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Pyrimethamine induces apoptosis of freshly isolated human T lymphocytes bypassing CD95/Fas molecule but involving its intrinsic pathway

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Abbreviations:

ALPS, autoimmune lymphoproliferative syndrome; $\Delta\Psi$, mitochondrial membrane potential; TCR, T cell receptor; FasL, Fas ligand; PBL, peripheral blood lymphocytes; HD, healthy donors; PBS, phosphate buffered saline; FBS, fetal bovine serum; PHA, phytohemagglutinin; IL, interleukin; DMSO, dimethyl sulphoxide; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; APC, allophycocyanin; PMA, phorbol myristate acetate; PFA, paraformaldehyde; IFN, interferon; PI, propidium iodide; JC-1, 5-5',6-6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide; FL, fluorescence channel; ELISA, enzyme-linked immunosorbent assay; Cyt C, Cytochrome C; SEM, scanning electron microscopy; TNFRSF, tumor necrosis factor receptor super family; sFASL, soluble FasL.

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Abstract

Pyrimethamine, a folic acid antagonist, may exert, in addition to anti-protozoan effects, immunomodulating activities including induction of peripheral blood lymphocyte apoptosis. However, the molecular mechanisms underlying this pro-apoptotic activity remain to be elucidated. Here we show that pyrimethamine, used at pharmacologically relevant concentration, induced *per se* apoptosis of activated lymphocytes via the activation of the caspase-8- and caspase-10-dependent cascade and subsequent mitochondrial depolarization. Importantly, this seems to occur independently from CD95/Fas engagement. The pro-apoptotic activity of pyrimethamine was further confirmed in a patient with autoimmune lymphoproliferative syndrome (ALPS), an immune disorder associated with a defect of Fas-induced apoptosis. In this patient, pyrimethamine treatment resulted in a “normalization” of lymphocyte apoptosis with a significant amelioration of laboratory parameters. Altogether these results suggest a mechanism for pyrimethamine-mediated apoptosis that seems to bypass CD95/Fas engagement but fully overlaps CD95/Fas-induced subcellular pathway. On these bases, a reappraisal of the use of pyrimethamine in immune lymphoproliferative disorders characterized by defects in CD95/Fas-mediated apoptosis should be taken into account.

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Introduction

The complex cascade of events triggered by apoptotic signals, including activation of specific caspases, depends on the nature of the activating stimuli (Hengartner, 2000). Two main cell death pathways have been recognized: the "mitochondrial" and the "death-receptor" pathways, involving caspase-9 and caspase-8, respectively (Schmitz et al., 1999; Fulda et al., 2001). Both pathways, however, converge towards specific mitochondrion activities (Fulda et al., 2001). In particular, according to the more credited theories, it would be changes of mitochondrial membrane potential ($\Delta\Psi$) to induce the main events of the apoptotic process among which the release of cytochrome c (Cyt C), the apoptosome formation and, finally, the chromatin clumping and DNA fragmentation.

Pyrimethamine (2;4-diamino-5-*p*-chlorophenyl-6-ethyl-pyrimidine) belongs to the group of anti-folate drugs blocking the enzyme dihydrofolate reductase essential for the synthesis of folic acid, a co-factor required for DNA synthesis. It is used in the treatment of infections caused by protozoan parasites such as *T. gondii* and *P. falciparum* (2000) (Winstanley, 2001). Accumulating evidences indicate that pyrimethamine can also modulate the immune response by suppressing the proliferation of mitogen- and antigen-stimulated human lymphocytes (Bygbjerg, 1985; Bygbjerg et al., 1986; Viora et al., 1996). In addition, a pro-apoptotic activity of pyrimethamine has been recently hypothesized by van der Werff Ten Bosch et al. who reported reversion of autoimmune lymphoproliferative syndrome (ALPS) with pyrimethamine (van der Werff ten Bosch et al., 1998; van der Werff Ten Bosch et al., 2002). ALPS is a rare immune disorder appearing in early childhood characterized by lymphadenopathy, splenomegaly, and autoimmunity along with expansion of circulating T cell receptor (TCR) α/β^+ CD4⁻CD8⁻ T cells (Sneller et al., 1992; Fisher et al., 1995; Rieux-Laucat et al., 1995). It generally associates with mutations of the Fas encoding gene and rarely with mutations of FasL (Fisher et al., 1995; Rieux-Laucat et al., 1995; Drappa et al., 1996; Wu et al., 1996; Bettinardi et al., 1997), caspase-8 (Chun et al., 2002) or caspase-10 (Wang et

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al., 1999) gene. A number of ALPS cases lacking of any detectable molecular defects were also described (Lenardo, 2003; Rieux-Laucat et al., 2003).

Up to date the fine mechanism(s) underlying the proapoptotic activity exerted by pyrimethamine on human lymphocytes remains to be elucidated, although the involvement of the mitochondrial apoptotic pathway has been suggested (van der Werff Ten Bosch et al., 2002). This hypothesis was consistent with previously reported data showing alterations of mitochondrial gene expression after incubation with pyrimethamine (Sprecher et al., 1995).

In this study we analyze in detail the apoptotic pathway triggered by pyrimethamine in peripheral blood lymphocytes (PBL) from healthy donors (HD) and an ALPS patient under pyrimethamine treatment. Results show that induction of apoptosis by pyrimethamine occurs through the activation of the caspase-8- and caspase-10-dependent cascade, independently from CD95/Fas engagement.

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Methods

Isolation and activation of PBL. Blood samples were obtained from 10 HD and an ALPS patient with informed consent. PBL were isolated from heparinized venous blood by Ficoll-Hypaque density-gradient centrifugation (Lympholyte-H; Cedarlane Laboratories, Hornby, Ontario, Canada) and washed twice in phosphate buffered saline (PBS), pH 7.4. PBL were then cultured in 24-well plates at a density of 1×10^6 cells/ml in RPMI-1640 medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Euroclone, Pero, Italy), 2mM glutamine (Sigma, St Louis, MO, USA) and 50 μ g/ml gentamycin (Sigma). The cells were activated for 72 h with 1 μ g/ml phytohemagglutinin (PHA; Sigma) plus 60 IU/ml of recombinant human interleukin (IL)-2 (Roche, Mannheim, Germany) and maintained in IL-2 (60 IU/ml) with refeeding every 48 to 72 h for up to 2 weeks before analysis, as previously described (Fisher et al., 1995).

Culture conditions and induction of cell death. Pyrimethamine (Sigma) was dissolved, immediately before experiments, in dimethyl sulphoxide (DMSO) and diluted in RPMI medium. Isolated human lymphocytes (resting or activated) were treated with different concentrations of pyrimethamine (80, 40, 4, and 0.4 μ g/ml). As a control, an equal volume of DMSO was added to lymphocytes. To activate the CD95/Fas pathway, an anti-human Fas IgM mAb (clone CH11; Upstate Biotechnology, Lake placid, NY, USA) was added to activated PBL at a concentration of 0.5 μ g/ml or, after 2 h pyrimethamine pre-treatment, at different concentrations (0.05, 0.5, and 5 μ g/ml). Pyrimethamine and CH11 treatments were also performed in cells pretreated for 2 h with: i) a neutralizing anti-human Fas IgG1 mAb (clone ZB4; 10 μ g/ml; Upstate Biotechnology); ii) the caspase-8 (z-IETD-fmk), caspase-10 (z-AEVD-fmk), caspase-9 (z-LEHD-fmk), and caspase-3 (z-DEVD-fmk) inhibitors (all from R&D Systems, Minneapolis, MN, USA). Caspase inhibitors were reconstituted in DMSO, diluted in RPMI medium according to the manufacturer's instructions and

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then used at different concentrations (10, 50, and 100 μ M). In consideration of the results obtained (maximal inhibition observed), only the results obtained with a 50 μ M concentration of different caspase inhibitors will be shown.

Flow cytometry analyses

Lymphocyte phenotyping and BCL-2 expression. For surface phenotyping, 500 μ l of whole blood were lysed using 10 ml of Ortho lysing reagent (Ortho-Clinical Diagnostics, Raritan, NJ, USA), washed, labeled with appropriate combinations of 4 mAbs for 30 min at 4°C, and fixed within 1 h from blood collection. Anti-CD3 fluorescein isothiocyanate (FITC), anti-CD45RA FITC, anti-HLA-DR FITC, anti-CD25 FITC, anti-CCR5 FITC, anti-CD95 FITC, anti-TCR α/β phycoerythrin (PE), anti-CD62L PE, anti-CD4 (peridinin chlorophyll protein) PerCP or allophycocyanin (APC), anti-CD8 PerCP were purchased from BD Immunocytometry Systems (San Jose, CA, USA). Direct staining with anti-TCRBV antibodies (IOTest Beta Mark, Immunotech, Marseille, France) was performed according to manufacturer's instruction. For cell surface quantitative evaluation of CD95L (Fas ligand, FasL), unconjugated mAb to CD95L (BD Pharmingen, San Diego, CA, USA) and FITC-conjugated goat anti-mouse IgG (BD Immunocytometry Systems) were also used. After staining, cells were washed once in PBS containing 2% FBS and analyzed on a flow cytometer. Analysis of cytokine production at the single cell level was performed as previously described (Pierdominici et al., 2003). Briefly, freshly isolated PBL were stimulated for 16 h with 1 μ g/ml ionomycin (Sigma) and 25 ng/ml phorbol myristate acetate (PMA; Sigma) in the presence of 10 μ g/ml brefeldin A to inhibit cytokine secretion. After a wash in PBS, cells were fixed with 4% paraformaldehyde (PFA) by incubation for 5 min at room temperature, permeabilized with FACS permeabilizing solution (BD Immunocytometry Systems) for 10 min, washed and stained. The following cytokine-specific mAbs from BD Immunocytometry Systems were used: FITC labeled anti-interferon- γ (IFN- γ , IgG2b),

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FITC labeled anti-IL-2 (IgG1) and PE labeled anti-IL-4 (IgG1). Surface phenotyping was performed with anti-CD4 APC and anti-CD8 PerCP. For BCL-2- expressing cells analysis, PBL were fixed and permeabilized as described above and stained with an anti-BCL-2 FITC mAb (clone124; DAKO, Glostrup, Denmark) for 30 min. Surface phenotyping was performed with anti-CD4 APC and anti-CD8 PerCP mAbs.

Apoptosis. Quantitative evaluation of apoptosis was performed by a double staining flow cytometry method using FITC-conjugated annexin V/propidium iodide (PI) apoptosis detection kit (MBL, Woburn, MA, USA) according to the manufacturer's protocol. FITC-conjugated annexin V positive cells were considered as cells in the early stages of apoptosis. Cells distinguished by their ability to take up both FITC-annexin V and PI were considered as cells in the later stages of apoptosis. Live cells were those negatively staining for FITC-annexin V and PI. For selected experiments, electronically gated CD4⁺ and CD8⁺ lymphocytes were considered.

Mitochondrial membrane potential ($\Delta\Psi$). $\Delta\Psi$ was studied by using the lipophilic cationic probe 5-5',6-6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1; Molecular Probes), as previously described (Cossarizza et al., 1995). Briefly, cells were incubated in complete medium for 10 min at room temperature in the dark with 10 μ g/ml JC-1 probe. JC-1 was dissolved and stored according to the manufacturer's instructions. At the end of incubation period, cells were washed twice in cold PBS, resuspended in a total volume of 400 μ l, and analyzed on a flow cytometer.

Detection of caspase-8, -10, -9 and -3activity. Activation state of the caspases 8, 9 and 3 was evaluated by using the CaspGLOW fluorescein active caspase staining Kit (MBL, Woburn, MA, USA) while that of caspase-10 by using the APO LOGIX_{TM} carboxyfluorescein caspase detection kit (Cell Technology, Mountain View, CA, USA). These kits provide sensitive means for detecting activated caspases in living cells and utilize specific caspase inhibitors (IETD-fmk for caspase-8, AEVD-fmk for caspase-10, LEHD-fmk for caspase 9 and DEVD-fmk for caspase-3) conjugated to FITC as the fluorescent marker.

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These inhibitors are cell permeant, nontoxic and irreversibly bind to caspase active form. The FITC label allows detection of activated caspases in apoptotic cells directly by flow cytometry. Untreated and treated PBL were incubated with FITC-IETD-fmk, FITC-AEVD-fmk, FITC-LEHD-fmk or FITC-DEVD-fmk for 1 h at 37 °C following the manufacturer's instruction. After this time samples were washed three times and immediately analyzed on a cytometer by using fluorescence (FL)-1 channel. Two additional experimental controls were also considered: i) samples prepared by pre-treating cells with specific caspase-8, -10, -9 or -3 inhibitors before pyrimethamine administration and ii) unlabelled PBL (negative control).

Enzyme-linked Immunosorbent Assays (ELISA)

Cytochrome C (Cyt C) release. Cyt C was analyzed in total extracts of activated PBL, cultured in the presence or absence of pyrimethamine using a sensitive and specific commercial ELISA kit (R&D Systems). Briefly, activated PBL were removed after 6, 12 24 and 48 h of pyrimethamine exposure, washed in cold PBS, lysed and centrifuged at 1000 g for 15 min. The supernatants were then assayed according to the manufacturer's instructions. The intensity of the light emitted was quantified by using a microplate reader at 450 nm, as the primary wavelength and 570 nm, as the reference wavelength. Cyt C concentration was expressed as ng/ml. The limit of sensitivity of the assay, as supplied by the manufacturer, was 0.31 ng/ml.

Soluble FasL (sFasL). Aliquots of PBL supernatants were removed 6, 12 24 and 48 h after pyrimethamine treatment and FasL release was analyzed by using a commercially available ELISA kit (R&D Systems) according to the manufacturer's instructions. The intensity of the color was measured using a microplate reader using 450 nm as the primary wavelength and 570 nm as the reference wavelength. As supplied by the manufacturer, the minimum detectable dose of FasL ranged from 1.01 to 8.05 pg/ml. FasL concentration was expressed as ng/ml.

IL-10 determination. Serum was removed rapidly and carefully from the red cells after clotting. Levels in serum of human IL-10 were determined using a commercially available ELISA kit

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(Euroclone, Wetherby, U. K.) according to the manufacturer's instructions. The intensity of the color was measured using a microplate reader using 450 nm as the primary wavelength and 650 nm as the reference wavelength. The limit of sensitivity of the assay as supplied by the manufacturer was 5 pg/ml.

Morphological studies

Static cytometry analysis. To visualize intracellular distribution of BCL-2 protein, PBL were collected and plated on poly-L-lysine coated slides, fixed for 30 min at room temperature in PBS containing 3.7% PFA, and permeabilized for 5 min with PBS containing 0.5% triton X-100. After washings, cells were incubated at 37° for 30 min with an anti-BCL-2 polyclonal antibody (Santa Cruz Biotechnology, CA, USA). Cells were then incubated with FITC-conjugated anti-rabbit IgG (whole molecule) (Alexa Fluor 488, Molecular Probes). Finally, all samples were mounted with glycerol-PBS (2:1) and observed by intensified video microscopy by using a Nikon Microphot fluorescence microscope equipped with a color chilled 3CCD camera (Zeiss, Germany). Normalization and background subtraction were performed for each captured image. Figures were obtained by the OPTILAB (Graftek, France) software for image analysis.

Scanning electron microscopy (SEM). PBL were collected and plated on poly-L-lysine coated slides, fixed with 2.5% gluteraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 20 min after different treatments as stated above. Following post-fixation in 1% OsO₄ for 30 min, cells were dehydrated through graded ethanols, critical point dried in CO₂ and gold coated by sputtering. The samples were examined with a Cambridge 360 scanning electron microscopy.

Patient clinical features. The *in vivo* pro-apoptotic effects of pyrimethamine were studied in a 12-year-old female with ALPS. Diagnosis of ALPS was made at the age of 10 years when immunological analysis showed: i) reduced Fas-mediated apoptosis of PHA/IL-2-stimulated T-lymphocytes (30%; control values > 50%), ii) increased percentage of circulating TCR α/β^+ CD4⁺

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CD8⁻ T cells (13%; control values <1 %), iii) high IL-10 serum level (247 pg/ml; control values <10 pg/ml). Genomic DNA was prepared from PBL and screened for Fas mutations using described methods (Rieux-Laucat et al., 1995). Sequence analysis of the tumor necrosis factor receptor super family 6 (TNFRSF6) gene revealed no mutation. Clinical patient history showed, by age 22 months, splenomegaly, neutropenia, thrombocytopenia, hypergammaglobulinemia with increased IgG serum levels, and recurrent urticarial rashes. Results of viral and bacterial serology were consistently negative. In the following years she showed severe episodes of Coombs-positive hemolytic anemia. Antinuclear (++++, homogeneous pattern) as well as IgG anticardiolipin (30 GPL vs. ≤ 10 GPL of controls) antibodies were present. She received many courses of steroids with transient effects. Thrombocytopenia and urticarial rash persisted after splenectomy performed when the patient was 9 years of age.

Pyr treatment was started with an oral dose of one tablet per week.

Data analysis and statistics. Flow-cytometric analyses were performed by a four-color multiparameter flow cytometer (FACScalibur, BD Immunocytometry Systems) using the Cell Quest Pro software. At least, 20,000 events were acquired. To determine marker expression on CD4⁺ and CD8⁺ cells, total lymphocytes were first identified and gated by forward and side scatter. The cells were then additionally gated for CD4 or CD8 expression. Appropriate isotypic negative controls were run in parallel. Statistical analysis was performed by Student's t test using Statview program for Windows. All apoptosis data reported in this study are the mean of at least five separate experiments ± SD.

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Results

Pyrimethamine induces apoptosis of human T lymphocytes

We first evaluated the dose-dependent effects of pyrimethamine on cell viability in resting or PHA/IL-2 activated PBL obtained from HD (Fig. 1A-C). In resting lymphocytes, only pyrimethamine concentrations ≥ 40 $\mu\text{g/ml}$ were capable of inducing significant apoptosis. Lower concentrations, corresponding to those detected *in vivo* at the steady state (0.4 $\mu\text{g/ml}$ - 4 $\mu\text{g/ml}$) (Weidekamm et al., 1982), did not demonstrate any pro-apoptotic activity in resting cells (<10%). Conversely, in activated lymphocytes, the pro-apoptotic effect of pyrimethamine was already remarkable at a concentration of 4 $\mu\text{g/ml}$ (41 ± 5 %), reaching values close to 80%-90% at higher doses. Control experiments carried out with DMSO alone did not display any pro-apoptotic activity (< 10%). In this set of experiments aimed at evaluating the dose-response curve of pyrimethamine, the expression of a key molecule of importance in the inhibition of apoptotic cell death pathway, i.e. the BCL-2 molecule, was also assessed. In fact, pyrimethamine-induced apoptosis was detected along with a downregulation of BCL-2 expression, both in resting (Fig. 1D) and activated lymphocytes (Fig. 1E). On the basis of these results we selected the dose of pyrimethamine 4 $\mu\text{g/ml}$ as an optimal concentration for further studies on activated lymphocytes. To better evaluate pyrimethamine-induced cell death, a time-dependent analysis of apoptosis was also carried out. We cultured PHA/IL-2 activated lymphocytes in the presence of pyrimethamine at different time points (6, 12, 24, 48, and 72 h), (Fig. 2). Depending on the HD examined, the time period in which most of the T cells were annexin V single positive was variable between 12 h and 24 h (data not shown). We thus decided to focus our attention to 24 h pyrimethamine treatment. At this time point lymphocytes from all HD were in fact undergoing apoptosis (annexin V/PI double positive cells, $p < 0.05$ vs. untreated cells). This clearly suggests a “fast” apoptotic execution phase in pyrimethamine-treated human lymphocytes. Apoptosis reached a maximum after 48 h ($p < 0.01$ vs.

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untreated cells). Percentage of necrotic cells was < 1% at all time points analyzed. Because it was previously demonstrated an active role of the CD95/CD95FasL system in apoptosis induced by anti-folate drugs, e.g. methotrexate (Debatin, 1999), we also evaluated the expression of CD95/Fas and FasL on the cell surface. Moreover, the release of FasL molecule in the growth medium was assessed. As shown in Table 1, as compared to untreated cells, a significant up-regulation of CD95/Fas expression was detected on the surface of activated lymphocytes after 48 h of pyrimethamine treatment ($p < 0.05$). Conversely, FasL expression and secretion remained unchanged at all time points studied, with the exception of FasL expression that showed a trend to an increase, although not significant, at 48 h of pyrimethamine treatment.

To exclude a direct involvement of CD95/Fas in pyrimethamine-induced apoptosis, PBL were treated with the ZB4 mAb, i.e. the CD95 neutralizing antibody (Fig. 3A,B). Interestingly, the percentage of apoptosis in pyrimethamine-treated PBL preincubated with ZB4 remained substantially unchanged. As expected, control experiments carried out with activated PBL exposed to anti-CD95/Fas mAb (clone CH11) showed that ZB4 pre-incubation was able to significantly reduce Fas-mediated apoptosis. These results seem to suggest that the pyrimethamine-induced apoptosis does not entail the engagement of CD95/Fas molecule.

The role of caspase-8 and caspase-10

In consideration of the well-known cascade of events occurring intracellularly after apoptotic triggering, further studies were then carried out on caspase cascade, i.e. the main actors of the cell death program (Shi, 2004). Thus, to study the activation of the initiator caspases (caspases 8, 10, 9) and the effector caspase-3, we incubated the activated PBL with pyrimethamine at different time points. Caspase activity was then evaluated by a fluorimetric method (Fig. 4). No significant caspase activity was found at early time points (6 and 12 h). Caspases 8, 10, 3 were found significantly activated after 24 h of pyrimethamine treatment (Fig 4A,B,D) while significant increase of caspase-9 was evident only after 48 h. More interestingly, at 24 h caspase-8 activity was

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more pronounced as compared to other caspases and statistical analyses clearly indicated a significant difference ($p < 0.01$) between the values obtained with caspase-8 with respect to all other caspases (Fig. 4A).

Next, to establish the involvement of caspase activation in pyrimethamine-mediated apoptosis, we preincubated the activated PBL with selective inhibitors of caspase-8 (z-IETD-fmk), caspase-10 (z-AEVD-fmk), caspase-9 (z-LEHD-fmk) and caspase 3 (z-DEVD-fmk). Pyr-induced apoptosis was significantly inhibited by caspase-8, -10 and -3 inhibitors (used at a concentration 50 μ M, see Methods), Fig. 4E. Accordingly, caspase activity was significantly decreased (Fig. 4A-D). Remarkably, i) caspase-9 inhibitor (z-LEHD-fmk) was unable to reduce pyrimethamine-triggered apoptosis (Fig 4E) and ii) preincubation of activated PBL with the caspase-8 inhibitor z-IETD-fmk abrogated caspase-9 activity after pyrimethamine treatment (Fig. 4G). This suggests that pyrimethamine-induced apoptosis occurred primarily through the caspase-8/-10-dependent pathway, i.e. *via* extrinsic pathway.

Since altogether the above reported data were consistent with the hypothesis of a common activation pathway shared by pyrimethamine and CD95/Fas, it was mandatory to determine whether exposure to pyrimethamine could influence Fas-induced apoptosis of activated T cells. In order to verify this hypothesis, activated lymphocytes were treated with pyrimethamine prior incubation with increasing doses of an agonist anti-CD95/Fas mAb (0.05, 0.5, and 5 μ g/ml), (Fig. 5). In the absence of pyrimethamine, the ability of anti-CD95/Fas mAb to trigger cell death increased with dose to reach $46 \pm 5\%$ at concentration of 5 μ g/ml. Higher doses of anti-CD95/Fas mAb did not further increase the number of apoptotic cells (data not shown). In the presence of pyrimethamine, an additive effect on Fas-mediated apoptosis was found at doses of anti-CD95/Fas mAb ≤ 0.5 μ g/ml. However, when pyrimethamine was used in the presence of a dose of 5 μ g/ml of anti-CD95/Fas mAb, the additive effect was not detectable, this suggesting a biochemical plateau of enzyme activity and a common apoptotic pathway.

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The role of mitochondria

To deeply characterize the apoptotic pathway triggered by pyrimethamine in lymphocytes, we also focused on the possible role of mitochondria, well-known regulators of cell death (Kroemer et al., 1997). In particular, previous studies suggested that hallmarks of apoptosis-associated mitochondrial modification are significant decrease in $\Delta\Psi$ (Ferri and Kroemer, 2001) (Matarrese et al., 2003) and Cyt C release from mitochondria into the cytosol. Therefore, time-dependent changes occurring in $\Delta\Psi$ and the release of Cyt C were analyzed in pyrimethamine-treated PBL. As shown in Fig. 6A, the increase of cells showing $\Delta\Psi$ loss, although already detectable after 24 h of pyrimethamine treatment, reached statistical significance as compared to untreated cells ($p < 0.01$) at 48 h. Interestingly, two hours pretreatment with caspase-8 and-10 inhibitors was capable of significantly decreasing the percentage of cells showing $\Delta\Psi$ loss in pyrimethamine-treated PBL (Fig. 6B). As a positive control, we used activated PBL treated with anti-Fas/CD95 but pre-incubated with caspase inhibitors (Fig 6B). Parallel analyses carried out on cytosolic cyt C (released from mitochondria) clearly indicated a significant release ($p < 0.01$) of this apoptogenic factor starting from 48 h treatment (Table 2).

The above reported results have been summarized in Fig. 7.

Clinical and laboratory improvement of ALPS by pyrimethamine treatment

In order to ascertain if the pro-apoptotic effects seen in *in vitro* experiments could have a role in the immunoregulatory activity exerted *in vivo* by pyrimethamine, we studied the CD95/Fas-induced apoptosis and BCL-2 expression in T cells from a representative ALPS patient undergoing pyrimethamine administration.

No toxicity was observed during pyrimethamine treatment. Hematological analyses showed a normalization of platelet count (264.000/ μ l) within one month. IL-10 level significantly decreased but remained high (from 247 pg/ml to 66.3 pg/ml); IgG level decreased from 3450 mg/dl to 2030 mg/dl (normal range for age-matched HD, 640-1909 mg/dl). There was no improvement of the

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recurrent urticarial rashes. Five months later, the therapy was stopped when the patient developed an episode of meningitis due to a *S. pneumoniae*. During hospitalization, she had also an acute episode of coxo-femoralis arthritis. During the following three months all treatments were stopped and she remained in a good clinical condition. Laboratory analyses were within the normal range with the exception of a platelets count of 41.000/ μ l.

Morphological scanning electron microscopy analysis of lymphocytes was conducted before and after one month of pyrimethamine treatment. This analysis, performed for the first time in an ALPS patient, provides useful information regarding some typical aspects of lymphocyte alteration and injury such as modifications of microvillous structures. What we found is reported in Fig. 8: typical cell surface thin protrusions, well visible in PHA/IL-2-activated lymphocytes from HD (A), were altered in the ALPS patient before pyrimethamine administration (B). The same evaluation, but carried out after pyrimethamine therapy, indicated the presence of lymphocytes with a morphology similar to that detected in HD (C). Apoptotic induction by antiCD95/Fas mAb clearly induced loss of microvillous structures and typical cell surface signs of cell death in HD (D) while surface structural features of lymphocytes from the ALPS patient before therapy appeared unchanged (E). Conversely, cells obtained after pyrimethamine treatment, once exposed to anti-CD95/Fas, underwent cell surface modifications typical of cell death as in HD (F). According to these results, flow cytometry analysis of activated T lymphocytes showed, before therapy, a significant impairment of Fas-induced apoptosis as compared to HD ($30 \pm 2\%$ vs. $49 \pm 3\%$, respectively, $p < 0.001$), (Fig. 8G). This defect was observed in both the $CD4^+$ ($20 \pm 2\%$ vs. $46 \pm 5\%$, $p < 0.001$) and, to a lesser extent, $CD8^+$ T cell subpopulations ($36 \pm 2\%$ vs. $53 \pm 6\%$, $p < 0.01$). However, a “normal” CD95/Fas surface up-regulation was demonstrated on PHA/IL-2 activated lymphocytes (median fluorescence intensity, 71 ± 2 in activated cells and 38 ± 1 in untreated cells) suggesting “qualitative” but not “quantitative” defects in the transmission of the death signal delivered to the Fas molecule. Consistent with the decreased rate of anti-CD95/Fas-induced apoptosis, the percentage of activated lymphocytes with $BCL-2^{\text{low}}$ expression was lower in the ALPS patient (Fig.

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8H) than in HD ($34 \pm 2\%$ vs. $53 \pm 5\%$, $p < 0.01$). This was more evident in $CD4^+$ ($27 \pm 2\%$ vs. $57 \pm 7\%$, $p = 0.002$) than in $CD8^+$ T cell subset ($33 \pm 2\%$ vs. $47 \pm 2\%$, $p < 0.01$). After 1 month of pyrimethamine treatment, the percentage of Fas-mediated apoptosis increased, reaching values not significantly different from those detected in HD. Interestingly, this was associated with a normalization of BCL-2 expression (Fig. 8I) as also confirmed by intensified video microscopy analyses (Fig. 8, L and M, before and after therapy, respectively). Finally, when the spontaneous apoptosis of activated T lymphocytes was considered, no significant differences could be demonstrated between HD and the ALPS patient both before and after therapy (percentage of apoptosis $< 10\%$ in all cases).

Flow cytometric analysis of T-cell subsets

An extensive immunophenotyping was performed in PBL from the ALPS patient administered with pyrimethamine (Table 3). Before therapy, a significant predominance of T cells with an effector memory phenotype ($CD45RA^+CD62L^-$, and $CD45RA^-CD62L^-$) and, consequently, a restriction of naive T cell pool ($CD45RA^+CD62L^+$) were detected within the $CD8$ subset. The expression of HLA-DR molecule was increased in both $CD4^+$ and $CD8^+$ T cells while that of CD95 was within the normal range. The pattern of activation-induced cytokine production was consistent with the predominant effector memory phenotype observed in the $CD8$ subset. In fact, as compared to age-matched HD, there was a significant increase of $CD8^+$ T cells polarized toward the production of IFN- γ (34% vs. $16 \pm 12\%$). As opposite, the frequency of $IL-2^+$ and $IL-4^+$ T cells fell within the normal range with the exception of a significant increase of $IL-4$ production in the $CD4$ subset (4% vs. $1 \pm 0.5\%$). Expansions of selected $CD8$ TCRBV families (BV3, BV13.6 and BV14) were also revealed by flow cytometry analysis.

When the absolute counts were considered a high lymphocyte T cell count was demonstrated in the ALPS patient (4331 cells/ μ l vs. $2000-2700$ cells/ μ l of age-matched HD). Consequently, the absolute count of $CD4^+$ and $CD8^+$ naive, memory and activated T cells was significantly increased

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as compared to HD. After one month of therapy the number of lymphocytes significantly reduced (from 4331 cells/ μ l to 3547 cells/ μ l) as well as that of CD4⁺ and CD8⁺ T cells although it remained above normal values. Finally, a normalization of CD8⁺ IFN- γ -expressing T cell count was detected.

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Discussion

In the present work we characterized the apoptotic pathway triggered by pyrimethamine in human lymphocytes. Parallel analyses carried out in T cells from HD and a patient with ALPS depicted the same scenario: pyrimethamine was able to induce CD95/Fas downstream caspase cascade.

Activated lymphocytes are known to be vulnerable to the “physiological” signal represented by triggering of CD95/Fas with the consequent activation of caspases 8 and 10. Our data indicate that, similarly, pyrimethamine induced apoptosis of activated T cells via a mechanism bringing into play the upstream caspases 8 and 10 although it did not require CD95/Fas engagement, as demonstrated by experiments performed with a specific mAb blocking CD95/Fas. According to this, pyrimethamine exerted its proapoptotic activity by sharing the same signaling cascade of CD95/Fas molecule and, when used in combination to a triggering anti-CD95/Fas mAb, it was able to exert a significant additive activity on Fas-mediated apoptosis.

Although our results suggest that the primary target for pyrimethamine is represented by caspase-8-driven cascade, we can not rule out the possibility that pyrimethamine may also directly act on mitochondria thus contributing to the powerful proapoptotic activity of the drug. Similarly to other anti-folate compounds, pyrimethamine led to mitochondrial membrane depolarization, a late event in the mitochondrially driven apoptosis cascade (Kroemer et al., 1997). To this regard it is interesting to consider that the expression of BCL-2, a regulator of mitochondrial proapoptotic activity, was downregulated by pyrimethamine. In addition, pre-exposure to antioxidizing drugs such as the N-Acetyl-Cysteine (NAC) was capable of partially protect lymphocytes from both the loss of $\Delta\Psi$ and apoptosis (our unpublished observations). Thus, our findings seem to indicate that mitochondria can represent a further target of pyrimethamine in addition to Fas caspase cascade. Therefore, pyrimethamine-mediated apoptosis may be considered as a multi-faceted process in which different inducers or regulators of apoptosis are simultaneously implicated. However, on the basis of the results obtained evaluating annexin V positivity and PI permeability, i.e. a rapid

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execution phase with the occurrence of double positive cells, the possibility of concomitant alternative forms of cell death, e.g. oncosis, can not be ruled out (Krysko et al., 2004; Scholz et al., 2005).

On the basis of these *in vitro* results, we also shepherded through an *in vivo* situation in order to achieve further information regarding the mechanisms responsible for the pro-apoptotic activity exerted by pyrimethamine. To this aim an ALPS patient, before and after pyrimethamine treatment, was studied. This patient had no detectable Fas mutations although presented a defective Fas-induced apoptosis. A weekly dose of pyrimethamine led, within one month of treatment, to a significant increase of Fas-induced apoptosis of both CD4⁺ and CD8⁺ activated T cells in association with a normalization of BCL-2 expression. According to these data, a significant reduction of lymphocyte counts was also detected. Our results are partially at variance with those reported by van der Werff Ten Bosch et al. (van der Werff Ten Bosch et al., 2002) who observed in ALPS patients under pyrimethamine treatment a reversion of lymphoproliferative signs but no changes in Fas-induced apoptosis of T lymphocytes. This may be partially explained by the probable diverseness of genetic defects responsible for the ALPS phenotype in the patients studied. In fact, two out of seven patients described by van der Werff Ten Bosch et al. had Fas mutations while the remaining five patients, as well as the ALPS patient presented in this report, had uncharacterized genetic defects in the apoptotic pathway. In addition, different experimental procedures could be also taken into account to explain such discrepancy.

Different factors may contribute to the normalization of Fas-induced apoptosis in the ALPS patient here studied. Our results are consistent with the above described proapoptotic effects of pyrimethamine bypassing the requirement of upstream caspases 8 and 10 molecules eventually defective in our ALPS patient (i.e. membrane/cytoskeleton interaction molecules or molecules involving in the DISC formation). Pyrimethamine could also normalize Fas-induced apoptosis through a downregulation of BCL-2 expression, eventually mediated by decreased IL-10 production. In fact, IL-10 induces the antiapoptotic protein BCL-2 in T cells (Cohen et al., 1997)

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and high circulating IL-10 levels have been detected in our as in other ALPS patients (Lopatin et al., 2001; van der Werff Ten Bosch et al., 2002). Finally, we can not rule out the possibility that an increase in FasL expression, as detected *in vitro* in pyrimethamine-treated lymphocytes, may contribute to the pyrimethamine-induced apoptosis *in vivo* after a prolonged exposure to the drug.

From a clinical point of view we retain our findings elicit some noticeable considerations. The intense pro-apoptotic effect exerted by pyrimethamine, in addition to its well-known antiproliferative activity (Bygbjerg, 1985; Bygbjerg et al., 1986; Viora et al., 1996), might possibly result in a consistent suppression of the immune responses. Therefore treatment with pyrimethamine should be carefully considered in patients suffering of immune defects in which a worsening of clinical conditions could be determined. To this regard it has been previously observed that HIV-1 infected patients treated with pyrimethamine as a primary prophylaxis against toxoplasmic encephalitis showed a higher mortality rate in comparison to untreated control patients (Jacobson et al., 1994).

In contrast, lots of diseases, sharing with ALPS similar immunopathogenic mechanisms, exist in which induction of apoptosis could be beneficial. We refer in particular to autoimmune diseases as well as genetic or infectious-induced immune disorders characterized by an increased and chronic lymphocyte proliferation. In all these conditions, the immunosuppressive effect of pyrimethamine could help in reversing the unbalanced lymphocyte homeostasis and therefore leading to an amelioration of clinical status. Furthermore, on these bases, a reappraisal of the use of pyrimethamine in certain lymphoproliferative disorders can be taken into account in the long run.

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Footnotes

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Legends for Figures

Fig. 1. Dose-dependent effects of pyrimethamine on PBL apoptosis and BCL-2 expression. Resting (A, D, C left column) and PHA/IL-2-activated (B, E, C right column) PBL from HD were incubated with pyrimethamine at different concentrations for 48 h. Apoptotic cells were evaluated by double staining with annexin V-FITC/PI. A, B, Results are expressed as the means \pm SD of 10 independent experiments: **, $p < 0.01$ and ***, $p < 0.001$, significance as compared to untreated control cells. D, E, Changes in apoptosis were accompanied by changes in BCL-2 intracellular content. C, Results obtained from activated PBL of a representative HD are shown. Numbers reported in the lower and upper right quadrants represent the percentages of annexin V single positive cells and annexin V/PI double positive cells, respectively. Pyr, pyrimethamine.

Fig. 2. Time course analysis of pyrimethamine-induced apoptosis. Apoptotic cells were evaluated by double staining with annexin V-FITC/PI after 6, 12, 24, 48, and 72 h of pyrimethamine-treatment. A, Results are expressed as mean \pm SD of 10 independent experiments. *, $p < 0.05$ and **, $p < 0.01$, significance as compared to untreated control cells. B, Results obtained from activated PBL of a representative HD are shown. Numbers reported in the lower and upper right quadrants represent the percentages of annexin V single positive cells and annexin V/PI double positive cells, respectively. Pyr, pyrimethamine.

Fig. 3. Pyrimethamine-induced apoptosis is not influenced by pretreatment with an anti-CD95/Fas neutralizing mAb (clone ZB4). Apoptotic cells were evaluated by double staining with annexin V-FITC/PI after 48 h incubation with pyrimethamine or an anti-CD95/Fas triggering (clone CH11; 0.5 $\mu\text{g/ml}$) mAb in the absence or presence of the neutralizing anti-CD95 antibody ZB4 (10 $\mu\text{g/ml}$). A, Results are expressed as the means \pm SD of 5 independent experiments. **, $p < 0.01$, significance as

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compared to untreated control cells; ++, $p < 0.01$, significance as compared to PBL treated with CH11 alone. B, Results obtained from activated PBL of a representative HD are shown. Numbers reported in the lower and upper right quadrants represent the percentages of annexin V single positive cells and annexin V/PI double positive cells, respectively. Pyr, pyrimethamine.

Fig. 4. Analyses of apoptotic cascade. A-D, Caspase activity. The activity of caspases 8, 10, 9, and 3 in pyrimethamine-treated activated PBL in the absence or presence of different caspase inhibitors (z-IETD-fmk, caspase 8 inhibitor; z-AEVD-fmk caspase 10 inhibitor; z-LEHD-fmk, caspase 9 inhibitor, and z-DEVD-fmk, caspase 3 inhibitor), respectively, was detected at different time points by a colorimetric assay. All tested caspase inhibitors were used at a concentration of 50 μ M. Reported values were obtained by considering the difference between caspase activity found in treated cells with respect to untreated control cells. Data are expressed as mean \pm SD of 3 independent experiments in triplicate. *, $p < 0.05$ and **, $p < 0.01$, significance as compared to untreated control cells. +, $p < 0.05$ and ++, $p < 0.01$, significance as compared to cells treated with pyrimethamine alone. E, Apoptotic cells were evaluated by double staining with annexin V-FITC/PI after pyrimethamine treatment in the absence or presence of inhibitor of caspase-8, caspase-10, caspase-9 and caspase-3. Data are expressed as mean \pm SD of 3 independent experiments in triplicate. *, $p < 0.05$ and **, $p < 0.01$, significance as compared to cells treated with pyrimethamine alone. F, Results obtained from activated PBL of a representative HD are shown. Numbers reported in the lower and upper right quadrants represent the percentages of annexin V single positive cells and annexin V/PI double positive cells, respectively. G, Preincubation with the caspase-8 inhibitor (z-IETD-fmk) abrogates caspase-9 activity after pyrimethamine treatment. Data are expressed as the means \pm SD of 3 independent experiments in triplicate. **, $p < 0.01$, significance as compared to cells treated with pyrimethamine alone. Pyr, pyrimethamine.

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Fig. 5. Pyrimethamine bolsters Fas-mediated apoptosis. Activated PBL were treated with pyrimethamine prior incubation with increasing doses of an agonist anti-CD95/Fas mAb (clone CH11). A, Reported values were obtained by considering the difference between the percentage of annexin V-FITC positive cells found in treated cells with respect to untreated cells. Results are expressed as the means \pm SD of 5 independent experiments. *, $p < 0.05$ and **, $p < 0.01$, significance as compared to untreated control cells; ++, $p < 0.01$, significance as compared to cells treated with CH11 alone at the corresponding concentration. B, Results obtained from activated PBL of a representative HD are shown. Numbers reported in the lower and upper right quadrants represent the percentages of annexin V single positive cells and annexin V/PI double positive cells, respectively. Pyr, pyrimethamine.

Fig. 6. Flow cytometry analysis of $\Delta\Psi$ performed by using JC-1 probe. A, Time course analysis of the modulation of mitochondrial membrane potential by pyrimethamine. Results are expressed as mean \pm SD of 10 independent experiments. *, $p < 0.05$ and **, $p < 0.01$, significance as compared to untreated control cells. B, Changes in $\Delta\Psi$ induced by anti-CD95/Fas mAb (CH11; 0.5 $\mu\text{g/ml}$), pyrimethamine, caspase-8 inhibitor (z-IETD-fmk), caspase-10 inhibitor (z-AEVD-fmk) alone or in various combinations. Caspase inhibitors were used at a concentration of 50 μM . Results are expressed as mean \pm SD of 5 independent experiments. *, $p < 0.05$ and **, $p < 0.01$, significance as compared to untreated control cells. +, $p < 0.05$ and ++, $p < 0.01$, significance as compared to cells treated with pyrimethamine or CH11 alone. Pyr, pyrimethamine.

Fig. 7. Scheme illustrating the possible pathways involved in pyrimethamine-induced apoptosis in activated PBL. In this pathway, pyrimethamine-induced apoptosis directly involves, as initiators caspases, caspases 8 and 10 without engaging CD95/Fas. This is followed by the typical executioner cascade, i.e. mitochondrial changes and downstream events. An additional activity of

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pyrimethamine on mitochondria cannot be ruled out. ↓, indicates a decreased expression; —|, indicates an inhibitory activity. Pyr, pyrimethamine.

Fig. 8. Fas-induced apoptosis and BCL-2 expression of lymphocytes from HD and ALPS patient before and after pyrimethamine therapy. A-F, Scanning Electron Microscopy analysis. Typical cell surface thin protrusions were detectable in PHA/IL-2-activated PBL from HD (A) but not ALPS patient before therapy (B). Similarly, once exposed to anti-CD95/Fas mAb, PBL displayed loss of microvillous structures and cell shrinking in HD (D) but not in ALPS patient before therapy (E). After pyrimethamine treatment, morphology similar to that of HD was observed both in untreated (C) and CD95/Fas-treated PBL (F) from ALPS patient. G, Apoptotic cells were evaluated by annexin V-FITC staining; **, $p < 0.01$ and ***, $p < 0.001$ significances as compared to HD cells; (t0), before therapy; (t1), after therapy. H and I, Flow and, L and M, Static cytometric analysis of BCL-2 expression before (H and L) and after (I and M) pyrimethamine treatment in ALPS patient. After therapy, apoptosis normalization was accompanied by a down regulation in BCL-2 intracellular content. neg, FITC-conjugated isotype control.

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TABLE 1

Time course analysis of cell surface expression of CD95/Fas and FasL, and FasL secretion. Cell surface expression of CD95/Fas and FasL was detected in activated PBL by flow cytometry analysis. Results are expressed as means \pm SD of the median values of the fluorescence intensity histograms from 5 independent experiments. sFasL was analyzed by using a commercially available ELISA kit in the supernatants of activated PBL. sFasL concentration are expressed as ng/ml.

Time (h)	CD95/Fas (median of fluorescence intensity)		FasL (median of fluorescence intensity)		sFasL (ng/ml)	
	medium	pyrimethamine	medium	pyrimethamine	medium	pyrimethamine
0	90 \pm 10		12 \pm 10		481 \pm 46	
6	90 \pm 13	86 \pm 14	12 \pm 11	14 \pm 13	nd	nd
12	86 \pm 14	96 \pm 12	9 \pm 6	11 \pm 8	381 \pm 42	379 \pm 21
24	76 \pm 2	94 \pm 2	9 \pm 6	11 \pm 8	345 \pm 68	329 \pm 52
48	71 \pm 10	115 \pm 17*	8 \pm 3	21 \pm 1	415 \pm 99	327 \pm 90

sFasL, soluble FasL; nd, not done; *, $p < 0.05$ as compared to untreated cells.

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TABLE 2

Time course analysis of the release of Cyt C. Cyt C was analyzed in total extracts of activated PBL using a sensitive and specific commercial ELISA kit. Results are expressed as the means \pm SD of 5 independent experiments. Cyt C concentration are expressed as ng/ml.

Time (h)	medium	pyrimethamine
0	2.5 \pm 1	
6	3.6 \pm 2	3.6 \pm 2
12	4.1 \pm 2	3.8 \pm 1.8
24	4.8 \pm 2	5.1 \pm 1.3
48	4.9 \pm 0.9	10.7 \pm 1.7**

*, $p < 0.05$ and **, $p < 0.01$ as compared to untreated cells

TABLE 3

Cytofluorometric analysis of CD4⁺ and CD8⁺ T cell subsets from an ALPS patient before and after pyrimethamine therapy. Percentage and absolute numbers in brackets of CD4⁺ and CD8⁺ T cell subsets from HD and the ALPS patient are shown. Data from HD are expressed as mean values \pm SD. ALPS patient was studied before (t0) and after 1 month (t1) of therapy. CD45RA⁺CD62L⁺ were defined as naive cells, CD45RA⁻CD62L⁺ as central memory cells, CD45RA⁻CD62L⁻ as effector memory cells and CD45RA⁺CD62L⁻ as terminally effector memory cells.

Cell subsets	HD	t0	t1
CD4 ⁺	39 \pm 6 (942 \pm 140)	34 (1472)	32 (1135)
CD4 ⁺ /CD45RA ⁺ CD62L ⁺	64 \pm 8 (598 \pm 117)	70 (1030)	60 (681)
CD4 ⁺ /CD45RA ⁻ CD62L ⁺	29 \pm 7 (270 \pm 67)	21 (309)	25 (284)
CD4 ⁺ /CD45RA ⁺ CD62L ⁻	1 \pm 0 (8 \pm 3)	3 (44)	3 (34)
CD4 ⁺ /CD45RA ⁻ CD62L ⁻	7 \pm 2 (65 \pm 23)	6 (88)	11 (125)
CD4 ⁺ /CD25 ⁺	21 \pm 3 (260 \pm 70)	19 (279)	20 (227)
CD4 ⁺ /CD95 ⁺	35 \pm 8 (358 \pm 48)	37 (545)	39 (443)
CD4 ⁺ /CCR5 ⁺	9 \pm 4 (99 \pm 28)	10 (147)	9 (102)
CD4 ⁺ /HLA-DR ⁺	5 \pm 2 (52 \pm 10)	12 (177)	14 (159)
CD4 ⁺ /IFN- γ ⁺	8 \pm 4 (82 \pm 54)	9 (132)	5 (57)
CD4 ⁺ /IL-2 ⁺	59 \pm 14 (757 \pm 271)	50 (736)	37 (420)
CD4 ⁺ /IL-4 ⁺	1 \pm 0.5 (12 \pm 4)	4 (59)	2.5 (28)
CD8 ⁺	21 \pm 2 (476 \pm 169)	24 (1039)	24 (851)
CD8 ⁺ /CD45RA ⁺ CD62L ⁺	73 \pm 11 (354 \pm 72)	41 (426)	41 (349)
CD8 ⁺ /CD45RA ⁻ CD62L ⁺	9 \pm 4 (42 \pm 17)	9 (93)	8 (68)
CD8 ⁺ /CD45RA ⁺ CD62L ⁻	11 \pm 5 (53 \pm 24)	20 (208)	19 (162)
CD8 ⁺ /CD45RA ⁻ CD62L ⁻	7 \pm 5 (35 \pm 28)	29 (301)	31 (264)
CD8 ⁺ /CD25 ⁺	3 \pm 0.6 (17 \pm 5)	2 (21)	1 (8)
CD8 ⁺ /CD95 ⁺	39 \pm 20 (242 \pm 68)	57 (592)	54 (459)
CD8 ⁺ /CCR5 ⁺	16 \pm 6 (90 \pm 7)	22 (229)	21 (179)
CD8 ⁺ /HLA-DR ⁺	13 \pm 10 (84 \pm 15)	27 (280)	27 (230)
CD8 ⁺ /IFN- γ ⁺	16 \pm 12 (69 \pm 54)	34 (353)	16 (136)
CD8 ⁺ /IL-2 ⁺	21 \pm 7 (92 \pm 29)	32 (332)	28 (238)
CD8 ⁺ /IL-4 ⁺	1 \pm 1 (1.5 \pm 1)	1 (10)	1 (8)

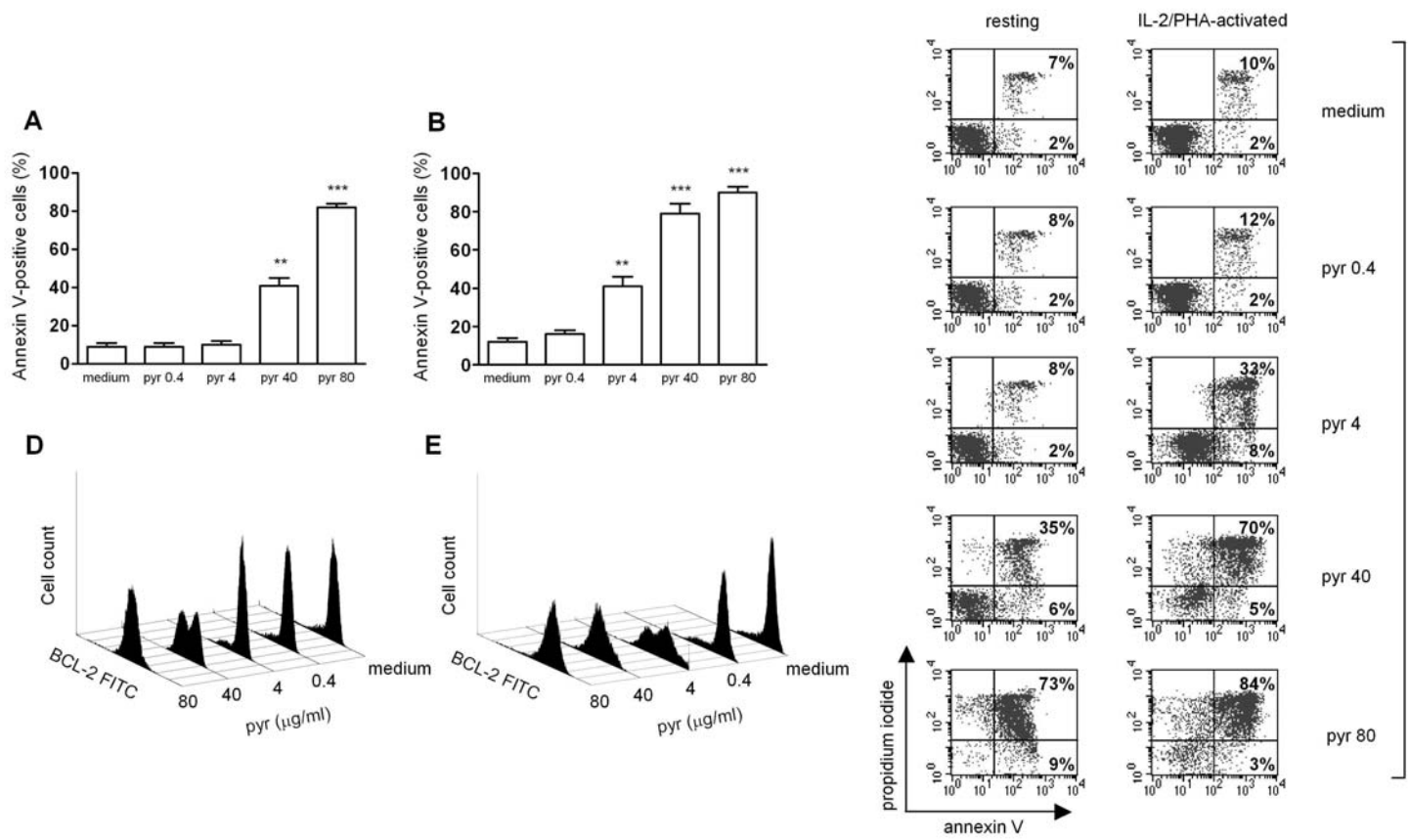
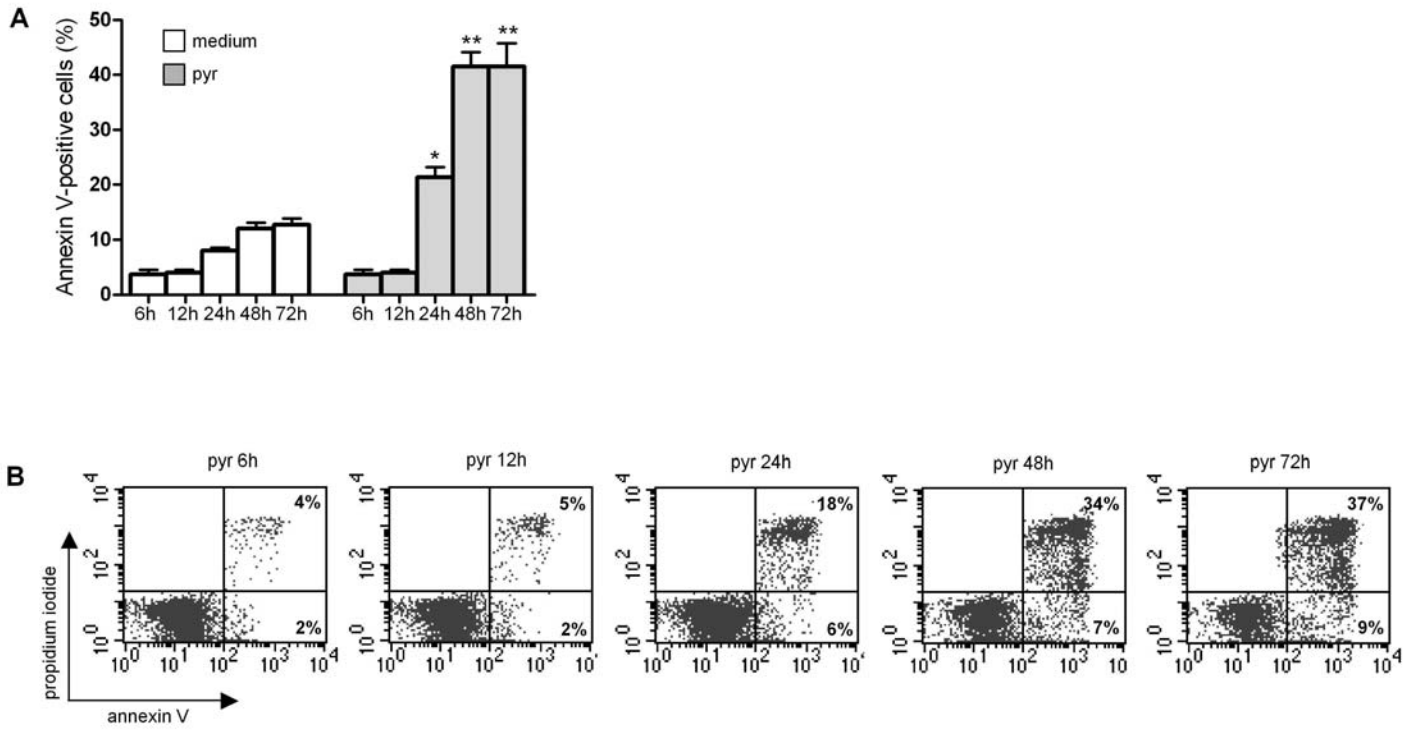


Fig. 1



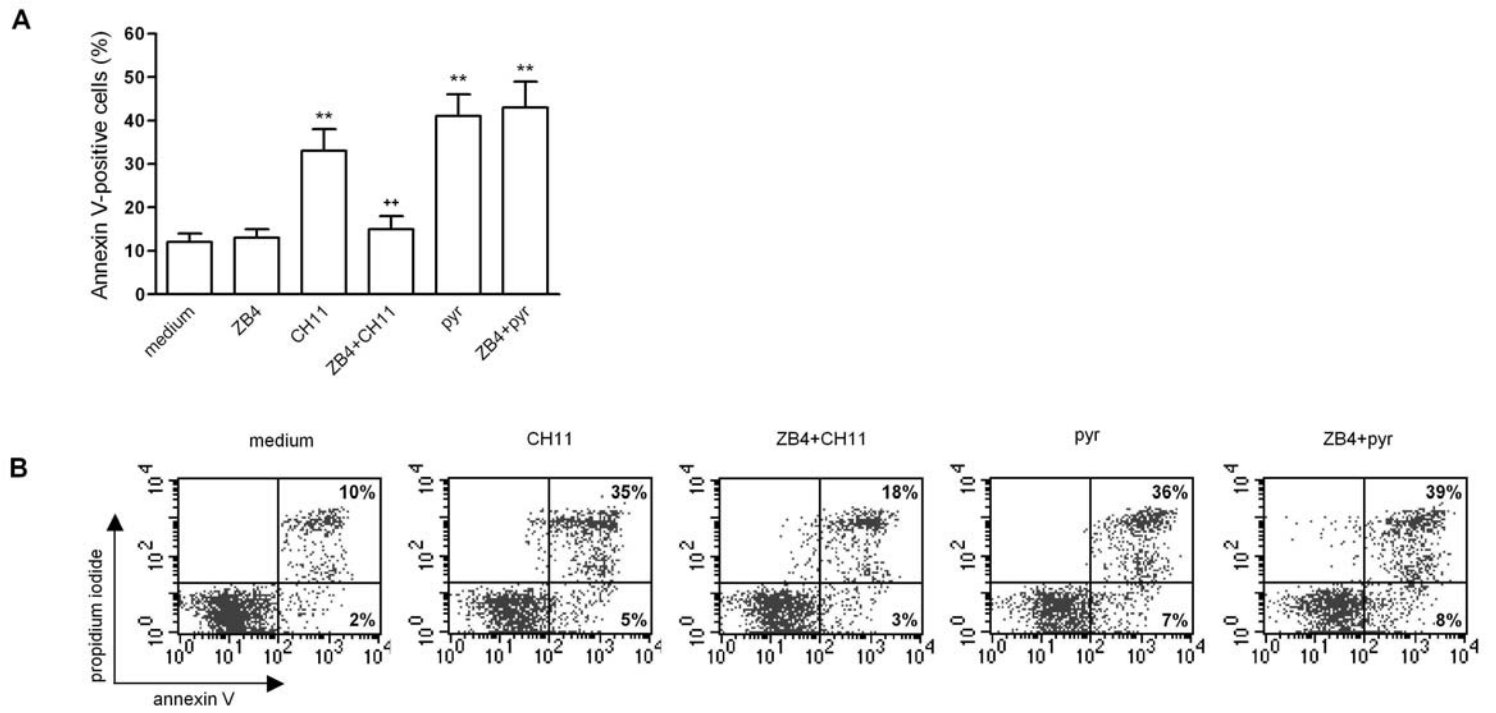
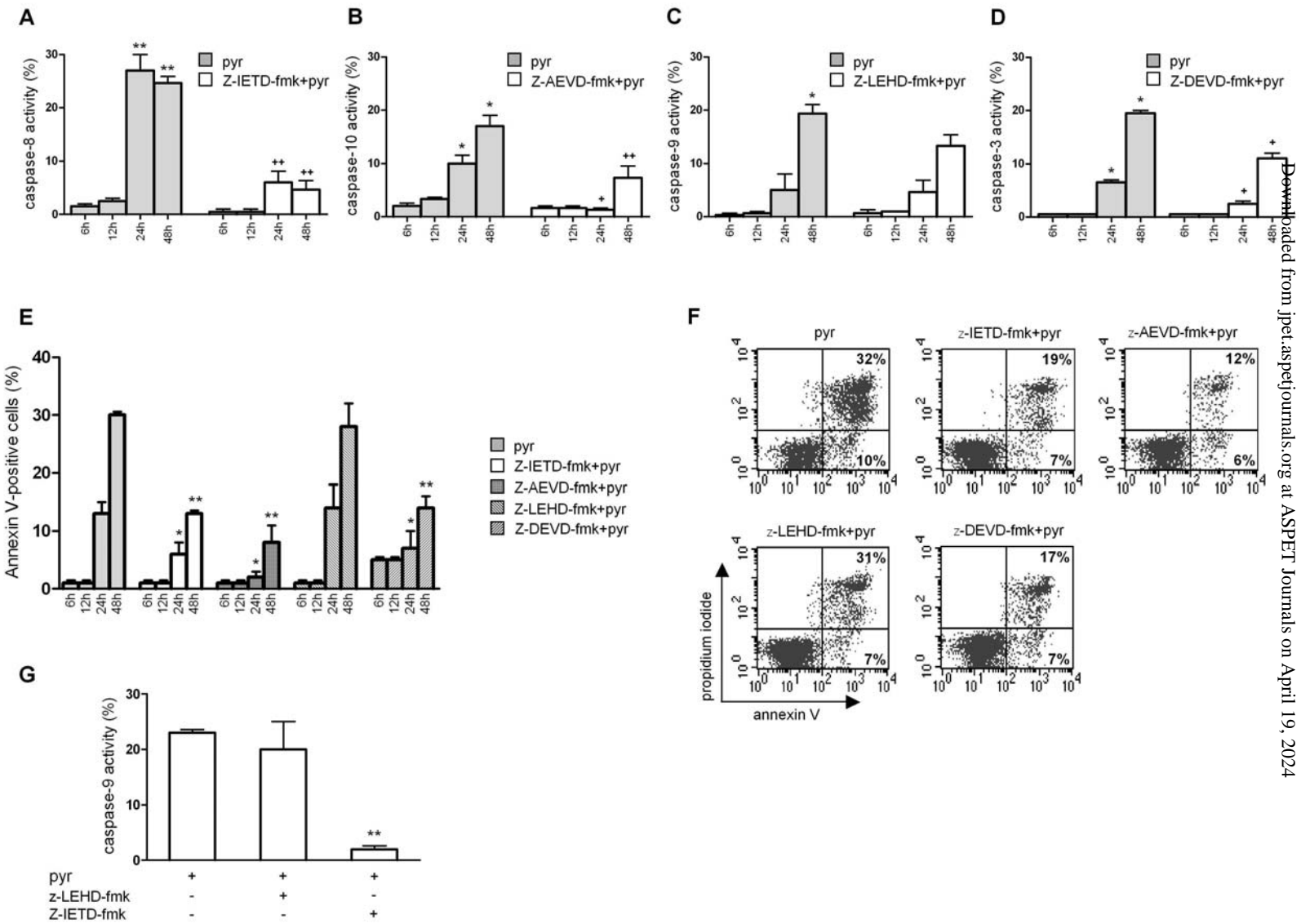


Fig. 3



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Fig. 4

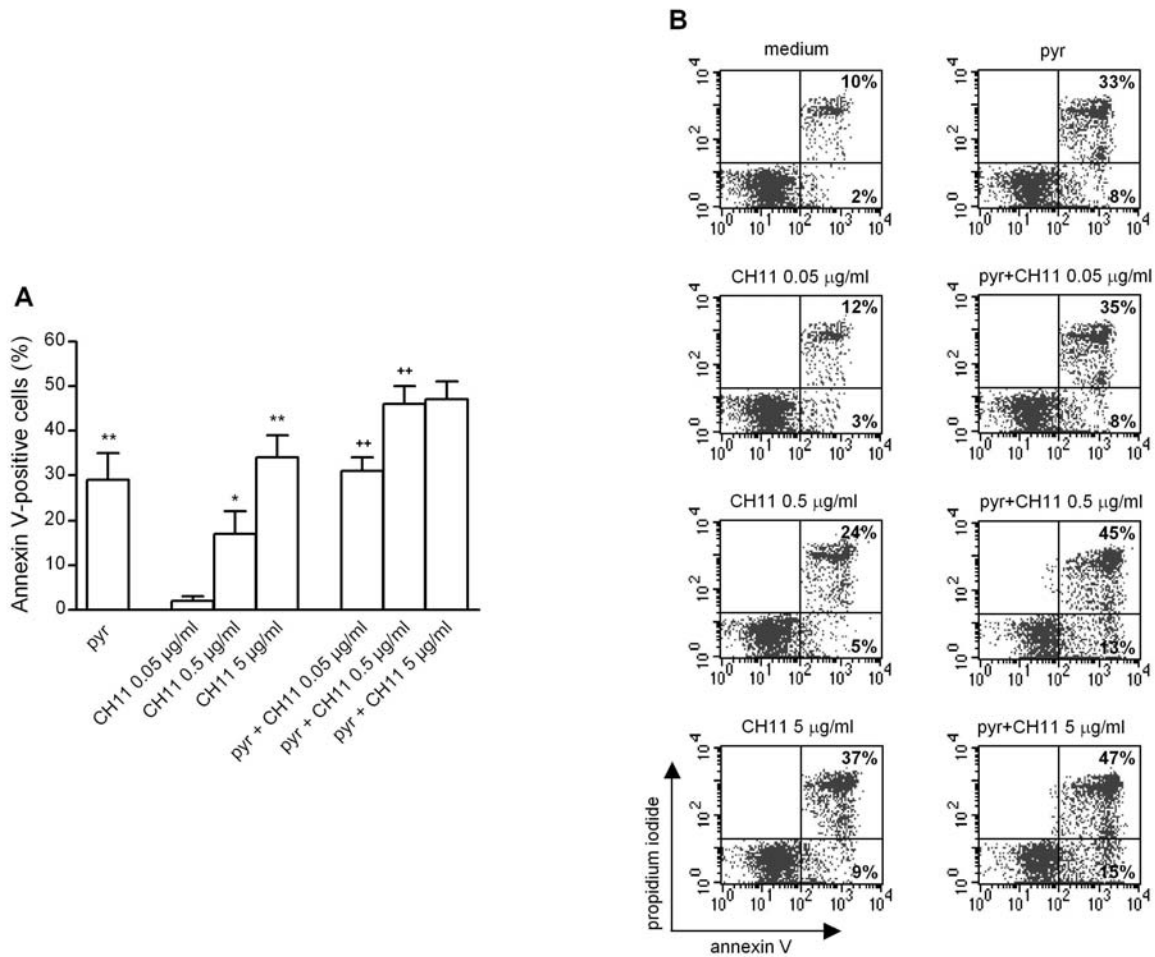


Fig. 5

