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Letter to the Editor

Activation-associated mitochondrial hyperpolarization hijacks T cells toward an apoptosis-sensitized phenotype

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Dear Editor,

Two different apoptotic pathways leading to activation of cell-specific programs have been hypothesized.[1] Cell types might therefore belong to two main prototypes and respond differently to apoptotic stimuli, eventually converging towards mitochondrially driven cascade.[2,3] This can be seen in cells of different histotypes and is characterized by loss of mitochondrial membrane potential (MMP) and opening of the mitochondrial megapore. This leads to the release of apoptogenic factors (cytochrome *c* and apoptosis-inducing factor), and downstream execution phase cascade involving apoptosome formation and caspase 9 activation. Caspase 3 activation and consequent cleavage of several substrates (e.g. poly-ADPribose-polymerase) are the final events.[3,4] Certain chemical, biological and physical agents have been described as affecting cell fate by directly acting on MMP.[5–7] Some of these 'mitochondriotropic' agents decrease MMP and apoptosis, while others hinder cell suicide.[8] Literature clearly suggests that activated human lymphocytes are highly sensitive to apoptotic triggers while resting T cells are 'refractory' to apoptotic stimulations such as monoclonal IgM anti-Fas antibodies (α-Fas).[9,10] In fact, activation of isolated human lymphocytes by using (i) phytohemagglutinin (PHA): (ii) anti-CD3 monoclonal antibody (α-CD3) and (iii) human immunodeficiency virus envelope protein gp120[11] in combined treatments with interleukin-2 (IL2) led to a significantly increased susceptibility to α-Fas-induced apoptosis (Figure 1). In the light of this activation-increased apoptotic proneness and the key role played by mitochondria in apoptotic cell death, a specific analysis of MMP was conducted. Three different probes were used: 3,3′-dihexyloxacarbocyanine iodide (DiOC6),[2] tetramethylrhodamine ester (TMRM)[12,13] and 5-5′,6-6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1).[14] These probes are commonly used in flow and static cytometry studies and yield overlapping results; they have therefore been considered interchangable. Experiments were performed in IL2-activated cells with or without the costimulatory molecules indicated above (PHA, α-CD3, gp120). Shepherding light through the MMP features of variously activated T cells, a striking behavior was detected. Compared to resting cells, median values of the fluorescence intensity histogram were significantly *increased* (corresponding to an increase of mitochondrial membrane polarization, Table 1B). This increase was positively correlated ($P<0.01$) with increased expression of different T-cell activation markers on the cell surface, for example, CD69, HLA-DR (Table 1A**)** and CD38 (not shown). These results were consistent with a more extensive qualitative and quantitative analysis of the polarization state of mitochondria obtained with JC-1 in resting and IL2-activated lymphocytes, shown in Figure 2, before and after α-Fas triggering. IL2-activated cells had a significantly higher percentage of *hyperpolarized* mitochondria ($59.7\pm5\%$) compared to resting cells ($26.1\pm3\%$) (compare Figure 2c with 2a, boxed areas). Similarly, parallel intensified video microscopy (IVM) qualitative analysis revealed a predominance of red fluorescence emission (typical of hyperpolarization) in IL2-activated (Figure 2d) compared to resting lymphocytes (Figure 2b). This increase was actually because of a hyperpolarization state of mitochondria and not because of increased dye uptake due to mitochondrial mass growth occurring during activation. In fact, cytofluorimetric analysis using the specific probe nonylacridine orange (5 μM NAO, Molecular Probes) clearly indicated that mitochondrial mass was substantially unchanged by T-cell activation (median values of the fluorescence intensity histograms were 49.1 ± 2 and 48.6 ± 2.5 for resting and activated lymphocytes, respectively). After 48 h α-Fas treatment produced the MMP loss typical of apoptotic cell death effector phase[15] in activated cells only. This is illustrated in Figure 2e (III quadrant), which shows a typical dot plot showing a number of cells with depolarized mitochondria. Similarly, IVM analysis also shows green fluorescent mitochondria (Figure 2f).

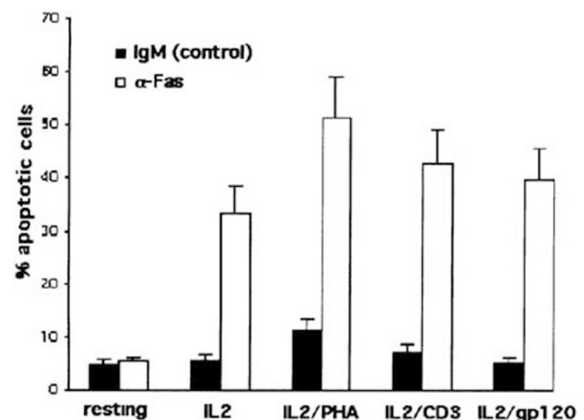

Figure 1 Quantitative cytofluorimetric analysis of apoptotic cells after double staining with annexin V-FITC/propidium iodide apoptosis detection kit (Eppendorf, Milan, Italy) in resting and activated T cells. Activation protocols considered: (i) 6000 IU IL2, 72 h; (ii) 6000 IU IL2+0.5 μg/ml PHA, 72 h; (iii) 6000 IU IL2+ 0.1 μg/ml α-CD3, 72 h; (iv) 6000 IU IL2+ 5 μg/ml gp120, 72 h). Apoptosis was induced with 500 ng/ml α-Fas (IgM anti-human Fas, clone CH11, Pharmingen) for 48 h (the same concentration of IgM was used as a control). Note that activation significantly ($P<0.01$) increased apoptotic susceptibility. Mean values $\pm$ S.D. from four different experiments are reported

Table 1 (**a**) Quantitative cytofluorimetric analysis of surface expression of activation markers CD69 and HLA-DR (antibody FITC- or PE-conjugated by Becton Dickinson, Mountain View, CA, USA) in resting and differently activated lymphocytes. (**b**) In the same samples, a parallel monitoring of MMP was performed by flow cytometry by using two different probes: DiOC6 (500 nM, green fluorescence) and TMRM (1 μM, red fluorescence) (Molecular Probes, Eugene, USA). Numbers in the table represent the median values of fluorescence intensity histograms. Note that upon activation with IL-2, lymphocytes display a significant increase of activation markers (CD69, HLA-DR) and a parallel (statistically correlated, $P<0.01$) increase of MMP

	Control		PHA		α-CD3		gp120	
	w/o IL2	with IL2	w/o IL2	with IL2	w/o IL2	with IL2	w/o IL2	with IL2
(A) activation markers								
CD69	5.1	8.9	13.2	29.7	6.2	29.7	4.7	8.3
HLA-DR	15.5	29.5	35.1	49.5	16.9	31.4	14.7	24.3
(B) MMP								
TMRM	70.6	106.5	65.7	115.3	81.7	105.5	74.4	103.3
DiOC6	17.7	34.9	21.0	30.1	28.3	34.7	19.6	31.1

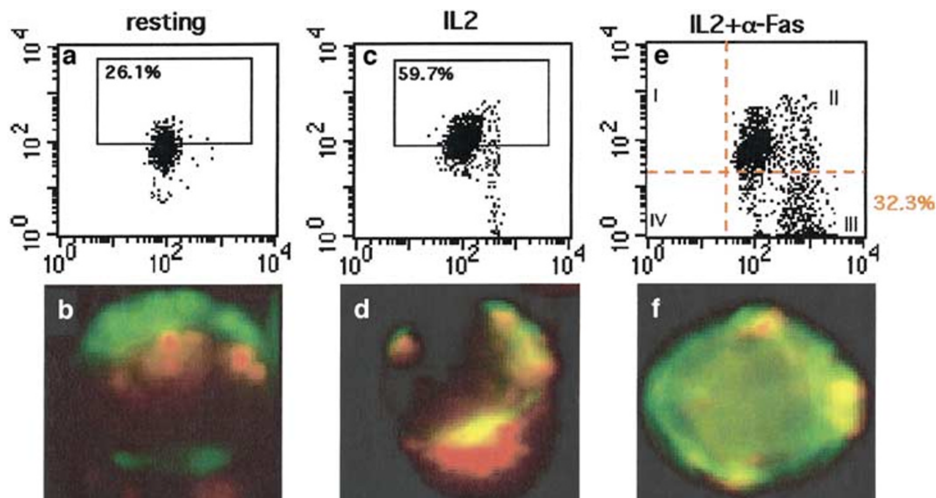

Figure 2 *Quantitative* (**a**, **c** and **e**) and *qualitative* (**b**, **d** and **f**) analyses of MMP of lymphocytes after JC-1 (10 μM, Molecular Probes) staining. Resting (**a** and **b**), IL2-activated (**c** and **d**) and α-Fas-treated IL-2-activated lymphocytes (**e** and **f**) as revealed by a FACScan cytometer or by an Intensified Video Microscopy System with a Nikon Microphot fluorescence microscope equipped with a CCD camera. Note: (i) the increased percentage of IL2-activated T cells with hyperpolarized mitochondria (compare **a** and **c**, boxed areas) and the typical features of apoptosis in Fas-triggered activated lymphocytes (**e**, the number in the III quadrant represents the percentage of cells with depolarized mitochondria); and (ii) the prevalence of red fluorescence (corresponding to J-aggregates in mitochondria, which increase when mitochondrial membrane becomes more polarized) in IL2-activated (**d**) with respect to resting lymphocytes (**b**). After treatment with α-Fas for 48 h, activated lymphocytes showed depolarized mitochondria as revealed by the prevalence of green fluorescence (**f**)

Agents capable of specifically influencing MMP homeostasis were therefore examined in terms of their ability to modulate apoptotic proneness in resting and activated T cells. Low doses were used of the protonophore uncoupler carbonyl cyanide fluorophenyl-hydrazone (FCCP),[16] known to hinder mitochondria hyperpolarization, and the antibiotic drug oligomycin (OLM), known to influence mitochondrial homeostasis by increasing MMP.[17] FCCP was ineffective in resting T cells, leaving the MMP largely unchanged (Figure 3a) and T cells remained refractory to α-Fas stimulation (Figure 3b). Conversely, FCCP was able to hinder the IL-2-induced MMP increase (Figure 3c, compare with Figure 2c) and, consequently, to protect IL2-activated T lymphocytes from α-Fas-induced late events, that is, MMP depolarization (Figure 3d, III quadrant). Apoptosis was accordingly significantly decreased (-81 ± 5%). OLM had the opposite effect. In resting T cells, OLM induced *per se* a significant mitochondrial membrane hyperpolarization state (Figure 3e). This was enough to hijack resting T cells towards a more apoptotic-prone phenotype. In fact, a significant increase in the percentage of apoptotic cells (+160 ± 12%) was detected compared to OLM-free resting T cells. In IL-2-activated T cells, where cytokine activation had already increased MMP (Figure 3g), OLM induced a significant further increase (+65%) of cells displaying typical signs of α-Fas-mediated apoptosis with respect to OLM-free cells (Figure 3H, III quadrant).

Overall, these results clearly indicated that (i) the ‘stabilizing’ effect of FCCP on MMP decreased apoptotic proneness, while (ii) the presence of a ‘mitochondriotropic’ drug such as OLM, inducing mitochondrial hyperpolarization (increasing MMP), led to increased apoptotic sensitivity. Accordingly, we also observed that freshly isolated T cells from naive

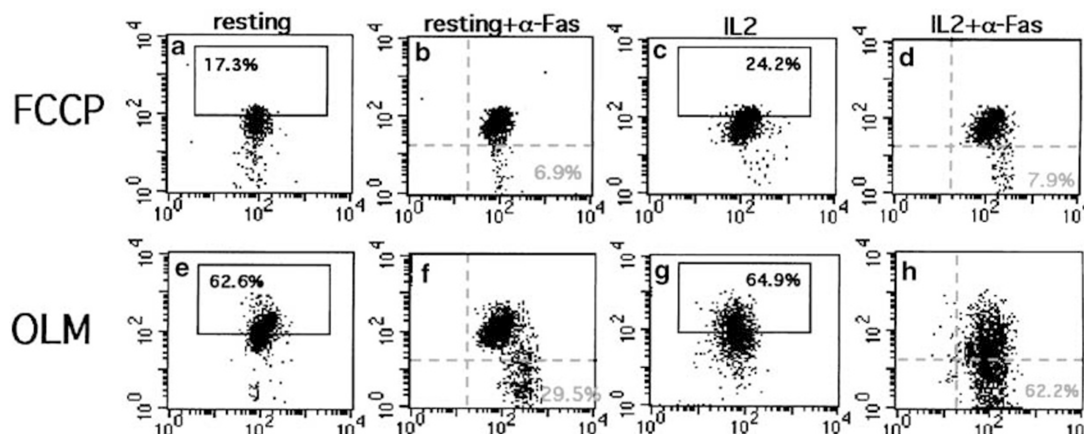

Figure 3 Effects of 'mitochondriotropic' agents on MMP of resting and IL2-activated lymphocytes before or after Fas-triggering. Note that FCCP: (i) was ineffective in resting cells (**a** and **b**) but ii) was able to 'stabilize' MMP of activated cells (**c**) and protect them from Fas-induced MMP loss (**d**). By contrast, OLM increased either the percentage of control cells with hyperpolarized mitochondria (**e** and **g**) or MMP loss of Fas-triggered cells (**f** and **h**) in both resting and activated lymphocytes. For flow cytometry pictures: abscissa, FL1 (green fluorescence, J-monomers); ordinate, FL2 (red fluorescence, J-aggregates). Boxed areas show percentages of lymphocytes expressing high red fluorescence (corresponding to J-aggregates that typically increased when mitochondrial membrane became *hyper*polarized). In quadrant III are cells with depolarized mitochondria (corresponding to apoptotic cells). All the samples were analyzed with a FACScan flow cytometer (Becton Dickinson) equipped with a 488 argon laser. At least 20 000 events were acquired. Data were recorded and statistically analyzed by a Macintosh computer using CellQuest Software. One representative experiment of four is shown

HIV-infected patients, which are constitutively activated and apoptosis prone,[18] also displayed a significantly higher percentage (27 $\pm$ 3%) of cells with hyperpolarized mitochondria compared to those from healthy donors (2.0 $\pm$ 1%) (manuscript in preparation). These results, together with the literature data obtained in lymphocytes from patients with Systemic Lupus Erythematosus[19] as well as in cell lines of different histotypes,[6,20–22] seem to suggest that the hyperpolarization state of mitochondria may represent an early key event in hijacking activated lymphocytes towards a *sensitized* phenotype.

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1. Hengartner MO (2000) Nature 407: 770 – 776.
2. Kroemer G *et al.* (1997) Immunol. Today 18: 44 – 51.
3. Fulda S *et al.* (2001) Oncogene 20: 1063 – 1075.
4. Green D and Kroemer G (1998) Trends Cell Biol. 8: 267 – 271.
5. Banki K *et al.* (1999) J. Immunol. 162: 466 – 470.
6. Matsuyama S *et al.* (2000) Nat. Cell. Biol. 2: 318 – 325.
7. Bossy-Wetzel E *et al.* (1998) EMBO J. 17: 37 – 49.
8. Olson M and Kornbluth S (2001) Curr. Mol. Med. 1: 91 – 122.
9. Parlato S *et al.* (2000) EMBO J. 19: 5123 – 5134.
10. Stahnke K *et al.* (2001) Blood 98: 3066 – 3073.
11. Capobianchi MR (1996) J. Biol. Regul. Homeost. Agents 10: 83 – 91.
12. Rasola A *et al.* (2001) Cytometry 45: 151 – 157.
13. Bedner E *et al.* (1999) Cytometry 35: 181 – 195.
14. Cossarizza A *et al.* (1995) Exp. Cell Res. 220: 232 – 240.
15. Kroemer G and Reed JC (2000) Nat Med. 6: 513 – 519.
16. Giovannini C *et al.* (2002) FEBS Lett. 523: 200 – 206.
17. Wang GQ *et al.* (2001) J. Biol. Chem. 276: 3610 – 3619.
18. Phenix BN and Badley AD (2002) Biochimie 84: 251 – 264.
19. Gergely *et al.* (2002) Arthritis Rheum. 46: 175 – 190.
20. Matarrese P *et al.* (2002) Am. J. Pathol. 160: 1507 – 1520.
21. Piacentini M *et al.* (2002) J. Neurochem. 81: 1061 – 1072.
22. Matarrese P *et al.* (2001) Biochem. J. 355: 587 – 595.