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Title: Life in the Fas lane: Differential outcomes of Fas signalling

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Abstract

Fas, also known as CD95 or APO-1, is a member of the tumour necrosis factor/nerve growth factor (TNF/NGF) superfamily. Although best characterised in terms of its apoptotic function, recent studies have identified several other cellular responses emanating from Fas. These responses include migration, invasion, inflammation and proliferation. In this review we focus on the diverse cellular outcomes of Fas signalling and the molecular switches identified to date that regulate its pro- and anti-apoptotic functions. Such switches occur at different levels of signal transduction, ranging from the receptor through to cross-talk with other signalling pathways. Factors identified to date including other extracellular signals, proteins recruited to the DISC and the availability of different intracellular components of signal transduction pathways. The success of therapeutically targeting Fas will require a better understanding of these pathways, as well as the regulatory mechanisms that determine cellular outcome following receptor activation.

Keywords: Fas/CD95, DISC, apoptosis, NFκB, non-apoptotic, regulation.

Introduction

Fas (CD95/APO-1) is a member of the tumour necrosis factor/nerve growth factor (TNF/NGF) superfamily [1,2]. Members of this family function in cellular differentiation, proliferation and activation. Following the discovery that mutations in Fas and Fas ligand (FasL/CD95L) were the underlying cause of the massive lymphadenopathy and autoimmune lymphoproliferative syndrome in *lpr* and *gld* mice, respectively, subsequent studies on the Fas system focused primarily on its role in apoptosis. Recent studies suggest however, that other cellular responses may emanate from Fas. Signalling through Fas has been shown to activate the three main mitogen-activated protein kinase (MAPK) pathways, p38, JNK1/2 and ERK1/2, as well as the transcription factor NFκB, leading to cell proliferation, migration and inflammation. However, the molecular mechanisms leading to activation of such pathways and hence the physiological outcome of Fas signalling are not fully understood. This review aims to outline what is known about these roles of Fas and the factors that regulate cell fate following Fas ligation.

Fas and FasL

Fas is a prototypical death receptor consisting of a N-terminal region containing three cysteine-rich domains (CRDs), with ligand binding occurring predominantly at the 2nd and 3rd CRDs, a transmembrane domain and an intracellular region containing an ~ 80 amino acid domain called the 'death domain' (DD) [3]. This DD is essential for transduction of the apoptotic signal. Fas is predominantly located at the cell surface, where it has been shown to pre-associate in homotrimers [4]. It is ubiquitously expressed in the body, but is particularly abundant in liver, heart, brain and colon tissues, and in activated mature lymphocytes [5-7].

In contrast to its receptor, Fas ligand has a much more restricted pattern of expression, being found mainly in haematopoietic cells and in immune privileged sites such as the eye and testis. FasL was originally discovered on cells of the lymphoid/myeloid lineage, including activated T cells and natural killer cells [8]. In the immune system, the FasL/Fas system plays an important role in the down-regulation of immune reactions, being involved in activation-induced cell death (AICD), a process whereby activated T cells are eliminated at the end of an immune response, as well as T cell and natural killer cell-mediated cytotoxicity [9]. In immune privileged sites, constitutively expressed FasL triggers apoptosis of activated inflammatory cells entering the sites, helping to protect these sites from a potentially disastrous inflammatory immune response [10].

Although synthesized as a transmembrane protein, FasL can be cleaved by metalloproteinases, releasing a soluble form (sFasL). The apoptotic activity of sFasL is far less than that of the membrane-bound form [11], and it appears in some instances to be a mechanism for downregulating at least part of its apoptotic activity [12]. Membrane-bound FasL can also be internalised and trafficked to secretory lysosomes. FasL secreted in these microvesicles is regarded as being biologically active [13,14]. Similar to Fas, FasL can also self-associate into a homotrimer, with binding of FasL homo-trimers to the pre-associated Fas inducing the formation of receptor micro-aggregates [15].

Fas-mediated apoptosis

Upon ligation by FasL, Fas receptors multimerize in the cell membrane which leads to a conformational change in the intracellular domain of the receptor [16]. This in turn leads to the recruitment of the DD-containing adaptor molecule Fas-associated death domain (FADD) through DD-DD interactions, with FADD in turn recruiting the initiator caspase, pro-caspase-8, forming a signalling complex called the death-inducing signalling complex (DISC). Formation of the DISC occurs within seconds after Fas ligation [17,18]. At the DISC, oligomerization of pro-caspase-8 occurs, facilitating its autoactivation through self-cleavage, followed by the release of active proteases [19]. Depending on which signalling pathway is activated following ligation of Fas, apoptosis proceeds through one of two pathways, either the extrinsic or intrinsic apoptosis pathway, with cells classified as either type I or type II [18,20]. In the extrinsic apoptosis pathway, following effective formation of the DISC, activated caspase-8 directly activates downstream effector caspases such as caspase-3, -6 and -7, resulting in the cleavage of a restricted set of target proteins and culminating in the apoptotic death of the cell (type I cells). Efficient formation of the DISC and caspase-8 activation was shown to require receptor internalisation, as blocking Fas internalization impaired DISC formation and apoptosis [21]. Indeed recruitment of FADD and pro-caspase-8 to the activated receptor occurred predominantly after the receptor had moved into an endosomal compartment [21].

In contrast, in type II cells, DISC formation and caspase-8 activation is reduced, and in these cells the mitochondria serve an essential role as signal amplifiers. This mitochondrial or intrinsic apoptotic pathway is activated by cleavage of the BH3-only pro-apoptotic protein Bid by caspase-8. Truncated Bid translocates to the mitochondria where, through its effects on members of the Bcl-2 family, it induces the release of pro-apoptotic molecules including

cytochrome c and second mitochondria-derived activator of caspase (SMAC). Caspase-9 is then activated upon association with cytochrome c and the apoptotic protease-activating factor 1 (Apaf-1) in a multiprotein complex known as the apoptosome. Activated caspase-9 activates the effector caspases, ultimately resulting in the apoptotic death of the cell.

Fas-mediated apoptosis in many cells has been shown to involve the redistribution and clustering of Fas in lipid rafts [22], followed by recruitment of the DISC components to these rafts [23-25]. This redistribution and clustering of Fas in lipid rafts can also be pharmacologically mediated, and is sufficient to mediate the activation of the apoptotic signal independent of the presence of FasL [22,23,26]. Indeed, re-distribution of Fas into lipid rafts has recently been shown to promote the conversion of cells from a type II to a type I-like phenotype [27].

Regulation of the level of X chromosome-linked inhibitor of apoptosis protein (XIAP) has also been recently shown to dictate whether a cell undergoes type I or type II Fas-mediated apoptosis [28]. XIAP is a potent inhibitor of caspase-3, -7 and -9 [28], and is itself inhibited by SMAC released by the mitochondria. Fas stimulation led to a reduction in the level of XIAP in type I cells, but an increase in XIAP in type II cells. Moreover, genetic ablation of XIAP in type II cells changed their phenotype to that of type I cells [28]. However, the mechanism responsible for this differential regulation of XIAP levels in response to Fas activation in type I and type II cells remains unclear.

Fas-induced proliferation

One of the first indications that the Fas system may have additional, non-apoptotic, functions came from studies showing that ligation of Fas augmented proliferation of CD3-activated primary T cells [29]. Sensitivity of human T cells to Fas-mediated apoptosis depends on their activation status. Naive T cells are resistant, with sensitivity to Fas-induced cell death developing 6–7 days after T cell activation [30], consistent with the important role of FasL/Fas in AICD. An unexpected finding, however, was that ligation of Fas could co-stimulate the proliferation of these naive T cells [29]. Further investigations suggested that the physiological response of T cells to Fas ligation is determined by the antigenic history of the cell and the availability of co-stimulation [31]. Activation of T cells requires two main signals; engagement of the TCR/CD3 complex (signal 1), together with a second co-stimulatory signal (signal 2), usually provided by the ligation of 'classical' co-stimulatory receptors including CD28. In the absence of exogenous rescue signals such as CD28 co-

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stimulation or T_H1 (IL-12) and T_H2 (IL-4) differentiation cytokines, the default response of TCR/CD3-stimulated naive CD4$^+$ T cells to Fas activation was apoptosis. In contrast, despite the absence of exogenous rescue signals, TCR/CD3-stimulated memory T cells proliferated more rapidly as a consequence of Fas ligation [31].

The outcome of Fas ligation was also proposed to depend on the strength or 'dose' of the anti-Fas agonist [32]. High concentrations of Fas agonists inhibited the proliferation of primary human naive CD4$^+$ T cells, while low doses of the very same Fas agonists promoted TCR-triggered NFκB and MAPK activation and enhanced T cell proliferation compared to conventional co-stimulation through the classical co-stimulatory CD28 molecule [32,33]. Such a low concentration of FasL is found on antigen-presenting cells (APC) at the onset of an immune response [34].Together these findings suggest that the FasL/Fas system may, in a cell context-specific manner, replace the conventional co-stimulatory signal required by naive T cells for proliferation (signal 2). Thus, in addition to its role in the contraction phase of an immune response (AICD), Fas/FasL may support T cell expansion at the onset of an immune response, as well as contributing to the increased efficiency and accelerated kinetics displayed during memory responses.

T cells are not the only cell type in which this classically pro-apoptotic molecule has been shown to modulate cell growth, with a variety of non-immunological cells including B cells, fibroblasts and tumour cells also shown to respond to Fas stimulation with enhanced proliferation [35-39]. For instance, reducing Fas or FasL expression in a variety of tumour cell lines was recently shown to reduce cell proliferation *in vitro*, with optimal tumour growth depending on constitutive signalling through Fas by FasL *in vivo* [38]. Enhanced tumour growth was proposed to be due to FasL-mediated activation of JNK and ERK, resulting in phosphorylation of Jun and increased expression of the *Egr1* and *Fos* transcription factors [37,38] (Fig. 1). Activation of Fas was also shown to promote liver regeneration following partial hepatectomy in mice [39]. This contrasts sharply with the massive hepatocyte apoptosis that occurs in mice following intraperitoneal injection of Fas-activating antibodies [40]. Ligation of Fas by FasL also induced proliferation in quiescent hepatic stellate cells, with the mitogenic signal shown to be due to FasL-induced phosphorylation of the epidermal growth factor receptor (EGFR), and subsequent ERK activation. At the same time, cell death was prevented by FasL-mediated inactivation of the Fas receptor by tyrosine nitration [41].

Multiple functions of Fas have also been described in neuronal cells. In addition to the induction of neuronal cell apoptosis, several studies have suggested that Fas may play a role

in regulating neuronal development, growth, differentiation and regeneration in the central nervous system [42]. Both Fas and FasL are widely expressed in the nervous system [43,44]. Instead of inducing neuronal apoptosis, ligation of Fas triggered neurite outgrowth and branching [7,45], and accelerated nerve regeneration following sciatic nerve injury *in vivo* [7]. In a model of traumatic brain injury, activation of Fas was initially detrimental, inducing apoptosis, but later in the course of the disease, was beneficial, promoting regeneration by mediating neuronal branching [46]. Fas-induced neurite growth was shown to require recruitment of ezrin, a molecule that links transmembrane proteins to the cytoskeleton, to Fas and subsequent activation of the small GTPase Rac1 [47]. FasL-mediated activation of ERK further promoted process elongation and branching, fine-tuning process morphology [7,47].

Additional evidence supporting a role for non-apoptotic Fas signalling in the nervous system under certain circumstances comes from *lpr* mice. Fas-deficient *lpr* mice develop autoimmune disease due to an accumulation of immature and mature T cells as a result of impaired Fas-mediated immune homeostasis. These mice also exhibit a variety of neurological defects, with such defects mostly attributed to this auto-immune disease [48]. However, despite the ability of potent immunosuppressive drugs to prevent the development of autoimmune disease in the *lpr* mouse, they had no effect on the neurological defects seen in these mice [42], suggesting that the neurological degeneration may be due to a lack of Fas-mediated proliferation and survival signals.

Fas-mediated suppression of T cell activation

In addition to the roles outlined thus far for FasL/Fas, a third function has been attributed to the FasL/Fas system specific to T cells – suppression of T cell activation (Fig. 2). Activation of T cells through the TCR triggers the recruitment of a series of tyrosine kinases and substrates to the TCR/CD3 complex, ultimately triggering the release of calcium (Ca^{2+}) from the endoplasmic reticulum stores into the cytoplasm. Emptying of these stores triggers an influx of extracellular Ca^{2+} through the opening of Ca^{2+} release-activated Ca^{2+} channels (CRAC) in the plasma membrane, resulting in sustained Ca^{2+} influx [49]. This elevation of intracellular free Ca^{2+} is one of the key triggering signals for T cell activation by antigen, resulting in the activation of the transcription factor NFAT and the subsequent production of IL-2. Cross-linking of Fas on T cells prior to stimulation with anti-CD3 mAb almost completely inhibited TCR-mediated Ca^{2+} influx [50]. This suggests that Fas-mediated inhibition of Ca^{2+} influx through CRAC channels could prevent T cell activation due to

impaired transcription factor activation and IL-2 synthesis, perhaps resulting in the induction of T cell anergy.

Further investigations revealed that the inhibition of TCR-mediated Ca^{2+} influx was mediated by acidic sphingomyelinase (aSMase) (Fig. 2) [51]. Crosslinking of Fas resulted in the activation of aSMase, which hydrolyses sphingomyelin, a ubiquitous membrane sphingolipid, to generate phosphocholine and ceramide [52]. Ceramide, or its metabolite sphingosine, was found to block Ca^{2+} influx through the CRAC channels in lymphocytes, impairing NFAT activation and IL-2 synthesis [51,53]. Thus, the upregulation of FasL that occurs during an immune response may, in addition to the induction of apoptosis of activated cells, further aid in the contraction phase of the immune response by preventing the activation of resting cells. Indeed, numerous viruses induce FasL expression in APC, with such APCs impaired in their ability to activate T cells [53], suggesting that this may represent a potential mechanism of viral immune evasion.

Together, these differing effects of Fas on T cells i.e. AICD, co-stimulation and suppression of T cell activation may play an important role in regulating the immune response, ensuring optimal T cell activation during the initial activation and expansion phase, whilst also being important for the termination of the immune response. For instance, FasL co-stimulation was shown to be necessary for maximal proliferation of cytotoxic T cells (CTL) *in vivo* [54]. Also, in an induced model of lupus, Fas on CD4^{+} T cells was shown to be important in providing help for CD8^{+} T cells, while Fas on CD8^{+} T cells was shown to be important in effector downregulation [55]. Moreover, CD4^{+} T cells and CD8^{+} T cells appear to exhibit different sensitivities to Fas-mediated apoptosis, with CD8^{+} T cells more sensitive than CD4^{+} T cells [56].

Induction of cell migration and invasion by Fas

Numerous studies have shown that signalling through Fas can promote cell migration and invasion, in particular in apoptosis-resistant malignant cells [57-59]. For instance, in pancreatic cancer cells TNF receptor associated factor 2 (TRAF2) was shown to not only protect cells from Fas-mediated apoptosis but also to strongly enhance Fas-mediated cell invasion [59]. Moreover, TRAF2 was recently shown to promote proteasomal degradation of active caspase-8, raising the signalling threshold for death receptor-induced apoptosis [60]. TRAF2 mediates signal transduction from members of the TNF receptor superfamily and is required for both TNFα and Fas- mediated activation of NFκB and JNK. NFκB can be

activated in cells via two main activating pathways [61]. The specific pathway linking Fas to NFκB is unclear, with studies implicating both the canonical [62,63] and non-canonical [64,65] NFκB activation pathways as being important for Fas-induced NFκB activation. The reasons for these opposing findings are unclear, but may be due to differences in the cell type analysed.

Activation of Fas in TRAF2 over-expressing cells resulted in enhanced activation of NFκB and AP-1, increased secretion of extracellular matrix-degrading metalloproteinases (MMPs), urokinase-type plasminogen activator (uPA) and IL-8, and subsequently increased invasion. Moreover, induction of cellular migration by Fas in both breast and ovarian cancer cells was shown to be far greater than that seen with other members of the TNF family, including TRAIL and TNFα, and required activation of NFκB, ERK1/2 and caspase-8 [57] (Fig. 3).

Several recent studies have further characterised the signalling pathways activated by Fas that mediate Fas-induced migration and invasion. Activated Fas was shown to recruit both the Src family kinase Yes and the p85 subunit of PI3 kinase (PI3K) in glioblastoma cells, leading to PI3K activation and the expression of MMPs such as MMP2 and MMP9 [66]. FasL also activated PI3K in macrophages and neutrophils, in this case via recruitment and activation of Syk kinase, leading to activation of MMP-9, and ultimately increased migration of the cells [67]. This increased secretion of MMPs is thought to play an important role in Fas-mediated invasion, facilitating cell migration through the extracellular matrix [68].

The formation of actin-driven cell protrusions are also thought to be required for Fas-induced invasion [68]. In colorectal cancer cells, activation of Fas by FasL induced the formation of actin-driven cell protrusions through Rac and the cofilin pathway [69]. Activated Fas promoted the phosphorylation of platelet-derived growth factor receptor-β (PDGF- β) by an unknown mechanism, leading to phosphorylation of phospolipase C γ1. This in turn ultimately resulted in the liberation of the actin-severing protein cofilin from the plasma membrane, initiating cortical actin remodelling and the formation of membrane protrusions [69]. Fas-activated Rac plays an important role in this process [47], with concomitant activation of the cofilin pathway and Rac driving the formation of cell protrusions required for migration and invasion [68] (Fig. 3).

Another protein recently shown to play a role in Fas-induced invasion is TRIP6 [70]. TRIP6 is a focal adhesion molecule that serves as a platform for the recruitment of a number

of molecules involved in actin assembly, cell motility and survival. Interestingly, TRIP6 was recently shown to antagonise binding of FADD to Fas, inhibiting apoptosis, whilst at the same time promoting activation of NFκB and cell migration [70] (Fig. 3).

In addition to normal physiological roles such as neurite outgrowth, Fas-mediated migration and invasion may also play an important role in tumour recurrence following treatment of colorectal liver metastases. Signalling through Fas by FasL was shown to play an important role in local tumour cell invasion and accelerated outgrowth of micrometastases following both radiofrequency ablation [71] and ischemia/reperfusion injury which can occur during partial liver resection [72]. Both of these treatments are used to treat individuals with colon cancer that has metastasized to the liver, suggesting that targeting Fas-induced migration and invasion may be of therapeutic benefit.

Fas as an activator of inflammation

Some of the first evidence that FasL/Fas signalling could trigger inflammation came from allograft studies of FasL over-expressing tissues and tumours [73,74]. In these studies, ectopic over-expression of FasL resulted in extensive neutrophil recruitment and graft rejection in many cases. Numerous studies have since demonstrated that activation of Fas signalling in a variety of non-lymphoid cells including colonic and lung epithelial cells [75-77], hepatocytes [78], synoviocytes [79], macrophages [80,81] and fibroblasts [82,83] can lead to the expression and release of inflammatory factors *in vitro* and *in vivo*. Such factors may in turn recruit inflammatory cells, exacerbating the inflammatory process. Accordingly, Fas/FasL-mediated inflammation has been implicated in playing an important role in the pathogenesis of several diseases, including acute respiratory distress syndrome [77], cystic fibrosis [84], arthritis [79,85] and cancer [86], all of which have an underlying inflammatory component. Moreover, many chronic inflammatory diseases are attenuated in mice lacking Fas or FasL [85,87,88].

Cytokines and chemokines induced by Fas include interleukin-6 (IL-6), IL-8, IL-1β, TNFα, MCP-1 (monocyte chemoattractant protein-1), IP-10 (interferon-gamma-induced protein 10) and prostaglandin E2 (PGE_2). IL-6, IL-1β and PGE_2 are multi-functional pro-inflammatory cytokines. IL-8 is a neutrophil chemoattractant while MCP-1 and IP-10 are chemotactic for monocytes and T cells, respectively. Fas-induced cytokine production predominantly involves the activation of NFκB and the p38, JNK and ERK MAPK signalling pathways [76,79] (Fig. 4). Several recent studies, however, have shown that MyD88 may also

play a role in this process. MyD88 is an adaptor molecule more commonly associated with inflammatory cytokine production by the Toll-like receptor (TLR) family of pattern recognition receptors and the interleukin-1 receptor (IL-1R) family. Similar to FADD, MyD88 also has a DD, allowing the potential binding of MyD88 to Fas via DD-DD interactions. Fas-induced KC (murine analog of IL-8) production by alveolar epithelial cells was shown to be dependent on MyD88 (Fig. 4) [77], while Fas-induced chemokine production by macrophages was also shown to be reduced in cells lacking MyD88 [89]. FADD-deficient cells also displayed enhanced TLR4- and TLR2-induced pro-inflammatory protein production [90]. This was proposed to be due to the interaction of FADD with MyD88 and IRAK1 (a TLR signalling molecule), with FADD reducing the stability of the MyD88-IRAK1 interaction, thereby attenuating the TLR4-induced signal [90].

Further evidence of cross-talk between the TLR, IL-1R and FasL/Fas pathways comes from studies showing that TLR4 and IL-1R1 signalling is reduced in *lpr* peritoneal macrophages and that blocking FasL/Fas interactions in macrophages suppresses LPS-induced (TLR4 agonist) and IL-1R1-induced inflammatory cytokine production [85]. LPS-activated macrophages also produce a large amount of IL-1β upon FasL stimulation [91], suggesting that TLR-mediated induction of cytokines and chemokines may in part be due to enhanced Fas-induced inflammatory cytokine/chemokine production. Consistent with this, Fas-deficient mice are resistant to LPS-induced lethality after *P. acnes* treatment [92], while TLR4 signalling is reduced in *lpr/lpr* and *gld/gld* peritoneal macrophages [85]. Moreover, interruption of Fas/FasL interaction suppressed LPS-induced macrophage production of IL-6 and TNFα [85]. Alternatively, signalling through Fas may regulate TLR-induced inflammation due to cross-talk between the signalling pathways downstream of the receptors. In the absence of Fas ligation, the Fas adaptor molecule, FADD was shown to interact with the TLR adaptor MyD88 in the cytoplasm, potentially blocking/limiting MyD88 signalling (Fig. 4) [85]. Further studies are required to elucidate the extent of the cross-talk between these pathways and to determine how important this cross-talk is in promoting Fas-induced inflammation.

Reverse signalling through FasL

Although predominantly described in terms of its ability to activate signalling through Fas, several studies have shown that FasL may also transduce a signal into the cell. This phenomenon of reverse or retrograde signalling has primarily been described in T cells, and

in this situation Fas acts as the ligand for membrane-bound FasL, transmitting a signal into the FasL-expressing cell. This reverse signal has been shown to be due to the recruitment of Src homology 3 (SH3) domain-containing proteins such as Fyn, Grb2 and the p85 subunit of PI3K to the cytoplasmic region of FasL [93,94] (Fig. 5). One of the first reports of reverse signalling in non-T cells was in cancer cells, with reverse signalling through FasL enhancing cell migration [95]. This enhanced migration was due to interaction of FasL with, and subsequent activation of, the hepatocyte growth factor receptor Met. FasL was shown to complex with Met and activate the Met signalling pathway in tumour cells, promoting tumour cell migration and metastasis [95]. Moreover, reverse signalling through FasL has also recently been shown to occur in the nervous system [96]. Stimulation of FasL on Schwann cells induced the secretion of nerve growth factor (NGF), with NGF secretion requiring the activation of the Src and the MAPK ERK1/2 pathways. NGF, in turn, stimulating neurite outgrowth *in vitro* [96]. Further work, however, is required to fully elucidate the physiological role of reverse signalling through FasL *in vivo*.

Regulation of the outcome of Fas signalling

What regulates the outcome of Fas signalling, and why Fas stimulation in some cells results in apoptosis, whilst in other cells a variety of non-apoptotic signalling pathways are activated, remains unclear. Several mechanisms have been proposed, with the final outcome potentially a combination of some or all of these mechanisms.

Soluble versus membrane-bound FasL:

The outcome of Fas signalling may potentially be determined by the form of the ligand that activates the receptor (Fig. 6a). Cleavage of membrane-bound FasL (mFasL) from the cell surface generates soluble FasL (sFasL). These forms of FasL differ greatly in their apoptotic activity, with sFasL being far less apoptotic than mFasL [12,97]. Indeed, cleavage of mFasL to sFasL may represent a potential mechanism to downregulate the activity of mFasL [12,97,98]. Thus, depending on the form of FasL that binds to Fas, different signalling pathways may be activated.

Consistent with this, in a mouse model of glaucoma, full length mFasL was shown to play a major role in retinal neurotoxicity, with administration of sFasL protecting against the death of the retinal ganglion cells [99]. Using lymphoma cells transfected with the different forms of FasL, Hohlbaum *et al* showed that sFasL not only blocked the apoptotic activity of

mFasL but also opposed the pro-inflammatory activity of mFasL *in vivo* [100]. mFasL also triggered the production of pro-inflammatory cytokines by monocytes and macrophages [81,101], with cardiac-specific expression of sFasL inhibiting activation of macrophages and pro-inflammatory cytokine production in a model of heart disease [102].

In addition to its role in down-regulating the activity of mFasL, sFasL may also potentially activate non-apoptotic signalling pathways. In a recent study using gene-targeted mice engineered to lack either sFasL or mFasL, mFasL was found to be essential for Fas-induced killing of target cells by T cells and for activation-induced cell death [103]. However, in contrast to the previous studies demonstrating that sFasL inhibited mFasL-mediated apoptosis and inflammation, in this study sFasL was proposed to be the form of FasL responsible for the pro-inflammatory function of FasL. Mice expressing sFasL alone contained abnormally elevated serum levels of pro-inflammatory cytokines including IL-6 and TNFα and developed a fatal auto-immune disease and hepatic tumours [103], consistent with the ability of sFasL to activate NFκB [103,104]. Moreover, in a mouse model of stroke, *gld* mice were found to have significantly improved neurological deficit scores [105]. *Gld* mice have a point mutation in the FasL gene that inactivates FasL function [106]. These mice had attenuated pro-inflammatory cytokine and chemokine production following cerebral ischemia. sFasL and JNK phosphorylation was decreased in *gld* mice, while in the ischemic cortex of the control B6 mice, the level of sFasL was greatly increased, suggesting that the reduction and dysfunction of sFasL in *gld* mice might contribute to the reduced inflammation seen in these mice, with sFasL potentially responsible for the FasL-induced inflammation seen [105].

Differences in the levels of receptor-interacting protein 1 (RIP1) in the cells analysed may account for these contradictory findings, with a recent study showing that RIP1 may play a role in modulating the differential outcome of signalling through sFasL and mFasL (Fig. 6*d*) [107]. RIP1 was shown to be required for the efficient activation of downstream caspases by Fas following stimulation with mFasL. Cross-linking sFasL bypassed the requirement for RIP1 by initiating the formation of larger, but less efficient DISC complexes than mFasL. It was proposed that when sFasL is associated with matrix proteins, such as may occur under physiological conditions, cell death does not occur due to the recruitment of less RIP1 to the DISC, thus favouring the activation of non-apoptotic signalling pathways [107].

For a variety of reasons, many studies fail to distinguish between mFasL and sFasL, or investigate the function of FasL using soluble FasL corresponding to the entire

extracellular domain rather than the natural cleavage product [38,63,108]. Moreover, FasL can be released from cells by at least two mechanisms. In addition to metalloproteinase-mediated cleavage of FasL from the cell surface to yield a truncated soluble product (sFasL), cell lines and activated T cells have been reported to release full-length FasL in the form of microvesicles [109,110]. ELISA assays don't distinguish between sFasL and full-length FasL in microvesicles, possibly confounding the findings regarding the predominant function of sFasL and mFasL, and making it difficult to determine whether non-apoptotic Fas signalling is predominantly induced by sFasL and/or mFasL. Finally many cells express both sFasL and mFasL, and the predominant activity may thus be determined by the relative expression of the different forms.

Regulation at the level of the receptor:

The response to engagement of the Fas receptor by FasL in some cells appears to be determined at the level of the receptor (Fig. *6b*), with studies showing that changes in receptor internalisation may play an important role in determining cell fate following Fas ligation [111]. Clathrin-mediated internalisation of Fas into endosomal compartments was shown to be required for optimum DISC formation and apoptosis to occur, with inhibition of internalisation resulting in Fas-induced activation of the NFκB and ERK signalling pathways [21,112]. Binding of Fas initiates the rapid clustering of Fas in lipid rafts which aggregate in large clusters that are internalised [113]. Post-translational palmitoylation of Fas was shown to be necessary for receptor localisation to lipid rafts, with mutations that prevented Fas palmitoylation resulting in a marked reduction in Fas translocation to lipid rafts, internalization and apoptosis [114]. Moreover, a conserved glycosphingolipid-binding motif located in the extracellular domain of Fas has been identified as being required for clathrin-dependent Fas internalization, with loss of function switching Fas towards its non-apoptotic functions [112].

The strength of the activating signal may also play a role in the outcome of Fas ligation [115]. Although a large number of tumours have somatic mutations in the Fas death domain, they rarely display loss of heterozygosity, suggesting the presence of one wild-type allele confers an oncogenic advantage. Indeed, induction of apoptosis has been shown to require two wild-type alleles of Fas, whereas the signalling threshold to activate NFκB is much lower, requiring the presence of just one wild-type allele [116]. Consistent with the strength of the activation signal playing an important role in determining cell fate, stimulation

of Fas in fibroblasts triggered proliferation in cells expressing low levels of the receptor, while those over-expressing the receptor died by apoptosis [36].

Finally, sialylation of the Fas receptor by the golgi glycosyltransferase, ST6Gal-1, was recently shown to inhibit apoptotic signalling by Fas by decreasing the ability of FADD to bind to Fas, as well as inhibiting receptor internalisation [117]. ST6Gal-1 is upregulated by oncogenic K-Ras [118], with oncogenic K-ras recently implicated in playing a role in the switch in the cell response of colon cancer cells following Fas activation [119]. In the presence of oncogenic K-Ras, binding of FasL to Fas triggered cell migration and invasion, whereas deletion of the K-Ras effector, Raf1, was sufficient to switch the cell response back to apoptosis, abrogating the metastatic activity of the receptor [119].

DISC-interacting proteins as determinants of cell fate:

FLICE-like inhibitory proteins (c-FLIP) have been shown to play a role in modulating the outcome of Fas engagement (Fig. 6*c*). c-FLIP is a family of alternatively spliced variants, three isoforms of which are expressed as proteins and comprise: Long (c-FLIP$_L$), Short (c-FLIP$_S$) and Raji (c-FLIP$_R$). All three can bind to the DISC, with c-FLIP$_S$ and c-FLIP$_R$ blocking apoptosis by inhibiting the activation of pro-caspase-8 [120]. The role of c-FLIP$_L$, however, in apoptosis is less clear [121]. In some cell contexts c-FLIP$_L$ has pro-apoptotic activity [122,123]. Ectopic expression of c-FLIP$_L$ at physiologically relevant levels was shown to enhance pro-caspase-8 processing in the DISC and promote apoptosis, while a decrease in c-FLIP$_L$ expression resulted in inhibition of apoptosis [123].

Numerous other studies, however, have demonstrated an anti-apoptotic function for c-FLIP$_L$, with c-FLIP$_L$ linked to activation of NFκB and the MAPK signalling pathways [79,124]. Inhibition of c-FLIP expression in rheumatoid synoviocytes resulted in a reduction in Fas-mediated NFκB activation [79], while T cells over-expressing c-FLIP$_L$ were shown to exhibit increased proliferative responses following stimulation with sub-optimal doses of antigen [125]. Over-expression of c-FLIP$_L$ was shown to promote the activation of the NFκB and Erk signalling pathways, together with the increased production of IL-2, via recruitment of adaptor proteins and kinases including TRAF-1, TRAF-2, RIP and Raf-1 to the DISC [126]. Similarly, in fibrotic lung myofibroblasts the switch from apoptosis to proliferation was shown to be mediated by c-FLIP$_L$ and required signalling through TRAF and NFκB [127], while overexpression of TRAF2 in pancreatic cancer cells not only protected the cells from Fas-mediated apoptosis but also led to the constitutive activation of NFκB and AP-1

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[128]. Moreover the change in response of hepatocytes to Fas ligation (proliferation versus apoptosis) correlated with higher levels of FLIP in regenerating versus resting hepatocytes after treatment with anti-Fas [39].

Finally, in several other studies FasL-induced NFκB activity was shown to be inhibited by all c-FLIP variants. Knockdown of c-FLIP resulted in enhanced NFκB activity, while over-expression of c-FLIP blocked Fas-induced NFκB activation [129-131]. Over-expression of c-FLIP suppressed Fas-induced RIP1 recruitment, a signalling protein that can interact with the IKK-γ (NEMO) subunit of the IκB kinase (IKK) complex, and ultimately promote NFκB activation [129].

The reason for these conflicting reports on the role of c-FLIP in Fas-mediated signalling is unclear, but may be due to differences in the dose of FasL used. The same amount of c-FLIP$_L$ was shown to slow down cell death induction following weak Fas stimulation but to accelerate cell death upon stimulation with high doses of FasL [122]. Alternatively, differences in the concentrations of the c-FLIP isoforms in the different cell types analysed may account for the different results obtained in the different cellular systems. Recent systems biology approaches have shown that the stoichiometry of c-FLIP proteins and pro-caspase-8 in the DISC may be of critical importance in the outcome of receptor triggering, with the balance between the levels of c-FLIP$_L$ and pro-caspase-8 determining the outcome of Fas activation [63,122,132].

Pro-caspase-8 has intrinsic catalytic activity, with this catalytic activity limited to pro-caspase-8 itself and c-FLIP. In contrast, mature caspase-8 does not cleave pro-caspase-8, but instead efficiently cleaves effector pro-caspases, triggering the apoptotic signal [133]. At the DISC, heterodimer formation of pro-caspase-8 and c-FLIP$_L$ results in pro-caspase-8 activation and in pro-caspase-8-mediated cleavage of c-FLIP$_L$, generating p43-FLIP [63]. p43-FLIP, in turn can interact with TRAF2, ultimately leading to the activation of NFκB [134]. However, both high and low concentrations of c-FLIP$_L$ inhibited p43-FLIP generation, and altering the ratio of pro-caspase-8 to c-FLIP$_L$ at the DISC was sufficient to alter the balance between apoptosis and NFκB activation [63].

In contrast to Fas-induced NFκB activation [129,135], activation of the MAPK signalling pathways by Fas requires processing of pro-caspase-8 to caspase-8, with caspase-8 activity shown to be necessary for activation of the MAPK pathways [129,132,136,137]. However, pro-caspase-8 is processed more rapidly in c-FLIP$_L$-pro-caspase-8 heterodimers

than in pro-caspase-8 homodimers [122], with the ratio of c-FLIP$_L$ to caspase-8 at the DISC defining the amount of activated caspase-8 generated at the DISC. Thus, despite the requirement for pro-caspase-8 or caspase-8 activity by NFκB [63] and MAPK [132], respectively for Fas-induced activation, the balance between c-FLIP$_L$ and pro-caspase-8 may regulate activation of both, suggesting that the composition of the Fas DISC may act as an important switch in determining whether apoptotic or non-apoptotic signalling prevails following Fas ligation.

In addition to signalling through FADD and pro-caspase-8, recent studies suggest that Fas may recruit and signal through TRIP6 (Fig. 6*d*). Activation of Fas was shown to induce cytoskeletal reorganization, leading to the association of Fas with the adaptor proteinTRIP6 [70]. In binding to Fas, TRIP6 interfered with FADD binding to the DD of Fas, thereby preventing apoptosis. At the same time, TRIP6 promoted NFκB activation and cell migration [70]. Another protein recently proposed to regulate Fas signalling specifically in lymphoid cells is Toso (Fig. 6*d*) [138]. Toso is an immune-specific cell surface protein. Originally described as an inhibitor of Fas-mediated apoptosis through its ability to bind to FADD [139,140], Toso was recently shown to associate with FADD in a RIP1-dependent fashion, not only preventing apoptosis, but also promoting the activation of NFκB and the ERK1/2 MAPK signalling pathway [138].

Together these studies suggest that the DISC and the signalling proteins recruited to the DISC play a major role in the outcome of Fas ligation, with the levels and/or activation of the proteins recruited determining the signalling cascade that predominates and thus playing a major role in determining the fate of the cell.

Non-DISC-interacting proteins as determinants of cell fate:

Recent studies have determined that the outcome of Fas ligation may also be mediated by non-DISC-interacting proteins such as mutated PI3K catalytic subunit α (*PIK3CA*) [141] and oncogenic K-Ras (Fig. 6*e*) [119]. In colon cancer cells, expression of mutated PI3K catalytic subunit α (*PIK3CA*) was shown to allow robust caspase-8 activation following Fas activation in the absence of apoptosis [141]. In these cells, mutant PIK3CA switched the Fas signal from apoptosis to NFκB activation and cell invasion. Enhanced cell invasion required caspase-8-mediated cleavage of the protein kinase rho-associated, coiled-coil containing protein kinase 1(ROCK-1), leading to remodelling of the actin cytoskeleton and invasion[141]. Oncogenic K-Ras, in turn, not only prevents Fas-mediated apoptosis, but also

promotes Fas-mediated cell motility and invasion [119,142]. Oncogenic K-Ras was shown to prevent caspase-8 processing [142], with the K-Ras effector, Raf1, essential for the switch from apoptosis to cell invasion [119]. The ability of these oncogenic proteins to switch the signalling output of Fas from apoptosis to that promoting invasion is consistent with the fact that many tumours retain expression of Fas, albeit at a reduced level [143], suggesting that expression of Fas is advantageous to the tumour [38].

Tissue Microenvironment in the regulation of Fas signalling:

The presence of cytokines and growth factors in the microenvironment could potentially play an important role in the outcome of Fas ligation (Fig. 6*f*). For instance, short-term cultured astrocytes express Fas but are resistant to Fas-mediated apoptosis and secrete IL-8 following Fas engagement. In the presence of interferon-gamma (IFNγ) however, resistant astrocytes become sensitive to Fas-mediated death [144]. Similarly, engagement of Fas, in combination with IFNγ, resulted in apoptosis of HT29 colon carcinoma cells, while Fas ligation alone resulted in IL-8 synthesis [145]. Exposure to growth factors such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF) prior to Fas activation may not only confer resistance to Fas-mediated apoptosis, and instead promote Fas-induced cell proliferation through activation of the PI3K/Akt pathway [146]. Cytokines may also promote Fas-mediated apoptosis, with IL-2 shown to enhance the sensitivity of colon cancer cells *in vitro* to Fas-mediated apoptosis [147]. Finally, cytokines in the microenvironment can enhance Fas signalling, with IL-7 shown to synergize with Fas to induce proliferation of sub-optimally activated T cells [148]. Thus, from a physiological standpoint, the response of cells to Fas ligation is likely to be influenced by the cellular microenvironment *in vivo*.

Conclusions

Studies of the Fas receptor have shed new light on the physiological function of this molecule, extending its function beyond that of the 'prototypical' death receptor to include proliferation, inflammation and invasion. The factors identified to date that determine the cellular response to Fas ligation are varied, ranging from the level of the receptor, to DISC-interactions, to microenvironmental signals. Given that excessive or insufficient apoptosis underlie, among others, autoimmune and neoplastic diseases respectively, detailed characterization of the mechanisms that determine the outcome of Fas signalling may identify potential targets to allow the switching of this outcome, as appropriate. This knowledge may

be particularly important in the development of novel therapeutic targets for neoplastic disease as these non-apoptotic signals of Fas first became particularly evident in apoptosis-resistant tumour cells.

Acknowledgements

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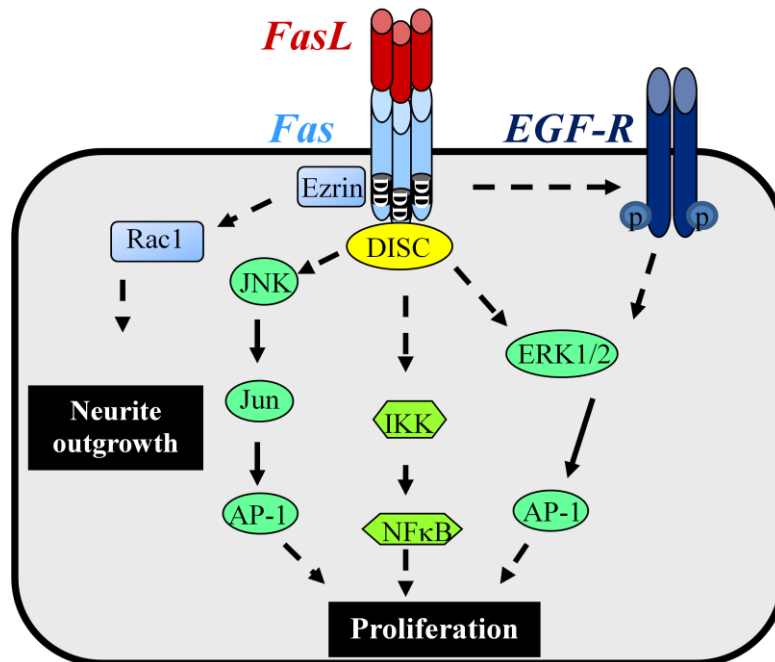
Figures

Fig.1   Mitogenic signalling pathways implicated in Fas-induced proliferation. Following Fas stimulation, formation of the DISC occurs. Fas-mediated activation of the NFκB transcription factor and the MAPK signalling pathways downstream of the DISC can promote cell proliferation. FasL can also induce ligand-dependent EGFR (epidermal growth factor receptor) activation and phosphorylation, which triggers mitogenic signalling through ERK. Neurite growth requires recruitment of ezrin to Fas and activation of the small GTPase Rac1. Dashed lines represent signalling pathways/intermediaries that not fully elucidated.
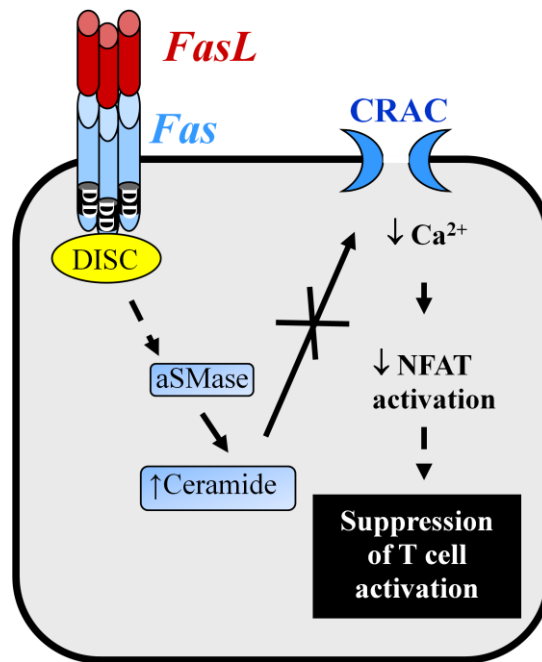
Fig.2 Fas-mediated suppression of T cell activation. In T cells, activation of acidic sphingomyelinase (aSMase) following Fas ligation can increase the level of ceramide in the cells, which in turn inhibits Ca^{2+} release-activated Ca^{2+} channels (CRAC) in the plasma membrane. Impairment of CRAC opening prevents sustained Ca^{2+} influx, leading to suppressed NFAT activation and impaired T cell activation. Dashed lines represent signalling pathways/intermediaries that not fully elucidated.
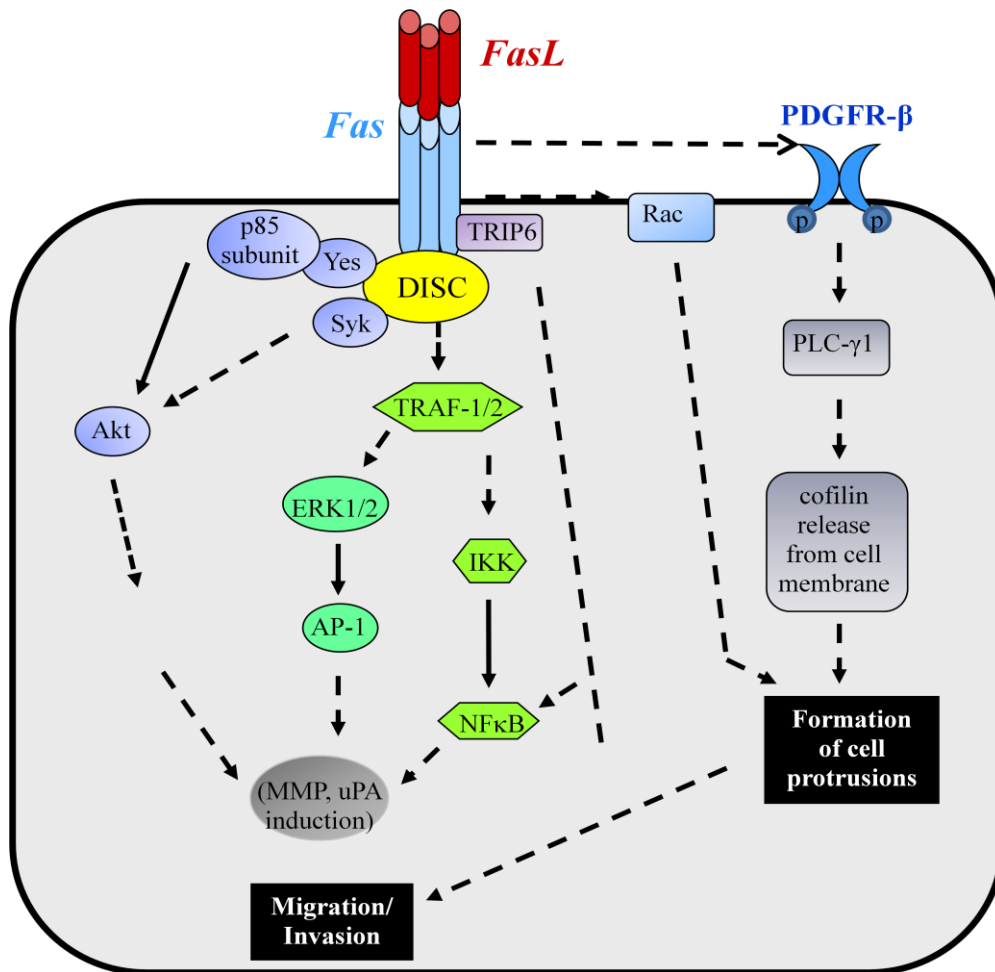
Fig.3 Signalling pathways implicated in Fas-mediated migration. Multiple signalling pathways have been shown to play a role in the induction of cell migration and invasion by Fas. Ligation of Fas stimulates platelet-derived growth factor receptor- β (PDGFR-β) tyrosine phosphorylation by an unknown mechanism, leading to phosphorylation of phospholipase C-γ1 (PLC-γ1), with the subsequent release of cofilin from the cell membrane. Together with Fas-activated Rac, this leads to the formation of cell protrusions. Fas activation also results in the recruitment of the p85 subunit of PI3K and the tyrosine kinases Yes and Syk to Fas, activating Akt and NFκB. Fas-induced NFκB and ERK MAPK activation may also lead to cell migration in a TRAF2-mediated fashion. Association of TRIP6 with Fas following Fas activation can inhibit apoptosis, and promote NFκB activation and cell migration. MMPs and uPA are among the genes most prominently upregulated in response to Fas ligation, and together facilitate migration and invasion. Dashed lines represent signalling pathways/intermediaries that not fully elucidated.
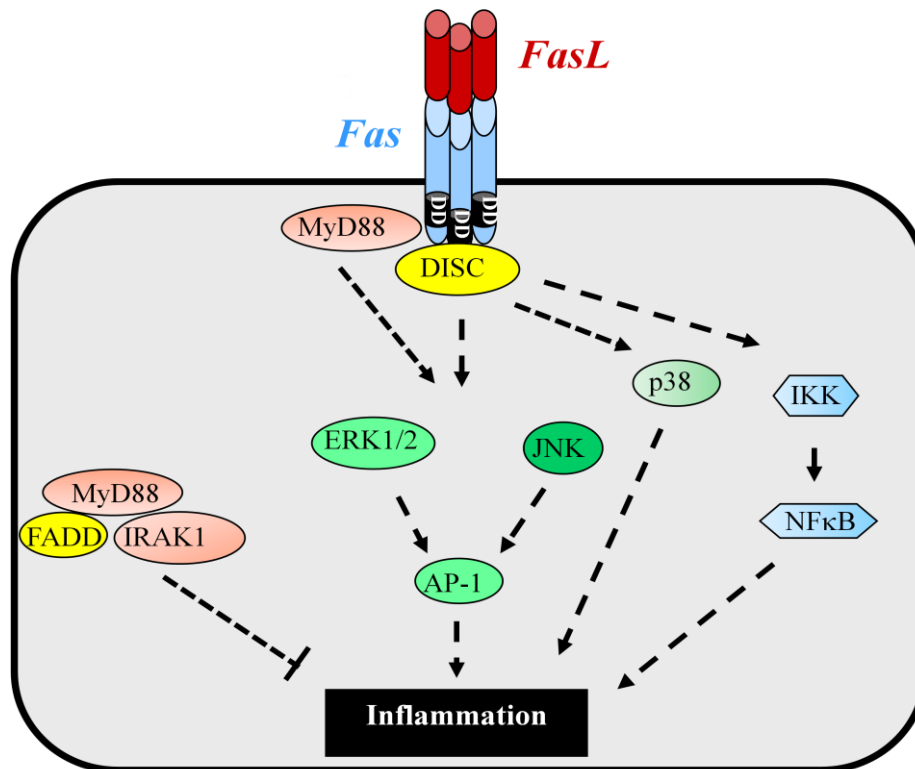
Fig.4 Signalling pathways involved in Fas-mediated inflammation. Activation of Fas may promote inflammation via activation of the p38, ERK and JNK MAPK signalling pathways and the NFκB transcription factor downstream of the DISC. Interaction of MyD88 directly with Fas may also activate the JNK and ERK MAPK signaling pathways, independently of FADD, and mediate Fas-induced inflammation. Alternatively, in the absence of Fas ligation, the Fas adaptor molecule, FADD may interact with MyD88 in the cytoplasm, potentially blocking/limiting MyD88 signalling. Dashed lines represent signalling pathways/intermediaries that not fully elucidated.
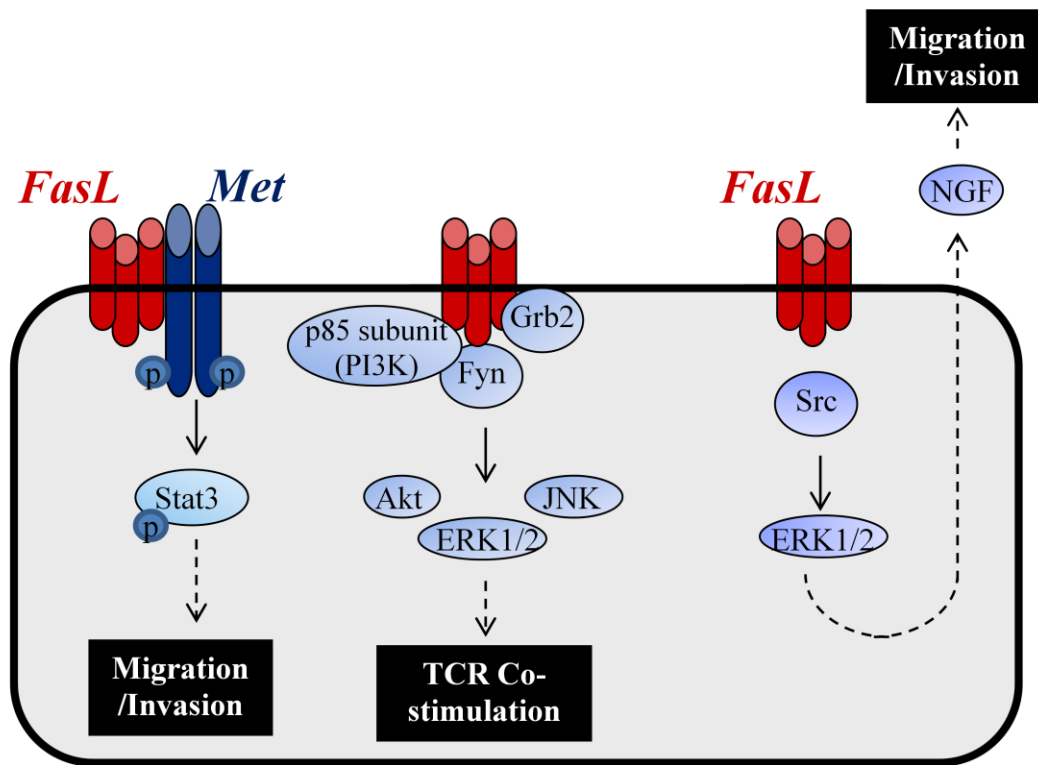
Fig.5 Reverse signalling through FasL. In addition to initiating signalling through Fas, membrane-bound FasL can also transmit a co-stimulatory signal in the reverse direction upon ligation of Fas, a process known as reverse or retrograde signaling. Binding of Src homology 3 domain-containing proteins such as Fyn, Grb2, and PI3K to FasL leads to the subsequent activation of the MAPK ERK1/2 signaling pathway. Interaction of FasL with the hepatocyte growth factor receptor, Met, can also activate the Met-Stat3 signaling pathway. Dashed lines represent signalling pathways/intermediaries that not fully elucidated.
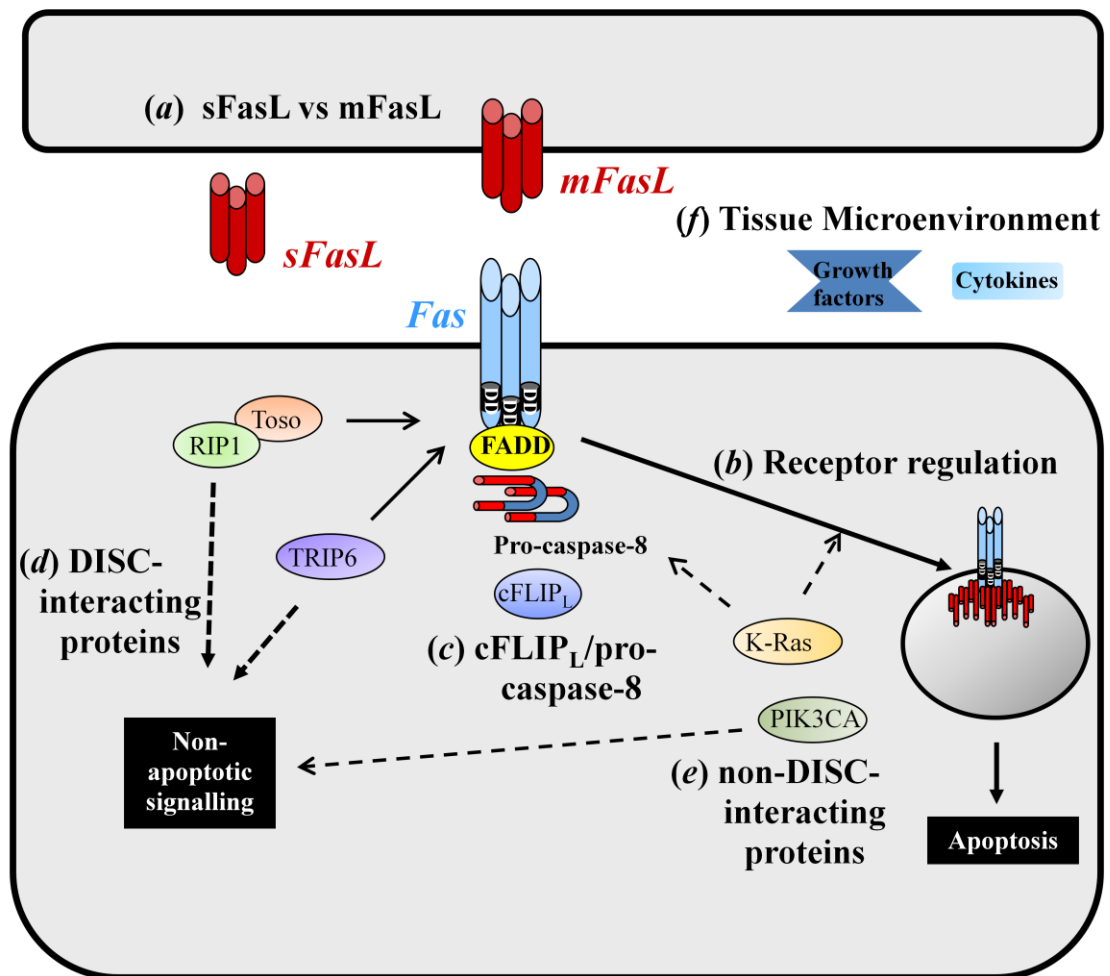
Fig.6 Multi-level regulation of the Fas signalling pathway. The outcome of Fas ligation is regulated at several points along the signalling pathway. (*a*) FasL may exist as either sFasL or mFasL, and they vary in their ability to induce apoptosis and/or non-apoptotic signalling. (*b*) At the receptor level, defective internalisation of the Fas signalling complex or the presence of mutations in one Fas allele may inhibit apoptosis and promote non-apoptotic signalling. (*c*) The balance between the ratio of cFLIP and pro-caspase-8 at the DISC may determine the outcome of Fas ligation. (*d*) Recruitment of proteins such as TRIP6 and Toso to the DISC can inhibit cell death, and promote the activation of non-apoptotic signalling pathways by Fas. (*e*) In addition to inhibiting apoptosis, oncogenic K-Ras and mutated PI3K catalytic subunit α (PIK3CA) promote Fas-mediated invasion through effects on receptor internalisation and the actin cytoskeleton. (*f*) Cell fate following Fas ligation was also shown to be regulated by growth factors and cytokines present in the cell microenvironment.