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Dynamin related protein 1-dependent mitochondrial fission regulates oxidative signalling in T cells

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ABSTRACT

In T cells mitochondria-derived reactive oxygen species (ROS) are indispensible for activation of the transcription factor NF-κB, expression of cytokines and the CD95 ligand (CD95L/FasL). Here we show that activation-induced ROS generation is dependent on mitochondrial fission. Inhibition of dynamin related protein 1 (Drp1) results in reduced ROS levels and transcriptional activity of NF-κB leading to diminished proliferation and CD95L-dependent activation-induced cell death (AICD). Upon stimulation Drp1 is S-nitrosylated, which is required for oxidative signalling, AICD and cytokine production. In conclusion, we describe a novel signalling pathway that links TCR-induced nitric oxide release to mitochondrial fission and oxidative signalling.

1. Introduction

T cell receptor (TCR) triggering induces a signalling cascade that leads to T cell activation and activation-induced cell death (AICD) [1]. Upon TCR engagement phospholipase Cγ1 produces inositol-3,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 raises intracellular calcium levels and leads to translocation of NF-AT into the nucleus. DAG triggers the Ras-MAP kinase cascade inducing activation of AP-1 and, in parallel, activation of protein kinase Cθ and subsequently the NF-κB translocation into the nucleus [2]. In parallel, PKC induce a shift in cellular metabolism and finally leads to the release of reactive oxygen species (ROS) [3]. ROS are essential for complete activation of NF-κB and AP-1 [4]. Several sources of ROS in TCR signalling have been described [5,6]. However, accumulating reports suggest that the mitochondrial respiratory chain is the source for early ROS production in T cells [7–10]. ROS signalling and subsequent transcription factor activation are indispensable for expression of interleukin (IL)-2 and the death ligand CD95L (FasL), which induces CD95 (Fas)-dependent apoptosis in pre-activated T cells (AICD) [6,11–13].

Mitochondrial function has been linked to mitochondrial morphology before [14]. In several cell types ROS production was correlated with increased fission [15–17]. In these settings oxidative stress was causative for mitochondrial fragmentation. Therefore, fission might be a way to cope oxidative stress. However, Yu et al. reported that under hyperglycemic conditions mitochondria undergo dynamin related protein 1 (Drp1)-dependent fission, which resulted in increased ROS release, demonstrating that fission also supports ROS formation [18].

In conjunction with T cell activation several functions of Drp1 have been described. Triggering of the CXCL-12 receptor resulted in increased fission and cell motility [19], whereas Drp1 was reported to be essential for TCR recycling at the immunological synapse [20]. These functions of mitochondrial dynamics were linked to local provision of energy-consuming processes like actin remodelling with ATP. However, the function of mitochondrial fission during T cell activation in respect to ROS production has not been investigated.

Abbreviations: AICD, activation induced cell death; CFSE, carboxyfluorescein succinimidyl ester; DAG, diacylglycerol; Drp1, dynamin related protein 1; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; IP_3, inositol 3,4,5-triphosphate; NAME, N^G-Nitroarginine methyl ester; NMMA, N^G-monomethyl arginine; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; TCR, T cell receptor

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Here, we show a novel signalling pathway, which links TCR signalling to mitochondrial fission, ROS release, and T cell activation.

2. Material and methods

Material and methods are given in the Supplementary data.

3. Results

3.1. Mitochondria undergo fission upon T cell stimulation

Mitochondrial fission was described to be a prerequisite for ROS production [18]. Therefore, we asked whether TCR stimulation results in Drp1-dependent mitochondrial fission. Jurkat cells were transfected with mitochondria-targeted YFP. Upon stimulation with agonistic CD3-engaging antibodies mitochondria showed a fragmented phenotype (Fig. 1A). Microscopic analysis of resting T cells was not possible due to the low numbers of clustered mitochondria (data not shown). Therefore, T cells were activated with PHA in the presence of IL-2. After TCR re-stimulation activated T cells showed increased mitochondrial fission (Fig. 1B and C). Determination of the form factor and aspect ratio of mitochondria further demonstrated mitochondrial fission upon stimulation (Fig. 1D and E).

Drp1 is the crucial factor for mitochondrial fission, which translocates from the cytosol to the outer mitochondrial membrane. Upon T cell stimulation with agonistic anti-CD3 antibodies an accumulation of Drp1 at mitochondria was observed indicating that elevated mitochondrial fragmentation occurs due to an increased fission rate rather than due to a decreased fusion rate (Fig. 1F).

3.2. Drp1 function is required for activation-induced ROS production and NF-κB activation

To investigate whether mitochondrial fission is required for activation-induced ROS generation in T cells we inhibited Drp1 by overexpression of dominant negative Drp1 (dnDrp1) [21] or by siRNA-mediated downregulation in Jurkat cells. ROS production was assessed using H_2DCF-DA. Expression of dnDrp1 or siRNA application reduced activation-induced ROS production (Fig. 2A and B). In addition, reduced ROS levels were observed, when primary T cells were stimulated with anti-CD3 antibodies in the presence of the Drp1 inhibitor Mdivi-1 (Fig. 2C).

ROS are essential for activation of NF-κB [4]. In line with reduced ROS production, overexpression of dnDrp1 (Fig. 2D), downregulation of Drp1 (Fig. 2E) or application of Mdivi-1 (Fig. 2F) led to diminished NF-κB activation as assessed with luciferase-reporter

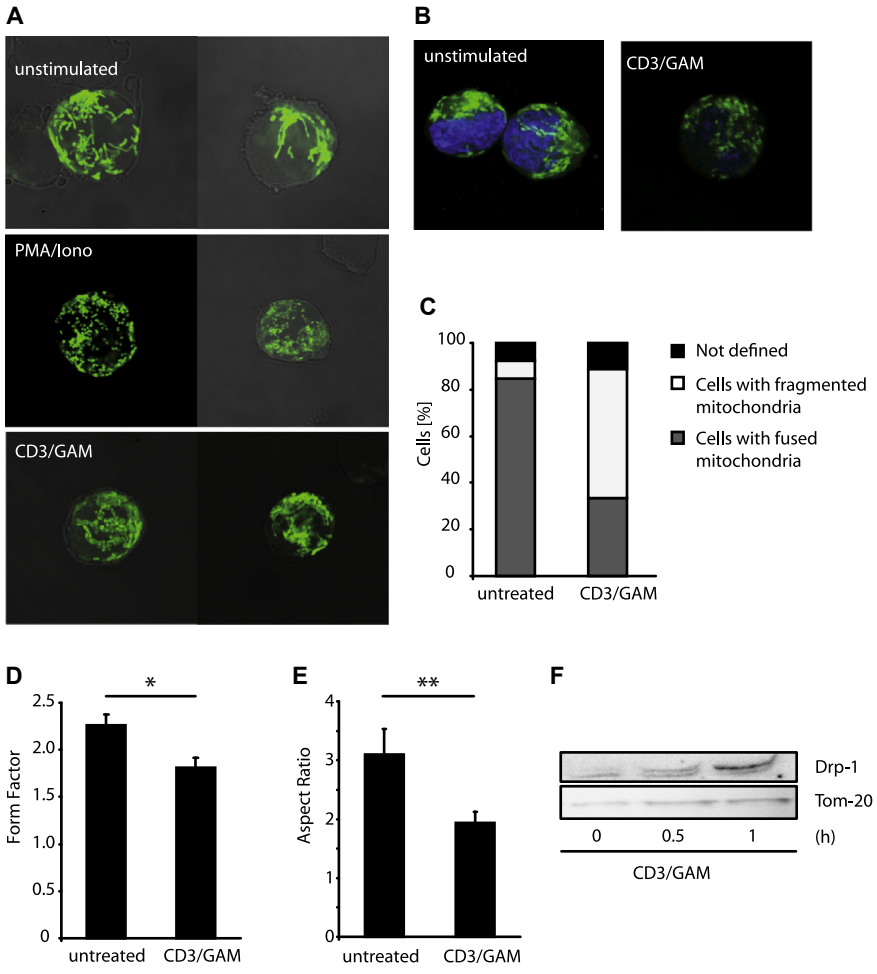

Fig. 1. Mitochondria undergo fission upon T cell stimulation. (A) Representative images of Jurkat cells transfected with mtYFP (upper panel) and stimulated for 45 min either with PMA (10 ng/ml) and iono (1 μM) (middle panel) or anti-CD3 and GAM (1 μg each, lower panel). (B–E) Pre-activated human T cells were stained with Mitotracker and DAPI before activation with anti-CD3 and GAM. (B) Representative image of unstimulated and stimulated pre-activated T cells. Cells with fragmented or fused mitochondria were counted in a blinded fashion (C) or analysed with ImageJ and form factor and aspect ratio were calculated (D,E). (F) T cells were stimulated with anti-CD3 and GAM (1 μg/ml each) for the indicated periods. Mitochondria were isolated by sorting with anti-Tom20 antibodies coupled to magnetic beads. Error bars represent S.E.M. ($^*P < 0.05$; $^{**}P < 0.001$, Student's *t*-test).

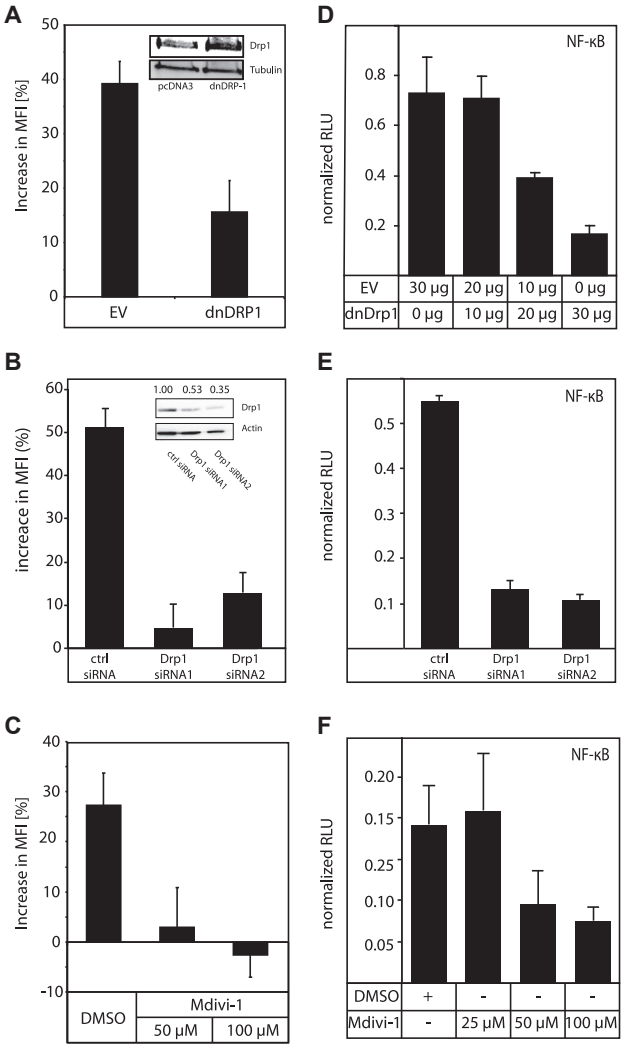

Fig. 2. Drp1 regulates mitochondrial ROS production and NF-κB activation upon TCR stimulation. (A and B) Jurkat cells were transfected with dnDrp1 or Drp1 specific siRNA. dnDrp1 overexpression (A) or downregulation (B) was determined by Western blot (inlay). ROS production in cells stimulated with PMA (10 ng/ml) was detected by H_2DCF-DA. ROS production is depicted as increase in MFI. (C) T cells pre-treated with Mdivi-1. ROS production in cells stimulated with anti-CD3 and GAM (1 µg/ml) was detected by H_2DCF-DA. (D–F) NF-κB activity was determined by luciferase reporter assays. Jurkat cells were transfected with the indicated amounts of EV or dnDrp1 (C), Drp1 siRNA (D) or pre-treated with Mdivi-1 (E) and activated with PMA (10 ng/ml) and ionomycin (1 µM) for 8 h. Results are shown as normalized relative light units (RLU). Data shown are representatives of three independent experiments. Error bars represent S.D.

assays. These results demonstrate that Drp1-mediated fission modulates TCR-induced ROS production and NF-κB activation.

3.3. Drp1 inhibition reduces TCR-dependent gene expression

Since NF-κB is an essential transcription factor for transcription of IL-2 [22] and CD95L [23], the influence of Drp1 inhibition on TCR-dependent gene expression and T cell function were investigated. Overexpression of dnDrp1 in Jurkat cells (Fig. 3A) and Drp1 inhibition by Mdivi-1 (Fig. 3B) reduced IL-2 mRNA levels. Further, T cells treated with Mdivi-1 showed a decreased secretion of IL-2 (Fig. 3C). Since IL-2 is required for the survival of activated cells we performed CFSE dilution assays in the presence of Mdivi-1 and detected less dividing cells (Fig. 3D).

TCR stimulation in pre-activated T cells and in Jurkat cells also induces CD95L upregulation and subsequent AICD [24]. As seen for IL-2 Mdivi-1 administration led to reduced CD95L transcription upon stimulation (Fig. 3E). In addition, AICD was reduced in dnDrp1 expressing Jurkat cells (Fig. 3F) and Mdivi-1-treated pre-activated T cells (Fig. 3G). However, Mdivi-1 was shown to inhibit mitochondrial outer membrane permeabilisation and cytochrome *c* release [25]. Therefore, inhibition of Drp1 might lead to less amplification of the CD95-dependent apoptosis pathway and reduced cell death could reflect a disturbed apoptosis machinery rather than reduced apoptosis initiation by diminished CD95L transcription. To analyse the apoptosis sensitivity of Mdivi-1 treated cells, cells were stimulated with anti-APO1, a CD95-agonistic antibody (Fig. 3H). CD95 triggering resulted in a similar extent of specific cell death in control cells compared to Mdivi-1 pre-treated ones demonstrating that the apoptosis machinery is not affected by Drp1 inhibition.

Thus, Drp1 is not only an essential factor for ROS release by mitochondria upon T cell stimulation, but it controls downstream events like cytokine expression, proliferation and AICD.

3.4. Drp-1 S-nitrosylation is required for ROS production

Drp1 activity and recruitment to mitochondria is regulated by several posttranslational modifications including S-nitrosylation. Nitric oxide and nitric oxide-derived species are involved in TCR signalling and their presence is essential for AICD [26–28].

To test, if nitric oxide signalling was required for activation-induced ROS production nitric oxide synthases were blocked with the NOS-inhibitors L-NAME and L-NMMA. Both inhibitors blocked ROS production, while inactive D-NAME had no effect (Fig. 4A–C). L-NAME further inhibited transcription of IL-2 and CD95L in Jurkat cells (Fig. 4D and E), indicating that nitric oxide signalling occurs upstream of ROS generation.

Cysteine 644 has been identified as S-nitrosylation site of Drp1. Mutation of this residue abrogates nitric oxide-induced cell death in neurons and results in diminished Drp1 oligomerisation and activity [29]. To elucidate, whether nitric oxide might influence ROS production in T cells *via* Drp1-mediated fission, mitochondrial fragmentation in the presence of L-NAME was analysed. Reduced fission was detected (Fig. 4F). In addition, biotin-switch assays were performed to detect S-nitrosylated proteins. Stimulated T cells showed higher levels of S-nitrosylation than control cells (Fig. 4G). In order to determine whether TCR-induced Drp1 nitrosylation has functional significance, Jurkat cells were transfected with $Drp1^{C644A}$, EV or $Drp1^{wt}$. $Drp1^{C644A}$ expression reduced activation-induced mitochondrial fission (Fig. S1A). In parallel, a minor decrease in ROS production was observed indicating that although Drp1 nitrosylation is modulating TCR-induced ROS production other posttranslational modifications might be involved (Figs. 4H and S1B). However, the reduction in ROS production in these cells was sufficient to inhibit NF-κB activation (Figs. 4I and S1C). Subsequently, the expression of IL-2 and CD95L was diminished (Figs. 4J and S1D and E) and cells showed less AICD (Fig. 4K).

4. Discussion

Several studies demonstrated a role for ROS in cell homeostasis and signalling [4]. In T cells ROS were identified as important second messengers. Paralleled by calcium influx into the cytosol ROS enable activation induced gene transcription [10–13]. TCR-dependent ROS production involves changes in activity of mitochondrial respiratory chain complexes and increased glycolytic flux [3]. In addition, Mitochondrial function was linked to morphology in different settings [14]. Therefore, we investigated, whether changes in mitochondrial morphology are also relevant in TCR signalling and ROS production. We observed that mitochondria undergo

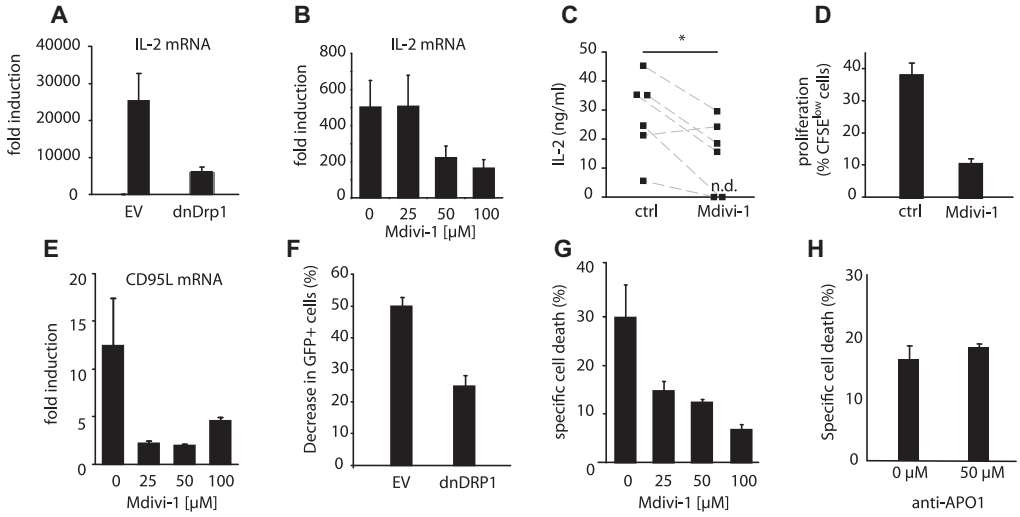

Fig. 3. Drp1 regulates cytokine release and AICD. (A) Jurkat cells transfected with dnDrp1 or empty vector (EV) were stimulated with PMA (10 ng/ml) and ionomycin (1 μM) for 2 h and IL-2 mRNA level was measured by qPCR. (B and C) T cells were pre-treated with Mdivi-1 and stimulated with anti-CD3 and GAM (1 μg each) and after 2 h treatment IL-2 mRNA level was determined by qPCR (B) or IL-2 secretion was measured by ELISA after overnight incubation. (D) CFSE-labelled T cells were pre-treated with Mdivi-1 and stimulated with anti-CD3, anti-CD28 and GAM (1 μg each). Percentage of proliferated, $CFSE^{low}$ cells was determined after 3 days. (E and F) Expanded T cells were pre-treated with Mdivi-1 and stimulated with anti-CD3 and GAM (1 μg each) and CD95L mRNA level was determined by qPCR after 2 h (E) or AICD was measured after 24 h by FSC/SSC exclusion (F). (G) Jurkat cells were transfected with GFP and dnDrp1 or EV and stimulated with PMA (10 ng/ml) and ionomycin (1 μM). After 24 h cell death was measured as decrease in GFP^+ cells. (H) T cells were pre-treated with Mdivi-1 and stimulated with 100 ng/ml the CD95 agonistic antibody anti-APO-1. Data shown are representatives of three independent experiments. Error bars represent S.D. ($^*P < 0.05$; paired Student's *t*-test).

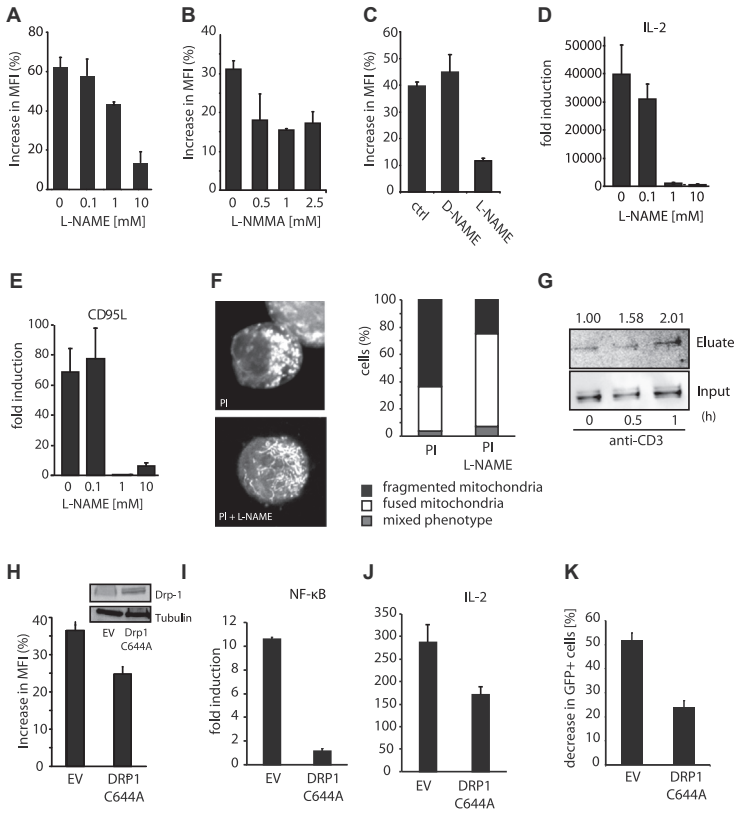

Fig. 4. Drp-1 S-nitrosylation is required for ROS generation. (A–C) Jurkat cells were treated with the general NOS inhibitors L-NAME (A), L-NMMA (B) and 10 mM L- or the inactive enantiomer D-NAME (C). ROS production was determined by H_2DCFDA staining. ROS production is depicted as increase in MFI. (D and E) Jurkat cells were treated with L-NAME and stimulated with PMA (10 ng/ml) and ionomycin (1 μM). IL-2 (D) and CD95L (E) mRNA expression was determined after 2 h by qPCR. (F) Jurkat cells were treated with 10 mM L-NAME and activated with PMA (10 ng/ml) and ionomycin (1 μM). 15 min before fixation cells were stained wit Mitotracker DeepRed. (G) Biotin Switch assay of T cells stimulated with anti-CD3 and GAM (1 μg/ml each). Numbers above Western blots represent relative nitrosylation at specified time points determined by densitometry. (H) Jurkat cells were transfected with $Drp1^{C644A}$ or empty vector (EV). ROS production after 1 h stimulation with PMA was detected with H_2DCF-DA. Overexpression was detected by Western blot (right panel). (I) NF-κB activity was measured by luciferase reporter assays. Transfected Jurkat cells were treated with PMA (10 ng/ml) and ionomycin (1 μM) for 8 h. (J) Jurkat cells transfected with $Drp1^{C644A}$ or EV were stimulated with PMA (10 ng/ml) and ionomycin (1 μM) for 2 h and IL-2 mRNA was measured by qPCR. (K) AICD of transfected Jurkat cells upon stimulation with PMA and ionomycin was measured as decrease in GFP^+ cells after 24 h. Data shown are representatives of three independent experiments. Error bars represent S.D.

fission upon T cell stimulation in a Drp1-dependent manner (Fig. 1). These morphological changes were a prerequisite for TCR-induced ROS production (Fig. 2). The connection of mitochondrial fission and the oxidative status of cells has been investigated in several studies. Application of exogenous hydrogen peroxide to myocytes [15] and endothelial cells [16] resulted in a drop of mitochondrial membrane potential and increased fission. Endogenous ROS production in various cell types induced by photodamage resulted in increased fission [17,30]. Fission could be prevented by administration of antioxidants, indicating that ROS are the trigger of mitochondrial fragmentation [17]. Other studies correlated fragmented mitochondrial networks with increased cellular oxidative stress [31]. While these observations may primarily account for pathophysiological conditions, induced short-term fission events were described to favour ROS release as a response to extracellular stimuli. Hepatoma cells treated with dexamethasone increased Drp1 expression and reduced Mfn1/2 levels, which led to mitochondrial fission. In parallel, oxygen consumption rose and more superoxide anions were produced [32]. Similarly, during hyperglycemic conditions, increased mitochondrial division coincides with elevated ROS levels. Importantly, under these settings inhibition of ROS production did not reduce mitochondrial fission, whereas inhibition of fission diminished ROS production [18,33]. In addition, alterations of mitochondrial structure were dependent on a signalling cascade involving calcium influx and MAPK activity [34]. Thus, ROS production can be a regulated event downstream of mitochondrial fission.

In the present study we observed that upon TCR stimulation Drp1 translocates to mitochondria. Further, inhibition of Drp1 led to reduced ROS production and T cell activation, indicating that increased fission, but not reduced fusion is responsible for mitochondrial fragmentation. Interestingly, others described that recombinant Drp1 increased mitochondrial ROS generation, when added to isolated mitochondria [35], further strengthening the pivotal role of Drp1 in the regulation of mitochondrial physiology. How changes in the mitochondrial fission and fusion balance translate to altered ROS production is unknown. However, it was hypothesized that in parallel to fission changes in the ultrastructure of the inner mitochondrial membrane can occur, which might change supercomplex formation and respiratory chain function [36]. Such changes have been described for activated T cells before [3] and the involvement of Drp1 in this process is a challenging subject of future investigations.

Drp1 inhibition by either overexpression of dnDrp1, siRNA-mediated Drp1 knockdown or the application of Mdivi-1 not only reduced ROS production but also NF-κB activation and IL-2 and CD95L expression. A similar mechanism was also described for the triggering of Toll-like receptor (TLR) 4 in microglia. Drp1 inhibition also blocked ROS-generation and NF-κB activation and reduced the expression of proinflammatory cytokines [37]. The role of Drp1 in TCR-signalling is controversial. Baixauli et al. reported that Drp1 inhibition reduced accumulation of mitochondria at the immunological synapse, which disturbed the assembly of the central supramolecular activation cluster (cSMAC) [20]. This failure of immune synapse formation did not result in reduced TCR activity, but increased PLCγ1 and ERK activation, which subsequently raised IL-2 secretion due to inefficient TCR/CD3 recycling. However, mitochondrial localisation at the immune synapse was also described to be a prerequisite for proper calcium influx and subsequent T cell activation [38]. Therefore, it is reasonable that mitochondria have complex functions in T cell activation and the termination of TCR responses. In particular, the use of different stimuli might help to distinguish these activating and inhibitory roles. We used activation by agonistic anti-CD3 antibodies or by PMA and ionomycin. Under these conditions the immunological synapse is not formed, but mitochondria are fragmented in a Drp1-dependent manner, which further activates ROS-dependent gene transcription resulting in IL-2 production, cell proliferation and AICD.

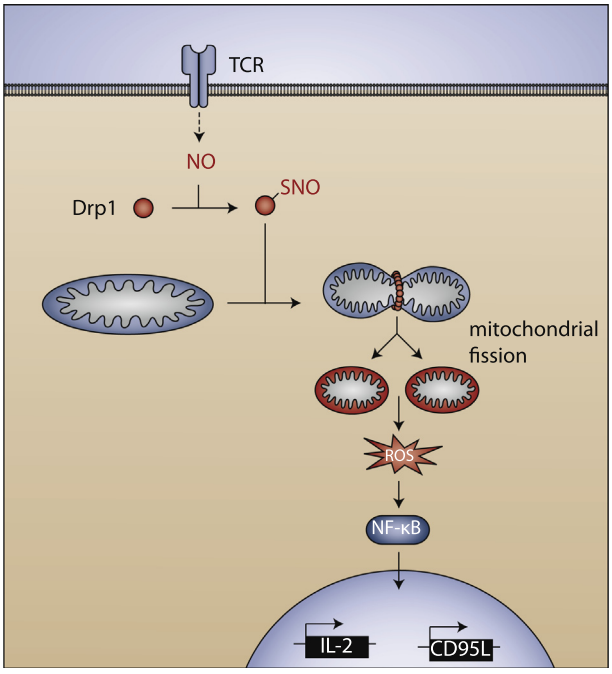

Fig. 5. TCR-triggered mitochondrial ROS production is dependent on Drp1. TCR stimulation-induced nitric oxide leads to S-nitrosylation of Drp1. Drp1 then translocates to mitochondria and initiates fission required for ROS release. ROS then act as second messengers for NF-κB-dependent expression of, e.g. IL-2 and CD95L.

Drp1 activity and localisation is tightly controlled by several posttranslational modifications [39]. Among those S-nitrosylation was shown to be required for Drp1-mediated fission as a response to elevated nitric oxide levels [29]. Although the mechanism, by which S-nitrosylation affects Drp1 is controversially discussed [40], overexpression of $Drp1^{C644A}$ rescued neurons from nitric oxide-induced fission and cell death [29]. In order to investigate whether Drp1 S-nitrosylation plays a role in T cells, the effects of nitric oxide on ROS production were determined. NOS inhibition resulted in reduced ROS release and cytokine expression (Fig. 4), demonstrating a function of nitric oxide upstream of ROS generation. These results are in line with previous publications, which reported, that nitric oxide is a critical second messenger on TCR signalling that modulates mitochondrial physiology [26,41] expression of IL-2 [28] and CD95L [27]. Even though nitric oxide production in T cells was low and its presence could only be indirectly detected by the presence of nitrated proteins (data not shown), it was sufficient to induce Drp1 nitrosylation (Fig. 4). Overexpression of $Drp1^{C644A}$ inhibited mitochondrial ROS production, NF-κB activation, cytokine transcription and AICD, proving its role in TCR-induced mitochondrial fission.

In conclusion, our observations hint a novel branch of TCR signalling, in which nitric oxide production results in a Drp1-dependent shift in mitochondrial morphology that allows ROS production and results ultimately in activation-induced gene expression (Fig. 5).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.03.029.

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