0.1 mM EDTA). Aliquots (10 μl) of the medium or cell lysates were incubated at 37°C for 3 h with 30 μM Ac-DEVD-AMC in 100 μl Assay Buffer (50 mM HEPES-NaOH buffer (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, and 10% glycerol), and the fluorescence was detected with an excitation wavelength of 360 nm and emission wavelength of 460 using a microplate reader (Infinite M200; Tecan, Männedorf, Switzerland). The specific caspase-3 activities were determined by subtracting the values obtained in the presence of 0.1 μM Ac-DEVD-CHO.

Western blot analysis. For western blotting, cells (5×10^5) were directly lysed by heating at 85°C for 30 min in 15 μl of sample buffer (30 mM Tris-HCl (pH 6.8), 1% SDS, 5% glycerol, 2.5% 2-mercaptoethanol, and 0.0005% BPB). Proteins were separated by 10–20% gradient SDS–PAGE, and transferred to PVDF membranes (Millipore). After blocking with blocking buffer (Tris-buffered saline containing 0.05% Tween 20 (TBST) supplemented with 5% non-fat dry milk or 4% Block Ace (DS Pharma Biomedical, Suita, Osaka, Japan)), the membranes were incubated at 4°C overnight with rabbit mAb against caspase-3 (clone 8G10; Cell Signaling) or mouse mAb against caspase-9 (clone 5B4; MBL, Nagoya, Japan) in blocking buffer. After washing with TBST, the membranes were incubated with HRP-conjugated anti-rabbit Ig or anti-mouse Ig antibody at room temperature for 1 h, and the proteins recognized by the antibody were visualized by a chemiluminescence reaction (Immobilon Western, Millipore). Western blotting with mouse mAb against BiP (Grp78) (clone 40; BD Biosciences) was performed as a loading control.

For immunoprecipitation, the culture supernatant was precleared by incubating twice at 4°C with a 1 : 1 mixture of nProtein A-Sepharose 4 Fast Flow (GE Healthcare, Uppsala, Sweden) and Protein G-Sepharose 4 Fast Flow (GE Healthcare) for 1.5 h. After removing Sepharose by centrifugation, the supernatant was incubated at 4°C overnight with rabbit mAb against the cleaved caspase-3 (clone 5A1E; Cell Signaling), followed by the incubation with Protein A-Sepharose. After washing with PBS containing 1% Triton X-100, the proteins bound to the beads were eluted by boiling in SDS sample buffer, separated by 10–20% SDS–PAGE, transferred onto a PVDF membrane, and subjected to western blotting with rabbit mAb against the cleaved caspase-3, followed by the incubation with HRP-conjugated Protein A (Bio-Rad, Hercules, CA, USA).

Conflict of interest

The authors declare no conflict of interest.

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The Many Roles of FAS Receptor Signaling in the Immune System

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FAS belongs to the subgroup of the tumor necrosis factor receptor (TNF-R) family that contains an intracellular "death domain" and triggers apoptosis. Its physiological ligand FASL is a member of the TNF cytokine family. Studies with mutant mice and cells from human patients have shown that FAS plays critical roles in the immune system, including the killing of pathogen-infected cells and the death of obsolete and potentially dangerous lymphocytes. Fas thereby functions as a guardian against autoimmunity and tumor development. FAS triggers apoptosis through FADD-mediated recruitment and activation of caspase-8. In certain cells such as hepatocytes, albeit not lymphocytes, FAS-induced apoptosis requires amplification through proteolytic activation of the proapoptotic BCL-2 family member BID. Curiously, several components of the FAS signaling machinery have been implicated in nonapoptotic processes, including cellular activation, differentiation, and proliferation. This review describes current understanding of Fas-induced apoptosis signaling and proposes experimental strategies for future advances.

Introduction

The cell surface-bound receptor FAS (also called APO-1 or CD95) was originally discovered as the target of two monoclonal antibodies that trigger apoptotic cell death in certain human tumor-derived cell lines in culture or as xeno-transplants in immunodeficient mice. Cloning of the gene encoding FAS revealed that it is a member of the tumor necrosis factor receptor (TNF-R) family (Itoh et al., 1991), which also includes the receptors for TNF, TRAIL (TNF-related apoptosis-inducing ligand), and those for other members of the TNF cytokine family. Accordingly, identification of the physiological ligand for FAS, called FAS ligand (FASL or CD95L) (Suda et al., 1993), through expression cloning demonstrated that it belongs to the TNF cytokine family. The discovery that certain spontaneous mutant mouse strains that develop lymphadenopathy and SLE (systemic lupus erythematosus)-like autoimmune disease carry homozygous defects in the genes encoding FAS (*Fas*$^{lpr/lpr}$ or *Fas*$^{lprcg/lprcg}$) (Watanabe-Fukunaga et al., 1992) or FASL (*Fasl*$^{gld/gld}$) (Takahashi et al., 1994) and the realization that a large fraction of human ALPS (autoimmune lympho-proliferative syndrome) patients have heterozygous inherited mutations in the *FAS* gene (Fisher et al., 1995; Rieux-Laucat et al., 1995) demonstrated that FASL-FAS signaling plays a critical role in the control of the immune system. ALPS patients (Straus et al., 2001) as well as the FAS- or FASL-deficient mice have an abnormally increased predisposition to lymphoma development, demonstrating that FASL-FAS signaling is also critical for tumor suppression, at least within the lymphoid compartment. FASL expressed on activated T lymphocytes or natural killer (NK) cells contributes to their ability to kill target cells, such as virus-infected or damaged cells. Abnormally increased FASL-mediated killing of healthy (FAS$^+$) bystander cells has been implicated in certain immunopathological states, such as hepatitis induced by extensive T cell activation. Pharmacological modulation of the FASL-FAS signaling machinery may therefore be a useful strategy for therapeutic intervention in certain diseases, but caution is warranted because administration of agonistic FAS-specific antibodies (Ogasawara et al., 1993) or FASL (Huang et al., 1999) cause extensive hepatocyte apoptosis and fatal hepatitis in mice. Detailed understanding of FASL-FAS signaling may allow the development of more subtle intervention strategies and, interestingly, one FAS-specific antibody was shown to cure *Fasl*$^{gld/gld}$ mutant mice from their lymphadenopathy without causing liver toxicity (Ichikawa et al., 2000).

Mechanisms of FASL-FAS Signaling Induced Apoptosis

It has been argued that researchers readily observe that FAS stimulation causes apoptosis because activators of FAS (agonistic antibodies or recombinantly produced forms of FASL) have been selected for this property and that we may therefore have overly emphasized this function of FASL-FAS signaling while underestimating other activities, such as induction of cellular proliferation or differentiation (Peter et al., 2007). However, activation of FAS by its physiological ligand, FASL, has certainly been proven to trigger apoptosis (Krammer, 2000; Nagata, 1997). For example, several studies have shown that cytotoxic T cells, which express FASL in membrane-bound form (mFASL) on their surface, can kill FAS$^+$ target cells, and neutralization of FASL with specific antibodies or FAS-Fc fusion proteins proved that this killing is mediated by FASL-induced activation of FAS (Krammer, 2000; Nagata, 1997). Moreover, restimulation of already activated normal T cells or T lymphoma-derived cell lines via their TCR (T cell antigen receptor) complex causes extensive suicide or fratricide, a process called activation-induced cell death (AICD). (It has recently been proposed to rename this process "restimulation-induced cell death" [RICD] in order to distinguish it more clearly from the cell death that can occur when naive T lymphocytes are stimulated for the first time with antigen or mitogen.) Experiments with *Fas*$^{lpr/lpr}$ or *Fasl*$^{gld/gld}$ mice or the aforementioned FASL blockers

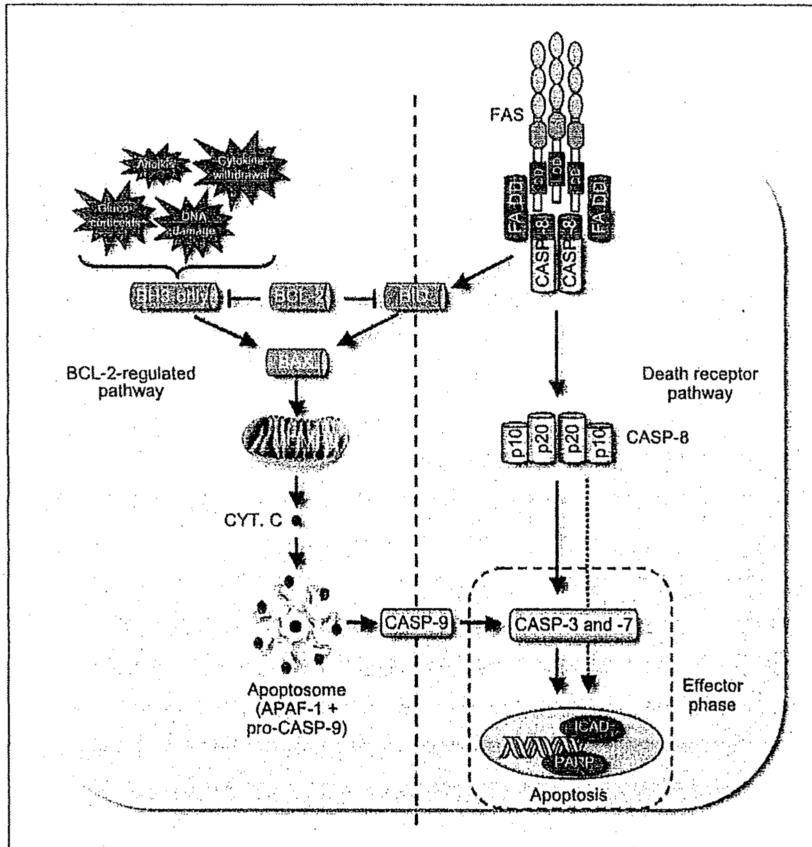

Figure 1. Death Receptor-Induced and Bcl-2-Regulated Apoptosis Signaling
This figure shows the two distinct but ultimately converging apoptosis signaling pathways in mammals. One is activated by so-called death receptors, members of the tumor necrosis factor receptor (TNF-R) family with an intracellular death domain, and requires FADD-mediated activation of caspase-8 (shown on the right). Auto-processing of caspase-8 results in a heterotetrameric form of the protein that is able to activate the zymogens of effector caspases, caspase-3 and caspase-7, eventually leading to cleavage of vital proteins within the cell, such as PARP or lamins, or activation of the DNase CAD (caspase-activated DNase) by cleavage of its inhibitor, ICAD (inhibitor of CAD). It is possible that caspase-8 may also proteolyze to a certain extent some of the classical substrates of effector caspases. The BCL-2-regulated pathway (shown on the left) is arbitrated by the complex interplay between pro- and antiapoptotic members of the BCL-2 protein family and involves mitochondrial outer membrane permeabilization (MOMP). MOMP results in release of cytochrome C from the intermembrane space of the mitochondria into the cytosol to initiate apoptosome formation resulting in APAF-1-mediated activation of the "initiator" caspase, caspase-9, further leading to proteolytic activation of the downstream effector caspases, caspase-3 and caspase-7. Abbreviations: BH3-only, BCL-2 homology domain 3 only protein; MOMP, mitochondrial outer membrane permeabilization; Cyt. C, cytochrome C; ICAD, Inhibitor of caspase activated DNase (CAD); PARP, Poly (ADP-ribose) polymerase.

showed that this apoptosis is caused (at least in part) by physiological FASL-FAS signaling (reviewed in Krammer [2000]; Nagata [1997]). It is widely believed that defects in this process underlie the lymphadenopathy and autoimmune disease that develop in mice and humans that have abnormalities in their genes encoding FAS or FASL (Krammer, 2000; Nagata, 1997).

Given that physiological FASL-FAS signaling can induce apoptosis, we first describe the mechanisms of this process, but nonapoptotic processes that are reportedly activated by FAS will also be discussed. Elegant biochemical studies have shown that ligation of FAS rapidly causes assembly of an intracellular "death-inducing signaling complex" (DISC) (Kischkel et al., 1995), which was shown to contain the aspartate-specific cysteine protease, caspase-8 (Boldin et al., 1996; Muzio et al., 1996), its adaptor/activator FADD (Boldin et al., 1995; Chinnaiyan et al., 1995), and its modulator c-FLIP (FLICE [i.e., caspase-8] inhibitory protein) (Irmler et al., 1997). The interaction between FAS and FADD requires homotypic interaction of "death domains" (DD) (Boldin et al., 1995; Chinnaiyan et al., 1995), which are present in the intracellular region of FAS also in FADD (Figure 1). The recruitment of caspase-8 by FADD is mediated (at least in part) by homotypic interaction of "death effector domains" (DED) (Boldin et al., 1996; Muzio et al., 1996), which are present in both proteins (Figure 1). Studies with gene-targeted mice and transgenic mice expressing a dominant-negative mutant of FADD (FADD-DN) or a viral enzymatic inhibitor of caspase-8 (CRMA, cytokine response modifier A) demonstrated that FADD-mediated activation of the proteolytic activity of caspase-8 is essential for FAS-induced apoptosis in many (possibly all) cell types (including lymphoid and other hemopoietic ones) both in vitro and within the whole animal (Kang et al., 2004, 2008; Newton et al., 1998; Salmena et al., 2003; Varfolomeev et al., 1998; Zhang et al., 1998). It is noteworthy that in human cells, FAS activation also causes recruitment of caspase-10 into the DISC and that in certain cell lines, caspase-10 activation was reported to contribute to apoptosis signaling. Mice do not have a gene encoding caspase-10, and because the activation and function of caspase-10 are thought to be very similar to those of caspase-8, we will not deal with caspase-10 in detail in this review. Caspase-8 activation within the DISC occurs in two steps. First, recruitment of FADD to the intracellular region of FAS promotes (via homotypic interaction of "death effector domain" [DED] recruitment) dimerization and a conformational change in caspase-8 within the DISC that allows caspase-8 to gain full enzymatic activity (Boatright et al., 2003). Second, active caspase-8 undergoes autoproteolytic processing, which allows the enzyme to leave the DISC and gain access to substrates in other cellular compartments (Boatright et al., 2003). Experiments with caspase-8-deficient (*Casp8*$^{-/-}$) mice carrying a BAC transgene encoding WT caspase-8 or a mutant of caspase-8 that has full enzymatic activity but cannot cleave itself demonstrated that autoproteolysis of caspase-8 is essential for FAS-induced apoptosis, at least in lymphoid cells and hepatocytes (Kang et al., 2008). This

indicates that upon activation, caspase-8 must leave the DISC to be able to trigger apoptosis, probably so that it can gain access to critical proteolytic substrates. Active caspase-8 can proteolytically activate downstream effector caspases, such as caspase-3 and caspase-7 (Figure 1), but (at least theoretically) this may also directly contribute to cell demolition by proteolysis of certain cellular proteins. During the later stages of apoptosis, effector caspases perform the bulk of the proteolysis of vital cellular proteins, such as structural components (e.g., lamins, gelsolin), and they can also activate certain processes that dismantle nonproteinaceaous cellular constituents (Salvesen and Dixit, 1997). For example, effector caspase-mediated cleavage of ICAD (inhibitor of caspase-activated DNase), which acts as both a chaperone and inhibitor of CAD (caspase-activated DNase), is critical for internucleosomal DNA cleavage, a hallmark of apoptosis (Salvesen and Dixit, 1997).

The c-FLIP protein, which structurally resembles caspase-8 (presence of DED but no enzymatic activity), appears to affect FAS-induced apoptosis in opposite ways depending on the extent of its expression (Figure 1). At low concentration it is thought to promote caspase-8 activation, possibly because caspase-8 binds to c-FLIP with higher affinity than to itself (i.e., in caspase-8 homodimerization). In contrast, at high concentration c-FLIP reduces the proteolytic activity of caspase-8, possibly by competing for binding to FADD, and thereby inhibits apoptosis (Boatright et al., 2003). Studies with gene-targeted mice confirmed that c-FLIP has a dual role, because its selective loss in T lymphocytes or fibroblasts accelerated their Fas-induced apoptosis (Zhang and He, 2005), although loss of c-FLIP in all tissues pheno-copied the embryonic lethality seen in *Casp8*$^{-/-}$ mice (Yeh et al., 2000), which is caused by loss of a nonapoptotic function of caspase-8 (Varfolomeev et al., 1998).

The proapoptotic BH3-only family member BID is a critical substrate of caspase-8. Its proteolysis allows the truncated BID (tBID) to translocate from the cytosol to the outer mitochondrial membrane, thereby unleashing its proapoptotic activity (Figure 1; Li et al., 1998; Luo et al., 1998). BH3-only proteins (BID, BAD, BIK [also called BLK or NBK], HRK [also called DP5], BIM [also called BOD], NOXA, Puma [also called BBC3], and BMF) form one of the two proapoptotic subgroups of the BCL-2 protein family. Their name derives from the fact that they share with each other and the BCL-2 family at large only the ~16–24 aa BH3 region (Huang and Strasser, 2000). The BH3-only proteins are essential for initiation of developmentally programmed cell death and stress-induced apoptosis and they are activated in a death stimulus- as well as cell type-specific manner (Huang and Strasser, 2000). BAX, BAK, and BOK belong to the second proapoptotic (often called the "BAX-BAK" or "multi-BH domain") subgroup of the BCL-2 family. These proteins contain three BH (BCL-2 homology) regions and although they share surprisingly extensive structural similarity to their prosurvival relatives, they are required for mitochondrial outer membrane permeabilization (MOMP) and activation of the caspase cascade in the "BCL-2-regulated" (also called "mitochondrial" or "intrinsic") apoptosis signaling pathway (Green and Kroemer, 2004). The antiapoptotic BCL-2 family members—BCL-2, BCL-XL, BCL-W, A1, and MCL-1—share with each other up to four regions of homology (BH1, 2, 3, and 4) and are essential for cell survival, functioning in a cell type-specific manner (Youle and Strasser, 2008). There are currently two models to explain the functional interactions of the three BCL-2 subgroups in apoptosis signaling. The "direct model" suggests that the BH3-only proteins bind and thereby activate BAX and/or BAK directly and that the prosurvival relatives function to bind and sequester the BH3-only proteins (Green and Kroemer, 2004). The "indirect model" proposes that in healthy cells BAX and BAK are kept in check by the prosurvival BCL-2 family members and that BH3-only proteins, when induced by apoptotic stimuli, bind with higher affinity to the prosurvival BCL-2-like proteins, thereby unleashing BAX and/or BAK (Youle and Strasser, 2008). Regardless, experiments with gene-targeted mice and biochemical studies have shown that caspase-8-mediated proteolytic activation of BID is essential for FAS-induced killing in hepatocytes (within the whole animal) (Kaufmann et al., 2007; Yin et al., 1999) and pancreatic β cells (in culture) (McKenzie et al., 2008). In contrast, BID is dispensable for Fas-induced apoptosis in lymphoid cells (both in culture and within the whole animal) and accordingly, in contrast to FAS- or FASL-deficient mice, *Bid*$^{-/-}$ mice do not develop lymphadenopathy or autoimmunity (Kaufmann et al., 2007, 2009). The reasons for this discrepancy in FAS-induced apoptosis signaling between so-called type 1 (e.g., lymphocytes) and type 2 (e.g., hepatocytes) cells are presently unclear (Scaffidi et al., 1998), but they may be due to differences in FAS aggregation or internalization or differences in the extent of caspase activation or the amount of substrates that must be proteolyzed for the cells to die (Peter et al., 2007).

Some time ago, it was proposed that FAS-induced apoptosis occurs not only when FASL-expressing killer cells engage FAS$^+$ target cells, but also when cells are treated with certain chemotherapeutic drugs or γ-radiation (Debatin and Krammer, 2004). It was suggested that these cytotoxic stimuli induce FASL expression (via ceramide induction) and/or increase surface membrane deposition of Fas via a P53-dependent mechanism, both processes ultimately leading to autocrine or paracrine FASL-FAS-induced apoptosis. Studies with cells from *Fas*$^{lpr/lpr}$ mice did, however, show that γ-irradiation and chemotherapeutic drugs do not require Fas to trigger apoptosis (Newton and Strasser, 2000). Moreover, experiments with primary cells from mutant mice lacking FADD (Newton et al., 1998; Newton and Strasser, 2000; Yeh et al., 1998; Zhang et al., 1998) or caspase-8 function (Salmena et al., 2003; Varfolomeev et al., 1998) demonstrated that "death receptor" signaling in toto is not required for these pathways to apoptosis. Although we cannot rule out the possibility that the mechanisms regulating apoptosis differ markedly among distinct tumor cells, expression of dominant-negative FADD, CRMA, or c-FLIP had no impact on chemotherapeutic drug-induced killing of a collection of lymphoma-derived cell lines, demonstrating that FADD and caspase-8 are dispensable for this process in at least certain cancers (Strasser et al., 1995, 2000). We surmise that increased expression of FAS, FASL, and certain other members of these receptor and ligand families in chemotherapeutic drug-treated or γ-irradiated cells is a consequence of cellular stress. Although this process is not required for the death of these cells, it may be exploited therapeutically as illustrated by the synergistic effects of TRAIL (APO-2L, a TNF cytokine family member) and certain chemotherapeutic drugs (e.g., 5-fluoro-uracil) in the killing of certain tumor cells (Ashkenazi et al., 1999).

Collectively, these observations demonstrate that FADD-mediated recruitment and activation of caspase-8 are essential for FAS- and all death receptor-induced apoptosis and that the BH3-only protein BID is critical for this cell killing in some, albeit not all, cell types.

The Role of FASL-FAS-Induced Apoptosis in Normal as well as Pathological Killing of Target Cells

Both classical $CD8^+$ cytotoxic T cells as well as a portion of activated $CD4^+$ T cells express FASL on their surface in membrane-bound form (mFASL). Cleavage by a metallo-protease causes shedding of FASL and the resulting soluble FASL, sFASL, can still bind FAS (Tanaka et al., 1995, 1998). Studies with various forms of FASL that were produced in *E. coli* or transfected mammalian cell lines have indicated that mFASL kills target cells via FAS activation much more potently than does sFASL (Schneider et al., 1998; Suda et al., 1997; Tanaka et al., 1998). This is consistent with the notion that extensive aggregation of multiple preassembled FAS trimers and not only FASL trimer-FAS trimer interaction is required for apoptosis induction (Siegel et al., 2000). There is also some confusion in the literature based on the findings that some cultured cell lines shed mFASL-bearing vesicles into the supernatant. Although such vesicles can kill FAS^+ cells in vitro (Schneider et al., 1998), the physiological relevance of this process is currently unclear.

Comparison of T cells lacking both functional FASL and perforin ($Fasl^{gld/gld}Prf1^{-/-}$) with $Prf1^{-/-}$ T cells demonstrated that these two cell death-inducing proteins account for most killing activity of cytotoxic T cells and NK cells (Kägi et al., 1994), possibly with some additional contribution by TNF-α. Whereas FASL normally contributes to the killing of virus-infected, damaged, or excess cells, abnormally increased FASL-induced apoptosis of cells that should not be killed has been implicated as a cause of certain immuno-pathological disorders. For example, it has been shown that acute graft-versus-host (GvH) disease in mice can be prevented by combined treatment with neutralizing antibodies to FASL plus TNF-α (Hattori et al., 1998). Moreover, FASL was found to be critical for the pathological killing of hemopoietic progenitors during cytomegalovirus (CMV) infection (Mori et al., 1997). Interestingly, transfer of lymphocytes from $Fas^{lpr/lpr}$ mutant mice into congenic recipients elicits fatal GvH. This is due to the fact that Fas-deficient T cells produce abnormally high amounts of mFASL, which kill FAS^+ target cells within the host (Watanabe et al., 1995), because transfer of lymphocytes from FASL-FAS doubly deficient ($Fasl^{gld/gld}Fas^{lpr/lpr}$) mice does not cause GvH disease (Zhu et al., 2000). When the Fas^{lpr} mutation was crossed onto the NOD background, it was observed that such NOD-$Fas^{lpr/lpr}$ mice do not develop diabetes and therefore concluded that FASL-FAS signaling is critical for the pathological destruction of islet β cells in type I diabetes (Chervonsky et al., 1997). The interpretation of this finding is, however, confounded by the complication that these animals rapidly develop profound lymphadenopathy and that $Fas^{lpr/lpr}$ (and $Fasl^{gld/gld}$) mice actually become highly immunocompromised when they develop lymphadenopathy. Indeed, experiments in which islets from NOD-$Fas^{lpr/lpr}$ mice were transplanted into NOD mice demonstrated that FASL-FAS signaling played only a minor role in β cell destruction in these diabetes-prone animals (Allison and Strasser, 1998). Conversely, experiments in which diabetogenic T cells from NOD mice were injected into NOD-$Fas^{lpr/lpr}$*scid/scid* recipients (precluding excess FASL expression in the host) showed that FAS contributes to β cell destruction, at least within this context (Su et al., 2000). Analysis of perforin-deficient NOD mice revealed that as for the physiological killing of virus-infected target cells, the perforin-granzyme system plays a major role in the pathological killing of β cells in type I diabetes (Kägi et al., 1997). On the basis of all these data, we conclude that perforin plays the principal and FASL a contributory role in β cell destruction during the development of type I diabetes.

Although FASL has been widely reported to be expressed predominantly (perhaps exclusively) in antigen-stimulated T lymphocytes (both $CD8^+$ and also some $CD4^+$ T cells) and NK cells (Krammer, 2000; Mabrouk et al., 2008; Nagata, 1997), some reports indicated that in certain tissues, such as the testis, thyroid, or eye, nonhemopoietic cells can express FASL and thereby kill infiltrating T cells to establish an immune-privileged niche (Bellgrau et al., 1995). However, subsequent studies with transplantation of pancreatic islets transgenically engineered to express FASL ectopically indicated that FASL expressed on nonhemopoietic cells may not be critical to establish immune privilege (Allison et al., 1997). It has also been proposed that FASL expression on cancer cells may render them refractory to immune attack by engendering them with the ability to kill FAS^+ tumor infiltrating lymphoid and myeloid cells, but this so-called "tumor counter-attack" hypothesis has also been questioned (Restifo, 2000). Indeed, enforced expression of FASL on certain tumor cells was found to enhance rather than delay their destruction in transplant recipients (Igney et al., 2000). At least some of the confusion concerning the expression of FASL may be due to the use of unreliable reagents for its detection. The generation of gene-targeted knock-in mice (and tumors derived from them) with inducible deletion of the *Fas* (Hao et al., 2004) or *Fasl* (Mabrouk et al., 2008) genes are expected to resolve some of these controversies.

The Role of FASL-FAS-Induced Apoptosis in Lymphocyte Homeostasis

Mice lacking FAS or FASL develop progressive lymphadenopathy, which predominantly involves the so-called "unusual" ($Thy\text{-}1^+CD4^-CD8^-TCR\alpha/\beta^+B220^+$) T cells, which are thought to derive from conventional T cells that have been repeatedly activated in vivo via their TCR complex (Krammer, 2000; Nagata, 1997). These mutant mice do, however, also accumulate several-fold increases in conventional $CD4^+$ as well as $CD8^+$ T lymphocytes and B lymphocytes (Krammer, 2000; Nagata, 1997). This led to the conclusion that FASL-FAS signaling plays a critical role in the homeostasis of the lymphoid system, most likely by killing unwanted cells at one or several developmental checkpoints. During both B as well as T lymphopoiesis in primary lymphoid organs, apoptosis plays a critical role in the killing of cells that are either "useless" or "potentially dangerous" (Strasser, 2005). For example, immature lymphoid cells that failed to productively rearrange the V, (D), and J gene segments encoding one of their IG or TCR chains and therefore cannot express surface antigen receptors (BCR or TCR) are deleted as are those expressing antigen receptors that bind self-antigens with high affinity (Strasser, 2005). Because TCR

stimulation-induced apoptosis (AICD) of T lymphoma-derived cell lines requires FASL-FAS signaling (reviewed in Krammer [2000]; Nagata [1997]), it was hypothesized that FAS-induced apoptosis may be critical for the deletion of autoreactive thymocytes and immature B cells in the bone marrow. Although some studies provided evidence that FAS is essential for the deletion of thymocytes specific for so-called endogenous or experimentally applied super-antigens (antigens that are presented by antigen-presenting cells [APC] to T cells as full proteins associated with MHC molecules and not like conventional antigens as peptide fragments embedded within the MHC), this is controversial (Villunger et al., 2004). More definitive experiments with TCR or BCR transgenic mouse models confirmed that FASL and FAS are dispensable for the deletion of autoreactive thymocytes (Sidman et al., 1992) as well as autoreactive B cells developing in the bone marrow (Rubio et al., 1996), respectively. Further studies with TCR transgenic mice showed that FAS is also not required for the rapid death of naive T cells that occurs upon their transfer into hosts expressing the cognate antigen (Davey et al., 2002). In fact, studies with transgenic or gene-targeted mice that lack FADD (Newton et al., 1998) or caspase-8 function (Salmena et al., 2003) selectively in T lymphoid cells showed that death receptor-induced apoptosis signaling in toto is not required for deletion of autoreactive thymocytes. Instead the process for killing autoreactive T and B cells during their development and even in their mature state relies on the BCL-2-regulated apoptotic pathway and is initiated by the proapoptotic BH3-only protein BIM (Bouillet et al., 2002; Davey et al., 2002; Enders et al., 2003).

Programmed cell death also plays a role in the shut-down of cellular as well as humoral immune responses (Figure 2; Sprent and Tough, 2001). In the case of acute immune responses, such as infection with a nonpersisting pathogen (or injection with a degradable antigen), foreign antigen-specific T and B cells can expand several hundred-fold in numbers (Sprent and Tough, 2001). Most differentiate into effector cells (cytotoxic as well as helper T cells and antibody-producing plasma cells), which help overcome the pathogens by killing them, either directly or indirectly through cytokine-mediated activation of cells of the innate immune system. A small number of activated B and T cells differentiate into so-called "memory" cells, which are long-lived and able to respond rapidly in response to challenge with the same pathogen, thereby providing the organism with long-lasting immunity (Sprent and Tough, 2001). After pathogens have been defeated, effector cells are no longer needed and in fact are potentially dangerous, so most undergo programmed cell death (Sprent and Tough, 2001). This apoptosis restores normal cellularity in peripheral lymphoid organs, thereby making space for the development of immune responses to subsequent infectious challenges. In addition, apoptosis of activated lymphocytes minimizes the collateral damage to healthy tissues that can be caused by the immune effector molecules (e.g., inflammatory cytokines, immune complexes, perforin-granzymes) (Sprent and Tough, 2001). In the case of chronic infections with persistent antigenic challenge, the killing of activated lymphocytes is critical to achieve a state of cohabitation between the pathogen and the host, so that the numbers of pathogens are kept at an acceptable titer and at the same limiting immune activation to minimize inadvertent destruction of vital tissues (Sprent and Tough, 2001).

When FASL and FAS were discovered to be critical for TCR stimulation-induced AICD of normal cycling T lymphoblasts and transformed T lymphoma-derived cell lines (reviewed in Krammer [2000] Nagata [1997]), it was widely speculated that FASL-FAS-induced apoptosis would also prove to be essential for the death of antigen-activated T and B cells during shutdown of immune responses. For the removal of activated T cells during an acute immune response, however, this model makes little sense. The induction of FASL on T lymphoid cells undergoing AICD requires TCR stimulation, but during termination of acute immune responses, T cells die after pathogens have been expelled, a time when no antigen is present to activate the TCR. Indeed, although it was initially reported that FASL and FAS are critical for the killing of activated T cells during termination of acute immune responses (e.g., in TCR transgenic mice injected with the cognate peptide), later experiments with mice injected with a single dose of the super-antigen staphylococcus enterotoxin B (SEB) (Hildeman et al., 2002) or challenged with herpes simplex virus (HSV, causing nonpersistent infection) (Pellegrini et al., 2003) demonstrated that FAS and TNF-R1 death receptor signaling are dispensable for this process. In fact, the killing of antigen-activated T cells during termination of acute immune responses appears to be initiated by a decline in the levels of cytokines that promote their survival (e.g., IL-2, IL-7), which triggers the BCL-2-regulated apoptotic pathway predominantly through activation of the BH3-only protein BIM (Figure 2; Hildeman et al., 2002; O'Connor et al., 1998; Pellegrini et al., 2003).

In contrast to acute immune responses, pathogens and their antigens persist during chronic immune responses, thereby potentially facilitating repeated stimulation of already activated T lymphocytes through their TCR, which would then lead to FASL upregulation and their FAS-induced suicide or fratricide (Krammer, 2000; Nagata, 1997). Indeed, the first clear demonstration for a role of FAS-induced apoptosis in the shut-down of a T cell immune response came from studies in which mice were repeatedly injected with SEB (Strasser et al., 1995), thereby mimicking a chronic infection. More recently, experiments with mice infected with persistent viruses (e.g., LCMV or mouse gamma herpes virus) showed that the killing of activated T cells during the ensuing chronic immune response requires both FASL-FAS and the proapoptotic BH3-only BCL-2 family member BIM (Figure 2; Hughes et al., 2008; Hutcheson et al., 2008; Weant et al., 2008). Thus, their death appears to be caused by FASL-FAS signaling, triggered by repeated TCR stimulation, and by the BCL-2-regulated apoptotic pathway, which presumably is initiated by growth factor deprivation-induced induction of BIM.

B lymphoid cells express Fas on their surface and can be killed by treatment with FASL or agonistic FAS-specific antibodies (Krammer, 2000; Nagata, 1997) and, accordingly, FASL-FAS signaling was found to play a critical role in the control of humoral immune responses (Figure 2). The consequences of FAS activation in B cells are critically influenced by the activity of other signaling pathways. BCR crosslinking or CD40 stimulation, particularly their combination, protect B lymphocytes from FAS-induced apoptosis (Rothstein et al., 1995). This protection appears to be mediated by activation of REL-NF-κB transcription factors, which activate expression of c-FLIP (Hennino

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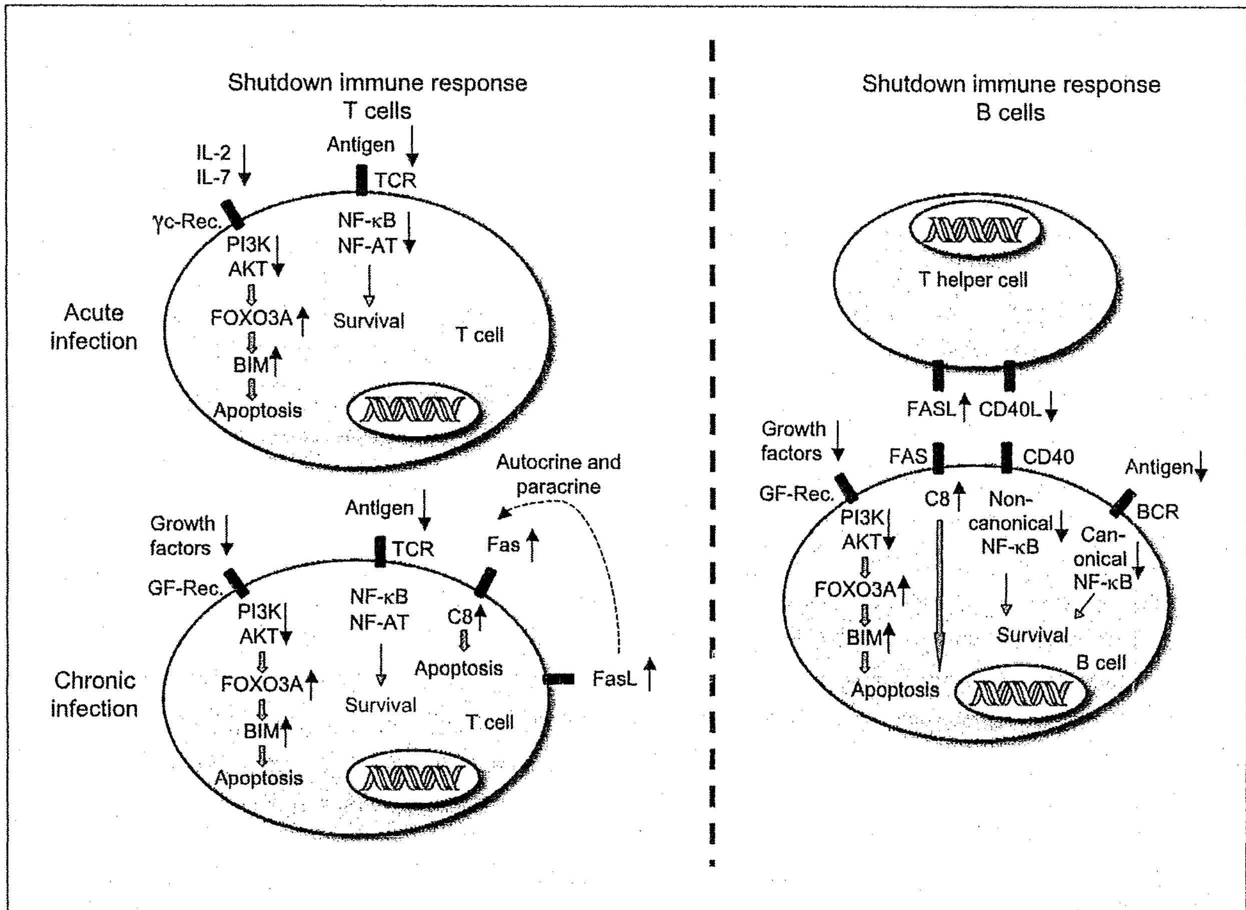

Figure 2. Fas-Mediated Death Receptor Signaling and Bcl-2-Regulated Apoptosis Signaling in the Killing of Activated T and B Lymphocytes
This figure shows the mechanisms that control FASL-FAS-induced apoptosis and BIM-dependent BCL-2-regulated apoptosis in activated T and B cells during shutdown of acute or chronic immune responses. During shutdown of an acute T cell immune response (left side, top), the reduction in the concentrations of cytokines, such as IL-2 and IL-7, leads to a reduction in PI3K and AKT. This causes activation of the transcription factor FOXO3A, which promotes transcriptional induction of the proapoptotic BH3-only protein BIM and consequently activation of the BCL-2-regulated apoptotic pathway. Furthermore, reduced antigen concentration leads to diminished TCR stimulation and consequently reduced activation of the REL-NF-κB and NF-AT transcription factors, which are known to promote expression of prosurvival proteins, such as BCL-2 or BCL-XL.
As in shutdown of acute T cell immune responses, during shutdown of a chronic T cell response (left side, bottom), the availability of growth factors is also reduced, leading to the activation of BIM and initiation of the BCL-2-regulated apoptotic pathway as described above. In addition, repeated TCR ligation triggers FASL expression on T cells leading to autocrine and/or paracrine stimulation of FAS, caspase-8 activation, activation of effector caspases, and consequently apoptosis as shown in Figure 1.
During shutdown of a B cell immune response (left side), FAS is induced on B cells and can be activated by FASL presented on T helper cells. This leads to B cell apoptosis when CD40 signaling is diminished because of reduced expression of CD40L on T helper cells, because this causes a reduction in the activity of the REL/NF-κB and NF-AT transcription factors, which normally promote expression of prosurvival proteins, such as c-FLIP. In addition, a reduction in the concentration of growth factors can lead to BIM upregulation as described above.
Abbreviations: γc Receptors, common gamma chain containing cytokine receptors (e.g., receptors for IL-2, IL-4, IL-7, IL-15); DISC, death-inducing signaling complex; GF-Rec., growth factor receptors; TCR, T cell antigen receptor; PI3K, phosphoinositide 3-kinases; BCR, B cell antigen receptor; C8, caspase 8.

et al., 2001). In fact, in combination with BCR-crosslinking and CD40 activation, FAS ligation may actually contribute to activation, proliferation, and differentiation of B cells, rather than trigger their apoptosis (Figure 2, and see below).

Germinal centers are anatomical niches within secondary lymphoid organs composed of B cells, specific CD4$^+$ T helper subsets, and follicular dendritic cells. In these locations, B cells and intrafollicular CD4$^+$ T cells recognizing the same antigenic compound are activated by interaction with each other and antigen retained on the follicular dendritic cells. This results in stimulation of B cells leading to their proliferation, hypermutation of the rearranged and expressed Ig variable region genes, and differentiation into antibody-secreting plasma cells on one hand and long-lived memory B cells on the other. During and after the germinal center reaction, activated B lymphocytes are subject to stringent selection. Only those B cells expressing high-affinity antibodies for the antigen (due to Ig V gene somatic hypermutation) can compete successfully for the limiting amount of antigen and T cell help (CD40L) and thereby gain survival signals through their BCR and CD40—low-affinity antibody-expressing B cells die by neglect. Theoretically, IGV gene hypermutation can give rise to B cells expressing BCRs specific

to self-antigens, and experiments with BCR transgenic mice showed that such autoreactive B cells are deleted by apoptosis (Shokat and Goodnow, 1995). FASL and FAS play critical roles in the control of B lymphocyte survival during the germinal center reaction (Figure 2; Krammer, 2000; Nagata, 1997). Activated B lymphocytes bearing low-affinity BCR are killed when FAS on their surface is ligated by FASL expressed on intrafollicular CD4$^+$ T cells and they fail to receive a prosurvival signal through their BCR and CD40 (Figure 2). In experimental models for deletion of autoreactive B lymphocytes within germinal centers, it was found that both FAS death receptor signaling as well as the BCL-2-regulated apoptotic pathway contribute to B cell killing (Figure 2; Rathmell et al., 1995). This is reminiscent of the finding that both of these pathways (the latter initiated by the proapoptotic BH3-only protein BIM) contribute to the killing of T cells that are chronically activated by pathogen-derived antigens or self-antigens (Hughes et al., 2008; Hutcheson et al., 2008; Weant et al., 2008). In further support of a collaboration of the FAS death receptor and the BCL-2-regulated apoptotic pathways in the deletion of autoreactive B and T cells in peripheral lymphoid organs and establishment of immunological tolerance, it was found that BCL-2 overexpression (Strasser et al., 1995) or loss of BIM (Hughes et al., 2008; Hutcheson et al., 2008; Weant et al., 2008) greatly accelerate and enhance lymphadenopathy and autoimmunity in FAS- or FASL-deficient mice. BIM was shown to play a critical role in the killing of activated B cells and IG-secreting plasma cells during shut-down of acute humoral immune responses (Fischer et al., 2007), and this BH3-only protein is probably activated by the decline in the concentration of growth factors (Figure 2).

Collectively, these results demonstrate that FAS and death receptor signaling in toto play no role in the deletion of autoreactive B or T cells during their development in primary lymphoid organs. Instead these pathways are critical, either alone or in combination with the BCL-2-regulated apoptotic pathway, for the killing of antigen-activated lymphocytes during termination of immune responses.

Unexpected Lessons from Deletion of Fas in Select Cell Types

Because FAS is expressed on a broad range of lymphoid, myeloid, and also nonhemopoietic cell types, it is not immediately clear in which cells FAS function must be lost to cause the lymphadenopathy and autoimmunity observed in FAS-deficient mice and humans (Krammer, 2000; Nagata, 1997). This important question has been addressed by generating FAS-deficient mice (*Fas$^{lpr/lpr}$*) that express a FAS transgene in either B and/or T cells (Fukuyama et al., 1998, 2002; Komano et al., 1999) or mice in which the sequences within the *Fas* gene that encode the death domain have been flanked by *loxP* sites and can therefore be deleted in a cell type-specific or inducible manner (via transgenic mice expressing the CRE recombinase under control of a cell type-specific or inducible promoter) (Hao et al., 2004, 2008). When interpreting data from the latter mice (and the *Fas$^{-/-}$* mice [Adachi et al., 1995]), it is important to remember that cells that have undergone CRE-mediated deletion of these sequences can still express FAS (devoid of the death domain) on their surface. It has been speculated that such a truncated FAS may still be able to exert some nonapoptotic functions either directly or indirectly by so-called "reverse signaling" via FASL (Peter et al., 2007; see also below). It must also be borne in mind that several of these experiments were performed on different inbred or even mixed genetic backgrounds, so the results may not always be directly comparable, because the impact of the *Fas^{lpr}* and the *Fasl^{gld}* mutations are greatly affected by genetic background. Nonetheless, these studies revealed many interesting aspects of the role of FASL-FAS signaling in the control of the normal immune system and prevention of autoimmunity (see references above). Transgenic expression of normal amounts of FAS in T cells of *Fas$^{lpr/lpr}$* mice prevented lymphadenopathy, in particular the accumulation of the unusual TCR-α/β^+CD3$^+$CD4$^-$CD8$^-$B220$^+$ T cells, but these animals still produced autoantibodies and developed SLE-like glomerulonephritis (Fukuyama et al., 1998). In contrast, transgenic expression of FAS on B cells protected *Fas$^{lpr/lpr}$* mutant mice from SLE-like autoimmunity although abnormal accumulation of T cells, including TCR-α/β^+CD3$^+$CD4$^-$CD8$^-$B220$^+$ ones, was not affected (Komano et al., 1999). Remarkably, with increasing age these mice developed a severe deficit in B cells and antibody-secreting plasma cells and this appeared to be due to the excess FASL produced by the abnormally accumulating T cells (Watanabe et al., 1995) triggering apoptosis of B lymphoid cells via the transgene-encoded FAS (Komano et al., 1999). Generation of *Fas$^{lpr/lpr}$* mutant mice that lack B cells because they carry a homozygous deletion of the J_H gene locus (precluding IGH expression) showed that B cells are required for the SLE-like autoimmunity but dispensable for the T cell-associated lymphadenopathy that is caused by defects in FAS (Shlomchik et al., 1994).

Selective loss of FAS on T cells (in *Cd4-Cre* or *lck-Cre* transgenic *Fas$^{loxP/loxP}$* mice) caused progressive lymphopoenia on the C57BL/6 background albeit to a lesser extent on a (C57BL/6xMRL) F1 background (Hao et al., 2004). This cell loss was mediated by the excessive FASL produced by the FAS-deficient T lymphocytes (Watanabe et al., 1995), because it could be prevented by injection with neutralizing antibodies to FasL (Hao et al., 2004). B lymphocyte deletion in these animals was probably mediated directly, as they express FAS on their surface, but such a process cannot explain the loss of T cells, since they lack FAS. Their progressive disappearance may be due to an indirect mechanism, which involves FASL-mediated killing of FAS$^+$ cells that deliver critical survival signals to T lymphocytes. B lymphocytes account for some, albeit clearly not all, of these survival signals, because T cell lymphadenopathy with CD4$^+$, CD8$^+$, as well as the unusual TCR-α/β^+CD3$^+$CD4$^-$CD8$^-$B220$^+$ T cells is substantially diminished (although not abrogated) in B cell-deficient *Fas$^{lpr/lpr}$* mutant mice compared to control *Fas$^{lpr/lpr}$* animals (Shlomchik et al., 1994).

Loss of FAS on B cells or on both B cells plus T cells (in *Cd19-Cre* transgenic or *Cd19-Cre-Cd4-Cre* bitransgenic *Fas$^{loxP/loxP}$* mice) elicited lymphadenopathy, hypergammaglobulinemia, and autoantibody production (Hao et al., 2004, 2008). All of these abnormalities developed in these animals, however, to a considerably lower extent and more slowly compared to animals with loss of FAS in all tissues or in all hemopoietic cells (Mx-Cre transgenic *Fas$^{loxP/loxP}$* mice injected with poly-IC). Interestingly, specific loss of FAS-mediated apoptosis in dendritic cells (with

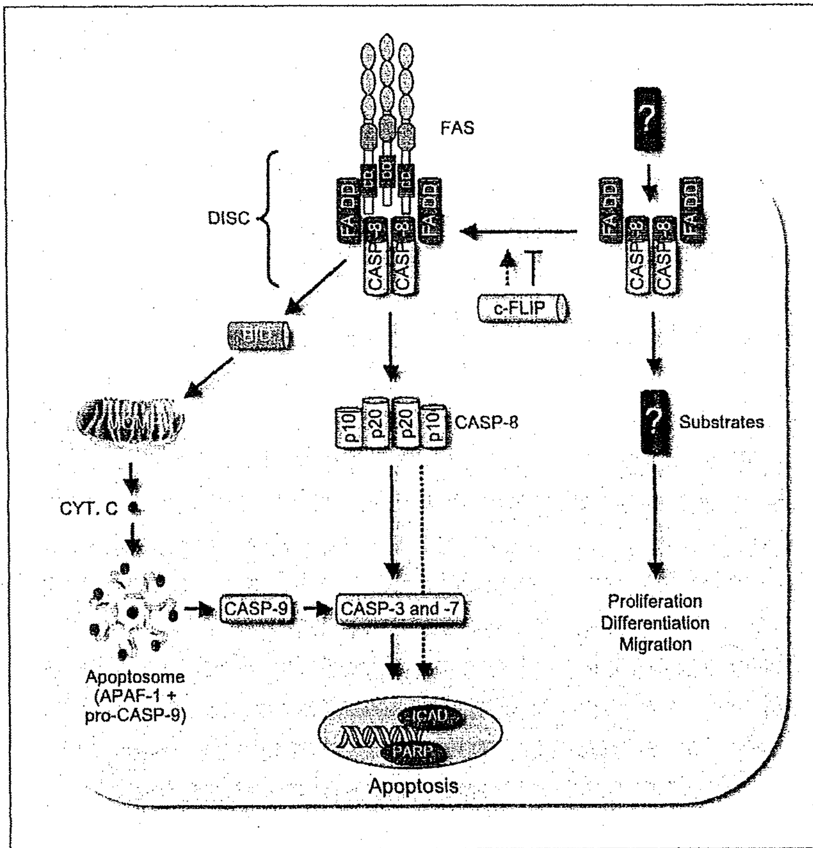

Figure 3. Nonapoptotic Signaling by Components of the Death Receptor Pathway

This figure shows the two distinct modes of caspase-8 activation: the well-known death receptor apoptotic pathway involving caspase-8 autoprocessing (shown on the left) followed by either direct activation of effector caspases or proteolytc activation of the BH3-only protein BID to activate the BCL-2-regulated apoptotic pathway (see also Figure 1). The caspase-8-related protein c-FLIP mainly acts as a catalytically inactive caspase-8 substitute competing for the binding to FADD, thereby limiting recruitment and activation of caspase-8 and thus blocking apoptosis initiation. However, c-FLIP has also been shown to promote caspase-8 recruitment and activation in certain circumstances. The right side illustrates the presently only poorly understood pathway by which FADD and caspase-8 promote cellular activation, proliferation, and differentiation without the need of self-processing. For this pathway, neither the upstream activators (death receptors?) nor the substrates of caspase-8 are identified. Abbreviations: DISC, death-inducing signaling complex; MOMP, mitochondrial outer membrane permeabilization; Cyt. C, cytochrome C; c-FLIP, cellular form of FADD-like IL-1β-converting enzyme-inhibitory protein; casp-8, caspase-8.

CD11c-CRE transgenic *Fas*$^{loxP/loxP}$ mice) also elicits certain features of autoimmunity, such as lymphoid hyperplasia and production of antinuclear autoantibodies (Stranges et al., 2007). Collectively, these results demonstrate that FAS imposes a barrier against lymphadenopathy and autoimmunity by acting not only in B and T cells but by functioning also in certain other hemopoietic and possibly nonhemopoietic cell types.

A recent study (with *IgHg1-Cre* transgenic *Fas*$^{loxP/loxP}$ mice, in which the *Fas* gene is deleted only in B cells that have switched from IGM to IGG production) has shown that FAS plays a particularly prominent role in the control of B as well as T cell homeostasis and autoantibody production in germinal center B cells (Hao et al., 2008). All immune defects caused by loss of FAS in these IGG$^+$ B cells could be prevented by loss of T cells (by deletion of the locus encoding the β chain of the TCR) or by blockade of the mutual costimulatory signals that are exchanged between activated B cells and activated CD4$^+$ T cells within germinal centers (by deletion of the *Cd28* gene) (Hao et al., 2008). These findings are consistent with the model (see also above) that antigen-stimulated B cells in germinal centers, which as a result of IGV gene somatic hypermutation express either a self-antigen-specific BCR or a BCR with low affinity for the immunogen, are killed by encountering FASL on activated intrafollicular CD4$^+$ T cells. It will be interesting to test this hypothesis by generating mutant mice that lack FASL exclusively within this T cell subset, and to examine how T cells recognize autoreactive B cells to kill them via FASL-FAS signaling.

Nonapoptotic Activities of Components of the Death Receptor Signaling Machinery

It is firmly established that several members of the TNF-R family, including some that are classified as death receptors (e.g., TNF-R1), and their corresponding ligands exert (either exclusively or in addition to their prodeath activity) nonapoptotic functions, such as the induction of cellular activation, proliferation, differentiation, or migration (Figure 3; Krammer, 2000; Nagata, 1997; Peter et al., 2007). Also for FAS activation, several investigations (even quite early ones in the course of studies of this receptor) have observed nonapoptotic consequences in a range of cell types. For example, FAS was reported to promote proliferation of human T lymphocytes as well as growth factor-deprived fibroblasts and maturation of dendritic cells in culture (reviewed in Peter et al. [2007]). Perhaps most impressively (and intriguingly), although injection of agonistic FAS-specific antibodies (Ogasawara et al., 1993) or FASL causes fatal hepatitis in mice (Huang et al., 1999), FAS stimulation (with agonistic FAS-specific antibodies) was reported to accelerate liver regeneration in mice subjected to partial hepatectomy (reviewed in Peter et al. [2007]). Interestingly, a delay in liver regeneration was seen in *Fas*$^{lpr/lpr}$ mutant mice as well as TNF-R1-deficient animals (reviewed in Peter et al. [2007]), implicating both of these death receptors in this process. The mechanisms by which FAS ligation stimulates cell proliferation and/or maturation are presently unclear, but the REL/NF-κB and MAP kinase signaling pathways have both been implicated (reviewed in Peter et al. [2007]). Interestingly, normal liver regeneration after partial hepatectomy was seen in *Fas*$^{lpr(cg)/lpr(cg)}$ mutant mice although *Fas*$^{lpr/lpr}$ mice exhibited a significant delay. The *Fas*$^{lpr(cg)}$ mutation causes an amino acid substitution within the death domain of

FAS (Watanabe-Fukunaga et al., 1992) that is thought to prevent recruitment of FADD into the DISC. This may indicate that FAS-induced cell growth, at least in hepatocytes, occurs by a mechanism that is independent of FADD and therefore probably also caspase-8. This idea is, however, contradicted by the finding that selective loss of caspase-8 in hepatocytes (in *Alb-Cre* transgenic *Casp8*$^{loxP/loxP}$ mice) impairs liver regeneration after partial resection (Ben Moshe et al., 2007).

Although the physiological importance of nonapoptotic signaling pathways activated by Fas remains controversial (Krammer, 2000; Nagata, 1997; Peter et al., 2007), it is now widely accepted that essential components of the death receptor machinery also perform critical nonapoptotic roles (Figure 3; Newton and Strasser, 2003). This was first discovered when it was observed that blocking the function of FADD does not only inhibit FASL-induced apoptosis of T lymphocytes but also impairs their activation and proliferation in response to mitogenic or antigenic stimulation (Newton et al., 1998; Zhang et al., 1998). Subsequently, it was found that FADD is also critical for pre-TCR-induced proliferation of T cell progenitors (pro-T3 and/or pro-T4 cells) in the thymus (Newton et al., 2000), TLR (Toll-like receptor)-mediated innate immune responses (Balachandran et al., 2004) (a process that notably also requires FADD in *Drosophila* [Hoffmann, 2003]), TLR-induced proliferation of B cells, and cytokine-induced proliferation of myeloid progenitors (Pellegrini et al., 2005). Experiments with gene-targeted mice in which the *Casp8* gene was deleted in a cell type-specific manner (with crosses with appropriate *Cre* transgenic mice) confirmed that most of these processes require not only FADD but also caspase-8 (Kang et al., 2004; Salmena et al., 2003). In addition, caspase-8 was found to be essential for cytokine-induced monocyte differentiation in culture (Kang et al., 2004) and, as already discussed above, for liver regeneration within the whole animal after partial hepatectomy (Ben Moshe et al., 2007). Remarkably, loss of caspase-8 (Varfolomeev et al., 1998), its activator FADD (Yeh et al., 1998), or its modulator c-FLIP (Yeh et al., 2000) in all tissues causes early embryonic lethality (~E10.5) because of nonapoptotic defects in vascular development and early hemopoiesis. It is presently not fully resolved whether these nonapoptotic processes require the enzymatic activity of caspase-8 or some other function of this protease, but there is evidence that the former may well be the case, at least for some. For example, in tissue culture, enzymatic inhibitors of caspase-8 were found to impair mitogen- or antigen-induced T cell proliferation (Kennedy et al., 1999) and cytokine-induced proliferation of myeloid progenitors (Pellegrini et al., 2005). We anticipate that the generation of gene-targeted mice that contain a mutation that disables the catalytic activity of caspase-8 may resolve this issue.

Accepting that FADD-mediated activation of caspase-8 is essential for the aforementioned nonapoptotic processes, one must ask the following two questions: (1) how are FADD and caspase-8 activated during these processes and (2) what intracellular nonapoptotic signaling pathways are triggered by caspase-8 (Figure 3)? Both of these important questions are currently unresolved and are considered by some as a "holy grail." Death receptors are the only presently known activators of FADD and caspase-8, and it is interesting to contemplate that low level ("tonic") autocrine or paracrine death ligand-death receptor signaling may play a critical role in cell fate determination during embryogenesis and in other scenarios (see above). Such tonic autocrine and/or paracrine TNF-TNF-R1 signaling has recently been found to play an essential role in the response of tumor cells to SMAC (second mitochondria-derived activator of caspases, also called DIABLO, direct inhibitor of apoptosis protein binding-protein with low pI) mimetic IAP (inhibitor of apoptosis protein) inhibitory drugs. There is, however, currently no evidence that death receptors and their ligands are essential for the established nonapoptotic functions of FADD and caspase-8. None of the gene-targeted mice lacking one or even two death receptors (FAS, TNF-R1, DR3, or TRAIL-R) or death ligands exhibit early embryonic lethality or defects in mitogen-induced activation and proliferation of B and/or T lymphocytes (reviewed in Newton and Strasser [2003]; Peter et al. [2007]). It remains, however, possible that there is greater functional overlap between death receptors than currently anticipated, and defects in vascular development during embryogenesis or mitogen-induced T cell proliferation, akin to those caused by loss of FADD or caspase-8, may become apparent only in mice lacking a combination of three or all four of these receptors or their ligands. Alternatively, there may be mechanisms for FADD and caspase-8 activation that are independent of death receptors (Figure 3). Perhaps biochemical studies, with pull-down of FADD and/or caspase-8 containing complexes from cells in which these proteins fulfil a nonapoptotic function, will be able to identify the mechanisms of their activation.

There is increasing evidence that FADD and caspase-8 must undergo different post-translational modifications and must be localized to different subcellular compartments depending on whether they mediate cell death (e.g., after treatment of T cells with FASL) or transduce cellular activation signals (e.g., after mitogenic stimulation of T cells) (O'Reilly et al., 2004). For example, a single amino acid within FADD was reported to be critical for its action in cell proliferation but apparently has no role in FADD-mediated apoptosis (Hua et al., 2003). Interestingly, T cells undergoing FASL-induced apoptosis contain a large amount of caspase-8 activity and most of it is found in the cytosol, whereas mitogenically activated T cells have considerably lower levels of caspase-8 activity, which is mostly concentrated in discrete foci at the plasma membrane (Koenig et al., 2008). Studies with mice expressing mutant forms of caspase-8 have shown that although autoproteolysis is required for the ability of this caspase to mediate FASL-induced cell killing, this processing is dispensable for its nonapoptotic functions (Figure 3; Kang et al., 2008). This is consistent with the notion that caspase-8 must be released from the DISC to gain access to critical substrates (i.e., the zymogens of the "effector" caspases) within the cytosol to effect cell killing, but must be retained at the plasma membrane to mediate its nonapoptotic functions. Identification of the substrates that are cleaved by caspase-8 near the plasma membrane is expected to greatly advance our understanding of its function in nonapoptotic processes. Although one study reported that caspase-8 is essential for TCR-ligation-induced REL/NF-κB activation, others found that REL/NF-κB, MAP kinase, and NFAT activation all occur normally in the absence of FADD or caspase-8 function in TCR- or TLR-stimulated T and B cells, respectively (Beisner et al., 2003; Newton et al., 2001; Salmena et al., 2003). It is

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also noteworthy, that for at least some of the processes in which FADD and caspase-8 play an essential nonapoptotic function, such as vascular development during embryogenesis, there is no evidence that REL/NF-κB plays a critical role. We find it remarkable how diverse the processes actually are in which FADD and caspase-8 play a critical nonapoptotic function, impacting on the responses of cells to ligation of cytokine receptors, antigen receptors, or TLRs. Interestingly, many of the nonapoptotic processes in which FADD and caspase-8 play a role involve transition of cells from the quiescent (G_0) into the cycling state. Therefore, and because pathways that are critical for cellular activation and proliferation (e.g., activation of REL/NF-κB, MAPK, NFAT) occur normally in their absence, we speculate that FADD and caspase-8 trigger a nonapoptotic function that modifies or facilitates the responses of other signaling pathways within cells. Alternatively (and not mutually exclusive), it is possible that FADD and caspase-8 play a critical role in the regulation of autophagy (Bell et al., 2008), a mechanism for procurement of energy and metabolites (e.g., in starved or stressed cells) that impacts on many cellular and developmental processes. As mentioned above, identification of binding partners for FADD and substrates of caspase-8 within mitogenically activated T cells or cytokine-stimulated monocytes may open an entire new area of investigation.

Collectively, these observations demonstrate that FADD and caspase-8 are not only essential for death receptor-induced apoptosis signaling but also have critical, albeit biochemically still ill-defined, roles in cellular activation, proliferation, and differentiation.

Conclusions and Perspectives

We now have a very good framework of understanding of the role of FASL-FAS-induced apoptosis in the control of the immune system and its critical function as a guardian against autoimmune disease and certain lymphoid malignancies. This knowledge is being exploited to develop novel cancer therapies, such as TRAIL-R-specific agonists or SMAC/DIABLO mimetics. Many anticancer drugs have proven to be efficacious in the treatment of (certain) autoimmune diseases (e.g., Rituximab, CD20-specific antibodies), so it is possible that the aforementioned compounds that are currently being developed may also become useful in the treatment of such diseases.

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LETTERS

Regulation of the innate immune response by threonine-phosphatase of Eyes absent

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Innate immunity is stimulated not only by viral or bacterial components, but also by non-microbial danger signals (damage-associated molecular patterns)[1]. One of the damage-associated molecular patterns is chromosomal DNA that escapes degradation. In programmed cell death and erythropoiesis, DNA from dead cells or nuclei expelled from erythroblasts is digested by DNase II in the macrophages after they are engulfed. *DNase II$^{-/-}$* (also known as *Dnase2a$^{-/-}$*) mice suffer from severe anaemia or chronic arthritis due to interferon-β (IFN-β) and tumour necrosis factor-α (TNF-α) produced from the macrophages carrying undigested DNA[2,3] in a Toll-like receptor (TLR)-independent mechanism[4]. Here we show that Eyes absent 4 (EYA4), originally identified as a co-transcription factor, stimulates the expression of IFN-β and CXCL10 in response to the undigested DNA of apoptotic cells. EYA4 enhanced the innate immune response against viruses (Newcastle disease virus and vesicular stomatitis virus), and could associate with signalling molecules (IPS-1 (also known as MAVS), STING (TMEM173) and NLRX1). Three groups have previously shown that EYA has phosphatase activity[5–7]. We found that mouse EYA family members act as a phosphatase for both phosphotyrosine and phosphothreonine. The haloacid dehalogenase domain at the carboxy terminus contained the tyrosine-phosphatase, and the amino-terminal half carried the threonine-phosphatase. Mutations of the threonine-phosphatase, but not the tyrosine-phosphatase, abolished the ability of EYA4 to enhance the innate immune response, suggesting that EYA regulates the innate immune response by modulating the phosphorylation state of signal transducers for the intracellular pathogens.

We previously showed that *DNase II$^{-/-}$* mouse embryonic fibroblasts (MEFs) express CXCL10 and IFN-β when they engulf *Cad$^{-/-}$* apoptotic cells[4]. We used this system to identify molecules involved in the DNA-induced innate immune response. A MEF complementary DNA library (about 20,000 clones) in a retrovirus-based expression vector was prepared, and divided into 400 pools. Retrovirus was produced for each group (see Methods), and used to infect *DNase II$^{-/-}$* MEFs. The MEFs were incubated with apoptotic *Cad$^{-/-}$* thymocytes, and CXCL10 levels in the culture supernatant were quantified. One pool of cDNAs that increased the concentration of CXCL10 from 402 to 1,414 pg ml^{-1} was subjected to sib-selection, which led to the identification of EYA4. When *DNase II$^{-/-}$* MEFs expressing mouse EYA4 were exposed to apoptotic thymocytes, they produced 3.7- and 25.7-fold more CXCL10 and IFN-β protein, and 8.3- and 11.3-fold more of their transcripts, than the parental cells, respectively (Fig. 1a, b and Supplementary Fig. 1). Overexpressing DNase II in the *DNase II$^{-/-}$* MEFs blocked the production of CXCL10 and IFN-β, indicating that the effect of EYA4 was on the signal transduction triggered by the undigested DNA.

When wild-type MEFs were transfected with *Escherichia coli* or mammalian DNA, or with polyinosinic-polycytidylic acid (hereafter poly(I:C)), they expressed *Ifnb* messenger RNA[1,8,9] (Fig. 1c). This response was enhanced 6–20-fold by EYA4. When Namalwa cells were infected with Newcastle disease virus (NDV), they expressed IFN-β in a dose-dependent manner (Fig. 1d): the level of IFN-β was 3.8- and 12.1-fold higher in two *Eya4*-expressing clones than in the parental cells. Accordingly, the titre of Vesicular stomatitis virus (VSV) produced in MEFs expressing EYA4 was about 10 times lower than that in the parental MEF (Fig. 1e). When fetal liver macrophages were intracellularly challenged with poly(I:C), they expressed IFN-β, and this was enhanced by EYA4 (Fig. 1f). On the other hand, the expression of IFN-β by the extracellular poly(I:C), lipopolysaccharide (LPS), or CpG oligonucleotide was not enhanced by EYA4. These results indicated that EYA4 specifically enhanced the innate immune response triggered by intracellular sensors. The mammalian EYA family is comprised of four members[10]. The poly(I:C)-induced expression of the *Ifnb* gene was 4–10 times higher in MEFs expressing EYA1, EYA2, EYA3 or EYA4 than in vector-transfected cells (Supplementary Fig. 2). Reduction of EYA expression with a mixture of short-interfering RNAs (shRNAs) against four human *EYA* genes in 293T cells diminished the poly(I:C)-induced *IFNB* expression, and this could be rescued by the expression of mouse EYA4 that does not carry the target sequence for shRNA (Fig. 1g).

Promoters of the *Ifnb* and *Cxcl10* genes carry interferon regulatory element (IRE) and NF-κB sites essential for their expression[11,12]. A deficiency of both *Irf3* and *Irf7* in MEFs completely abrogated the apoptotic-cell-induced production of CXCL10 in EYA4-expressing cells, and co-expression of IRF3 enhanced the effect of EYA4 (Fig. 2a). The poly(I:C)-induced activation of IRF3 (its phosphorylation at Ser 388 (ref. 13) and dimerization[14]), as well as the activation of NF-κB were enhanced by EYA4 (Fig. 2b, c and Supplementary Fig. 3). IPS-1 works as an adaptor[15,16] for the RIG-I (also known as DDX58) and MDA5 (IFIH1) system; MyD88 and TRIF (TICAM1) are adaptors for the TLR system[8]. The IPS-1-mediated activation of the *Ifnb* promoter[15] was 6.1-fold stronger in EYA4-expressing MEFs than in wild-type MEFs. In contrast, the expression of EYA4 had no effect on the MyD88- and TRIF-induced activation of the *Ifnb* promoter (Fig. 2d). When EYA works as a co-transcription factor for the myogenin (*Myog*) gene, it translocates from the cytoplasm to the nucleus[17]. In contrast, EYA4 in MEFs remained in the cytoplasm after stimulation with poly(I:C) (Fig. 2e). When Flag-tagged EYA4, and haemagglutinin (HA)-tagged IPS-1, STING[18] or NLRX1 (refs 19, 20) were expressed in 293T cells, IPS-1, STING and NLRX1 interacted with EYA4 (Fig. 2f and Supplementary Fig. 4). The endogenous IPS-1 interacted with EYA4 transiently after poly(I:C) treatment (Fig. 2g), suggesting that the interaction between EYA4 and IPS-1 is stimulus-dependent.

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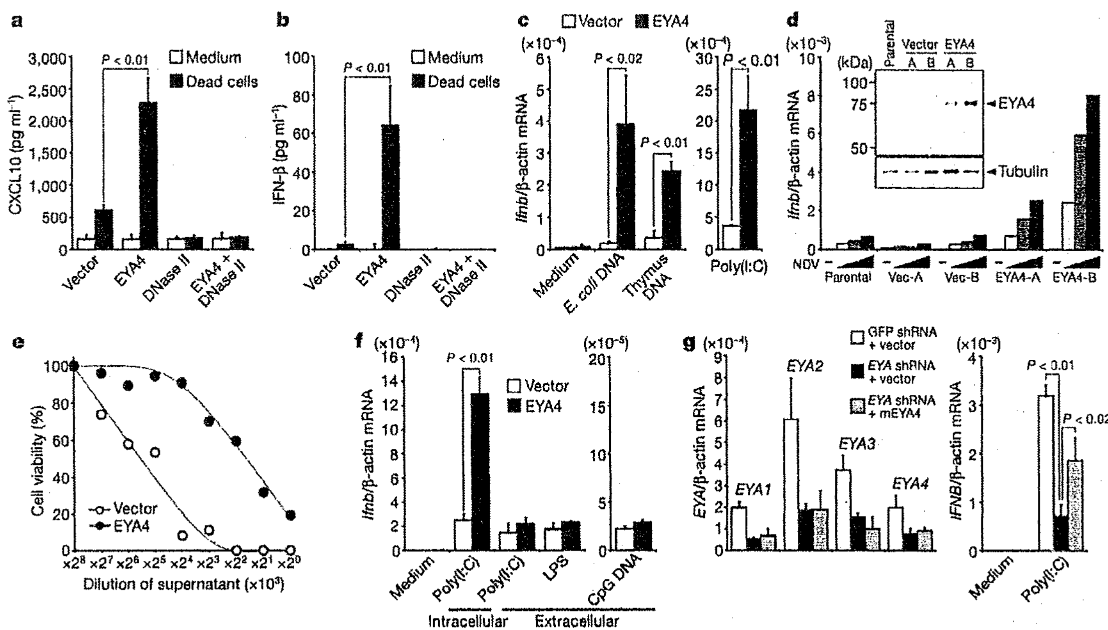

Figure 1 | Identification of EYA4 as a regulator for innate immunity. **a, b,** *DNaseII*$^{-/-}$ MEFs that expressed EYA4, DNase II, both EYA4 and DNase II, or vector control were incubated with medium or dead cells, and the concentrations of CXCL10 (**a**) and IFN-β (**b**) were quantified. **c,** MEFs plus either vector control or EYA4 were transfected with 100 ng ml^{-1} *E. coli* or calf thymus DNA, or with 10 ng ml^{-1} poly(I:C), and levels of *Ifnb* mRNA were quantified relative to β-actin mRNA levels. **d,** Parental, and vector- or EYA4-Namalwa cells (two clones each, A and B), were challenged with NDV, and *Ifnb* mRNA was quantified at 8 h. Inset, cell lysates were analysed by western blotting. Tubulin was included as a loading control. **e,** MEFs plus vector control or EYA4 were infected with 0.001 multiplicity of infection (m.o.i.) of VSV for 12 h. Cytopathic activity in the supernatants was determined. Values are the means from sextuple samples. **f,** Fetal liver macrophages, infected with EYA4 retrovirus, were transfected with 100 ng ml^{-1} poly(I:C), or extracellularly treated with 100 ng ml^{-1} of poly(I:C), 1.0 ng ml^{-1} LPS, or 1.0 μM CpG, and *Ifnb* mRNA levels were quantified at 6 h. **g,** 293T was transfected with shRNA for GFP, or a mixture of shRNA for human *EYA1–4*, with or without pEF-mEYA4. At 72 h, *EYA* mRNA was quantified (left). The cells were transfected with 1.0 μg ml^{-1} of poly(I:C), and *IFNB* mRNA levels were quantified (right). All error bars are s.d.

To characterize the phosphatase activity of EYA, mouse EYA4 was expressed in 293T cells and purified (Fig. 3a). As reported with the C-terminal domain of mouse EYA3 (ref. 6), EYA4 showed tyrosine-phosphatase activity with an artificial peptide under acidic conditions (pH 6.0). Its threonine-phosphatase was potentiated under basic pH, with an optimum pH of around 8.0 (Fig. 3b). The tyrosine-phosphatase required bivalent cations (Mg^{2+} or Mn^{2+}) (Fig. 3c), and $ZnCl_2$ (1 mM) and EDTA (5 mM) inhibited this activity (Supplementary Fig. 5). On the other hand, the threonine-phosphatase did not require a metal ion (Fig. 3d), and was not inhibited by 10 mM EDTA or EGTA (data not shown), but $ZnCl_2$ inhibited its activity (Fig. 3d). NaF inhibited both the tyrosine- and threonine-phosphatases, whereas calyculin and okadaic acid inhibited the threonine-phosphatase but not the tyrosine-phosphatase activity (Fig. 3e, f). Under optimal conditions, the K_m and K_{cat} were similar between tyrosine- and threonine-phosphatases, and they were comparable to those for protein phosphatase 2A, protein phosphatase 2B, and protein phosphatase 2C (Supplementary Fig. 6). Other mouse EYA family members, produced in 293T cells (Supplementary Fig. 7), also showed both tyrosine- and threonine-phosphatase activity (Supplementary Fig. 8). None of the members showed phosphatase activity against phosphorylated peptides of (pS)EEEEE and (pT)EEEEE, confirming that EYAs are not acid or alkaline phosphatases. The threonine-phosphatase activities of mouse EYA3 and EYA4 were confirmed by preparation in the wheat-germ cell-free system (Supplementary Fig. 9).

The tyrosine-phosphatase activity of the EYA proteins was previously assigned to the haloacid dehalogenase (HAD) domain in the C-terminal half[6,7]. Although its N-terminal domain is less well conserved, an alignment of the amino acid sequences revealed a conserved motif (Supplementary Fig. 10) in which six tyrosine residues are well conserved. We produced three EYA4 mutants: DYY, Y4 and D352N (Fig. 4a, b). For DYY, Ala was substituted for Asp 246, Tyr 247 and Tyr 250, and for Y4, Ala was substituted for Tyr 258, Tyr 261, Tyr 262 and Tyr 267; for D352N, Asn was substituted for Asp 352. As reported[6,7], Asp352Asn lost its tyrosine-phosphatase activity (Fig. 4c), but its threonine-phosphatase activity was unaffected (Fig. 4d). The tyrosine-phosphatase activity of DYY and Y4 was comparable to that of wild-type EYA4, but their threonine-phosphatase activity was severely affected, with Y4 showing the most disruption. This was also true with peptides from the C-terminal region of RNA polymerase II (Supplementary Fig. 11). When EYA3 was divided into an N-terminal and C-terminal domain, the C-terminal domain carried the tyrosine-phosphatase, but not the threonine-phosphatase, activity (Supplementary Fig. 12). In contrast, its N-terminal domain showed threonine-phosphatase activity, but negligible tyrosine-phosphatase activity. These results apparently contradict the previous report that assigned the threonine-phosphatase of EYA to the HAD domain[5]. When Asp352Asn and Y4 mutants were expressed in mouse fetal liver macrophages (Fig. 4e), Asp352Asn enhanced poly(I:C)-induced IFN-β expression as efficiently as the wild-type EYA4, whereas Y4 severely lost this ability (Fig. 4f). This was in contrast to the effect of the Y4 mutation on the ability of EYA4 to enhance the SIX4-mediated transcription of the myogenin gene promoter (Supplementary Fig. 13). The point mutations in the N or C termini did not affect its ability to interact with IPS-1 (Fig. 4g), suggesting that the threonine-phosphatase is critical for the ability of EYA4 to enhance the innate immune response.

Here we have reported that EYA4 interacts with IPS-1, STING and NLRX1, which are on the mitochondrial outer membrane or endoplasmic reticulum[15,18,19,21], and stimulates the IRF3-mediated transcription of the *Ifnb* and *Cxcl10* genes. A recent report indicates that EYA dephosphorylates a tyrosine residue of histone H2AX, and renders the cells resistant to genotoxic-agent-induced apoptosis[22]. These results indicate that EYA has dual functions: one to regulate the chromatin structure using its tyrosine-phosphatase, the other to

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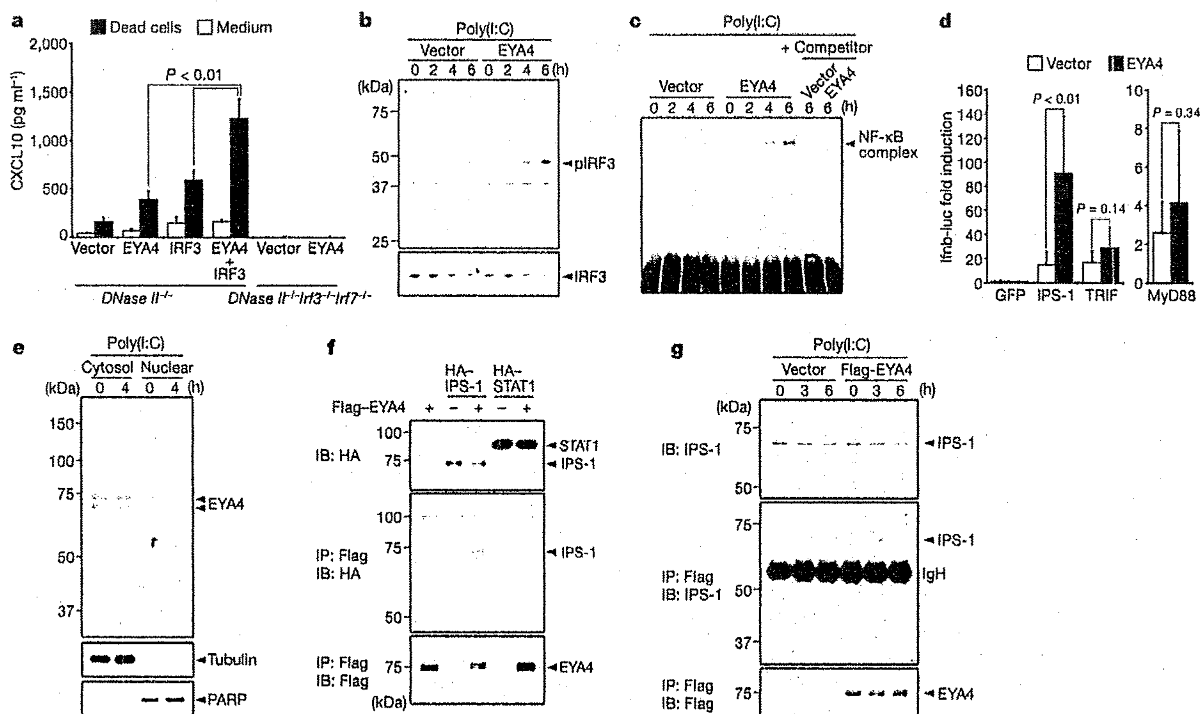

Figure 2 | Activation of the signalling cascade by EYA4. a, *DNase II*$^{-/-}$ or *DNase II*$^{-/-}$ *Irf3*$^{-/-}$ *Irf7*$^{-/-}$ MEFs expressing EYA4 and/or IRF3, were cultured with medium or dead cells, and CXCL10 levels were determined. **b, c,** MEFs containing vector control or EYA4 were transfected with 1 μg ml^{-1} poly(I:C) for the indicated times. The cell lysates were analysed by western blot for phosphorylated-IRF3 (pIRF3) (**b**), or nuclear extracts were analysed by electrophoretic mobility shift assays (EMSAs) for NF-κB (**c**). **d,** MEFs containing vector control or EYA4 were transfected with pGL3-Ifnb-luc, and pEF-EGFP, pEF-IPS-1, pEF-TRIF or pEF-MyD88. The luciferase activity is shown relative to the EGFP value. **e,** MEFs were treated with 1.0 μg ml^{-1} poly(I:C) for 4 h, and cell extracts were fractionated into cytoplasmic and nuclear fractions and analysed by western blotting. Two bands for EYA4 are probably due to alternative splicing[29]. The fractionation of the cell extracts was confirmed by blotting for tubulin and PARP. **f,** 293T cells were co-transfected with the vector for Flag–EYA4 and HA-tagged IPS-1 and STAT1. Cell lysates were immunoprecipitated (IP) with anti-Flag and analysed by western blotting. Cell lysates are shown at the top (5% of the input). IB, immunoblot. **g,** MEFs plus vector control or EYA4 were transfected with 1.0 μg ml^{-1} poly(I:C) for the indicated times. Cell lysates were immunoprecipitated with anti-Flag and analysed by western blotting. Cell lysates are shown at the top (10% of input). All error bars are s.d.

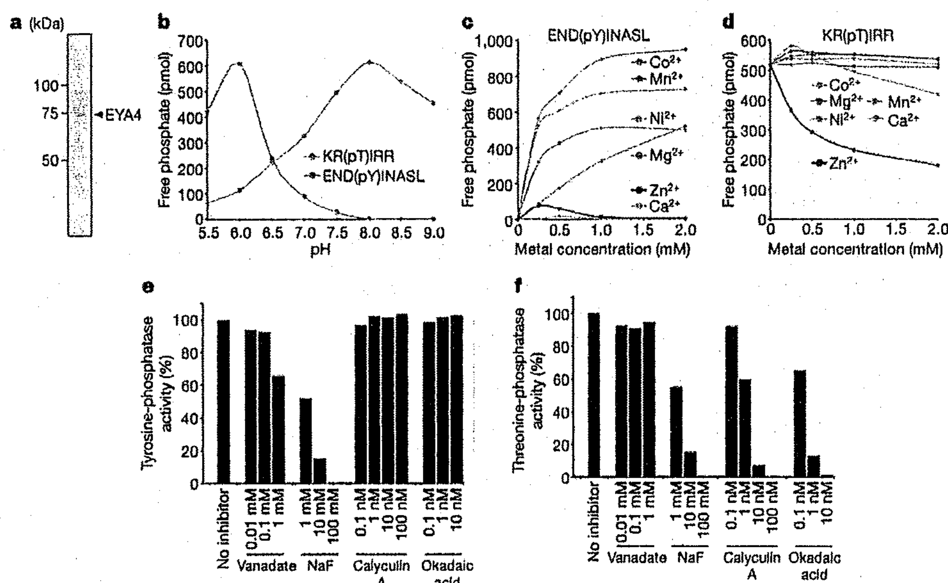

Figure 3 | Two different phosphatase activities in mouse EYA. a, Mouse EYA4 expressed in 293T, was purified and analysed by SDS–PAGE. **b–d,** Dephosphorylation with END(pY)INASL and 5 pmol EYA4 (**b, c**), or with KR(pT)IRR and 2.5 pmol EYA4 (**b, d**) at the indicated pH (**b**) or in the presence of metal ions (**c, d**) is shown. **e, f,** Dephosphorylation of RRLIEDAE(pY)AARG (**e**) or SDQEKRKQI(pT)VRGL (**f**) by 1.25 pmol EYA4 in the presence of phosphatase inhibitors is shown.

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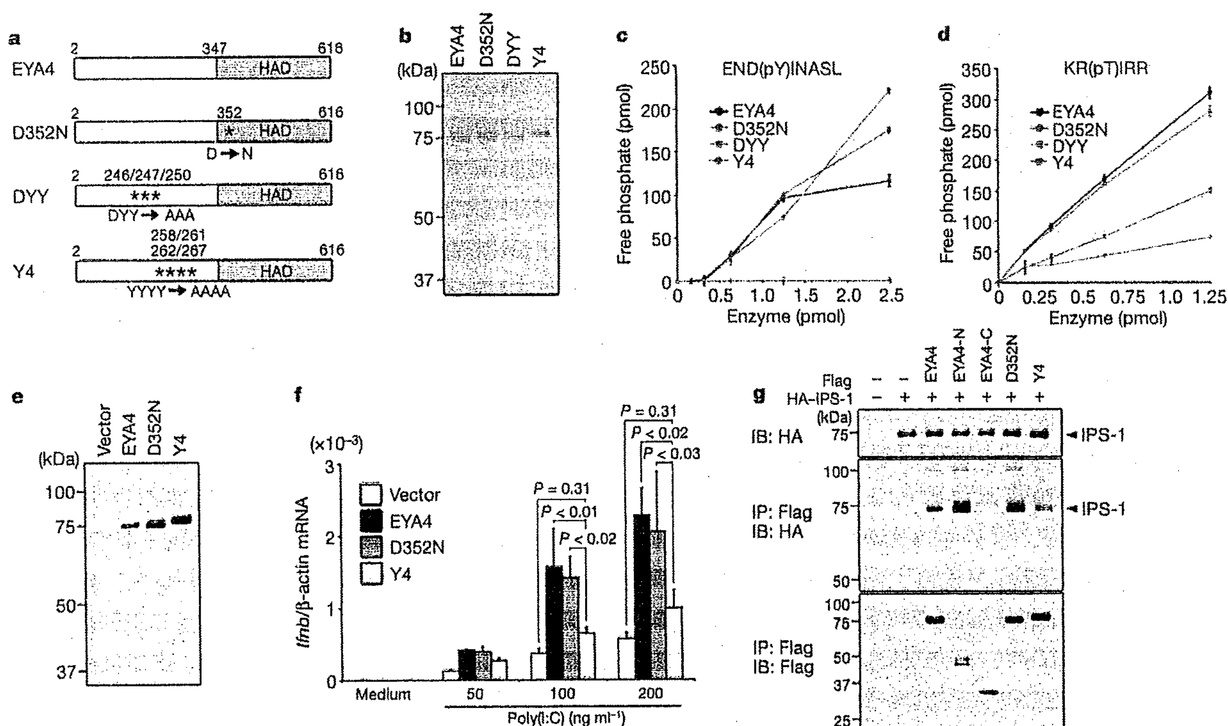

Figure 4 | The threonine-phosphatase activity of EYA4 is required for the innate immune reaction. **a**, Diagrams of EYA4 and its mutants are shown. **b**, EYA4 and its mutants, produced in 293T cells, were purified and analysed by SDS–PAGE. **c**, **d**, Dephosphorylation by EYA4 and its mutants is shown. **e**, **f**, Fetal liver macrophages were infected with EYA4 retrovirus. Cell lysates were analysed by western blot using an anti-Flag antibody (M2) (**e**). The cells were transfected with poly(I:C) for 6 h, and *Ifnb* mRNA levels were quantified relative to β-actin levels (**f**). **g**, 293T cells transfected with vectors for Flag–EYA4 or its mutants, and with HA–IPS-1. Cell lysates were immunoprecipitated (IP) with anti-Flag, followed by western blotting. Shown at the top is 10% of the input. All error bars are s.d.

regulate the innate immune response in the cytoplasm using its threonine-phosphatase. It will be interesting to study how the two phosphatase activities of EYA are regulated, and how the cellular localization of EYA is determined. The N-terminal region of EYA for the threonine-phosphatase had no apparent similarity with other phosphatases, yet its activity could be inhibited by okadaic acid. As found between protein phosphatase 1 and protein phosphatase 2A[23], the tertiary structure of the N-terminal domain of EYA may be similar to other threonine phosphatases. Several kinases (RIP1 (also known as RIPK1), IKK-ε (IKBKE), TBK1, IKK-α (CHUK) and IKK-β (IKBKB)) are involved in RIG-I–MDA5-mediated signal transduction[24]. It was previously proposed[25] that binding of viral RNA to RIG-I–MDA5 recruits signalling molecules (TRADD, TRAF3 and NEMO (IKBKG)), to form a large complex. It is possible that EYA is one of the components of this large complex, and regulates the signalling by modulating their phosphorylation state (Supplementary Fig. 14). Genes in the *EYA* family are often mutated in various autosomal-dominant disorders associated with branchial arch malformations, hearing loss, and ocular segment anomalies[26]. Mutations can be found throughout the EYA molecule[27], and some of these mutations have no effect on the tyrosine-phosphatase activity or on the interaction with a SIX family member[28]. Whether any of the mutations found in genes of the *EYA* family in human patients affect the threonine-phosphatase activity remains to be determined.

METHODS SUMMARY

Expression cloning. Double-stranded cDNA with poly(A) RNA from MEFs was inserted into a retroviral vector, and introduced into *E. coli* to generate a library (400 pools of 50 clones). Plasmid DNA from each pool was introduced into packaging cells, and the culture supernatant was used to infect *DNaseII*$^{-/-}$ MEFs. The MEFs were allowed to engulf apoptotic *Cad*$^{-/-}$ thymocytes, and CXCL10 in the culture supernatant was quantified by ELISA. The positive pools were subjected to sib-selection.

Molecular biology, biochemistry and cell biology. Macrophages and MEFs were transformed by retrovirus-mediated transfection. Namalwa cells expressing mouse EYA4 were established by electroporation with EYA4 expression vector. The MFG-E8-mediated system was used for engulfment of apoptotic cells. The Fugene system was used to introduce nucleic acids into cells. IFN-β and CXCL10 were quantified by ELISA. The promoter activity of the *Ifnb* and myogenin genes was determined with Dual-Luciferase Reporter Assay System (Promega). Real-time PCR was performed using a LightCycler 480 system (Roche Diagnostics). The shRNA expression plasmids for human *EYA1*, *EYA2*, *EYA3* and *EYA4* were purchased from OriGene.

Production of recombinant EYAs. For recombinant EYA, the Flag-tag was ligated at the 5′ end of *Eya*, and inserted into the pEF-BOS vector. A point mutation was introduced by recombinant PCR. The expression vector was introduced into human 293T cells, and the recombinant protein was purified using anti-Flag M2 gel. In some cases, EYA was synthesized using the wheat-germ cell-free system, and purified.

Phosphatase assay. Phosphorylated synthetic peptides were custom-synthesized at the Toray Research Center, MBL, and Invitrogen. To assay the phosphatase activity, EYA was incubated with 400 μM phosphorylated peptide at 37 °C for 60 min, and released phosphate was quantified by a colourimetric method using malachite green-molybdate.

Statistical analysis. Results were statistically analysed using an analysis of variance (ANOVA) test.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions Y.O. screened the cDNA library, and identified EYA4 as a regulator of the innate immune reaction. T.S. produced recombinant EYA in 293T cells and wheat-germ extracts, biochemically characterized, and found its threonine-phosphatase activity. S.N. designed the research and wrote the paper.

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