FasL cross-linking inhibits activation of human peripheral T cells

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Abstract

Activation of resting T cells in vitro is triggered by combined TCR and CD28 engagement and can be modulated by simultaneous ligation of various other surface receptors. Although the Fas ligand (FasL) is best known for its capacity to initiate cell death in Fas-bearing cells, it has recently been implicated in the regulation of T cell activation. Thus, a cross-talk between the TCR and FasL is likely, but far from being biochemically elucidated. We now report that FasL engagement by immobilized but not soluble FasFc fusion protein and anti-FasL polyclonal antibody blocks the activation of human peripheral T cells even in the presence of CD28 co-stimulation. The data presented here stress the importance of the Fas/FasL system for signal initiation via the TCR–CD3 complex and provide further arguments for a retrograde signaling capacity of FasL or a crucial role of Fas as a co-stimulatory molecule.

Introduction

At least two signals are required for activation of resting T lymphocytes. Signal one emerges from the engagement of the TCR–CD3 complex by antigen presented on a self-MHC molecule. In vitro, this first signal can be mimicked by antibodies against the TCR–CD3 complex (i.e. CD3ε). Highly purified T cells, however, strictly depend on the co-stimulatory signal for full activation. This second signal might be provided to naive T cells through the constitutively expressed ‘classical’ co-stimulatory receptors CD28 or CD278 (ICOS). These members of the Ig superfamily become phosphorylated by TCR-dependent Src-related kinases to recruit PI3 kinase and subsequently form multimolecular complexes that amplify the TCR signal and initiate gene transcription, cytokine and cytokine receptor expression and proliferation.

Over the past years, however, it has been reported that several other surface molecules also affect TCR-dependent cellular activation. In the present study, we focused on Fas ligand (FasL, CD95L, TNFSF6), a member of the tumor necrosis factor superfamily (TNFSF) as a potential co-stimulator for primary human T cell activation. FasL is a type II transmembrane protein that was identified as the ligand for Fas/CD95/Apo-1 with a death-promoting activity in the context of activation-induced cell death (AICD) (1–3). Although members of the TNFSF might be shed as soluble cytokines, signal induction seems most efficient in a cell–cell contact-dependent manner. This in turn argues for a second function of the ligands when engaged by the corresponding receptors. A reverse or retrograde signaling capacity has meanwhile been documented for several ligands of the family including CD27 ligand (CD27L), 4-1BB ligand (4-1BBL), OX40 ligand, CD30 ligand (CD30L), CD40 ligand (CD40L) and FasL (4–8).

Interestingly, TNFSF members are highly homologous within the extracellular TNF homology domains, but differ substantially in their cytoplasmatic regions. Importantly, most TNFSF proteins do not contain any of the classical receptor-associated tyrosine-based activation or inhibition motifs, nor apparent docking sites for enzymes or adapter proteins. Therefore, in most cases it remains unclear how retrograde signaling via TNF family members cooperates with other receptor-induced signaling pathways. Six TNFSF members (TNF, CD27L, CD30L, CD40L, 4-1BBL and FasL) harbor a cytosolic casein kinase (CK) I substrate motif. To date, however, CK activity and phosphorylation of that motif were experimentally proven only for TNF and suggested for murine FasL (9, 10).

In all studies, retrograde FasL signal transduction required cross-linking in the presence of sub-optimal TCR–CD3 stimulation (7, 11, 12), indicating that FasL retrograde signaling

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somewhat directly interferes with the TCR–CD3-triggered signal. Reverse signaling via FasL is most likely mediated by the unique cytoplasmic region. In contrast to all other members of the TNFSF, the intracellular part of FasL contains a conserved polyproline docking site for protein–protein interactions [see (13, 14) for review]. Although we and others have identified a series of FasL-interacting proteins including Src kinases, the p85 subunit of PI3 kinase, the adapter proteins Nck, Grb2 and others (15–17), the pathways that might enable the reverse signaling of FasL are still poorly defined.

Sun and colleagues recently reported that triggering of FasL by cross-linked FasFc induces FasL phosphorylation and the association with Src homology 3 (SH3) domain containing molecules such as Fyn, the p85 subunit of PI3 kinase and Grb2 leading to co-stimulation of murine-activated CD8+ T cells. In their hands, the PI3 kinase-dependent Akt pathway and ERK1/2 and JNK phosphorylation were enhanced upon FasL cross-linking, resulting in activation of transcription factors including NF-AT and AP-1, enhanced proliferation and cytokine production (11). In this scenario, both, the proline-rich domain as well as the CKI motif seems to be important elements since mutations or deletion of the two regions prevented optimal FasL co-stimulation (10). It should be mentioned, however, that in contrast to the co-stimulatory action of Fasl on murine CTLs, FasL seems to inhibit proliferation, cell cycle progression and IL-2 production of freshly isolated murine CD4+ T cells (12).

In order to elucidate these contradictory findings and to address the molecular mechanism of reverse signaling, we established a system to study the co-stimulatory properties of FasL on freshly isolated human T cells. We now report that FasL engagement also modulates activation of primary human T cells. We show that ligation of FasL by plate- or bead-bound FasFc fusion protein or anti-FasL polyclonal antibody (pAb) abolishes TCR–CD3-induced signal initiation and proliferation. With regard to a potential TCR to FasL signal cross-talk, we describe a reduced ERK1/2, LAT, PLCγ and ZAP-70 phosphorylation associated with a block in cell cycle progression. Although the exact mechanism of the FasL to TCR signal cross-talk needs further clarification, our data point to an important role of the Fas/FasL system in T cell activation. The dramatic inhibition of T cells by the presence of FasFc fusion proteins or FasL pAb in fact argues for a direct retrograde signaling capacity but indicates at the same time that also the Fas receptor (CD95) could be an important co-stimulatory receptor for T cell activation.

Methods

Reagents and antibodies

Antibodies included the anti-CD28 mAb (CD28.2, IgG1), anti-FasL mAb (NOK-1, IgG1), anti-CD25 mAb (2A3, IgG1), anti-CD69 mAb (L-78, IgG1), anti-CD56 mAb (B156, IgG1), all from BD Biosciences (San Diego, CA, USA), anti-TCRβ (MHAB01-4, IgG1; Caltag Laboratories, Hamburg, Germany), anti-CD3 mAb (OKT3, IgG2a; Cilag, Sulzbach, Germany), anti-CD4 (13B8.2, IgG1) and anti-CD8 (B9.11, IgG1) from Dianova (Hamburg, Germany), anti-FasL C-20 (pAb, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-FasL-PE (NOK-1, IgG1; Caltag Laboratories), anti-human IgG (Fc specific, clone CG-7; Sigma, Munich, Germany) and rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Human IgGFc was purchased from Bethyl Laboratories (Montgomery, TX, USA). In some experiments, a cocktail of different staphylococcal enterotoxin (SE) superantigens A, B, C, D and E (Toxin Technologies, Sarasota, FL, USA) was used. TCR-independent stimulation was performed with phorbolester phorbol 12-myristate 13-acetate (PMA) (10 ng ml−1; Sigma) and ionomycin (500 ng ml−1; Calbiochem, Darmstadt, Germany). In other experiments, OKT3/anti-CD28/IgGFc- or OKT3/anti-CD28/FasFc-loaded M-450 epoxy beads (Dynal, Hamburg, Germany) were used at a ratio of two cells per bead. To this end, 25 μl of beads were loaded with a total of 5 μg Ab/fusion protein. Standard culture medium was RPMI 1640 with 10% pooled human AB serum (DRK, Baden-Baden, Germany), 100 U ml−1 penicillin, 100 μg ml−1 streptomycin, 2 mM glutamine and 25 mM HEPES (Biochrom, Berlin, Germany). For some experiments, fetal bovine serum (FBS) (Biochrom) was used instead of AB serum.

Cells

PBMC were isolated fromuffy coat preparations of healthy donors. To purify E-rosetted (E+) human T lymphocytes, we performed rosette formation with neuraminidase-treated sheep red blood cells. For further isolation of CD4+ T cells, 40 μg ml−1 anti-CD4 or anti-CD8 and 10 μg ml−1 anti-CD56 antibodies were added to 20 × 10^6 ml−1 (E+) T cells. After 30 min, the cells were washed and incubated with freshly prepared rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) at 37°C for 60 min. Dead cells were removed by Ficoll gradient centrifugation, and the remaining cells were recovered and analyzed for surface expression of CD4 and CD8. Alternatively, CD4+ and CD8+ T cells were magnet-activated cell sorting (MACS) purified from PBMC using respective kits for negative selection from Miltenyi Biotec (Bergisch Gladbach, Germany). The purity of individual populations was examined by flow cytometry and reached routinely at least 95–99% of the desired phenotype.

Generation and purification of FasFc fusion proteins

Human embryonic kidney 293 (HEK 293) cells were kept in DMEM (Invitrogen) with 10% FBS, antibiotics, L-glutamine and HEPES buffer solution. For purification of FasFc fusion proteins, serum-free FreeStyle™ expression medium (Invitrogen) was used. The cDNA sequence encoding the extracellular part of the human Fas protein (CD95, aa 1–173) was cloned into a pcDNA3.1-derived human IgG Fc-tag expression vector provided by Harald Wajant (Würzburg, Germany). Fusion proteins were produced from transiently transfected HEK 293 cells and dialyzed against PBS. The fusion proteins were checked by SDS–PAGE and Coomassie Blue staining. Bioactivity of FasFc was tested by inhibition of Fas-mediated lysis on Jurkat cells or AICD of T cell clones and immunoprecipitation of FasL from stably transfected K562 (KFL-9, provided by David Kaplan, West Grove, PA, USA). Reverse signaling via FasL is most likely mediated by the unique cytoplasmic region. In contrast to all other members of the TNFSF, the intracellular part of FasL contains a conserved polyproline docking site for protein–protein interactions [see (13, 14) for review]. Although we and others have identified a series of FasL-interacting proteins including Src kinases, the p85 subunit of PI3 kinase, the adapter proteins Nck, Grb2 and others (15–17), the pathways that might enable the reverse signaling of FasL are still poorly defined.
Flow cytometry
Cells were stained with FITC- or PE-conjugated antibodies and incubated for 30 min on ice. After three washes, samples were fixed and analyzed in a flow cytometer using the Cell-quest analysis software from Becton Dickinson. FasL expression on resting T lymphocytes was tested in a time course with or without stimulation. A total of 10^5 freshly isolated T cells were incubated in untreated wells or in wells coated with OKT3 (1 μg ml\(^{-1}\)) and anti-CD28 (5 μg ml\(^{-1}\)). At the indicated time points, the cells were carefully recovered and immediately stained with FasL-PE mAb or mouse IgG1-PE as control. Cell viability was determined by propidium iodide (PI) exclusion with 2.5 μg ml\(^{-1}\) PI (Serva, Heidelberg, Germany) in PBS for 10–15 min prior to FACS analysis.

Confocal microscopy
A total of 10^6 freshly isolated (E\(^+\)) T cells were washed with PBS and spread on poly-L-lysine-treated coverslips at 4°C for 30 min. Adherent cells were fixed and permeabilized with cold methanol (−20°C) for 5 min and stained with anti-FasL mAb NOK1 followed by Alexa Fluor 488-conjugated goat anti-mouse antibody (Molecular Probes, Leiden, The Netherlands). Mouse IgG1 served as isotype control. Samples were analyzed on a LSM 510 laser scanning microscope with LSM Software (Zeiss, Jena, Germany).

[\(^{3}\)H]thymidine incorporation
To determine the effects of FasL engagement on TCR-CD3-induced proliferation, wells of 96-well flat-bottomed tissue culture plates were coated for 2 h at 37°C with 100 μl of OKT3 (1–2 μg ml\(^{-1}\)), with or without anti-CD28 (5 μg ml\(^{-1}\)) or with or without SE superantigen cocktail (0.5 ng ml\(^{-1}\)). FasFc or human IgGFc control protein (20 μg ml\(^{-1}\)) was included in the coating solution as indicated. Pre-treated culture plates were washed three times with PBS/5% FBS, before freshly isolated PBMC, unseparated T cells (E\(^+\)), CD4\(^+\) and CD8\(^+\) T cells were plated at 100 000 cells per well in culture medium. On day 3, cells were pulsed with [\(^{3}\)H]thymidine (1 μCi per well) and cultured for additional 16–18 h. Samples were harvested and [\(^{3}\)H]thymidine incorporation was assessed in a β-counter (Inotech, Dottikon, Switzerland). All experiments were done at least in triplicates. Values given in graphs are mean values with standard deviations.

CFSE distribution assay
We also used 5-(6-)carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling to analyze proliferation and cell cycle progression. To this end, purified CD4\(^+\) T cells were incubated at 10^8 cells ml\(^{-1}\) with 5 μM CFSE (Fluka, Buchs, Switzerland) in PBS for 10 min at 37°C. The labeling reaction was terminated by adding 5–10 ml of ice-cold RPMI for 5 min on ice. The cells were washed three times with RPMI 10% FBS and re-suspended in RPMI 10% AB serum for cell

Fig 1. FasL expression on freshly isolated human peripheral blood T cells. (A) Unseparated (E\(^+\)) T cells were stained as detailed in the Methods with anti-FasL-PE or the PE-labeled isotype control Ab. The shown histogram plot is zoomed on the first two logs of the relative fluorescence intensity. (B) Time course kinetics up to 2 h of FasL expression on unstimulated (−) and OKT3 plus anti-CD28-treated (+) T cells. (C) Freshly isolated (E\(^+\)) T cells were additionally analyzed for FasL expression by confocal microscopy. An overview (left panels, bar indicating 20 μm) and two cells at higher magnification (right panels, bar indicating 2 μm) are shown.
culture. A total of 1 × 10^6 CFSE-labeled T cells per well were seeded in 24-well plates and incubated for 2–4 days with or without anti-CD3 plus anti-CD28 in the presence or absence of FasFc, anti-FasL pAb or Fc control protein. In some experiments, cells were stimulated with PMA and ionomycin instead of OKT3 and anti-CD28. Human recombinant IL-2 (10 U ml⁻¹, EuroCetus, Frankfurt, Germany) was added as indicated. Proliferation of CFSE-labeled CD4⁺ T cells was visualized by incremental loss of CFSE fluorescence analyzed by flow cytometry.

SDS–PAGE, immunoprecipitation and western blotting

SDS–PAGE and western blotting were used to analyze the phosphorylation state, e.g. mitogen-activated protein kinase (MAPK) in purified CD4⁺ T cells. Briefly, 2–3 × 10^6 cells were stimulated as indicated and lysed in NP-40 lysis buffer [1% Nonidet P40, 20 mM Tris- HCl, pH 7.4, 150 mM NaCl with aprotinin (10 μg ml⁻¹), leupeptin (10 μg ml⁻¹), 1 mM phenylmethylsulphonylfluoride, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM sodium pyrophosphate (all from Sigma)]. A total of 2–3 × 10^6 CD4⁺ T cells were separated on 4–12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose (Hybond C-Extra; GE Healthcare, Munich, Germany). Protein loading and efficacy of transfer were visualized with Ponceau S (Sigma). The blots were blocked with non-fat dry milk, washed, stained with antibodies against ZAP-70 (Santa Cruz Biotechnology), phospho-Lck (pY505) (Biosource, Nivelles, Belgium), phospho-ERK1/2, ERK1/2, phospho-LAT (pY171) and phosho-PLCγ (all from New England Biolabs, Frankfurt, Germany) and developed with HRP-conjugated secondary antibodies and ECL (GE Healthcare).

For immunoprecipitation, 10 × 10^6 cells were stimulated for 5 min at 37°C, lysed in NP-40 lysis buffer, centrifuged and pre-cleared with protein G-Sepharose (GE Healthcare) for 1 h at 4°C. The pre-cleared supernatants were incubated for 24 h at 4°C with phospho-specific (Y319) ZAP-70 antibody (GE Healthcare) and analyzed by immunoblotting using the phospho-specific (Y319) anti ZAP-70 antibody.

Results

FasL expression on freshly isolated T lymphocytes

A role of FasL as a co-stimulatory or accessory molecule for T cell activation was initially suggested from in vivo experiments with generalized lymphoproliferative disease (gld) and lymphoproliferation (lpr) mutant mice and recently substantiated by biochemical analyses of potentially involved signaling pathways in isolated murine CD8⁺ T cells (7, 11, 12, 18, 19). Of note, FasL reverse signaling was only observed in association with sub-optimal TCR-CD3 stimulation (11).

As shown in Fig. 1, FasL surface expression was readily detectable on freshly isolated human T lymphocytes. Compared with isotype controls, staining with PE-labeled anti-FasL mAb NOK-1 resulted in a homogenous shift in fluorescence intensity (Fig. 1A). This is consistent with published data, where FasL was detected on the murine T cells using enzymatic amplification staining, a flow cytometric technique for amplifying weak surface expression. In time course

Fig. 2. Cross-linked but not soluble FasL agonists inhibit T cell activation. PBMC were cultured in triplicates for 3 days in 96-well plates coated with 1 μg ml⁻¹ anti-CD3 mAb OKT3 or 0.5 ng ml⁻¹ SE superantigen. FasFc or Fc control protein (both 25 μg ml⁻¹) were either added as soluble factors (A) or bound to the plates together with the TCR ligand (B and C). Experiments with cells of three different donors stimulated with either OKT3 (B) or SE (C) are presented. Proliferation was determined by [³H]thymidine incorporation. Purified human Fc fragment of IgG served as a control.
experiments, only a mild transient increase in expression was observed 5–10 min after stimulation by plate-bound anti-CD3 and anti-CD28 antibodies (Fig. 1B). This increase in expression resembled the rapid activation-dependent surface appearance of FasL that we previously reported for activated human T cells or NK cells (20), indicating that in freshly isolated peripheral T cells a fraction of FasL might already be associated with preformed storage vesicles that are transported to the immunological synapse upon activation (21–26). When freshly isolated human T cells were analyzed by confocal microscopy, it appeared that FasL was mainly localized at the cell membrane, with some protein associated with a vesicular compartment in the vicinity of the cell surface (Fig. 1C).

FasL ligation per se does not lead to apparent signal induction

We first tested whether freshly isolated or pre-activated human T cells respond directly to FasL engagement. To this end, different T cell populations were stimulated with soluble FasFc fusion protein or soluble anti-FasL pAb. After short-term incubation, western blotting was performed to analyze pathways frequently triggered by ligation of surface receptors including the TCR–CD3 complex. In agreement with recently published data on murine T cells (11), no changes in overall tyrosine phosphorylation, ERK1/2 or Akt phosphorylation were observed by direct FasL stimulation of freshly isolated PBMC, purified T cells or CD4+ T cell clones (data not shown). Moreover, pre-treatment or simultaneous incubation of cells with soluble FasFc or anti-FasL pAb had no effect on subsequent TCR-driven short-term protein phosphorylation (data not shown) and did not alter the proliferative capacity when cells were stimulated with anti-CD3 mAb (OKT3) or SE superantigen (Fig. 2A).

Cross-linked FasFc inhibits activation of freshly isolated PBMC

Bidirectional modulation of signaling pathways often requires cross-linking of the receptors involved. Therefore, the subsequent experiments were designed to mimic cross-linking of the TCR–CD3 complex and/or FasL at the same time. To this end, tissue culture wells were coated with low concentrations of OKT3 or SE with or without FasFc fusion or Fc control protein. As shown in Figures 2B and 2C for three different donors, OKT3- or superantigen-induced proliferation of freshly isolated PBMC was dramatically inhibited. As mentioned, in the same setting, soluble FasFc fusion protein added to cells stimulated by plate-bound TCR–CD3 agonists only led to a very mild inhibition of [3H]thymidine uptake (Fig. 2A).

FasL engagement inhibits proliferation of both CD4+ and CD8+ T cells

Since previous observations in mice suggested a differential role for FasL in CD4+ and CD8+ T cells (18), we purified CD4+ or CD8+ cell populations by negative selection from (E+) T cells. In all experiments, we consistently found that the anti-CD3- or the anti-CD3 plus anti-CD28-triggered proliferation of E+, CD4+ and CD8+ T cells was substantially inhibited in the presence of plate-bound FasFc (Fig. 3). The purity of individual populations (95–97%) did not change during the culture period of up to 4 days. The inhibitory effect suggested that cross-linking of FasL might either deliver a strong negative signal to block TCR–CD3

![Fig. 3. FasL ligation blocks proliferation of human CD4+ and CD8+ T cells. Proliferation of unseparated (E+) T cells (A) and CD4+ (B) or CD8+ T cells (C) was tested. Cells were stimulated with 1 μg ml⁻¹ cross-linked anti-CD3 mAb OKT3 (CD3) or OKT3 plus 5 μg ml⁻¹ anti-CD28 mAb (CD3/CD28). Plate-bound FasFc but not Fc control protein blocked proliferation of unseparated (E+) and CD4+ or CD8+ T cells. All proliferation assays were done in triplicates and results are shown as mean with standard deviations.](https://academic.oup.com/intimm/article-abstract/21/5/587/727663)
signaling or, alternatively, interfere with a co-stimulatory Fas–FasL interaction required for full activation of freshly isolated CD4+ and CD8+ T cells.

The initiation of T cell activation is blocked by co-stimulation with plate-bound FasFc

As mentioned, we did not observe major differences in the overall reactivities of purified human CD4+ and CD8+ T cells. Therefore, we used MACS-purified CD4+ T cells in most of the following assays. As shown for a representative experiment in Fig. 4, we consistently found that the anti-CD3- (data not shown) or the anti-CD3 plus anti-CD28-triggered proliferation of CD4+ T cells was substantially inhibited in the presence of plate-bound FasFc. The dramatic inhibition of proliferation was associated with substantial differences in cluster formation as judged by microscopic inspection of individual wells and in blast formation as shown by the scatter plots in FACS analyses (Fig. 4A). As expected, during the initial 3 days of culture, cells kept in medium remained small and round while stimulation led to an increase in granularity and size (Fig. 4A, lower panels) and formation of proliferation clusters (Fig. 4A, upper panels). While cells in medium alone or in the presence of IgGFc proliferated vigorously in response to CD3 stimulation or CD3–CD28 stimulation, the presence of immobilized FasFc almost completely abolished proliferation. In order to quantify whether FasL ligation affects all cells or only a fraction in a given culture well, we performed CFSE labeling of primary CD4+ T cells prior to stimulation as described. FACS analyses for CFSE dilution were performed at different time points between 2 and 4 days. As expected, after 72 h (data not shown) and more striking after 87 h (Fig. 4B), we observed that CD3 activation alone resulted in a moderate induction of cell divisions, which could be enhanced by co-stimulation with anti-CD28 mAb (data not shown). Immobilized IgGFc used as a control did not have a profound effect on activation-induced proliferation. In contrast, the presence of plate-bound FasFc drastically reduced the number of cell divisions in the whole CD4+ T cell population, once more highlighting the importance of FasL for the induction of T cell activation. Of note,

![Fig. 4](https://academic.oup.com/immtol/article-abstract/21/5/587/727663/592)

**Fig. 4.** FasFc inhibits the proliferation of human CD4+ T cells. Purified freshly isolated CD4+ T cells were stimulated for 3 days in 24-well tissue culture plates coated with anti-CD3 mAb OKT3 (2 µg ml⁻¹) plus anti-CD28 (5 µg ml⁻¹) in the presence or absence of FasFc or Fc control protein (both 20 µg ml⁻¹). Cells were cultured in RPMI medium with 10% (v/v) human AB serum (for detail see Methods). (A) Upper panel: microphotographs show the increase in cell size and cluster formation as observed by inverse light microscopy after 3 days of stimulation. Lower panel: blastogenesis was monitored on the basis of cell size (forward scatter) and granularity (side scatter) determined by FACS analysis. (B) Freshly isolated human CD4+ T cells were labeled with CFSE at a final concentration of 5 µM and stimulated as indicated. CFSE-labeled cells were measured and analyzed by flow cytometry after 87 h of stimulation. Results from one out of eight independent experiments with different donors are shown. (C) PI staining of CD4+ T cells at day 3 of incubation. (D) Surface expression of CD25 and CD69 was assessed after 48 h of incubation in the absence (unstimulated) or presence of OKT3 plus anti-CD28 and indicated stimuli by staining with PE-conjugated anti-CD25 or anti-CD69 mAb followed by FACS analysis. The percentages of CD25 or CD69 positive cells are shown for one representative experiment out of five with different donors.
as illustrated by PI staining (Fig. 4C), the lack of cell proliferation was not due to enhanced cell death.

To rule out that the inhibitory effect of the FasFc fusion protein was due to a sterical interference with anti-CD3 and anti-CD28 signals, we also incubated TCR-stimulated cells in presence of plate-bound anti-FasL pAb. Also under such conditions, FasL ligation resulted in a complete block of proliferation (Fig. 5). In addition, we pre-coated culture wells with anti-human IgGFc antibodies before adding FasFc or Fc control protein. However, also this ‘increased cross-linking’ did not alter the inhibitory effect of FasFc (Fig. 6).

**Early block of T cell activation by FasFc**

In order to get an indication at which point FasFc interferes with T cell activation, we analyzed the expression of the two standard activation markers CD69 and CD25. Cells were treated as described and the surface expression of CD69 and CD25 was analyzed by flow cytometry (Fig. 4D). In line with the blockade of CD3- or CD3–CD28-induced T cell proliferation, in the presence of plate-bound FasFc fusion proteins, the inducible expression of CD69 and CD25 was also abolished. Notably, exogenous IL-2 was not able to overcome the FasFc-mediated inhibition of CD4⁺ T cell proliferation, whereas CD3/CD28-triggered activation was further enhanced (Fig. 7).

**Suppression of TCR internalization by FasL cross-linking**

TCR ligation is associated with subsequent receptor internalization, recycling and degradation. It is believed that TCR translocation to an endosomal compartment potentiates T cell activation by generating essential signal platforms. As shown in Fig. 8, FACS analysis using an anti-TCR mAb clearly revealed that FasFc co-stimulation significantly reduced the TCR internalization.

**The TCR-FasL cross-talk occurs upstream of PKC and calcium mobilization**

To more specifically determine where a TCR to FasL cross-talk could take place within the TCR-dependent signaling cascade, we treated cells with the protein kinase C (PKC) activator PMA in combination with ionomycin. We stimulated purified CD4⁺ T cells labeled with CFSE in the presence or absence of FasFc fusion protein as described before, or with PMA (10 ng ml⁻¹) and ionomycin (500 ng ml⁻¹). After 3 days of incubation, we observed that PMA/ionomycin-treated T cells proliferated more vigorously than anti-CD3/anti-CD28-stimulated cells (Fig. 9).

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**Fig. 5.** Co-stimulation with anti-FasL pAb blocks TCR-induced proliferation. Freshly isolated primary CD4⁺ T lymphocytes were labeled with 5 μM CFSE and 1 × 10⁶ cells were stimulated or not in 24-well tissue culture plates with plate-bound anti-CD3 mAb OKT3 (2 μg ml⁻¹) plus anti-CD28 (5 μg ml⁻¹) in presence or absence of the pAb anti-FasL (C-20) (10 μg ml⁻¹). At day 4, the cell division pattern of CFSE-labeled cells was analyzed by flow cytometry. (A) Forward scatter/side scatter (FSC/SSC) dot plots to demonstrate blastogenesis. (B) CFSE profiles. (C) FSC/FL-2 dot plots indicating cell viability after PI (FL-2) staining. The results are from one representative experiment out of three experiments performed with cells from different donors.
Under these conditions, FasFc was not able to inhibit the proliferation induced by PMA and ionomycin, suggesting that the potential TCR to FasL cross-talk associated with the blockade of T cell activation is likely to be independent of or to occur upstream of PKC activation and calcium mobilization.

Reduced MAPK, LAT, ZAP-70 and PLCγ activation by FasL-mediated signaling

We next investigated whether the phosphorylation of the MAPK ERK1/2 and the further upstream located phospholipase Cγ, the adapter protein LAT, the tyrosine kinase ZAP-70 and the Src kinase Lck were affected by FasL co-stimulation. To this end, freshly isolated human CD4+ T cells were stimulated in the presence or absence of OKT3/CD28/IgGFc beads or OKT3/CD28/FasFc beads between 5 min and 2 h. The cells were lysed in NP-40 lysis buffer and analyzed by western blotting using an anti-phospho-PLCγ, anti-phospho-ERK1/2, anti-phospho-LAT, anti-phospho Lck and an anti-ERK1/2 mAb as a loading control (Figs 10 and 11A). To analyze the phosphorylation of the tyrosine residue 319 of activated ZAP-70, we immunoprecipitated and blotted phosphorylated ZAP-70 with a phosho-specific antibody (Fig. 11B). Rebrobing of whole cell lysates with a ZAP-70 antibody confirmed comparable protein loading whereas the inhibition by FasL ligation was verified by the decreased phosphorylation of the MAPK ERK1/2 within the same lysate (Fig. 11B).

Taken together, we detected significant phosphorylation of LAT, ZAP-70, PLCγ and ERK1/2 in TCR-triggered cells but not or to a much lower degree in T cells co-stimulated with FasFc (Figs 10 and 11). In contrast, regarding the inhibitory phosphorylation site of Lck (Tyr 505), in the very same lysates, Lck proved to be equally phosphorylated in freshly prepared T lymphocytes with or without activation. We thus conclude that the inhibition of T cells by bead-bound FasFc co-stimulation takes place not only before MAPK activation but rather at a very early stage of T cell activation, upstream of ZAP-70, LAT and PLCγ phosphorylation and therefore certainly prior to the expression of activation markers.

Discussion

Following analyses of antigen- or TCR-CD3-driven activation and selection in wild-type and Fas (lpr, lpr58) or FasL (gld) mutant mice, an alternative role of FasL as a co-stimulatory
or accessory molecule for thymocytes and mature T cells became evident (7, 12, 18, 19, 27). T cells derived from such mice are resistant to Fas/FasL-induced cell death and, therefore, the mice develop lymphocyte hyper-proliferation or accumulation, splenomegaly and autoimmune diseases (28–30). The aforementioned reports also indicated a dual function of FasL as a signal transducer for murine CD4+ and CD8+ T cells. While CD4+ cells showed a reduction in TCR-CD3-induced cell cycle progression and later entered apoptosis, CD8+ cells obviously received a positive co-stimulatory signal through FasL to progress through the cell cycle and to acquire cytotoxic effector function (7, 18, 19). Thus, CD8+ T cell lines derived from gld mice were repressed in antigen-specific proliferation compared with CD8+ T cell lines expressing wild-type FasL. In contrast, antigen-specific CD4+ T cells from gld mice obviously proliferated more vigorously upon stimulation than the wild-type counterparts, indicating that FasL engagement might inhibit the proliferation of wild-type CD4+ T cells (12). The observation that the expression of functional FasL is required for a controlled T cell activation in mice strongly suggested a reverse signaling capacity that might regulate a FasL to TCR–CD3 cross-talk. In a more recent study, Fink et al. (10, 11) attempted to identify the molecular basis of FasL-mediated co-stimulation in murine CD8+ T cells and suggested the amplification of several classical signaling pathways by ligation of FasL, as detailed in the Introduction.

In order to investigate FasL reverse signaling in freshly isolated human T lymphocyte subsets, we first showed that FasL is readily detectable on freshly isolated human T lymphocytes (Fig. 1). In time course experiments, we observed a transient mild increase in expression indicating that also in freshly isolated T cells, a fraction of FasL might be associated with storage vesicles (20). By microscopical inspection, it appeared that FasL mainly localizes at the cell membrane, while a minor fraction associates with a vesicular compartment in the vicinity of the cell surface (31).

Implying the retrograde signaling capacity of FasL, we wondered whether ligation of FasL on primary human T lymphocytes would interfere with the induction of T cell proliferation by TCR-CD3 stimulation. It is important to note that in all our...
experiments with freshly isolated human T lymphocytes, we observed a similar negative regulation of TCR- or TCR–CD28-induced T cell activation in response to FasL ligation, independent of the Tcell phenotype (Fig. 3). Also, for all donors tested, we determined a major reduction of proliferation of freshly isolated PBMC or purified T cell subsets in the presence of plate-bound FasFc. Clearly, FasL cross-linking was absolutely required to exert this inhibition since soluble FasFc fusion protein had no effect. This inhibitory effect was also seen using immobilized anti-FasL pAb (Fig. 5). Furthermore, it did not matter whether the cells were stimulated with anti-CD3 alone or in combination with anti-CD28. In all cases, proliferation was significantly reduced by FasL cross-linking. This result is in part compatible with the reported observations for murine CD4+ T cells (12). However, whereas Desbarats et al. could overcome the inhibitory effect of FasL by adding exogenous IL-2, we did not observe a different outcome of FasL ligation on the TCR-dependent activation of primary human T cells in the presence or absence of IL-2 (Fig. 7). The inability of IL-2 to circumvent the inhibitory effect of FasFc indicates that the mechanism of action is different from other inhibitory co-stimulators, i.e. CTLA-4 (32, 33) and that the absence of IL-2 does not correlate with the observed block in cell cycle progression.

Quite surprisingly, the clear and almost complete negative regulation of TCR activation by FasL described in this study has not been reported earlier. In contrast, as mentioned, Sun et al. (11) recently published that FasL acts as an amplifier for TCR signals at least in activated murine CD8+ T cells. Is this apparent contradiction due to methodological differences or perhaps species-specific effects? To eliminate the possibility of different co-stimulatory conditions, we also performed the aforementioned experiments with primary human T cells in the presence of pre-coated anti-human IgGFc antibodies to more efficiently cross-link FasFc (Fig. 6). However, even under these conditions, we obtained the very same result as using the standard protocol in the absence of anti-human IgGFc antibodies (Fig. 4).

To further characterize the inhibitory effect of FasFc co-stimulation, we asked whether FasL ligation is able to block the proliferation initiated by the PKC activator PMA and the calcium ionophore ionomycin. Under these conditions, FasFc did not prevent T cell activation, indicating that potential FasL reverse signaling acts upstream of PKC and calcium mobilization. We also investigated the activation of MAPK in TCR-triggered cells in the presence or absence of cross-linked FasFc. Compatible

![Fig. 9.](image_url) TCR-FasL cross-talk acts upstream of PKC and calcium mobilization. After purification of primary human CD4+ T cells, the cells were labeled with 5 μM CFSE and stimulated or not with 2 μg ml⁻¹ anti-CD3 mAb OKT3 plus 5 μg ml⁻¹ anti-CD28 (upper panels) or PMA (10 ng ml⁻¹) and ionomycin (500 ng ml⁻¹) (lower panels) in the presence or absence of FasFc or Fc control protein (both 20 μg ml⁻¹). At day 3, proliferation was analyzed by flow cytometry. One experiment out of three is shown.

![Fig. 10.](image_url) Reduced phosphorylation of PLCγ and ERK1/2 upon FasFc co-stimulation. Purified human CD4+ T cells were stimulated or not with OKT3/CD28/IgGFc beads or OKT3/CD28/FasFc beads for the indicated time points and lysed in 1% NP-40 lysis buffer. Lysates from 2 × 10⁶ cells were separated by SDS-PAGE and analyzed by western blotting using antibodies against phospho-PLCγ1, phospho-ERK1/2, ERK1/2 and phospho-Lck. Similar results were observed in more than three separate experiments using T cells purified from different donors.
with the complete block in cell cycle progression, we detected phosphorylated ERK only in TCR-stimulated cells and not in T cells co-stimulated with FasFc (Figs 10 and 11). Since FasFc had no effect on PKC/ionomycin treatment, but blocked TCR internalization and inhibited MAPK and ZAP-70 activation as well as the phosphorylation of LAT and PLCγ, our data indicate that FasFc treatment inhibits T cell activation at a very early stage of T cell activation, possibly by an interaction between adapter or signaling molecules at the level of the formation of the TCR-dependent activation complex.

Although the present data might argue for a direct retrograde signaling capacity either by an inhibitory signal through FasL itself or a FasL-mediated inhibition of the TCR signal, it could also be that the observed effects of FasFc fusion proteins merely represent an inhibition of a potential stimulatory Fas signal. In this context, it was shown that Fas engagement with agonistic anti-Fas mAb (34) or Fas ligation on murine naive CD4+ T cells activated by FasL present on dendritic cells (35) augment T cell activation. However, low expression seems sufficient to exert such a reverse signal. In a physiological setting, Fas expressed on APCs might be more relevant for the modulation of T cell activation via FasL than as a death receptor for e.g. dendritic cells (39, 40). Along this line, FasL but not Fas was shown to be involved as an accessory molecule in positive selection of thymocytes during T cell development and maturation (27). Again, although in the thymus both FasL and Fas are expressed, it is still controversial whether the molecules interact in situ (27, 41, 42). How in this scenario the inhibitory effect of FasL ligation might be turned off is completely unknown. However, it is clear that the T cell surface composition and thereby the signaling threshold dramatically change during the initial activation period.

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**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AICD</td>
<td>activation-induced cell death</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>4-1BBL</td>
<td>4-1BB ligand</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CD27L</td>
<td>CD27 ligand</td>
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<tr>
<td>CFSE</td>
<td>5-(6-)carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CK</td>
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</tr>
<tr>
<td>E+</td>
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</tr>
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<td>FasL</td>
<td>Fas ligand</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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**Fig. 11.** FasL engagement inhibits TCR-induced phosphorylation of ZAP-70 and LAT. (A) Resting human CD4+ T lymphocytes were stimulated or not with OKT3/CD28/IgGFc beads or OKT3/CD28/FasFc beads for the indicated time points and lysed in 1% NP-40 lysis buffer. The lysates were investigated by western blotting using antibodies against phospho-LAT, phospho-ERK1/2 and ERK1/2. (B) Freshly isolated CD4+ T cells were stimulated as indicated for 5 min. Lysates of 10 × 10⁶ cells were pre-cleared by protein G-Sepharose and subjected to immunoprecipitation using an anti-phospho-ZAP-70 (Tyr319) mAb. After separation by SDS-PAGE, phosphorylation of ZAP-70 was detected by immunoblotting using the phospho-ZAP-70-specific mAb (upper panel). Comparable protein input was confirmed by blotting whole cell lysates for ZAP-70 (middle panel). The anti-phosho-ERK1/2 mAb was used to verify the inhibition of T cell activation by FasFc within the same lysates. Results are representative of at least three independent experiments.
T cell activation controlled by FasL

gld  generalized lymphoproliferative disease
lpr  lymphoproliferation
MACS  magnet-activated cell sorting
MAPK  mitogen-activated protein kinase
pAb  polyclonal antibody
PI  phosphatidylinositol
PKC  protein kinase C
PMA  phorbil 12-myristate 13-acetate
SE  staphylococcal enterotoxin
TNF  tumor necrosis factor
TNFSF  tumor necrosis factor superfamily

References