun kinase (JNK) pathway and activation of AP-1 and NF κ B. Both transcription factors then contribute to induction of FasL promoter activity since mutation of one or the other binding site abrogates DNA damage-induced promoter activity [86,91]. The role of JNK in stress-induced FasL expression has further been confirmed in studies by Faris et al. [87,88].

NFAT, AP-1 and NF κ B are all transcription factors, which become readily activated upon cell activation, even in resting T cells. Nonetheless, it is clear that pre-activated T cells express FasL much more efficiently than resting T cells. Much of our attention has thus been focusing on this aspect of FasL regulation. A fundamental difference between resting and pre-activated cells is that the latter ones are in cell cycle, and therefore, express a different pattern of transcription factors and regulatory proteins. A transcription factor crucially involved in cell cycle regulation is c-Myc. c-Myc is often deregulated in tumor cells and promotes the expression of various genes required for proliferation. Paradoxically, c-Myc has also been implicated in regulation of apoptosis (reviewed in [92]). For example, down-regulation of c-Myc by antisense oligonucleotides or dominant negative mutants efficiently blocks AICD in T cell hybridomas [93,94]. This effect of c-Myc on T cell apoptosis appears to be specifically on the regulation of FasL expression. We have found that inhibition of c-Myc by various means (i.e. antisense oligonucleotides, TGF β , dominant negative mutants, drugs) blocks activation-induced FasL expression, but not Fas expression or Fas-induced apoptosis [83,95,96]. Surprisingly, c-Myc acts directly on the promoter through interaction with a non-canonical binding site in the FasL promoter [97]. It is tempting to speculate that the requirement for a cell cycle-regulated transcription factor, such as c-Myc, may ensure that FasL is only efficiently expressed in primed cycling T cells. The cooperation between c-Myc and activation-induced transcription factors such as NFAT and NF κ B, may further guarantee stimulus-specific expression of FasL in cytotoxic T cells and may prevent bystander killing due to uncontrolled FasL expression by other proliferating cells.

6. Post-transcriptional regulation of FasL

Post-transcriptional regulation is well known for the prototypical member of the TNF superfamily, namely TNF α

Fig. 3. Intracellular storage of FasL in activated cytotoxic T cells. (A) Immunohistochemical detection of FasL in activated T cells in vivo. (B) Intracellular detection of FasL in granule-like structure (arrows) in T cells by confocal microscopy. (A and B) Small intestinal mucosa, experimental acute graft-vs.-host disease. Bar: 10 μ m.

itself. TNF α was initially identified as a soluble cytokine and only later recognized to be expressed as a transmembrane protein. TNF α is specifically cleaved by the metalloprotease TACE (TNF α -converting enzyme) [98,99]. Specific modification of transmembrane TNF α at three different sites prevents the processing to its soluble form [100]. Similarly to TNF α most other family members, including FasL, can be processed to soluble molecules. In contrast to TNF α , however, FasL loses its apoptosis-inducing activity when processed by (yet to be identified) metalloproteases [37–39] (Fig. 2). Although soluble FasL still forms trimer and binds to the Fas receptor it fails to induce cell death. Thus, retention by cell membranes appears to be crucial for the FasL bioactivity, and only further cross-linking of soluble FasL by antibodies can restore its apoptosis-inducing activity. Interestingly, soluble FasL is even potent antagonist of membrane-bound FasL, presumably due to cell surface down-regulation of the Fas receptor upon binding of soluble FasL [37,39]. The specific induction of NF κ B and anti-apoptotic gene products by soluble FasL has also been suggested [101]. Intriguingly, soluble FasL has been detected in many different diseases [102–104], suggesting that the cleavage of FasL may represent a disease-regulating mechanism.

Recent findings have demonstrated another potentially important post-transcriptional regulation of FasL. Immunohistochemical analysis of FasL expression in T cell lines revealed that FasL is not only expressed on the cell surface or in the Golgi apparatus, i.e. in the process of being transported to the cell surface, but also in secretory vesicles. More surprisingly, in CD8⁺ T and NK cells, intracellular FasL co-localized with perforin and granzymes in the same cellular compartments [58]. This suggests that in long-term activated lymphocytes FasL is synthesized and stored in granule-like structures and can be rapidly released upon restimulation by target cells, similarly to perforin and

granzyme B (Fig. 2). The lack of requirement for de novo protein synthesis may thus allow cytotoxic T cells to kill more rapidly and efficiently [62]. This sorting of de novo synthesized FasL to secretory lysosomes appears to be mediated by a specific proline-rich domain in the cytoplasmic tail of FasL, which is lacking in other members of the family [105]. Interestingly, this domain specifically interacts with the SH3 domains of Fyn and Lck [106,107], which may link the degranulation process of intracellularly stored FasL to TCR-derived signals.

While these observations have been made in in vitro long-term T cell cultures, we have recently found evidence that preformed stored FasL may also play a crucial role in FasL-mediated cytotoxicity in vivo. During experimental acute GvHD, we have observed that functional FasL expression upon ex vivo restimulation of T cells became gradually protein synthesis-independent and failed to be blocked by CsA. This was attributed to the increasing accumulation of FasL protein in granule-like vesicles, which could also be observed in the affected tissue of the diseased animals ([59]; Fig. 3). Interestingly, when analyzing FasL protein expression in GvHD T cells by Western blotting, we observed a considerable amount of full-length protein, presumably derived from the intracellular stores, as well as a truncated form, which most likely represents the remaining cytoplasmic tail upon cleavage of membrane FasL by metalloprotease. Thus, FasL activity in T cells, in particular under pathophysiological conditions, may be tightly regulated by transcriptional as well as post-transcriptional events, such as intracellular storage and antagonism by soluble FasL.

7. Concluding remarks

Since the initial description of the *gld* mouse and the cloning of FasL, hundreds of publications have described

an enormous variety of biological effects mediated by this death-inducing ligand. Many of these findings helped us to better understand the biology of this molecule and the physiology of our body, but many of these descriptions may also turn out to be in vitro only findings with no significance in vivo. Nonetheless, it is currently clear that FasL is an important effector molecule in many immunopathologies. Thus, the regulation of FasL expression and bioactivity by drugs and specific inhibitors may represent an attractive goal of therapeutic intervention in many diseases.

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