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Forum Review

Metabolic Switches of T-Cell Activation and Apoptosis

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ABSTRACT

The signaling networks that mediate activation, proliferation, or programmed cell death of T lymphocytes are dependent on complex redox and metabolic pathways. T lymphocytes are primarily activated through the T-cell receptor and co-stimulatory molecules. Although activation results in lymphokine production, proliferation, and clonal expansion, it also increases susceptibility to apoptosis upon crosslinking of cell-surface death receptors or exposure to toxic metabolites. Activation signals are transmitted by receptor-associated protein tyrosine kinases and phosphatases through calcium mobilization to a secondary cascade of kinases, which in turn activate transcription factors initiating cell proliferation and cytokine production. Initiation and activity of cell death-mediating proteases are redox-sensitive and dependent on energy provided by ATP. Mitochondria play crucial roles in providing ATP for T-cell activation through the electron transport chain and oxidative phosphorylation. The mitochondrial transmembrane potential ($\Delta\Psi_m$) plays a decisive role not only by driving ATP synthesis, but also by controlling reactive oxygen species production and release of cell death-inducing factors. $\Delta\Psi_m$ and reactive oxygen species levels are regulated by the supply of reducing equivalents, glutathione and thioredoxin, as well as NADPH generated in the pentose phosphate pathway. This article identifies redox and metabolic checkpoints controlling activation and survival of T lymphocytes. *Antioxid. Redox Signal.* 4, 427–443.

INTRODUCTION

PROGRAMMED CELL DEATH or apoptosis represents a physiological mechanism for elimination of unwanted cells upon completion of immune responses. During development of the immune system, potentially autoreactive lymphocytes are removed by apoptosis (29). Pathological processes, such as infection by human immunodeficiency virus type 1 (HIV-1), may also deplete T cells via apoptosis (136). Lymphocytes have developed complex signaling networks that mediate survival, activation, or proliferation or trigger apoptosis in response to changes in the extracellular or intracellular microenvironment. T lymphocytes are primarily activated through the T-cell receptor (TCR) following interaction with a specific peptide/major histocompatibility antigen complex on the antigen-presenting cell. The outcome of TCR engagement depends on concomitant signaling through co-stimulatory molecules (CD28, CD40L, LFA-1, CD2) and cytokines (98). Intracellular signal transduction is mediated via protein tyrosine kinases (LYN, SYK) and phosphatases [(CD45, SHP-1, phospholipase Cγ1 (PLCγ1)] leading to cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2), and Ca^{2+} mobilization. Then a secondary cascade of kinases, protein kinase C (PKC) and protein kinase A (PKA), activate transcription factors, NFAT, nuclear factor-κB (NF κB), activator protein-1 (AP-1), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK), and initiate cell proliferation and cytokine production. By contrast, engagement of cell-surface death receptors [tumor necrosis factor (TNF), Fas] leads to activation of caspases. These cell-death proteases participate in a cascade that culminates in cleavage of a key set of structural proteins, enzymes, and chromosomal DNA, thus resulting in disassembly of the cell. Both cell proliferation and apoptosis are energy-dependent processes. Energy in the form of ATP is provided through glycolysis and oxidative phosphorylation. The mitochondrion, the site of oxidative phosphorylation, has

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long been identified as a source of energy and cell survival. The synthesis of ATP is driven by an electrochemical gradient across the inner mitochondrial membrane maintained by an electron transport chain and the membrane potential (negative inside and positive outside). A small fraction of electrons react directly with oxygen and form reactive oxygen species (ROS). Although ROS have long been considered as toxic by-products of aerobic existence, evidence is now accumulating that controlled levels of ROS modulate various aspects of cellular function and are necessary for signal-transduction pathways, including those mediating apoptosis (24, 62, 63, 86, 104, 105, 155). Disruption of the mitochondrial membrane potential has been proposed as the point of no return in apoptotic signaling (158, 173, 182). Mitochondrial membrane permeability is subject to regulation by an oxidation–reduction equilibrium of ROS, pyridine nucleotides (NADH/NAD + NADPH/NADP), and reduced glutathione (GSH) levels (30). Regeneration of GSH by glutathione reductase from its oxidized form, GSSG, depends on NADPH produced by the pentose phosphate pathway (PPP) (115). ROS levels and $\Delta\Psi_m$ are regulated by the supply of reducing equivalents from PPP. Metabolic fluxes between glycolysis and the PPP are particularly relevant for balancing cellular requirements for energy and ROS production. This review will delineate redox and metabolic pathways controlling the T cell's fate in response to activation and apoptosis signals.

REDOX CHECKPOINTS OF T-CELL ACTIVATION

Stimulation of the TCR by an MHC peptide complex initiates intracellular signaling events that lead to lymphokine production and clonal expansion. The antigen-binding αβ or γδTCR is associated with a multimeric receptor module comprised of the CD3 γδε and TCR ζ chains. The cytoplasmic domains of CD3 and ζ chains contain a common motif, $YXX(L/I)X_{6-8}YXX(L/I)$, termed immunoglobulin receptor family tyrosine-based activation motif (ITAM), which is crucial for coupling of intracellular tyrosine kinases (85). Binding of $p56^{lck}$ to CD4 or CD8 attracts this kinase to the TCR ζ/CD3 complex, leading to phosphorylation of ITAM. Phosphorylation of both tyrosines of each ITAM is required for SH-2-mediated binding by zeta-associated protein-70 (ZAP-70) or the related SYK. ZAP-70 is activated through phosphorylation by $p56^{lck}$. Activated ZAP-70 and SYK target two key adaptor proteins LAT and SLP-76 (85). Oxidative stress causes diminished phosphorylation and displacement of LAT from the cell membrane of T cells (58). Phosphorylated LAT binds directly to PLCγ1. Further downstream, PLC γ1 controls hydrolysis of PIP_2 to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG stimulates PKC, which, in turn, activates PKA. Activity of PKCs is susceptible to oxidative modifications (52). The N-terminal regulatory domain contains zinc-binding autoinhibitory cysteine-rich motifs. Oxidization of these cystidine residues relieves self-inhibition, enhances PKC activity, and signals for cell growth. By contrast, antioxidant selenocompounds and vitamin E analogues selectively target the C-terminal catalytic domain, inhibit PKC activity, and thus generally interfere with cell growth (52). PKC represents a group of serine/threonine kinases that regulate a diversity of signaling pathways, including programmed cell death (108). Activation of PKC by phorbol esters has been shown to prevent or enhance apoptosis in a cell type-specific manner (108). During apoptosis, activation of caspases results in proteolysis of cellular substrates, which leads to the characteristic morphologic and biochemical changes (112, 124). In particular, PKC-δ (40) and PKC-θ may be activated by caspase-mediated cleavage in apoptotic cells (34) (Fig. 1).

Phosphorylation of inositol lipid second messangers is mediated by phosphatidylinositol 3′-hydroxyl kinase (PI_3K). The stimulatory effect of the TCR alone on PI_3K activity is small. Concurrent triggering of the CD28 co-stimulatory molecule by its ligands CD80 or CD86 is required for optimal PI_3K activation. IP_3 binds to its receptors in the endoplasmic reticulum, opening Ca^{2+} channels that release Ca^{2+} to the cytosol. Increased cytosolic Ca^{2+} concentration activates the serine/threonine phosphatase calcineurin, which dephosphorylates the NFAT. Dephosphorylated NFAT can translocate to the nucleus where it promotes transcription of interleukin-2 (IL-2) in concert with AP-1, NFκB, and Oct-1. Although activities of AP-1 and NFκB are increased by oxidative stress (14), both thiol insufficiency and H_2O_2 treatment suppress calcineurin-mediated activation of NFAT (46). Thus, expression of cytokines, *i.e,*. IL-2 (with AP-1 and NFAT motif-containing promoter) and IL-4 (with AP-1-less NFAT enhancer), can be selectively regulated by oxidative stress, depending on the relative activation level and binding of transcription factors (144). LAT and SLP-76 also play essential roles in TCR-mediated activation of the Ras family GTPases. LAT recruits Grb-2 and associated Son of Sevenless (SOS), a guanine nucleotide exchange factor that activates Ras. Oxidative stress also activates c-Src and ERK1/2. ROS-mediated formation of Ras-GTP is dependent on activity of two redox-sensing kinases Fyn and JAK2, but not c-Src (1). Alternatively, ROS-mediated activation of ERK5 is mediated by c-Src independent from Fyn (2). Redox-activated Ras recruits PI_3K to the plasma membrane (35). Activated Ras-GTP also turns on mitogen-activated protein (MAP) kinases [MAPK/apoptosis signal-regulating kinase 1(ASK1)/ERK]. By recruiting Vav, Cbl, Gads, and Nck, SLP-76 contributes components to the LAT signaling complex, mediating rises in cytosolic Ca^{2+} concentration and IL-2 production upon TCR engagement. MAP kinases phosphorylate Elk1, which mediates transcription of the Fos gene. Fos is a component of AP-1, which regulates expression of IL-2 and other cytokine genes.

CD3/CD28-induced activation of Rac, Vav1, PKCθ, $p56^{lck}$, $p59^{fyn}$, JNK, and NFκB are enhanced by oxidative stress (68). Activation of JNK is mediated through Src and Cas kinases (186). Oxidative stress promotes p53/56lyn-mediated phosphorylation and activation of p72Syk and subsequent increases in cytosolic Ca^{2+} concentration (142). As noted above, localization of LAT to the cell membrane is impeded by oxidative stress, thus resulting in T-cell hyporesponsiveness (58).

Expression of IL-2 and its receptor are both dependent on activation of NFκB (50, 144). Balance of ROS production and endogenous synthesis of antioxidants, such as thioredoxin (TRX) (125), GSH, and NADPH, are critical for controlling susceptibility to IL-2-dependent proliferative (165) and cell death signals (10, 172). Shifting of the redox equilibrium toward oxidation stimulates translocation of NFκB into the nucleus and enhances DNA binding by Sp1, allowing transcription from IL-2 gene promoter (144). Activation of NFκB is controlled by the IκB family of inhibitor proteins (137). In the cytosol, IκB is bound to NF-κB, preventing its translocation to the nucleus. Redox-mediated phosphorylation and subsequent

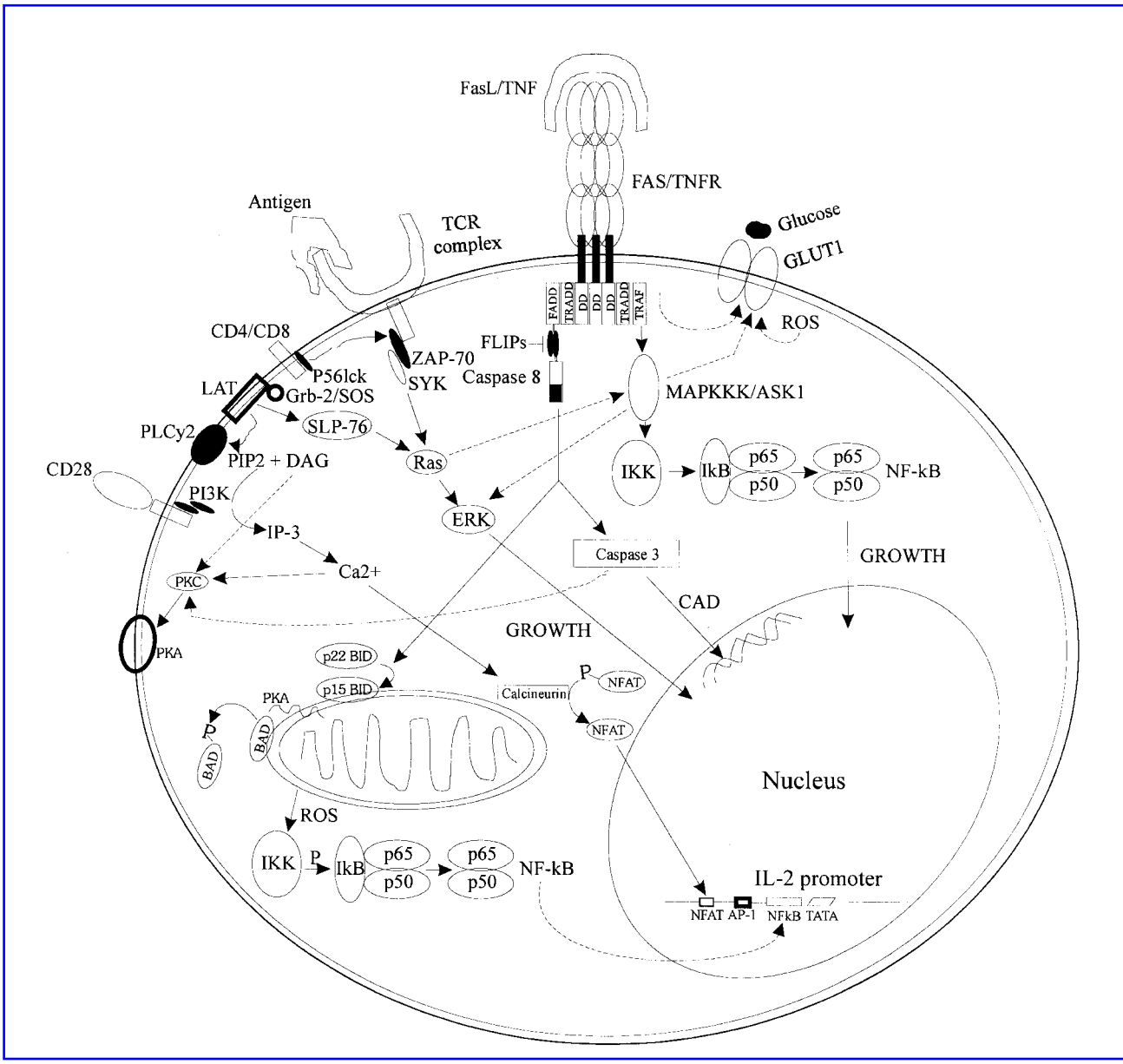

FIG. 1. Schematic overview of redox signaling and apoptosis pathways in HIV-infected cells. HIV-1 *tat* promotes elongation of viral RNA by RNA polymerase II (RPII), enhances viral RNA transcription by redox-mediated stimulation of NFκB, Sp1, AP-1, and JNK activities. *Tat*-induced ROS and vpr-mediated changes in $\Delta\Psi_m$ increase susceptibility to apoptosis triggered by oxidants, TNF, and Fas ligand (FasL). Expression of the Fas and TNF receptors is enhanced by the *nef* protein. Activation of caspase-8 through Fas signaling and mitochondrial injury leads to the release of executioner caspase-activating factors. This process is inhibited by bcl-2 and its viral homologues. The process is enhanced through cleavage of bcl-2 by HIV-1 protease. Fas ligand (FasL) crosslinks the Fas receptor (Fas/Apo1/CD95), which recruits an adapter protein with a Fas-associated death domain (FADD). Viral FLIPs (vFLIPs) possess a death effector domain similar to those of FADD and caspase 8 and, thus, interrupt Fas signaling. Although not shown, vFLIPs may also block TNF receptor-mediated signaling through FADD shared by both the Fas and TNF pathways. Upon recruitment of caspase-8, its oligomerization causes self-cleavage and activation of downstream effector caspases (148). Caspase-3-activated DNase (CAD) causes host cellular DNA fragmentation (42). Phosphorylation of BAD by mitochondria-anchored PKA results in antiapoptotic sequestration of BAD into the cytosol.

degradation of IκB releases NFκB and allows its nuclear translocation (75, 101, 150, 168). Activation of NFκB may also occur by a redox-dependent degradation of IκB without prior phosphorylation (89). Once NFκB is translocated in the nucleus, its binding to the NFκB motif may also be redox-controlled (37, 67, 132, 133) (Fig. 2). The NFκB/Rel protein family of transcription factors contain a cysteine residue within their arginine-rich DNA-binding domain (37). TRX stimulates DNA-binding activity of NFκB through reduction of a disulfide bond involving Cys^{62} of the p50 subunit of NFκB (114). Along the same line, elevated levels of GSSG inhibit DNA-binding activity and transcriptional activation by NFκB (47). Thus, TRX and GSSG have important regulatory roles in NFκB-mediated IL-2 gene transcription (Figs. 1 and 2).

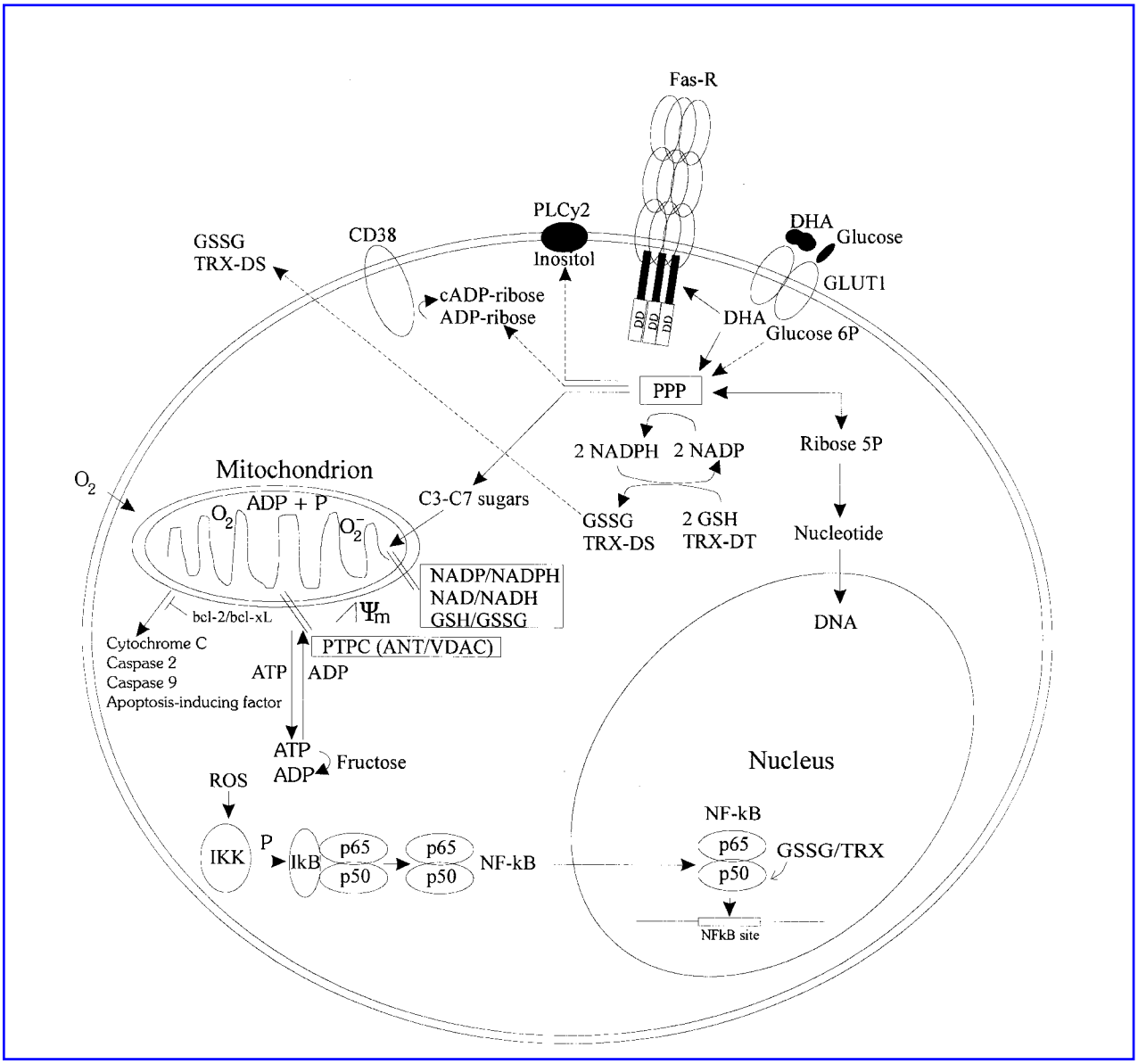

FIG. 2. Metabolic control of T-cell activation and cell death through $\Delta\Psi_m$, ROS levels, and the PPP. Intracellular antioxidants GSH and TRX-DT are regenerated at the expense of NADPH supplied primarily through metabolism of glucose via the PPP. Glutathione reductase and TRX reductase synthesize GSH and TRX-DT at the expense of NADPH. Formulation of the PPP and its efficiency to provide NADPH are dependent on the expression of G6PD and TAL. $\Delta\Psi_m$ is controlled by intracellular GSH/NADH/NADPH levels, integrity of the permeability transition pore complex (PTPC) largely comprised of adenine nucleotide translocator (ANT; inner membrane), voltage-dependent anion channel (VDAC; outer membrane), and translocation and dimerization of pro- and antiapoptotic bcl-2 family members in the intermembrane space. Secreted TRX functions as a chemoattractant for proinflammatory neutrophils and macrophages. Controlled increase of reactive oxygen intermediate levels activates NFκB and promotes cell growth. GSSG and TRX enhance binding of NFκB to DNA. Among PPP products, ribose 5-phosphate is required for nucleotide and DNA synthesis and supports cell growth, C3–C7 sugars influence mitochondrial function and ROS production, inositol and ADP-ribose serve as precursors for second messengers, inositol phosphates and cADP-ribose, respectively. DHA is imported through GLUT1. DHA is metabolized through the PPP, thereby enhancing GSH levels. DHA also increases surface expression of Fas-R.

METABOLIC CONTROL OF T-CELL ACTIVATION

ROS production modulates T-cell activation, cytokine production, and proliferation at multiple levels (63, 159). In turn, intracellular levels of ROS are controlled by a balance of mitochondrial ROS production and concentration of reducing equivalents, GSH, ascorbate (126), and TRX (125). Whereas production of GSH and TRX requires *de novo* peptide/protein synthesis, regeneration of their oxidized forms is dependent on NADPH provided by metabolism of glucose through the PPP (136) (Fig. 2). Dehydroascorbate (DHA), the oxidized form of vitamin C, is the major transport form of ascorbate in T cells, primarily via the hexose transporter GLUT-1 (174). Intracellularly, DHA is regenerated into ascorbate at the expense of GSH (119) and, ultimately, NADPH produced by the PPP (115). Another important product of the PPP is ribose 5-phosphate, a building block of DNA and other nucleotides involved in intracellular signaling (61) and energy metabolism (23). During T-cell activation, increased concentrations of inositol phosphates require *de novo* synthesis from glucose 6-phosphate (175). Energy for T-cell activation is dependent on synthesis of ATP (23). Further, mobilization of Ca^{2+} from intracellular stores is mediated by ADP-ribose (61). Increased glucose uptake is facilitated by up-regulation of glucose transporters via Ras and src proteins (44). Manganese superoxide dismutase (Mn-SOD) plays a critical role in neutralization of ROS produced in mitochondria. A targeted disruption of the Mn-SOD 2 locus leads to embryonic or neonatal lethality in mice. Reconstitution of hematopoiesis with Mn-SOD knockout fetal liver cells in lethally irradiated wild-type mice resulted in increased protein oxidative damage of erythroid progenitor cells and hemolytic anemia, whereas T and B lymphocytes and myeloid cells showed normal development (45).

EXTRACELLULAR SIGNALS TRIGGERING T-CELL APOPTOSIS

In response to antigenic stimulation, doubling time of T cells may be as short as 4.5 h; thus, a single T cell could give rise to 10^{12} cells and potentially double the total number of T cells in a human being within 1 week (98). Clonal expansion is continuously downsized, and potentially autoreactive cells are eliminated via apoptosis. An array of signals through the TCR, costimulatory molecules, cell-death receptors, lymphokines, and other circulating metabolites such as adenosine, ATP, nitric oxide, and ROS can trigger T-cell death. Thus, crosslinking of the Fas and other death receptors may lead to different outcomes. For example, Fas transduces an activation signal and stimulates proliferation in freshly isolated peripheral blood lymphocytes (PBL) (3, 120) or in certain tumor cell lines (135). The mechanisms of processing biologically opposing signals through Fas stimulation have not been precisely determined. Execution of these extracellular signals is dependent on the intracellular milieu. Most resting T cells are resistant to apoptosis. Even engagement of cell-surface death receptors, *e.g.,* Fas/CD95, results in activation of resting T cells (3). In contrast, death signaling through the APO-1/Fas/CD95 antigen (177) and the structurally related cell-surface receptor for TNF (92, 111) is accelerated in activated T cells. Prestimulation with CD3 or mitogenic lectins sensitizes peripheral blood T lymphocytes to apoptotic signaling through the Fas receptor (3, 11, 120, 123). Stimulation of freshly isolated PBL for up to 3 days with Fas antibody is not cytotoxic (11). However, incubation of PBL for as few as 20 min to 6 h with 1 μg/ml Fas antibody, Con A (11), or co-stimulation with CD3/CD28 monoclonal antibodies increased $\Delta\Psi_m$ [detected by 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) and 5-5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) fluorescence in Fig. 3] without increasing ROS levels [measured by dihydrorhodamine 123 (DHR)/D123 and hydroethidine (HE) fluorescence in Fig. 3]. Three to 7 days after CD3/CD28 (Fig. 3) or Con A stimulation, ROS levels dramatically increased, conferring sensitivity to Fas-induced apoptosis (11). Thus, mitochondrial hyperpolarization, occurring 20 min to 6 h after T-cell activation, and elevation of ROS levels, detectable 3–7 days after T-cell activation, represent metabolic switches resulting in cell death upon Fas stimulation.

Cell-surface expression of the Fas receptor is also redox-sensitive (100, 134). Export of the Fas receptor from the Golgi complex to the cell surface appears to be enhanced by p53 (15). GSH levels and Fas expression are concurrently enhanced by DHA, suggesting that oxidative folding in the endoplasmic reticulum rather than an overall oxidative stress facilitates cell-surface expression (141). ROS regulate TCR-mediated activation-induced apoptosis as well (71). Sustained stimulation through the TCR increases ROS production (71, 105). Expression of Fas ligand is mediated via the redox-sensitive NFκB (82). Therefore, cognate interaction between the antigen and the corresponding TCR can increase oxidative stress and promote activation-induced cell death via the Fas receptor system.

Stimulation of the Fas or the TNF1 receptor can activate a second pathway, mediated by DAXX, which can activate the JNK cascade through the redox-dependent ASK1 (27, 73, 129). ASK1 can undergo ROS-mediated multimerization in response to Fas or TNF stimulation (53). In nonstressed cells, ASK1 is associated with TRX. The TRX-bound form of ASK1 cannot multimerize and remains inactive as a kinase (145). Stimulation of the Fas or TNF receptor results in a ROS-dependent dissociation from TRX and multimerization of ASK1 required for kinase activity. Such activation of ASK1 can be prevented by antioxidants (145). Through death-domain (DD) containing proteins, TNF receptor interacts with TNF receptor-associated factor-2 (TRAF2), which in turn activates IκB kinase (IKK) via the MAP kinase kinase kinase (MAPKKK)/ASK1 cascade. Subsequently, IKK activation leads to phosphorylation-mediated degradation of IκB and translocation of NFκB into the nucleus. This constitutes a potential survival signal triggered through the Fas/TNF receptors.

Cytokines, particularly IL-2, are critical in determining T-cell fate. Interestingly, expression of IL-2 and the receptor for IL-2 is dependent on activation of redox-sensitive transcription factor NFκB (50). Balance of ROS production and endogenous synthesis of antioxidants, such as TRX (125), GSH, and NADPH, are critical for controlling susceptibility to IL-2-dependent proliferative (165) and cell-death signals (10, 172). Once IL-2-stimulated cells enter late G1 or S phase,

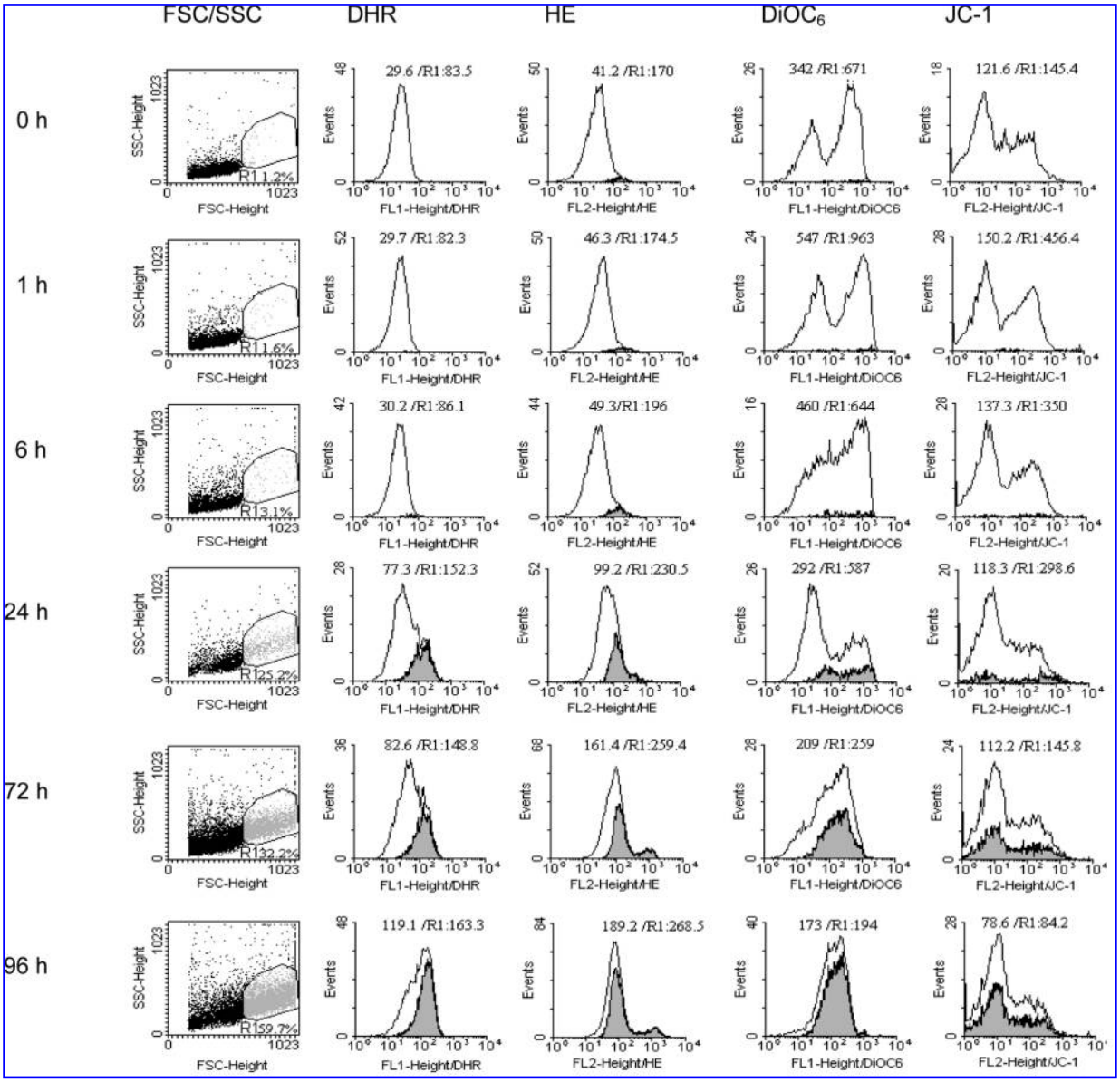

FIG. 3. Effect of CD3/CD28 stimulation on $\Delta\Psi_m$ and reactive oxygen intermediate levels. Normal PBL were incubated in Petri dishes precoated with 1 μg/ml/well CD3 monoclonal antibody (OKT3 clone CRL 8001 from ATCC, Rockville, MD, U.S.A.) for 1 h at 37°C. CD28 co-stimulation was performed by addition of 500 ng/ml monoclonal antibody CD28.2 (Pharmingen, San Diego, CA, U.S.A.) for 96 h. Subsequently, cells were stained with DHR, HE, $DiOC_6$, or JC-1, and analyzed by flow cytometry. In response to CD3/CD28 stimulation, forward and side scatter (FSC/SSC) dot plots show an increase in the percentage of cells undergoing activation/blast transformation (R1, region 1 in gray). Open histograms show fluorescence of all live cells. Dead cells and debris were excluded from the analysis by electronic gating based on FSC/SSC measurements. Fluorescence of cells in R1 is shown in gray. Values over histograms indicate mean channel fluorescence of live cells and R1 cells, respectively.

they become exquisitely susceptible to apoptosis. This susceptibility may be caused not only by increased ROS production, but also by diminished levels of cellular Fas-linked inhibitory proteins (cFLIPs) (4). Withdrawal of antigen, IL-2, or IL3 can trigger cell death as well. These latter pathways are more sensitive to inhibition by the Bcl-2 family of proteins (83, 98). Several lymphokines, IL4, IL-7, and IL-15, that share a common γ chain with IL-2 can prevent apoptosis induced by IL-2 withdrawal (98).

IL-12 and IL-18 play particularly important roles in Th1 development (33), type I CD8+ effector T cells (131) and $CD44^{high}$/CD8+ memory T cells (167). IL-18, originally described as interferon-γ-inducing factor, also promotes expression of the Fas ligand (33) and, thus, enhances Fas-mediated apoptosis of tumor cells by asialo GM1+ NK cells (66). Indeed, Il-18-induced NK activity is abrogated in *gld* mice lacking functional Fas ligand. IL-12 synergizes with IL-18 not only in promoting Th1 development, but also via increas-

ing perforin production and perforin/granzyme B-mediated cytotoxicity (66). Co-stimulation of activated T cells by IL-12 and IL-18 increases expression of both the Fas receptor and its ligand, resulting in activation-induced cell death inhibitable by wide-spectrum caspase blockers (97).

INTRACELLULAR PROCESSING OF APOPTOSIS SIGNALS

Delivery of signals through the APO-1/Fas/CD95 antigen and the structurally related TNF family of cell-surface death receptors has emerged as a major pathway in elimination of unwanted cells under physiological and disease conditions (124). Fas and the type I TNF receptor may mediate cell death via cytoplasmic DD shared by both receptors (76, 161). Signaling through the receptors involves the assembly of a death-inducing signaling complex with IL-1β converting enzyme (ICE)/caspase-1-like activity (20, 41, 107, 112, 163). The process of death by Fas stimulation starts out with the activation of caspase-8 [Fas-associated death domain-like ICE (FLICE)/MACHα1/Mch5] recruited via its N-terminal death effector domain to the death-inducing signaling complex (20, 122). Sequential activation of ICE/caspase-1, caspase-3, and related cysteine proteases results in the proteolysis of several cellular substrates, which leads to the characteristic morphologic and biochemical changes of apoptosis (112, 124). Processing of Fas signaling can be inhibited by death effector domain-containing inhibitory proteins, termed FLICE inhibitory proteins [FLIPs; vFLIPs for viral and cFLIPs for cellular forms (4, 18, 164)].

Increased production of ROS was demonstrated in TNF (70, 118, 151) and Fas-mediated cell death (9, 60, 81, 170, 178, 182). Disruption of the $\Delta\Psi_m$ has been proposed as the point of no return in apoptotic signaling (158, 173, 182). Interestingly, elevation of $\Delta\Psi_m$, mitochondrial hyperpolarization, and ROS production precede phosphatidylserine (PS) externalization and a disruption of $\Delta\Psi_m$ in Fas- (11) and H_2O_2-induced apoptosis of Jurkat human leukemia T cells and normal human PBL (140). These observations were confirmed and extended to p53 (103), TNFα (54), and staurosporine-induced apoptosis (149). Elevation of $\Delta\Psi_m$ is independent from activation of caspases and represents an early event in apoptosis (11, 103). Pretreatment with caspase inhibitors, DEVD, Z-VAD, and Boc-Asp, completely abrogated Fas-induced PS externalization, indicating that activation of caspase-3, caspase-8, and related cysteine proteases was absolutely required for cell death (41, 107, 148, 163). ROS levels were partially inhibited in DEVD-treated Jurkat cells, suggesting that caspase-3 activation, perhaps through damage of mitochondrial membrane integrity, contributes to ROS production and serves as a positive feedback loop at later stages of the apoptotic process. Nevertheless, ROS levels remained significantly elevated after pretreatment with caspase inhibitors. This suggested that activation of caspase-3 or caspase-8 was not required for increased ROS production and $\Delta\Psi_m$ hyperpolarization. By contrast, DEVD, Z-VAD, and Boc-Asp blocked PS externalization and decline of $\Delta\Psi_m$ in annexin V-positive Jurkat cells, suggesting that disruption of $\Delta\Psi_m$ (2) was a relatively late event with respect to ROS production and $\Delta\Psi_m$ hyperpolarization (1) and depended on activation of caspase-3 and related proteases. The precise mechanism by which Fas and TNF signaling leads to changes in $\Delta\Psi_m$ and ROS levels remains to be defined. Cleavage of cytosolic bid by caspase-8 generates a p15 C-terminal fragment that translocates to mitochondria. This may represent the initial insult to mitochondria in the Fas/TNF pathway (59) (Fig. 1).

MITOCHONDRIAL CHECKPOINTS IN T-CELL APOPTOSIS: $\Delta\Psi_M$, ATP SYNTHESIS, AND ROS PRODUCTION

Mitochondrial hyperpolarization appears to be the earliest change associated with Fas (11), H_2O_2 (140), HIV-1 (10), p53 (103), TNFα (54), and staurosporine-induced apoptosis (149). Elevation of $\Delta\Psi_m$ is also triggered by activation of the CD3/CD28 complex (Fig. 3) or stimulation with Con A (11). Therefore, elevation of $\Delta\Psi_m$ or mitochondrial hyperpolarization represents an early but reversible switch not exclusively associated with apoptosis. With $\Delta\Psi_m$ hyperpolarization and extrusion of H^+ ions from the mitochondrial matrix, the cytochromes within the electron transport chain become more reduced, which favors generation of ROS (157). Thus, mitochondrial hyperpolarization is a likely cause of increased ROS production and may be ultimately responsible for increased susceptibility to apoptosis following T-cell activation.

Disruption of the $\Delta\Psi_m$ is the point of no return in all apoptosis pathways (148, 158). It is associated with release of cytochrome *c* that activates apoptosis-activating factor 1 (Apaf-1), which in turn activates caspase-9 and caspase-3 (102). Sequential activation of effector/executioner caspases such as caspase-3, caspase-6, and caspase-7 leads to cleavage of key factors necessary for cell survival. Caspase-3, formerly called CPP-32 (148), cleaves IκB-α between Asp^{35} and Ser^{36} (13). Cleavage by caspase-3 blocks the ability of IκB-α to undergo phosphorylation-induced degradation. Thus, accumulation of IκB-α would inhibit activation of NFκB and their responsive genes (13). Substrates of caspases also include structural proteins such as lamins, actin, fodrin, poly(ADP-ribose) polymerase (148), PKC (34, 40), and caspase-activated DNase (CAD). CAD degrades the genomic DNA by cleavage at regular (180–200 bp) intervals (42, 148).

Proteins of the Bcl-2 family are localized to membranes of distinct organelles, including mitochondria (55, 86). They play key roles in maintenance of $\Delta\Psi_m$ and mitochondrial membrane integrity (55) (Figs. 1 and 2). This apoptosis-regulatory function may be related to their ability to form ion channels (59). Both the proapoptotic (Bax, Bad) and anti-apoptotic members (Bcl-2, Bcl-X_L) of the family can form ion-conducting channels in lipid membranes (59). Bax can create a channel in the outer mitochondrial membrane, thus releasing cytochrome *c* and other caspase-activating moieties into the cytosol. Bcl-2 and Bcl-X_L inhibit this process through dimerization with Bad or Bax. Activity of Bad is controlled by a mitochondria-anchored PKA (Fig. 1) (65). Enforced dimerization of proapoptotic Bax or Bad results in diminished membrane potential and increased production of

ROS. By contrast, overexpression of Bcl-2 or Bcl-X_L will antagonize these effects by forming heterodimers with Bax or Bad. Bcl-2 overexpression also inhibits release of cytochrome *c* from mitochondria (84 ,185). Oncogenic viruses possess Bcl-2-like proteins, which are thought to contribute to their transforming potential (59).

REGULATION OF $\Delta\Psi_M$ by HIV-1

Alterations of $\Delta\Psi_m$ occur early in T cells of HIV-infected patients (31). Decrease of $\Delta\Psi_m$ was associated with elevated TNFα or HIV gag 24 levels and compensated by *in vitro* incubation with antioxidants (31). Mitochondrial damage was also shown in uninfected but activated T cells, possibly explaining their increased susceptibility to cell death (25). Indeed, activation of normal peripheral blood T cells with mitogenic stimulation alone elicited changes in $\Delta\Psi_m$ (11) and increased susceptibility to Fas-induced apoptosis (120). Proapoptotic effects of the HIV-1 *vpr* protein may also be mediated by disturbing $\Delta\Psi_m$, thus causing dysfunction of the mitochondrial respiratory chain (109). *Vpr* can form cation-selective ion channels across lipid bilayers and consequently perturb transmembrane potentials (138). The C- terminus of *vpr* binds to the adenine nucleotide translocator in the permeability transition pore complex of the mitochondrial membrane (Fig. 2), thus causing rapid dissipation of $\Delta\Psi_m$ (78). The HIV-1 *nef* protein inhibits activity of Ca^{2+}-dependent K^+ channels in human glial (87) and T-lymphoid cells (187), and interferes with signaling through the TCR ζ chain (184) and the Fas as well as TNF receptors (183). *Nef* associates with ASK1, a common signaling intermediate of the Fas and TNF pathways, inhibiting both of these apoptosis pathways (48). In HIV-infected cells, *nef* also increases expression of the Fas ligand, leading to increased killing of bystander cells. Thus, *nef* may play a key role in protecting HIV-infected host cells while selectively killing bystander cells (48). It is presently unknown whether *nef* can directly influence $\Delta\Psi_m$. Cleavage of Bcl-2 by HIV-1 *protease* may contribute to increased ROS levels and apoptosis (156) via disruption of $\Delta\Psi_m$.

ATP SWITCH IN SELECTING CELL-DEATH PATHWAYS

The mitochondrion, the site of ATP synthesis via oxidative phosphorylation, has long been identified as a source of energy and cell survival. The synthesis of ATP is driven by an electrochemical gradient across the inner mitochondrial membrane maintained by an electron transport chain and the membrane potential (negative inside and positive outside). Activity of caspases requires ATP to the extent that depletion of ATP by inhibition of F_0F_1-ATPase with oligomycin (96) or exhaustion of intracellular ATP stores by prior apoptosis signals, Fas stimulation (96), or H_2O_2 pretreatment, leads to necrosis (95). Thus, intracellular ATP concentration is a key switch in the cell's decision to die via apoptosis or necrosis (96). Metabolic depletion of ATP by fructose enhances CD95-mediated apoptosis and inhibits TNF-induced liver injury (94). Extracellular ATP and adenosine have long been known to be cytotoxic. Adenosine may act through P1 purinoreceptors, raising intracellular Ca^{2+} and causing oxidative stress. ATP could also activate ectoenzymes as a phosphate donor or, through stimulation of $P2X_7$ purinoreceptors, deplete intracellular K^+, resulting in activation of caspases (28). Sustained increase of intracellular Ca^{2+} concentration mediates coupling of ATP production to metabolic need during T-cell activation (99). In fact, increased cytosolic Ca^{2+} concentration could be responsible for elevation of $\Delta\Psi_m$, which is a primary driving force of ATP synthesis (93). As noted above, further reduction of the cytochromes within the electron transport chain by $\Delta\Psi_m$ hyperpolarization favors increased ROS generation (157). Subsequent damage to mitochondrial membranes leads to release of mitochondrial Ca^{2+} stores and a secondary increase of cytosolic Ca^{2+} concentration (153). Ca^{2+} serves as a cofactor for activation of caspases and endonucleases during the execution phase of apoptosis (116).

ROS SWITCH

A small fraction of electrons (<5%) that flow through complexes I and III escape, react directly with oxygen, and form ROS. Controlled production of ROS is required for various T-cell signal-transduction pathways. ROS levels also depend on neutralization by enzyme systems (superoxide dismutase, catalase) and availability of reducing equivalents. ROS can elicit positive responses, such as lymphocyte activation (63, 71) and proliferation (159). In turn, convergence of multiple signaling pathways raising ROS levels increases susceptibility to apoptosis (24, 72, 79, 104). Thus, ROS level may represent a critical switch in the cell's decision to execute activation pathways, increase expression of redox-sensitive (IL-2) versus redox-insensitive (IL-4) lymphokines (144), or proceed with an apoptosis pathway upon concurrent death signals. ROS production can lead to mitochondrial depolarization, which may in turn lead to further ROS release, termed ROS-induced ROS release (RIRR) (188). ROS-induced mitochondrial depolarization may be transient and reversible in the presence of antioxidants, in particular GSH. In the absence of antioxidants, RIRR leads to oxidative burst, dysruption of $\Delta\Psi_m$, and apoptosis of cardiac myocytes (188). Although not yet shown, RIRR is also likely to operate as a self-amplifying mechanism during T cell apoptosis.

Whereas oxidative stress generally favors apoptosis, excessive ROS production may actually be inhibitory. The caspases themselves are cysteine-dependent enzymes, and as such appear to be redox sensitive (64, 152). Prolonged and excessive oxidative stress may actually limit caspase activity, inhibit apoptosis, and favor cell death via necrosis (64).

GLUCOSE SWITCH

Glucose transport and metabolism have been identified as targets of regulatory impact in several apoptosis pathways, such as Fas (17) and HIV signaling (154). Glucose transport can be lost within minutes of the stimulation of Jurkat cells

with Fas antibody (17), thereby blocking the cell's ability to combat oxidative stress and diminishing the survival of Fas-stimulated cells. Of note, oxidative stress alone results in elevated glucose transporter 1 (GLUT1) expression (88). Accordingly, increased oxidative stress in HIV-infected cells is accompanied by enhanced expression of GLUT1 (154). Increased expression of GLUT1 may also be responsible for accumulation of vitamin C in HIV-infected cells (143). The oxidized form of vitamin C, DHA, is transported into the cell via GLUT1 (174). Intracellularly, DHA is reduced back to vitamin C at the expense of GSH (119). Under physiological conditions, vitamin C has a predominantly antioxidant role (26). Prooxidant and proapoptotic effects of vitamin C may be related to hydroxylation (169) and/or formation of ascorbyl radicals (146). In the event of oxidative stress, vitamin C can be further oxidized to ascorbate free radical (121), which may explain its toxicity for HIV-infected cells (143). Additionally, metabolites of glucose and vitamin C, a six-carbon sugar itself, may also contribute to cell death. Ribose 5-phosphate and other short chain sugars are directly capable of inducing apoptosis (12, 16). High glucose concentration can also lead to oxidative stress (39, 43, 57, 130), NFκB activation, and cell death (38). Hyperglycemia or elevated extra- and intracellular glucose concentrations result in oxidative stress through autooxidation and activation of the polyol pathway, causing progressive cellular and parenchymal tissue damage in patients with diabetes (21).

THE ROLE OF THE PPP IN REDOX SIGNALING AND MAINTENANCE OF THE $\Delta\Psi_M$

Changes in mitochondrial membrane integrity, leading to the release of cytochrome *c* and other caspase-activating factors (148), appear to be the point of no return in the effector phase of apoptosis (102). Mitochondrial membrane permeability is subject to regulation by an oxidation–reduction equilibrium of ROS, pyridine nucleotides (NADH/NAD + NADPH/NADP), and GSH levels (30) (Fig. 2). Regeneration of GSH from its oxidized form, GSSG, depends on NADPH produced by the PPP (115). In fact, a fundamental function of PPP is to maintain glutathione in a reduced state, thereby protecting sulfhydryl groups and cellular integrity from emerging oxygen radicals. Regeneration of the dithiol form of another antioxidant, TRX, is mediated by TRX reductase at the expense of NADPH (74). Activity of catalase is also dependent on NADPH (115).

Metabolism of glucose through the PPP provides (a) NADPH for reductive biosynthetic reactions and regeneration of reducing equivalents, GSH, TRX, and ascorbate and (b) ribose 5-phosphate for nucleotide biosynthesis. The PPP comprises two separate, oxidative and nonoxidative, phases. Reactions in the oxidative phase are irreversible, whereas all reactions of the nonoxidative phase are fully reversible. The two phases are functionally connected. The nonoxidative phase converts ribose 5-phosphate to glucose 6-phosphate for utilization by the oxidative phase, and thus, indirectly, contributes to generation of NADPH. Different enzymes are rate-limiting in the two phases. The oxidative phase primarily depends on glucose 6-phosphate dehydrogenase (G6PD) (181), whereas transaldolase (TAL) is the rate-limiting enzyme for the nonoxidative phase (69). Formulation of the PPP and its efficiency to provide NADPH is dependent on the expression of G6PD (91, 147, 166) and TAL (9–11, 117, 127, 180). TAL overexpression lowers G6PD and 6-phosphogluconate dehydrogenase (6PGD) activities and NADPH and GSH levels and renders the cell highly susceptible to apoptosis induced by serum deprivation, H_2O_2, nitric oxide, TNFα, and Fas signaling. When TAL levels are reduced, G6PD and 6PGD activities and GSH levels are increased and apoptosis is inhibited. TAL activity profoundly impacts the balance between the two branches of PPP and the ultimate output of NADPH and GSH (9). These findings are consistent with a dominant role for TAL within the metabolic network that controls the propagation of biochemical signals (127). Along the same line, up-regulation of TAL activity by phosphorylation results in depletion of NADPH and oxidative stress in fibroblasts isolated from patients with xeroderma pigmentosum or transformed by simian virus 40 (91).

Levels of TAL expression can determine the extent of mitochondrial ROS production, changes in $\Delta\Psi_m$, and subsequent caspase activation, PS externalization, and cell death during HIV infection (10). Overexpression of TAL accelerated HIV-induced oxidative stress, protease activation, PS externalization, and cell death in two human CD4+ T cell lines. In contrast, suppression of TAL activity abrogated these effects and blocked HIV-induced cell death. Thus, increased mitochondrial ROS production is a defining step in HIV-induced apoptosis and may serve as a possible target in the development of new therapeutics against HIV disease (136).

Signaling through the APO-1/Fas/CD95 antigen (177) and the structurally related cell-surface receptor for TNF (92, 111) is accelerated in activated T cells. Elevation of $\Delta\Psi_m$ and ROS levels precedes externalization of PS, disruption of $\Delta\Psi_m$, and cell death, in both Jurkat human T cells and PBL. Changes in $\Delta\Psi_m$ and ROS levels can be controlled by TAL through the supply of reducing equivalents from the PPP. Overexpression of TAL accelerated Fas-induced ROS production, $\Delta\Psi_m$ elevation, activation of caspase-8 and caspase-3, proteolysis of poly(ADP-ribose) polymerase, and PS externalization, whereas suppression of TAL diminished these activities. A series of caspase inhibitor peptides, DEVDCHO, Z-VAD.fmk, and Boc-Asp.fmk, block Fas-induced PS externalization, disruption of $\Delta\Psi_m$, and cell death, showing that these changes are sequelae of caspase activation. By contrast, caspase inhibitors do not affect Fas-induced elevation of ROS levels and $\Delta\Psi_m$. Early increases in ROS levels and $\Delta\Psi_m$, as well as dominant effect of TAL expression on activation of caspase-8/FLICE, the most upstream member of the caspase cascade, suggest a pivotal role for redox signaling at the initiation of Fas-mediated apoptosis.

ASCORBATE SWITCH

Ascorbic acid, or vitamin C, is important as a cofactor of several biosynthetic enzyme reactions and widely used as an antioxidant (19, 119). However, vitamin C also has prooxi-

dant properties and may cause apoptosis of human lymphocytes and myelogenous leukemia cell lines (5, 139). Under physiological conditions, vitamin C has a predominantly antioxidant role (26). Prooxidant and proapoptotic effects of vitamin C may be related to hydroxylation (169) and/or formation of ascorbyl radicals (146).

Humans and other primates lack gulonolactone oxidase, a key enzyme for ascorbic acid biosynthesis and, therefore, it must be provided from external sources (128). Even in ascorbic acid-synthesizing species, the majority of cells need ascorbic acid from the outside (51). Vitamin C is absorbed from the gastrointestinal tract in the form of ascorbic acid and circulates in the blood as ascorbate at pH 7.4. Ascorbate and its oxidized form, DHA, are taken up by transporters and facilitated diffusion in a cell type-specific manner (51). DHA is the major transport form of ascorbate in T lymphocytes and other blood cells, primarily via the hexose transporter GLUT1 (174). Within the cell, DHA is regenerated into ascorbate at the expense of GSH (119). However, ascorbate cannot regenerate GSH from its oxidized from, GSSG. GSSG is reduced to GSH at the expense of NADPH, which is produced by the PPP (115). Unless reduced back to ascorbate, DHA is rapidly hydrolyzed into 2,3-diketo-L-gulonate and decarboxylated to L-xylonate and L-lyxonate (80). In turn, these five-carbon sugars can enter the nonoxidative branch of the PPP (6, 22).

As recently discovered (140), DHA stimulates the activity of PPP enzymes TAL, G6PD, and 6PGD, elevates intracellular GSH levels, and increases resistance of Jurkat human T cells to H_2O_2-induced cell death. Whereas the antioxidant and prooxidant properties of vitamin C have long been recognized, existence of DHA at low concentrations in plant and animal cells was only regarded as evidence of ascorbate oxidation (160, 179). However, DHA is not merely an oxidized and transport form of vitamin C. DHA stimulates the PPP and GSH levels and inhibits H_2O_2-induced cell death of cells susceptible to the prooxidant properties of vitamin C.

A metabolic relationship between the GSH/GSSG and ascorbate/DHA redox couples has long been recognized (119). A maximum of 3.3-fold GSH elevation was observed after 48 h of stimulation with 800 μM DHA. DHA may be a more potent stimulator of GSH levels than *N*-acetylcysteine. GSH levels could only be increased up to twofold by treatment with *N*-acetylcysteine, at an optimal concentration of 3 m*M*, in Jurkat cells (9). Thus, the intracellular presence of DHA may not solely represent an accumulation of the oxidized form of vitamin C, but it may have a role in influencing GSH levels through the PPP. These changes were accompanied by an increase in G6PD protein levels with respect to actin levels, suggesting that DHA can stimulate gene expression. Carbon flux via the PPP has been implicated in regulating expression of other glucose-metabolizing enzymes, pyruvate kinase (36), glucose 6-phosphatase, and phosphoenolpyruvate carboxykinase (113). Although, xylulose 5-phosphate has been proposed as a key intermediate, its impact on the PPP and potential involvement of xylulose 5-phosphate metabolites remain to be determined (36, 113). GSH and GSSG levels were not affected by pretreatment of Jurkat cells with DHA for <24 h. Thus, the possibility that increased PPP enzyme activities were triggered by a DHA-induced oxidative stress could be clearly excluded. Stimulation by DHA of activities of NADPH-generating enzymes, G6PD and 6PGD, may be responsible for elevation of GSH levels. Genetically enforced augmentation of G6PD activities can increase GSH levels and resistance to apoptosis signals (9, 10, 147). In turn, increased GSH can directly stimulate expression of γ-glutamylcysteine synthetase, the rate-limiting enzyme of *de novo* GSSG synthesis (171), and further support GSH production.

Surprisingly, increased GSH levels did not protect against Fas-mediated apoptosis (141). In contrast, after DHA preincubation, Fas-induced mitochondrial membrane hyperpolarization and cell death were dramatically accelerated in both H9 and Jurkat cells. DHA increased cell-surface Fas receptor expression in both cell lines. The increased surface expression was not accompanied by a change in total Fas protein levels, thus excluding increased gene expression.

The native structure of the Fas receptor is dependent on formation of disulfide bonds. Point mutations affecting cysteine residues of Fas are incompatible with apoptotic signaling (76, 134). Folding into the correct three-dimensional structure through crosslinking of cysteine residues is catalyzed by protein disulfide isomerase (PDI) (49). PDI activity is particularly relevant for folding of proteins with cysteine-rich domains, such as Fas (49). Although GSSG has also been proposed as an oxidant cofactor for PDI, recent evidence shows that the glutathione system provides reducing rather than oxidizing power for protein folding in the endoplasmic reticulum (8, 32). Interestingly, PDI also catalyzes the reduction of DHA to ascorbate (176), and DHA is an oxidant cofactor of PDI-mediated folding in the endoplasmic reticulum (7). Whereas expression of Fas was stimulated by DHA, cell-surface CD4 density was not affected in DHA-treated cells. Of note, the extracellular domain of the Fas receptor has 20 cysteine residues (77), whereas that of CD4 only contains six cysteines (110). The presence of cysteine-rich domains may explain selective up-regulation of Fas expression by DHA. In summary, DHA and its sugar metabolites are potential targets for development of selective apoptosis regulators.

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ABBREVIATIONS

AP-1, activator protein-1; ASK1, apoptosis signal-regulating kinase 1; CAD, caspase-activated DNase; cFLIP, cellular Fas-linked inhibitory protein; DAG, diacylglycerol; DD, death domain; DHA, dehydroascorbate; DHR, dihydrorhodamine 123; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; ERK, extracellular signal-regulated kinase; FLICE, Fas-associated death domain-like ICE; FLIP, FLICE inhibitory protein; GLUT1, glucose transporter 1; G6PD, glucose 6-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; HE,

hydroethidine; HIV-1, human immunodeficiency virus type 1; ICE, interleukin-1β-converting enzyme; IKK, IκB kinase; IL, interleukin; IP_3, inositol 1,4,5-trisphosphate; ITAM, immunoglobulin receptor family tyrosine-based activation motif; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MAPKKK, MAP kinase kinase kinase; Mn-SOD, manganese superoxide dismutase; NFAT, NFκB, nuclear factor-κB; PBL, peripheral blood lymphocytes; PDI, protein disulfide isomerase; 6PGD, 6-phosphogluconate dehydrogenase; PI_3K, phosphatidylinositol 3′-hydroxyl kinase; PIP_2, phosphatidylinositol 4,5-biphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PPP, pentose phosphate pathway; PS, phosphatidylserine; RIRR, Ros-induced ROS release; ROS, reactive oxygen species; SOS, Son of Sevenless; TAL, transaldolase; TCR, T-cell receptor; TNF, tumor necrosis factor; TRAF2, TNF receptor-associated factor 2; TRX, thioredoxin; vFLIP, viral FLICE-inhibitory protein; ZAP-70, zeta-associated protein-70; $\Delta\Psi_m$, mitochondrial transmembrane potential.

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