Review Article

Indian J Med Res 138, November 2013, pp 577-590

Role of apoptosis-inducing factor (Aif) in the T cell lineage

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Received December 26, 2012

Multiple checkpoints regulating finely balanced death-versus-survival decisions characterize both thymic development and peripheral homeostasis of T lymphocytes. While exploring the mechanisms of T cell death involved at various stages during the life of a T cell, we have observed and reported a variety of non-redundant roles for apoptosis inducing factor (Aif), a mitochondrial flavoprotein. Aif is ubiquitously expressed in all cell lineages and functions as an NADH oxidase in its mitochondrial location. It is released following the mitochondrial death signals, whereupon it translocates to the nucleus, binds to DNA and causes large-scale DNA fragmentation. During T cell development, Aif is important for developing thymocytes to navigate the double negative (DN)3 to DN4 transition (beta-selection), via its oxidoreductase property which protects the rapidly proliferating cells from death due to reactive oxygen species (ROS). In peripheral mature T cells, Aif deficiency leads to an increased susceptibility of T cell blasts to activation induced cell death (AICD), possibly mediated by its antioxidant function, and decreased sensitivity to neglect-induced death (NID). Thus, Aif seems to have pro-apoptotic and anti-apoptotic roles in the same lineage in different contexts and at different stages. Surprisingly, in the closely related B lymphocyte lineage, Aif deficiency does not result in any abnormality. These findings generate the possibility of specific T cell dysfunction in human disease caused by Aif deficiency, as well as in mitochondriopathies due to other causes. Also, these data raise questions regarding the basis of lineage-specific consequences of the dysfunction/deficiency of apparently ubiquitous molecules.

Key words Apoptosis - lymphocyte development - mitochondria - ROS - T lymphocytes - thymus

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Introduction

Successful immune activation and its resolution depend on a variety of crucial checkpoints regulating the death-versus-survival decisions in the immune cells[1]. Since such physiological regulatory checkpoints are important for both normal development of immune cells[2,3] as well as for removal of excess cells following immune responses[4], understanding the mechanisms of death regulating these becomes relevant. Our recent work[5,6] indicates that the apoptosis-inducing factor (Aif), a mitochondrial flavoprotein, has interestingly pleiotropic roles in regulating cell death in the T cell lineage, both during the T cell development and in the peripheral T cell pool, functioning both pro- and anti-apoptotically in T lineage cells in the context of different death triggers. These apparently opposing physiological functions of Aif in cells of the same lineage raise interesting questions on many issues relevant to the understanding of mitochondrial biology, regulation of apoptosis, lineage specificity of mitochondrial dysfunction and pathobiology of cell death in general.

An overview of T cell development and survival of peripheral T cells is presented below with emphasis on the points of regulation of cell death. The mechanisms of major modes of cell death are summarised with some indications for caspase-independent cell death, followed by discussions of our data supporting the role of Aif in mediating death in the T cell lineage. Finally we discuss the relevance of our findings in the context of the possible consequences in human mitochondrial diseases.

T cell development in thymus - a game of death and survival

The precursors of T cells, the pluripotent stem cells generated in the bone marrow, migrate to the thymus and further differentiate, obtaining trophic signals from the thymic stromal microenvironment[7]. In the thymus, thymocyte differentiation proceeds along well-defined developmental stages characterized by functional maturation, phenotypic alterations and carefully balanced survival-or-death decisions[8]. The ultimate outcome of thymocyte differentiation is the generation of a T cell repertoire bearing somatically diversified functional T cell receptors (TCRs) on their surface which can recognize antigenic epitopes in the context of self MHC (major histocompatibility complex) molecules.

The earliest stages of T-lineage cells in the thymus are phenotypically negative for CD4 and CD8 (double-negative; DN). DN thymocytes sequentially pass through four defined stages: DN1, DN2, DN3 and DN4[8]. The TCR genes of DN1 cells, the earliest stage expressing c-Kit and CD44 but not CD25, have not yet started rearrangement of the TCRβ chain locus and are still in germline configuration. DN2 cells (CD44+ c-Kit+ CD25+) begin Dβ-Jβ rearrangement at their TCRβ chain locus and DN3 cells (CD44-, c-Kit-, CD25+) have ongoing V β-DJ TCRβ gene rearrangements. The DN3 cells test the functional capability of the newly rearranged TCRβ chain by pairing it with a surrogate monomorphic TCRα chain to form a TCR, the expression of which, along with that of the CD3 complex of molecules on the membrane, delivers proliferative and developmental signals necessary to proceed to the DN4 and onwards to the CD4+CD8+ (double positive; DP) stage. If the TCRβ chain rearrangement is unsuccessful, cells die off instead. The proliferative burst accompanying successful progression from DN3 to DN4 and DP stages is referred to as β-selection[8].

DP thymocytes further undergo TCRα locus rearrangement, which if successful, results in the generation of thymocytes bearing functional TCRs which are subjected to positive and negative selection processes guided by the specificity or avidity of interactions with peptide-MHC complexes in the stromal microenvironment[9]. In addition, depending on which MHC class the T cell is capable of recognizing, these become specialized to eventual cell fates of distinct effector subsets, namely, the 'helper' T cells (CD4+; capable of recognizing target epitopes bound on MHC class II) and cytotoxic T cells (CD8+: capable of recognizing targets bound to MHC class I).

Since the generation of somatically diversified antigen-recognition receptors (TCRs) by recombination is a process with a great deal of uncertainty and since MHC is an extremely polymoprhic locus in all mammalian populations, a large number of developing thymocytes have no TCRs or non-functional TCRs incapable of recognizing targets presented in the context of available self MHC molecules[8]. These categories of thymocytes which are superfluous and not usable are removed by regulated cell death pathways. In addition, T cells which have strong affinities to self epitopes can also be generated during the random assembly of TCRs, which are deleted from the developing repertoire, thereby avoiding significant autoimmunity.

These basic principles govern the organization of the thymus, the path taken by developing T lineage cells as they proceed through various developmental stages and their phenotypic and functional attributes.

Survival-versus-death decisions of T cells in the periphery

The naïve CD4 and CD8 T cells egressing the thymus home to the peripheral lymphoid organs and circulate until, if ever, these are activated by target epitopes presented on the antigen-presenting cells (APCs). On activation, naïve cells proliferate and differentiate further into effector T cells as well as to memory T cells which remain in a resting state for further activation in response to any re-exposure to the cognate antigen. Depending on circumstances, the numbers of activated cells during immune responses as well as resting cells surviving after immune responses are regulated by death-survival decisions[10].

Naive T cells which egress from the thymus survive in the periphery for several weeks[11]. A balance between the loss of ageing naïve T cells and the constant output of young naïve T cells from the thymus maintains the naïve T cell pool numbers and their polyclonal TCR repertoire[12]. Naive T cells depend on a variety of extrinsic signals to survive in the periphery including those delivered by the cytokine interleukin (IL)-7[13] and tonic interactions of TCRs with self-peptide/MHC ligands[14,15]. On the other hand, naïve cells that get activated proliferate and assume an effector phenotype eventually undergo contraction[16], failure of which leads to excessive activation of adaptive immune system and end-organ damage. Similarly, occasional naïve T cells with affinity to self-peptide/MHC complexes that escape thymic deletion encounter their targets in the periphery and begin responding, but die off quickly in a form of T cell death referred to as activation-induced cell death (AICD)[17], which is mediated by the extrinsic signalling pathway of apoptosis via engagement of death receptors (Fas, TNF-R1, TRAILR)[18]. AICD is measured in-vitro by generating activated T cells and re-stimulating them with ligation of CD3 or with phorbol myristic acid (PMA)/ionomycin in the presence of IL-2[19,20].

What are the molecular changes following TCR ligation which lead to the sensitization of activated T cells to apoptosis? On TCR stimulation, T cells shift the balance to an apoptosis-sensitive state by increasing the transcription of CD95L[17], which mediates CD95 ligation in the same or neighbouring cells, leading to the formation of death induced signalling complex (DISC)[21], which further activates downstream caspases (caspase 8 and 10). CD95 ligation can also lead to amplification of death signalling by cleavage of the BH3-only protein Bid, its association with Bak/Bax complex then leading to mitochondrial outer membrane permeabilization, release of cytochrome c, formation of signalling complexes comprised of cytochrome c and Apaf-1 (apoptosome) and further activation of executioner caspases (caspase 3 and 7)[22]. These pathways are further regulated by regulatory proteins like cellular FLICE [Fas Associated Death Domain (FADD)-like IL-Iβ - converting enzyme] inhibitory protein (c-FLIP)[22] and inhibitor of apoptosis (IAP)[23] and by crosstalk between the extrinsic and intrinsic pathways.

What are the molecular mechanisms by which FasL levels are upregulated in activated T cells? Evidence points to multiple possible signalling pathways through which FasL transcription is enhanced following TCR ligation. TCR ligation can lead to an increase in intracellular calcium levels, activation of calcineurin which dephosphorylates nuclear factor of activated T-cell (NFAT) leading to its nuclear translocation and binding to regulatory elements in the FasL promoter[18]. TCR ligation can lead to reduction in c-FLIP levels which sensitizes the DISC complex and activates apoptosis. FLIP, a homologue of caspase 8 which lacks enzymatic activity, is highly expressed in resting T cells[24]. Cells undergoing activation are sensitized to apoptosis by IL-2 mediated downregulation of c-FLIP levels[25]. In the induction of CD95L on activated T cells, the role of reactive oxygen species (ROS) is increasingly being appreciated[18,26]. The levels of ROS are increased in T cells upon activation[27]. ROS mediates calcium flux and drives activation of calcineurin, dephosphorylation of NFAT which subsequently increases the transcription of FasL. The role of ROS as an intermediate downstream of TCR ligation necessary for the upregulation of FasL is further supported by the observations that antioxidants as well as mitochondrial function inhibitors are capable of blocking the induction of FasL mRNA following TCR ligation. Exogenously added H_2O_2, on the other hand, can induce FasL mRNA even in the absence of TCR ligation[18].

Reactive oxygen species in activated T cells can originate from excess production of ROS or reduced antioxidant function in the activated T cell. Excess production of ROS is presumed to be due to the enhanced metabolic demand that accompanies T cell

activation and/or cell division which is met by increased oxidative phosphorylation at the respiratory complex and subsequent leakage of electrons and generation of superoxide free radicals[28], although there is some evidence that extra-mitochondrial NADPH oxidases can also generate ROS in T cells[29] as is also seen in neutrophils, as well as from peroxisomal enzymes. There is so far little literature addressing comparison of the levels and activities of anti-oxidant enzymes in the resting or activated T cells. Thus, available evidence mostly points to the source of ROS in activated T cells being mitochondria-derived in response to enhanced metabolic demands.

Electron leakage occurs predominantly from complex 1 of the respiratory chain. Inhibition of complex 1 assembly prevents ROS generation and consequently, AICD[30]. The generation of superoxide from complex 1 is triggered by translocation of PKC-theta, a component of the T cell receptor proximal signalling complex, to the mitochondria[30].

T cell death can occur during the contraction phase independent of the death receptor-mediated mechanisms that characterize AICD. Such death has been variously described in the literature as neglect induced death (NID), trophic signal withdrawal death (TSWD) and activation cell autonomous death (ACAD)[4]. The former terminologies (NID, TSWD) emphasize cytokine or antigen withdrawal leading to lack of triggering of common gamma chain of cytokine receptors as the main trigger for this form of death[19], whereas the term ACAD stresses upon it as a programmed and regulated form of death. NID controls the numbers of memory T cells surviving the initial attrition following infection and immunization. This form of death is mediated by the pro-apoptotic B cell lymphoma protein 2 (BCL2) interacting mediator of cell death (BIM)[31] and a closely related protein p53 upregulated modulator of apoptosis (PUMA)[32]. Bim is an activator BH3-only protein which activates multidomain proapoptotic proteins Bax and Bak. PUMA acts by displacing pro-apoptotic proteins like Bax and Bak from their Bcl-2 bound state and causes outer membrane permeabilization. Thus, NID is mediated by intrinsic pathway of apoptosis converging on the mitochondria leading to release of apoptotic signals from the mitochondria[33].

Evidence for caspase independent death pathways in T cells

Several studies point to the possibility of caspase independent death pathways in T cells[34,35]. *In vitro* experiments have shown that pan-caspase inhibitors do not completely prevent apoptosis in T cells exposed to death stimuli[36]. Human peripheral T cells activated with anti CD2 and staurosporine showed no protection from apoptosis in the presence of benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (Z-VAD.fmk), a broad spectrum peptide caspase inhibitor[36]. Similarly, pan-caspase inhibitors do not seem to prevent NID of the memory T cells and do not prevent the contraction phase of expanded CD8 T cells following viral infection[37]. Treatment with pan-caspase inhibitors in mice, which, in spite of causing hepatocyte protection from anti FasL induced apoptosis, did not affect the contraction of CD8 cells or the recall responses to secondary virus infection[37], which suggests that NID could be caspase independent. In general, extrinsic pathways of apoptosis are caspase dependent in their execution. Intrinsic pathway leads to release of mitochondrial death effectors[38] of which cytochrome c and SMAC/DIABLO are caspase-dependent in their actions. Aif and endonuclease G are caspase-independent[39] and are capable of translocating to the nucleus and causing DNA cleavage[40]. The death of melanoma-specific cytotoxic T cells was shown to be unaffected by caspase inhibition and was shown to be mediated by nuclear translocation of Aif from the mitochondria[41]. These suggestions prompted us to explore the possibility that Aif mediates death in the T cell lineage.

Aif: a molecule with pro-apoptotic and anti-apoptotic functions

Apoptosis-inducing factor was discovered as the first protein that regulates caspase-independent apoptosis[42,43]. Aif is a flavoprotein that functions as an NADH oxidase and uses FAD as a co-factor[44]. In healthy cells Aif is N-terminally anchored to the inner mitochondrial membrane and remains confined to intermembrane space. Aif is synthesized as a 67 kDa protein and contains a mitochondrial localization signal in the N-terminus. In the mitochondria, a mitochondrial peptidase cleaves the N-terminal signal and the mature 62 kDa Aif protein is generated. Aif normally functions as a NADH oxidase and the crystal structure of Aif reveals an oxidoreductase-like folding. Aif-null embryonic stem cells and HeLa cells show a quantitative reduction in complex I subunits along with reduction of complex I activity and a partial reduction in complex III activity. It is thus presumed that Aif is important for the structural and functional organization of complex I and probably of complex III[45]. In addition

to the oxidoreductase function of Aif, it has a clear functional domain for DNA binding activity[46]. On receiving apoptotic signals, Aif is cleaved from the inner mitochondrial membrane, released out into the cytosol following mitochondrial outer membrane permeabilisation (MOMP) (Fig.), translocates into the nucleus, binds to DNA and causes widespread chromatin condensation and cleavage. The signals that cleave and release Aif into the cytosol are only partially elucidated, however, cysteine proteases including calpains and certain cathepsins are presumed to play an important role[48]. Oxidative stress and DNA damage leads to activation of Poly ADP ribose polymerase (PARP), a nuclear enzyme that synthesizes Poly ADP ribose (PAR) at the expense of ATP and NAD+[49]. Activated PARP triggers DNA repair and when cellular injury is extensive, can trigger apoptosis through various pathways. Aif is one of the effectors for PARP-mediated cellular death[50]. Aif leakage can be caspase-dependent or caspase-independent depending upon the context[42,51]. Cytosolic Aif causes further damage to mitochondria and amplifies the release of Aif[42].

Aif-null genotypes show early embryonic lethality in mice. However, in the spontaneously generated Harlequin (Hq) strain of mice, a retroviral insertion in the first intron of the Aif gene causes poor mRNA splicing and a resultant reduction in Aif protein levels to 10-20 per cent of normal levels[52]. Hq mice are viable and show various organ-specific disease phenotypes

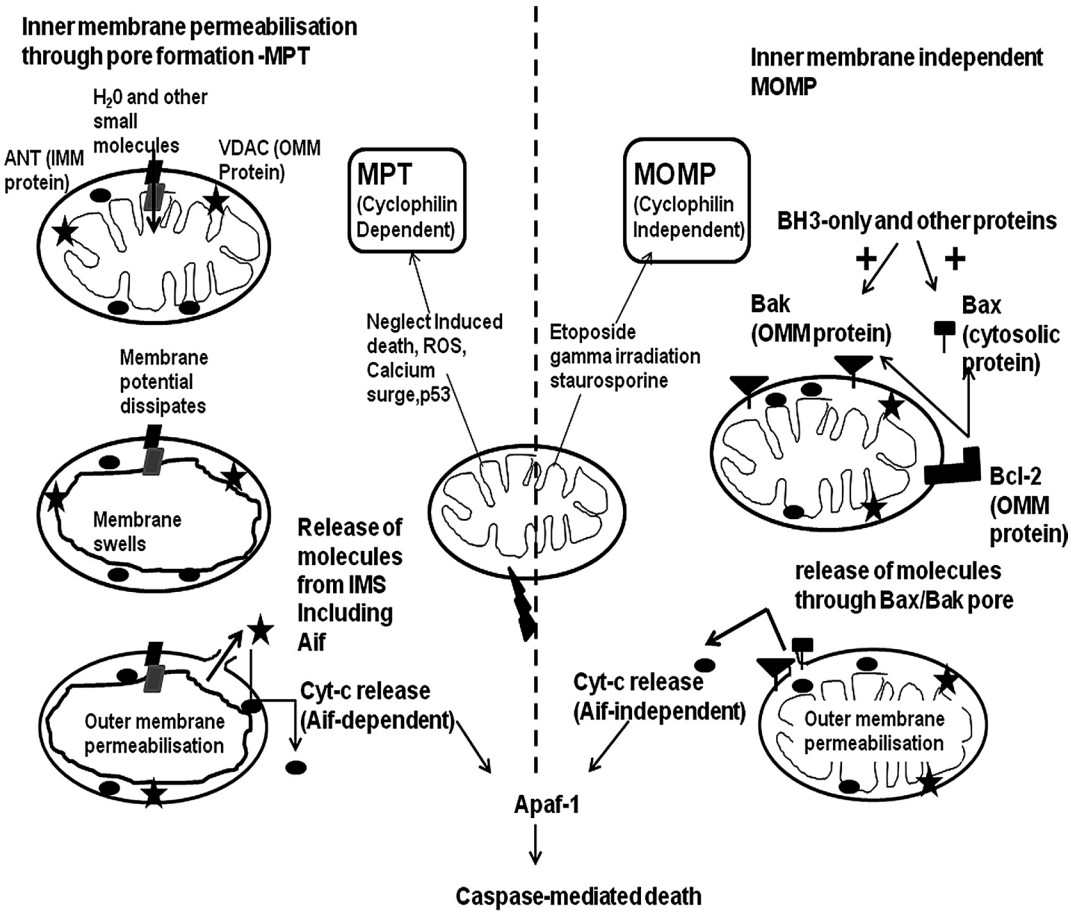

Fig. Potential role of Aif in intrinsic pathway of apoptosis. During apoptosis, mitochondrial leakiness is mediated by two distinct pathways – mitochondrial outer membrane permeabilization (MOMP) and mitochondrial permeability transition (MPT). MPT follows the cyclophilin dependent assembly of ANT and VDAC channels and results in release of death-effector molecules from inner membrane space, including Aif. In addition to its role in DNA binding and fragmentation, Aif amplifies further release of other effector molecules like cytochrome c from the mitochondria. MOMP is cyclophilin independent and release of effector molecules from inner mitochondrial space is mediated by Bax/Bak pores. (Adapted from Ref. 83). Key: Aif, apoptosis inducing factor; ANT, adenine nucleotide transporter; MPT, mitochondrial permeability transition; VDAC, voltage dependent anion channel; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; MOMP, mitochondrial outer membrane permeabilization; IMS, inter membrane space; Apaf-a, apoptosis protease activating factors-1.

(Table) including cerebellar degeneration leading to ataxia and retinal degeneration leading to blindness[52]. Thus Hq mice resemble human mitochondriopathies due to complex I deficiency[53]. In addition, tissue-specific knockout of the Aif gene in the skeletal muscle and heart leads to myocyte atrophy and dilated cardiomyopathy, respectively[54]. Neuronal degeneration in Hq mice is accompanied by abnormal apoptosis of neurons. Various cell types of Aif-hypomorphic Hq mice including neurons[52], cardiomyocytes[55] and pancreatic β cells[56] were found to have enhanced susceptibility to hydrogen peroxide-induced oxidative stress. Hq mice also develop enhanced cardiomyocyte damage in response to ischaemia reperfusion injury following surgical aortic ligation[55]. These observations have led to the hypothesis that Aif probably acts as a peroxide scavenger in cells in addition to its role in mitochondrial bioenergetics. However, this claim has been questioned by some studies in Aif-deficient cell lines[57,58] and Hq neurons[59], which have shown conflicting results in the regulation of cellular ROS levels by Aif.

Aif also has pro-apoptotic activities resulting from its DNA-binding and cleavage abilities following apoptotic signals. However, all death triggers leading to apoptosis do not necessarily include Aif-mediated death pathway. For example, Hq mice are protected from cerebral ischaemia-reperfusion damage when compared to wild type (WT) mice[60-62], an effect which is opposite to what is seen in the heart, where Hq mice develop larger infarcts on cardiac ischaemia-reperfusion injury[55]. Although Aif-null mouse embryos show lethality, they do not seem to have increased cell numbers[63]. Cerebellar granule cells from Hq mice show partial resistance to cell death induced by serum-withdrawal[52,63]. Cortical and hippocampal neurons from Hq mice are resistant to death induced by N-methyl-D-Aspartate (NMDA) and glutamate[64-66], which reflects the results of *in vivo* excitotoxic studies showing protection of hippocampal neurons of Hq mice from kainic acid-induced seizures[64]. Intracellular ROS levels possibly regulate the cleavage and release of Aif which is supported by an observation that treatment with an Manganese Superoxide Dismutase (MnSOD) mimetic attenuated nuclear translocation of Aif and ischaemic cell death in neurons[67]. Hq mice are also resistant to hepatic necrosis induced by paracetamol toxicity[68]. Relatively less is known regarding the molecular signals that lead to Aif cleavage and translocation to the nucleus. Elucidation of cellular signalling pathways which selectively cleave and translocate Aif from mitochondria will help to understand the specific pro-apoptotic role of Aif better. Thus, it is interesting that Aif-mediated death appears to be cell-lineage dependent, trigger-specific and context dependent.

Aif controls death in the T cell lineage

As mentioned previously, AICD and NID are the two distinct forms of death in peripheral T cells. Since there are suggestions in the literature that NID is caspase independent in its execution[34], and since Aif is one of the factors released from mitochondrion which causes caspase independent death, NID was evaluated in Aif-hypomorphic Hq and wild type (WT) T cells. NID is evaluated *in vitro* by an assay in which T cell blasts from splenocytes are generated by anti CD3 stimulation, followed by a short resting phase in complete medium. Live blast cells are then cultured in the absence of IL-2[5]. NID was observed to be lower in Hq T cell blasts in comparison to WT cells[5]. Cell death in WT T cell blasts was accompanied by Aif translocation to the nucleus as well as cytochrome c release from mitochondria, whereas, in Hq T cell blasts, cytochrome c release was delayed. In addition to its role in nuclear translocation and DNA fragmentation, Aif is known to cause mitochondrial damage and release of other death molecules like cytochrome c from the mitochondria[42]. Further, cytochrome c release has been shown to be dependent on Aif in the context of T cell death mediated by HIV-1[69]. Hence, the finding that release of cytochrome c is delayed in Hq T cells undergoing NID[5] leads to the conclusion that Aif helps in release of cytochrome c (Figure). That NID was dependent on Aif for its culmination was further confirmed by the finding that caspase 9 (which gets activated by cytochrome c-Apaf complex) activity was greater in the WT T cells than in Hq T cells[70].

Thus, Aif seems to be significantly non-redundant for NID in peripheral T cells. This raised the question of whether Aif is non-redundant in T cells for other forms of mitochondrial death as well. To pursue this further, T cell blasts from Hq and WT mice were evaluated for deaths induced by etoposide, staurosporine and gamma irradiation (all leading to mitochondrial death pathways via outer membrane permeabilization; Figure) or endoplasmic reticulum (ER) stress triggered by thapsigargin. However, in these assays, Hq and WT T cell blasts showed similar susceptibility to death, suggesting that Aif is redundant in these forms of death[5]. The death triggers leading to massive mitochondrial damage would be expected to release all the mitochondrial death effector molecules into the cytoplasm simultaneously and hence may not be

Table. Effects of Aif deficiency on various organs

Tissue/Organ	Phenotype due to Aif deficiency	Probable mechanism and evidence
A. Cytoprotective functions of Aif		
Brain	Cerebellum: degeneration of cerebellar granular and purkinje cells.	Enhanced oxidative stress in the absence of Aif - Aif deficient granule cells (cerebellum) show enhanced susceptibility to death in the presence of peroxide
	Multifocal degenerative lesions in thalamus, striatum and cortical neurons accompanied by glial activation.	
Retina	Retinal degeneration and loss of inner and outer nuclear layers.	Enhanced oxidative stress in the absence of Aif – Aif deficient neuronal cells in retina shows positive staining for 8-OHdG, a marker of oxidatively damaged DNA
Pancreas	Beta cell apoptosis and loss of beta cells.	Oxidative stress in beta cells in the absence of Aif - Aif deficient beta cells shows increased susceptibility to hydrogen peroxide induced apoptosis
Heart	Enhanced susceptibility to pressure overload induced decompensation.	Increased oxidative stress in the absence of Aif – Treatment with EUK-8, a superoxide dismutase and catalase mimetic reverses cardiac decompensation
	Enhanced susceptibility to ischaemic-reperfusion injury.	Increased oxidative stress in the absence of Aif - mitochondria from Hq hearts show reduced ability to scavenge free radicals.
	Dilated cardiomyopathy (in muscle-specific Aif knockout mice).	Increased oxidative stress in the absence of Aif – Cardiac muscle from Hq mice show increased lipid peroxidation products
Skeletal muscle	Atrophy (in muscle-specific Aif knockout mice).	Increased oxidative stress in the absence of Aif – increased catalase levels observed in skeletal muscle in Hq mice
Skin	Loss of hair follicles.	Increased oxidative stress in the absence of Aif – Hair follicles show increased superoxide anion levels
Immune system	DN3 to DN4 progression block in T cell development.	Increased oxidative stress caused due to multiple replicative cycles during beta-chain selection.
	Higher activation induced cell death (AICD) in peripheral T cells.	Aif acts as a mitochondrial peroxide scavenger. H_2O_2 treatment enhances AICD in Hq.
B. Pro-death functions of Aif		
Brain	Protection from ischaemic injury, Protection of periventricular neurons from ionizing radiation. Protection of hippocampal neurons from kainic acid induced seizures.	Neuronal ischemia, ionizing radiation and excitotoxicity lead to selective release of Aif, its nuclear translocation and cell death.
Liver	Protection from paracetamol induced hepatotoxicity.	No definitive evidence shown
Immune system	Lower neglect induced death (NID)	NID is mediated by Aif release following MPT.

Note: The phenotypes described are from harlequin mice unless otherwise mentioned. The effects described in A are expected to be reversed in transgenic mice consisting of only a functional oxido-reductase domain of Aif and those described in B are expected to be reversed in transgenic mice consisting of Aif with a functional DNA-binding activity alone.
Source: Refs 52-56
8-OHdG, 8-hydroxy-z'deoxyguanosine; Hq, Harle quin; AICD, activation induced cell death; MPT, mitochondrial permeability transition

critically dependent on Aif for its execution. However, those forms of death which cause MPT (mitochondrial permeability transition) and early Aif release would be expected to be prevented in Aif-deficient T cells, leading to a delay in cytochrome c release. This appears to be the case for NID. Treatment with cyclosporin A (CsA), which binds cyclophilins and inhibits the formation of permeability transition pore (PTP) in the mitochondria, was able to prevent NID in WT T cell blasts, suggesting that Aif release occurs through PTP formation and regulates cyt c release (Figure)[5]. On the other hand other death triggers like etoposide were not sensitive to CsA, suggesting that cyt c release in those situations is Aif-independent. Further, the effect appeared to be lineage-specific, since susceptibility of peritoneal macrophages to death induced by lipopolysacharide (LPS) and interferon (IFN)-gamma was similar in Hq and WT cells[5].

The second form of T cell death, AICD, is a death receptor-mediated pathway, unlike NID. When T cell blasts were examined for their sensitivity to AICD, Hq T cell blasts were found to show greater levels of death[5,70]. As mentioned earlier, mitochondrial ROS generation is known to be a signalling intermediate in mediating AICD by a variety of mechanisms. Hence, WT T cell blasts, on treatment with a superoxide dismutase (SOD) mimic, Manganese (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), show reduction of AICD. MnTBAP has an additional peroxynitrite scavenging role in addition to SOD mimetic role; however, treatment with NO synthase inhibitors does not reduce AICD in WT T cell blasts suggesting that the superoxide scavenging role of MnTBAP is specifically responsible for the protection observed[5,70]. Consistent with this, T cell blasts from mice heterozygous for a deletion in the mitochondrial isoform of superoxide (MnSOD) also showed enhanced AICD[5].

Aif has an oxido-reductase function and can act as a ROS quencher. Hence the lack of antioxidant activity of Aif is likely to be responsible for enhanced AICD in the Aif-deficient Hq mice. This possibility is further supported by the observations that treatment of Hq T cell blasts with EUK-134, ROS quencher and SOD mimic, reversed the enhanced AICD seen in them. However, T cell blasts from Hq mice were not protected by MnTBAP, suggesting that the activity of Aif is downstream of peroxide generation. When Hq and WT T cell blasts were treated with exogenously added hydrogen peroxide or DMNQ (a superoxide generator), Hq T cell blasts were found to be more susceptible to death[5]. These findings are consistent with a peroxide scavenging role of Aif, as has been previously suggested[52]. Moreover, during AICD, Aif localizes to the mitochondria and does not translocate to the nucleus as seen in NID[5]. Thus the activity of Aif in mitochondria seems to be responsible for limiting the AICD seen in WT T cell blasts.

By acting as an antioxidant, Aif leads to excessive activation of the extrinsic pathway of apoptosis, leading to greater caspase 8 activation as seen in Hq T cell blasts when compared to WT blasts. The enhanced susceptibility to AICD appears to be due to multiple contributory mechanisms involving the extrinsic pathway including enhanced expression of CD95L, increased secretion of TNF-alpha and enhanced susceptibility to DR-mediated death[5]. Such findings on Hq T cell blasts were replicated in small interfering RNA (siRNA)-mediated Aif-knockdown in normal activated human T cells[5], suggesting that the effect seen in Hq T cells are not due to altered developmental programming but due to Aif deficiency at the time of the assay.

The model that comes up from the data on NID and AICD in the Hq mice is that Aif functions as both pro-apoptotic and anti-apoptotic molecule, specifically in T cells. Following induction of death due to cytokine withdrawal (NID), Aif acts as a pro-death molecule by its early release from mitochondria, nuclear translocation and propensity to cause further damage to mitochondria and release of cytochrome c. On the other hand, in the cells of the same lineage, death in another context, namely AICD, shows Aif in its cytoprotective function by acting as a ROS scavenger.

When splenic cell counts are compared between WT and Hq mice, Hq spleens show lesser cellularity[5,70]. The reduction in cell number is due to reduction in frequency and numbers of T cells and not of B cells or macrophages. Hence T cells seem to be preferentially affected in Aif deficiency. Analysis of naïve versus memory stages in peripheral T cells reveal substantially higher frequencies of CD44 high effector/memory T cells in the periphery in the Hq mouse[5,70]. This may be a consequence of protection of activated repsonding Hq T cells from NID leading to accummulation of more memory T cells and perhaps their slower attrition as well. In addition, naïve cell numbers were drastically lower in peripheral lymphoid organs in Hq mice, which in turn could be a consequence of enhanced AICD and/or reduction in thymic output.

Aif regulates death during T cell development

The substantial reduction in the naïve T cell numbers in Hq mice prompted us to look for the possibility of defective thymic development in Hq mice. Thymic cellularity is drastically lower in Hq mice and when the various developmental stages in thymus are compared, there is a reduction in the numbers and frequencies of double positive (DP) thymocytes, single positive (SP) CD4 and SP CD8 cells. Double negative (DN) cell numbers are less disparate between WT and Hq mice, and higher in frequency in the Hq thymus[6]. This suggests a block in the transition from DN to DP stage during development. More specifically, the frequencies of DN3 thymocytes are higher in the Hq thymus in comparison to WT and the absolute numbers of DN3 cells are similar in Hq and WT cells, while there is a marked reduction in the absolute cell numbers and frequencies of DN4 cells in Hq thymus[6]. Thus there is a developmental block in the DN3 to DN4 transition in the Hq thymus. This is due to Aif deficiency in the thymocytes themselves, since mixed bone marrow chimeras made with Hq and WT donor marrow in WT recipients showed that Hq donor marrow was selectively deficient in generating the T cell lineage beyond the DN3 stage[6].

As mentioned previously, DN3 to DN4 transition occurs after a successful and productive TCRβ recombination (β-selection); thus, beta-selection is affected in Aif-deficiency. Hq and WT DN3 cells show similar frequencies of expression of intracellular TCRβ protein, the usage of various TCR-Vβ gene segments is similar in Hq and WT mice, and provision of an already recombined TCR transgene does not rescue the thymic defect of Hq mice, indicating that the TCRβ recombination step as such is not defective in Hq mice[6]. The rapid proliferative burst during β-selection is associated with the increased levels of cellular ROS and DN3 and DN4 cells from Hq mice have higher ROS levels as well as greater freuqencies of dead cells. These data indicate that Aif-deficiency in the T cell lineage may lead to inability to hande ROS stress during beta-selection leading to cell death and loss. In support of this model, treatment of Hq mice with the antioxidant small molecule EUK-134 led to a substantial rectification of the Hq thymic defect[6].

Dissecting the roles of Aif in the T cell lineage

Aif has pro-apoptotic activity in the form of its ability to bind DNA and cause fragmentation, as well as cytoprotective activity by virtue of its oxidoreductase function. In the T cell lineage, both these activities appear to play non-redundant roles in mediating cellular death under different contexts. Using transgenically expressed Aif, either the wild-type molecule or point mutants deficient in either DNA-binding or oxido-reductase functions, we have dissected the role these activities play in various T lineage cell defects found in the Hq mouse[6]. For example, the block in the DN3 to DN4 stage transition in Hq thymocytes appears to be due to the lack of antioxidant function of Aif leading to inability of Aif-deficient T cells to quench high levels of intracellular ROS levels during β-selection. Similarly, the enhanced AICD observed in the Hq T cell blasts appear to be due to the sensitization of T cell blasts to death by high ROS levels in the absence of oxido-reductase function of Aif. On the other hand, the absence of DNA binding Aif activity in Aif-deficient Hq T cells seems to protect them from NID, leading to the accumulation of memory T cells. This suggests that distinct functions of Aif, namely the oxido-reductase and DNA-binding activities, are responsible for modulating death in T cells under different contexts.

In addition, there can be complex interactions due to homeostatic interplay of various organ systems which cannot be explained by the effect of a single action on a single lineage. For example, Hq mice show a loss of β cells of pancreas[56], but paradoxically a significantly lower fasting and post-prandial blood glucose levels by glucose tolerance test. This can be explained by the effect of Aif deficiency in the muscle and liver, where, a reduction in oxidative phosphorylation and in metabolism leads to beneficial effects including enhanced sensitivity to insulin and resistance to diet-induced obesity[71]. Since Aif has separable oxido-reductase and DNA-binding properties, it is possible to hypothesise that these two distinct sets of organ-specific abnormalities characterized by increased or decreased cell death, observed in Hq mice are dependent on the loss of either the oxido-reductase or the DNA-binding function of Aif (Table).

It is, therefore, quite probable that these distinct functions of Aif make distinct contributions to its physiological and pathological roles in different tissues. Among the various morphological and functional alterations in various tissues of Hq mice, some are due to enhanced cell death; for example, cerebellar degeneration due to granule cell apoptosis and purkinje cell degeneration[52], retinal degeneration due to loss of ganglion cells and inner and outer nuclear layers[52], multifocal degenerative neuropathology in

cortex[72], atrophy and impaired regeneration in skeletal muscle[54,73,74], cardiac ventricular dysfunction and susceptibility to ischaemia[55], β cell loss in pancreas[56] and loss of hair follicles in the skin leading to hair loss[75]. These abnormalities are likely to be due to enhanced cell death, quite possibly mediated by the unrelieved oxidative stress (Table). However, there is no definitive proof for this. On the other hand, some cell lineages in Aif-deficient Hq mice are protected from death mediated by triggers such as paracetamol in hepatocytes[68], ischaemic injury in cerebral neurons[60-62], ionizing radiation in brain subventricular zone neurons[76], glutamate toxicity in neurons or kainic acid-induced excitotoxicity in hippocampus[64-66]. These may well be the contexts in which Aif is selectively released from the mitochondria to contribute to the intrinsic pathway of apoptosis (Table). However, there is so far no clarity regarding whether nuclear translocation of Aif post-release from mitochondria is essential in these situations, or whether Aif-mediated cytochrome c release is the basis of the contribution of Aif to cell death during these events. Further, whether the DNA-binding function of Aif (or at least, the residue/s in Aif regulating DNA binding) is essential for Aif-mediated cytochrome c release from mitochondria also remains an open question.

The Hq mouse - a model of human mitochondriopathy

The Aif-deficient Hq mouse is an established mouse model for mitochondriopathy as disease phenotypes in the Hq mice (cerebellar degeneration, retinal degeneration, cardiomyopathy, skeletal muscle atrophy) closely resemble many human mitochondriopathies[53]. Human mitochondriopathies are a heterogenous group of systemic or organ-specific diseases resulting from mutations in nuclear or mitochondrial DNA affecting mitochondrial oxidative phosphorylation[77]. Human mitochondriopathies due to several mutations affecting nuclear and mitochondrial DNA have been described; however, there is still a large number of cases for which a known mutation cannot be ascribed to. Complex I deficiency disorders comprise around 30 per cent of human mitochondriopathies and the molecular lesion in majority of these remain unknown. The phenotype of Hq mice as well as the heterogeneity in the organ-specific disease manifestation makes it an interesting model to explore complex I dysfunction disorders as well as diseases caused by other molecular dysfunctions.

Although Aif has been well known in the field of cell death research, Aif mutations were not known as a cause of human mitochondriopathy till recently. One study group specifically looked for mutations in Aif gene in 90 patients with idiopathic complex I disorders and found not a single case with Aif deficiency[78,79]. A recent report by Ghezzi *et al*[80] attributes an X-linked mitochondrial encephalopathy to Aif mutation. The pathogenic mutation is a deletion in a trinucleotide coding for Arginine in the human *AIFM1* gene located on the X chromosome. Unlike the Hq mouse which develops late onset ataxia and blindness, human Aif mutation causes severe, early onset encephalomyopathy, psychomotor development abnormalities and skeletal muscle atrophy. Remarkably, human Aif mutation causes a marked reduction of respiratory complex 3 and 4 activities in fibroblasts and multi-complex deficiency in skeletal muscle, unlike the predominantly complex 1 deficiency in Hq mice[80]. The mutant Aif (R201del) increased DNA-binding ability in *in vitro* assays, and the neurological signs were partially improved by riboflavin administration[80].

Consequences of mitochondriopathies for T cell functioning in human disease

If Aif-deficiency-mediated mitochondriopathy leads to mouse T cell lineage dysfunction, the clinically relevant question is: is there any T cell lineage dysfunction in human aif mutations, or in other human mitochondriopathies? T cell dysfunction has not been investigated so far in human mitochondriopathies but it is possible that energy-intensive lymphocytes can show some dysfunction due to mitochondrial abnormalities. In one report, it was seen that paediatric mitochondriopathy patients had recurrent infections and poor vaccine take[81]. However, it is possible that T cell dysfunction is specific to Aif deficiency and, therefore, will only be seen in Aif-related mitochondriopathies. That would be the case if the Hq mouse T cell lineage dysfunction was predominantly due to loss of the unique pro-apoptotic DNA-binding function of Aif. However, in the Aif-hypomorphic Hq mice, major abnormalities in the T cell lineage, such as thymic involution and enhanced AICD in peripheral T cells, appear to be due to loss of the anti-apoptotic Aif functions, which are mediated, at least in some instances, through regulation of cellular redox status by functioning as a peroxide scavenger and/or modifying mitochondrial oxidative phosphorylation via effects on assembly of respiratory chain complex I. Consistent with this, mice deficient in mitochondrial superoxide dismutase (SOD2), a major regulator of mitochondrial oxidant levels, show enhanced T cell AICD[5]. Further, the optic neuronal degeneration found

in Leber's hereditary optic neuropathy (LHON), a mitochondriopathy, is mimicked in the SOD2-deficient mouse[82] which also shows T cell lineage defects[5]. Human mitochondriopathies also lead to alterations in cellular redox status. Therefore, it is plausible to hypothesise that T cell dysfunction may occur in human mitochondriopathies, and this hypothesis has to be evaluated in future studies. Interestingly, blood lymphocytes from LHON patients showed increased apoptosis susceptibility to 2-deoxy-ribose-induced oxidative stress[83]. The rarity of mitochondriopathies, the difficulty in demonstrating mitochondrial lesion at the molecular level and modification of disease phenotype by mitochondrial heteroplasmy are some of the caveats that would be encountered in such exploratory studies.

Conclusions and future perspectives

In the context of functional consequences of mitochondrial dysfunction in the immune system, it remains to be explored whether there can be further uncharacterized functional defects in the various subsets of innate and/or adaptive immune cells. In the innate immune cells, for example, would there be alterations in the innate microbicidal functions leading to differences in outcome to infections? Would there be alterations in the differentiation patterns of naive T helper cells while being primed with antigen presenting cells having different cellular ROS levels? It is interesting to note that the abnormalities found in the T cell lineage in the Hq mice do not occur in the closely related B lineage cells, suggesting the redundancy of Aif, at least for some outcomes, in the B cell lineage. This selectivity of T lineage abnormality cannot be explained by differences in the Aif transcript levels between the B lineage and the T lineage cells, since microarray data (*http://www.immgen.org/index_content.html*) suggest that the two lineages have similar Aif transcript levels. Equally remarkable is the finding that it is specifically the β- selection developmental stage (DN3 to DN4) of T lineage cells that is affected. Other developmental stages in the T cells which are also highly proliferative and susceptible to ROS mediated stress do not show enhanced death in Hq mice. Microarray data also show a surge in Aif transcript levels at the DN3-DN4 stage that may be possibly contributing to the stage-specific effect of Aif deficiency in the Hq mice. Surprisingly, a surge in the Aif transcript levels is also observed at the equivalent pro-B cell stages in the B-cell lineage, in spite of which, Aif deficiency has no effect on B cell development. This leads to the questions as to what decides the variability in cellular susceptibility to ROS mediated injury across different lineages and developmental stages. It is interesting to speculate that the baseline housekeeping functions of ROS scavenging enzymes such as superoxide dismutase, catalase or peroxidase may be differently organized in various developmental subsets and lineages.

One of the intriguing aspects of pathophysiology of human mitochondriopathies is the remarkable variability in the clinical presentation and tissues affected. This has been traditionally ascribed to the variable energy demands of each tissue as well as to the non-uniform segregation of abnormal mitochondria during embryogenesis (heteroplasmy). The findings from the Hq mouse model make it possible that quantitative variations in the expression of housekeeping genes, in metabolic functions and in stress-response pathways across different developmental stages and cell lineages may be hitherto underappreciated contributing factors for the pathophysiological consequences of deficiency/ dysfunction of a ubiquitous and essential molecule.

Acknowledgment

The work on which this review is based was supported in part by grants from the Department of Biotechnology (to S.R., A.G., V.B., and S.S.M.) and the Department of Science and Technology, Government of India (to S.S.M., A.G., V.B. and S.R.), and the National Institutes of Health, USA (to J.M.D.) and the Arkansas Biosciences Institute, Littlerock, AR, USA (to J.M.D.).

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