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Volatile Anesthetics Induce Caspase-dependent, Mitochondria-mediated Apoptosis in Human T Lymphocytes In Vitro

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Background: **Volatile anesthetics modulate lymphocyte function during surgery, and this compromises postoperative immune competence. The current work was undertaken to examine whether volatile anesthetics induce apoptosis in human T lymphocytes and what apoptotic signaling pathway might be used.**

Methods: **Effects of sevoflurane, isoflurane, and desflurane were studied in primary human $CD3^+$ T lymphocytes and Jurkat T cells *in vitro*. Apoptosis and mitochondrial membrane potential were assessed using flow cytometry after green fluorescent protein-annexin V and $DiOC_6$-fluorochrome staining. Activity and proteolytic processing of caspase 3 was measured by cleaving of the fluorogenic effector caspase substrate Ac-DEVD-AMC and by anti–caspase-3 Western blotting. Release of mitochondrial cytochrome c was studied after cell fractionation using anti–cytochrome c Western blotting and enzyme-linked immunosorbent assays.**

Results: **Sevoflurane and isoflurane induced apoptosis in human T lymphocytes in a dose-dependent manner. By contrast, desflurane did not exert any proapoptotic effects. The apoptotic signaling pathway used by sevoflurane involved disruption of the mitochondrial membrane potential and release of cytochrome c from mitochondria to the cytosol. In addition, the authors observed a proteolytic cleavage of the inactive p32 procaspase 3 to the active p17 fragment, increased caspase-3–like activity, and cleavage of the caspase-3 substrate poly-ADP-ribose-polymerase. Sevoflurane-induced apoptosis was blocked by the general caspase inhibitor Z-VAD.fmk. Death signaling was not mediated *via* the Fas/CD95 receptor pathway because neither anti-Fas/CD95 receptor antagonism nor FADD deficiency or caspase-8 deficiency were able to attenuate sevoflurane-mediated apoptosis.**

Conclusion: **Sevoflurane and isoflurane induce apoptosis in T lymphocytes *via* increased mitochondrial membrane permeability and caspase-3 activation, but independently of death receptor signaling.**

SURGICAL trauma and general anesthesia can modulate immune function by different mechanisms during the perioperative period.[1] In particular, there is a transient activation of proinflammatory and antiinflammatory responses associated with a modulation of lymphocyte function *in vitro* and *in vivo*.[2–5] Contradictory results have been obtained with respect to how anesthetics influence lymphocyte number and function. Inhalation anesthetics such as halothane and nitrous oxide provoked peripheral lymphocytopenia in patients undergoing elective hysterectomy, whereas sevoflurane exposure had the opposite effect, leading to increased lymphocyte numbers but decreased neutrophil numbers.[6,7] In another study, sevoflurane did not influence the rate of neutrophil apoptosis during minimally invasive surgical procedures during general anesthesia.[8]

Apoptosis is a genetically determined and active form of cell death that plays an essential role under physiologic and pathologic conditions throughout the embryonic development and later life of multicellular organisms.[9] It can be initiated by extracellular or intracellular signals. Apoptotic cells shrink, bleb on their surface, and are fragmented into membrane-enclosed apoptotic bodies that are rapidly phagocytosed by neighboring cells.[10] In apoptosis, a specific class of cysteine proteases, called *caspases*, are activated in an amplifying proteolytic cascade.[11] The inactive procaspases are constitutively expressed in healthy cells, and an eventual low enzymatic activity is kept in check by inhibitors of apoptosis proteins. In response to apoptotic stimuli, caspases become activated by adapter-mediated clustering (initiator caspases) or by proteolytic processing (effector caspases).[12]

Two major apoptotic signaling pathways are known. The first is triggered by tumor necrosis factor α–like ligands that bind to specific cell surface death receptors. When the receptors are activated, they transmit through their cytoplasmic domain the apoptotic signal *via* the adapter molecule "Fas-associated protein with death domain" and the initiator caspase 8 directly to the processing and activation of caspase 3. Previous investigations have shown a correlation among increased CD95/Fas ligand (FasL) expression, down-regulation of survival-signaling factors, and peripheral lymphocyte apoptosis in surgical patients.[13–16]

The second apoptosis signaling pathway is mediated through the increased permeability of the outer mitochondrial membrane.[17] This leads to the release of apoptogenic factors from the intermembrane space of mi-

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tochondria into the cytosol, where they activate caspases.[17] One factor, cytochrome c, binds to the adapter Apaf-1 and recruits procaspase 9 into a heptameric high-order structure, the apoptosome. By that, caspase 9 gets activated and cleaves and activates caspase 3. Yet other mitochondrially released factors, such as Smac/DIABLO, bind and neutralize the caspase inhibitors of apoptosis proteins and thereby activate caspases indirectly. Increased mitochondrial membrane permeability leads to the disruption of the mitochondrial membrane potential with the consequence of forming reactive oxygen species.[17]

The CD3 cell population encompasses all known T-cell populations, including CD4, CD8, and T_{h1} or T_{h2}, which play a crucial role in the defense against nosocomial infections.[18] The effects of anesthetics on these cells have been investigated in a few *in vitro* studies and provided some evidence for apoptosis induction in immunocompetent cells. (1) When HL-60 cells, a cultured human promyelocytic $CD4^+$ leukemia cell line, or primary lymphocytes from healthy volunteers were treated with propofol, concentration-dependent apoptosis with caspase-3, -6, -8, and -9 activation and mitochondrial cytochrome c release was observed.[19,20] (2) Expression of Fas and FasL were significantly increased in pancuronium-treated lymphocytes compared with control cells.[21] (3) Halothane inhibits phytohemagglutinin-induced RNA and protein synthesis in human lymphocytes.[22] (4) Halothane, sevoflurane, and isoflurane induced apoptosis in human peripheral lymphocytes *in vitro*, which may be mediated through the suppression of interleukin-2 receptor expression, but the precise signaling mechanism in T lymphocytes remains to be identified.[23,24]

The aim of this study was to determine whether sevoflurane, desflurane, or isoflurane induce apoptosis in human T lymphocytes *in vitro* and what apoptotic signaling pathway might be involved.

Materials and Methods

Reagents

The following anesthetics were used: sevoflurane, isoflurane (Abbott, Wiesbaden, Germany) and desflurane (Baxter, Unterschleißheim, Germany). All other reagents were purchased from Sigma (Deisenhofen, Germany) unless specified otherwise.

Cell Culture

Jurkat T cells (ACC 282; DSMZ, Braunschweig, Germany) and primary human $CD3^+$ T lymphocytes were isolated and grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% glutamine, and 50 mg/ml penicillin and streptomycin (all from Gibco-BRL, Karlsruhe, Germany) in a humidified atmosphere containing 5% carbon dioxide at 37°C as previously described.[5] Wild-type (clone A3), "Fas-associating protein with death domain"–, and caspase-8–deficient Jurkat T cells were kindly provided by John Blenis, Ph.D. (Professor of Cell Biology, Department of Cell Biology, Harvard Medical School, Boston, Massachusetts).[25]

Exposure to Volatile Anesthetics and Experimental Protocol. Jurkat T cells or primary $CD3^+$ T lymphocytes were exposed to either air or a volatile anesthetic–air mixture using a 12-l airtight glass chamber as previously described.[5] The chamber atmosphere was kept continuously saturated with water at 37°C. Gas was prepared using a gas mixing unit by directing a 95% air–5% carbon dioxide mixture at 6 l/min through calibrated vaporizers (Dräger, Lübeck, Germany) that were placed at the entrance of the chamber. In preliminary experiments, we observed that the different culture conditions used in our experiments did not influence apoptosis in T cells during a period of 48 h, *i.e.*, cell viability was similar in a standard incubator and in the sealed chamber flushed with a 95% air–5% carbon dioxide mixture. Volatile anesthetic concentrations were monitored at the chamber exit port using a halogen monitor (PM 8050; Dräger). Concentrations of the inhalation anesthetics dissolved in the cell culture medium were measured by a fully automated solid-phase microextraction procedure followed by gas chromatography and mass spectrometry (CTC Combi PAL autosampler, Agilent model 6890 Series plus Gas Chromatograph with Agilent 5973 N Mass Selective Detector; Chromtech, Idstein, Germany). Using an external standard method, calibration curves were achieved for sevoflurane, desflurane, and isoflurane.[5]

For each exposure condition, a control sample was obtained simultaneously from T cells cultured in a standard 95% air–5% carbon dioxide incubator without anesthetic exposure. At the end of each experiment, both supernatants and the cells were frozen immediately in liquid nitrogen and stored at −80°C until protein extraction.

Isolation of Crude Mitochondria and Cytosols

Cells were harvested, washed in phosphate-based saline solution, and resuspended in extraction buffer containing 10 mM mannitol, 70 mM sucrose, 20 mM HEPES, 1 mM EDTA, 2 mM dithiothreitol, 100 μM phenyl-methyl-sulfonyl-fluoride, 10 μg/ml leupeptin, 400 ng/ml pepstatin, 10 μg/ml aprotinin, and 5 μg/ml cytochalasin B. The cells were homogenized for 10 s with a Dounce homogenizer and centrifuged at 2,000*g* for 5 min to eliminate debris and unbroken cells. The supernatants were centrifuged at 20,000*g* for 15 min. The resulting crude mitochondrial pellet was resuspended in lysis buffer containing 20 mM Tris HCl, 2 mM EGTA, 2 mM EDTA, and 6 mM β-mercaptoethanol plus 1% sodium-dodecyl-sulfate. Further centrifugation of the supernatant at

100,000*g* for 15 min yielded the cytosolic fraction. The fractions were aliquoted and kept at −80°C.

Quantitation of Cytochrome c Release

The amount of cytochrome c released from mitochondria into the cytosol was measured in the cytosolic fraction by Western blotting (a 1:1,000 dilution of cytochrome c; Alexis Corp., Gruenberg, Germany; cat. No. ALX-804-122) and for enzyme-linked immunosorbent assay (Active Motif, Rixensart, Belgium; cat. No. 48006). A 1:500 dilution of a cyclooxygenase-1 antibody (Alexis Corp.; cat. No. ALX-210-710) was used for protein normalization.

Sodium-dodecyl-sulfate-polyacrylamide Gel Electrophoresis and Western Blotting

Total cell extracts of Jurkat T lymphocytes (30 μg) were boiled in Laemmli sample buffer and subjected to 15% sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis. Before transfer, gels were equilibrated for 15 min in cathode buffer (25 mM Tris, 40 mM glycin, 10% methanol). Using a semidry blotting apparatus (Bio-Rad Laboratories, München, Germany), proteins were transferred at 0.8 mA/cm^2 for 1 h onto Immobilon P membranes (Millipore Corp., Eschborn, Germany), preequilibrated in methanol (15 s), ddH_2O (2 min each side), and anode buffer II (25 mM Tris–10% methanol). Equal loading and transfer were confirmed by Bradford measurement and by amido black staining of the membranes at the end of the procedure. Nonspecific binding sites were blocked by immersing the membrane into blocking solution (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20 [vol/vol] containing 5% milk powder; Fluka, Buchs, Switzerland) for 1 h at room temperature. Membranes were washed in blocking solution and incubated in a 1:2,000 dilution of anti–p32-caspase-3 antibody (cat. No. 9662) and a 1:1,000 dilution of anti–p17-caspase-3 antibody (cat. No. 9661; all from Cell Signaling Technology Inc., Beverly, MA) in blocking solution plus 5% bovine serum albumin overnight at 4°C, followed by extensive washing with blocking solution. Bound antibody was decorated with goat–anti-rabbit/horseradish peroxidase conjugate (Amersham Pharmacia, Freiburg, Germany), diluted 1:2,000 in blocking solution for 30 min at room temperature. After washing four times (5 min each), the immunocomplexes were detected using ECL Western blotting reagents (Amersham Pharmacia) according to the manufacturer's instructions. Exposure to Kodak XAR-5 films (Stuttgart, Germany) was performed for 15 s to 1 min.

Fluorogenic Caspase Activity Assay

Total protein cell extracts (10 μl; 50 μg) were mixed with 90 μl assay buffer (100 mM HEPES, pH 7.5, 2 mM dithiothreitol, 2 mM phenyl-methyl-sulfonyl-fluoride). The respective fluorogenic substrate for caspase-3 and Ac-DEVD-AMC (1 μl; 60 μM; Alexis Corp.) was added, and the fluorescence was measured at 30°C for 30 min in a Microplate Spectra Max Gemini XS reader (Molecular Devices, Sunnyvale, CA) at 380/460 nm.

Microscopic Analysis of Hoechst, GFP-Annexin V, and Propidium Iodide Staining

After treatment with anesthetics, suspension CD3$^+$ primary T cells were washed twice with phosphate-buffered saline and incubated with 3 μg/ml green fluorescent protein (GFP)-annexin V, 2.5 μg/ml propidium iodide, and 2 μg/ml Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) in annexin V binding buffer (10 mM HEPES–NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) for 15 min. The cells were then viewed under an Axiovert inverse fluorescence microscope (Zeiss, Jena, Germany), and pictures were taken with a Contax 167 MT camera (YASHICA Kyocera GmbH, Hamburg, Germany) at magnifications of 400× and 1,000×. Nuclear staining by Hoechst stain was revealed using an ultraviolet filter (excitation, 365 nm; emission, 480 nm).

Flow Cytometric Analysis

After treatment with anesthetics, Jurkat T cells were washed in phosphate-buffered saline and resuspended in 50 μl binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.9) containing 12.27 μg/ml GFP-annexin V and 0.5 μg/ml propidium iodide. Samples were incubated in the dark at room temperature for 15 min. Subsequently, 450 μl binding buffer was added, and the percentage of early apoptotic lymphocytes was measured using a flow cytometer (FACS®-CaliburFCM; Becton Dickinson, Heidelberg, Germany). Lymphocytes were gated using forward scatter and side scatter, and fluorescence intensity was measured in 2×10^5 lymphocytes. The fluorescence intensity of GFP-annexin V was measured at the fluorescence 1 channel, and the fluorescence intensity of propidium iodide was measured at the fluorescence 3 channel.

Determination of Mitochondrial Membrane Potential and Reactive Oxygen Species Generation

The mitochondrial membrane potential was analyzed using the fluorochrome stain $DiOC_6(3)$ (Molecular Probes). In brief, after sevoflurane exposure, 10^5 cells were stained in a solution with 40 nM $DiOC_6(3)$ for 30 min. Staining was quantified by fluorescence 1 and scatter characteristics using a FACS®-Calibur flow cytometer (Becton Dickinson). The fluorescence was measured by flow cytometry.

Quantitative and Statistical Analysis

Differences in measured variables between the experimental conditions were assessed using one-way analysis of variance on ranks followed by a nonparametric Student-Newman-Keuls test for multiple comparisons. Re-

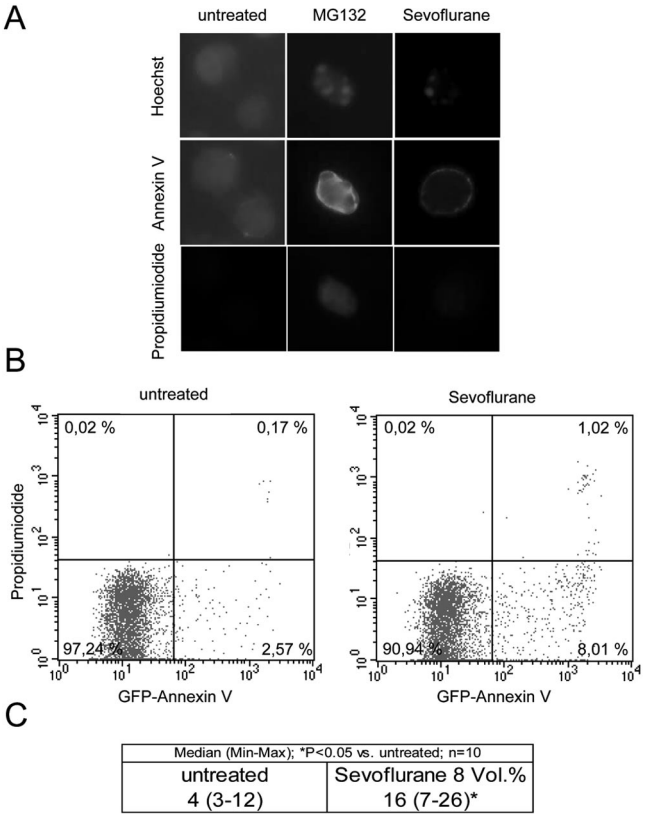

Fig. 1. Effect of sevoflurane on the apoptotic morphology of CD3$^+$ T lymphocytes. (*A*) Fluorescence microscope (magnification × 1,000) and (*B*) flow cytometric analysis of green fluorescent protein (GFP)-annexin V and propidium iodide staining of primary CD3$^+$ T lymphocytes after sevoflurane exposure (8 vol% for 24 h). *A* also shows nuclear morphology by Hoechst 33342 staining and 1 μM MG132 as a positive control for apoptosis. The data shown are representative of six independent experiments. (*B*) Flow cytometric analysis of GFP-annexin V and propidium iodide staining of CD3$^+$ T lymphocytes exposed to sevoflurane (8 vol%). (*C*) Statistical analysis of the flow cytometric data. Data are presented as the median and 25–75% and 95% confidence intervals of 10 independent experiments. * *P* < 0.05 *versus* untreated cells.

sults were considered statistically significant if *P* was less than 0.05. The tests were performed using the SigmaStat software package (Jandel Scientific, San Rafael, CA).

Results

Sevoflurane and Isoflurane but Not Desflurane Exposure Leads to Apoptotic Features in T Cells

Exposure of freshly isolated CD3$^+$ T lymphocytes to sevoflurane (8 vol%) or the known apoptosis inducer MG132 (1 μM, positive control) for 24 h provoked the cell surface exposure of phosphatidylserine (GFP-annexin V staining) and nuclear fragmentation (Hoechst staining) (fig. 1A), two typical features of apoptosis. Necrosis was excluded by propidium iodide negativity (fig. 1A, lower panel). To quantify apoptosis in response to sevoflurane, we performed a flow cytometric analysis of the GFP-annexin V/propidium iodide–stained CD3$^+$ T cells. As shown in a representative experiment in figure 1B, 8 vol% sevoflurane exposure increased the number of GFP-annexin V–positive T cells (2.57 to 8.01%; lower right quadrant) without seriously perturbing membrane integrity (propidium iodide staining, 0.12 to 1.02%; upper right quadrant). Although this increase was small, it was significant (fig. 1C; median [range]: 16% [7–26%]* *vs.* 4% [3–12%] untreated control cells; n = 10; * $P < 0.05$). To determine whether induction of apoptosis was specific for a particular anesthetic, we performed additional GFP-annexin V/propidium iodide flow cytometric experiments using Jurkat T lymphocytes exposed to either desflurane (18 vol%; fig. 2A) or isoflurane (5 vol%; fig. 2B) for 24 h. In contrast to sevoflurane (fig. 1B), desflurane did not trigger any phosphatidylserine exposure or plasma membrane disruption (fig. 2A). As with sevoflurane (fig. 1B), isoflurane increased the number of GFP-annexin V–positive, apoptotic cells from 0.7 to 4.8%, although more propidium iodide–positive necrotic cells were detected as well (3.5 to 8.1%; fig. 2B). These data indicate that two types of T cells, primary CD3$^+$ T lymphocytes and Jurkat T lymphocytes, underwent apoptosis after exposure to sevoflurane or isoflurane.

Sevoflurane and Isoflurane Provoke Caspase-3 Processing, Increased Caspase-3 Activity, and Cleavage of PARP in a Dose-dependent Manner

To investigate whether the apoptotic effects of the anesthetics were dose dependent and associated with the activation of the crucial effector caspase, caspase 3, we exposed Jurkat T cells to various doses of sevoflurane (2.5, 5, and 8 vol%), isoflurane (1.5, 2.5, and 5 vol%), and desflurane (6, 12, and 18 vol%) and studied the processing of p32 procaspase 3 to its active p19/17 forms and the cleavage of its major substrate poly-ADP-ribose-polymerase (PARP) by immunoblotting. As shown in figure 3A, desflurane induced neither caspase-3 processing nor a significant cleavage of PARP from the p116 to the p85 form. In contrast, processed p19/17 caspase 3 and increased abundance of the p85 PARP product were detected at 5 vol% isoflurane and 5 and 8 vol% sevoflurane (figs. 3B and C). To confirm and quantify caspase-3 activation, we performed a caspase-3 activity assay of the cytosols of these T cells by using a specific fluorogenic caspase-3 substrate (DEVD-AMC). Jurkat T cells exposed to desflurane showed activity comparable to that of untreated control cells (fig. 3D; n = 10 each). In contrast, cells exposed to sevoflurane or isoflurane showed a significant increase in caspase-3 activity (fig. 3D; $P < 0.05$ *vs.* untreated cells, n = 10 each). Therefore, T-cell death induced by sevoflurane and isoflurane was associated with caspase-3 activation, which confirms that it proceeded *via* apoptosis.

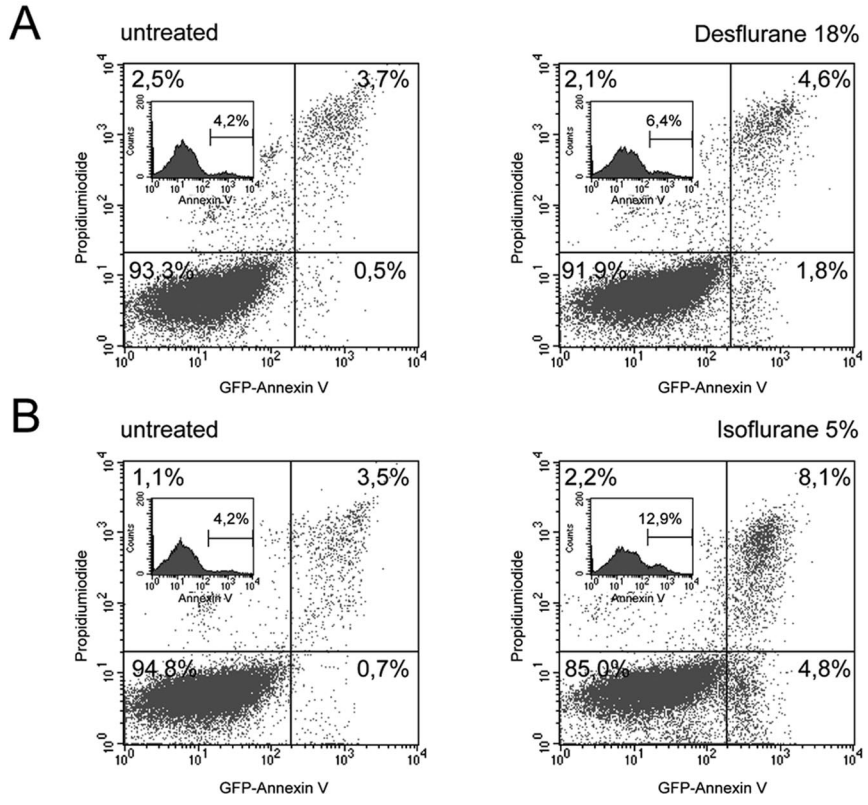

Fig. 2. Phosphatidylserine exposure and plasma membrane integrity after exposure of Jurkat T lymphocytes to desflurane (*A*) and isoflurane (*B*). Flow cytometric analysis of green fluorescent protein (GFP)-annexin V and propidium iodide staining of Jurkat T lymphocytes exposed to desflurane (18 vol%; *A*) or isoflurane (5 vol%; *B*) for 24 h. (*Insets*) Histogram of GFP-annexin V staining. The data shown are representative of six independent experiments.

Sevoflurane-induced Apoptosis Is Caspase Dependent

Based on the current findings, it was interesting to investigate whether sevoflurane-induced apoptosis was dependent on caspases or could also be executed in a caspase-independent manner. For that purpose, we performed quantitative flow cytometric analysis after GFP-annexin V/propidium iodide staining and caspase-3 and PARP processing by immunoblotting in the presence of absence of the pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk). As shown by a representative experiment in figures 4A–C, sevoflurane (8 vol%) induced apoptosis (fig. 4B; lower right quadrant; 5.3% *vs.* 1.5% control) and secondary necrosis (fig. 4B; upper right quadrant; 9.1% *vs.* 3.3%) that was fully blocked by 100 μM Z-VAD.fmk treatment (fig. 4C; 1.1% lower right and 3.9% upper right). Statistical analysis of the sum of apoptotic and secondary necrotic cells of 10 independent experiments revealed that the changes were highly significant (comparison sevoflurane-treated *vs.* untreated T cells: 15% [10–23%]* *vs.* 4% [3–12%] and comparison sevoflurane-*vs.* Z-VAD.fmk/sevoflurane-treated: 15% [10–23%]* *vs.* 6% [5–6%], n = 10; * $P < 0.05$). Z-VAD.fmk (100 μM) alone had no influence on the GFP-annexin V/propidium iodide staining (data not shown). Concomitant with this finding, Z-VAD.fmk prevented the formation of the p19/p17 active forms of caspase 3 and the p85 cleavage product of PARP induced by sevoflurane (fig. 4D). These data show that sevoflurane requires caspases for the induction of apoptosis in Jurkat T cells.

Sevoflurane-induced Apoptosis Is Not Mediated via *the Death Receptor Pathway*

We next wanted to know which apoptosis signaling pathway is used by sevoflurane. To assess the extrinsic Fas/CD95 receptor–induced pathway, we preincubated Jurkat T cells with a neutralizing anti-Fas/CD95 receptor antibody (anti-Fas/CD95; 1 μg/ml; cat. No. MD-11-3; MBL, Woburn, MA) 1 h before sevoflurane exposure and performed GFP-annexin V/propidium iodide flow cytometric analysis. These experiments demonstrated that sevoflurane-induced apoptosis and secondary necrosis could not be abolished by pretreatment with the anti-Fas/CD95 receptor antibody (fig. 5A *vs.* B *vs.* C). By contrast, as expected, FasL-induced cell death was entirely blocked by preincubating with the neutralizing anti-Fas antibody (fig. 5D *vs.* E). The anti-Fas antibody alone did not affect cell death at all (fig. 5F). To confirm our results and investigate whether death receptor signaling in general had any impact on sevoflurane-induced apoptosis, we analyzed phosphatidylserine exposure and propidium iodide positivity in Jurkat T cells deficient for "Fas-associating protein with death domain" or caspase 8, two components that are absolutely required for death receptor signaling.[25,26] As shown in figure 6, similar numbers of GFP-annexin V– and/or propidium iodide–positive cells were counted irrespective of whether untreated or sevoflurane-treated cells were wild type, "Fas-associating protein with death domain" deficient, or caspase-8 deficient (6% [2–8%] of the untreated cells *vs.* 16% [10–21%] of the sevoflurane-treated wild-type T cells [fig. 6A *vs.* B], 9% [5–12%] *vs.*

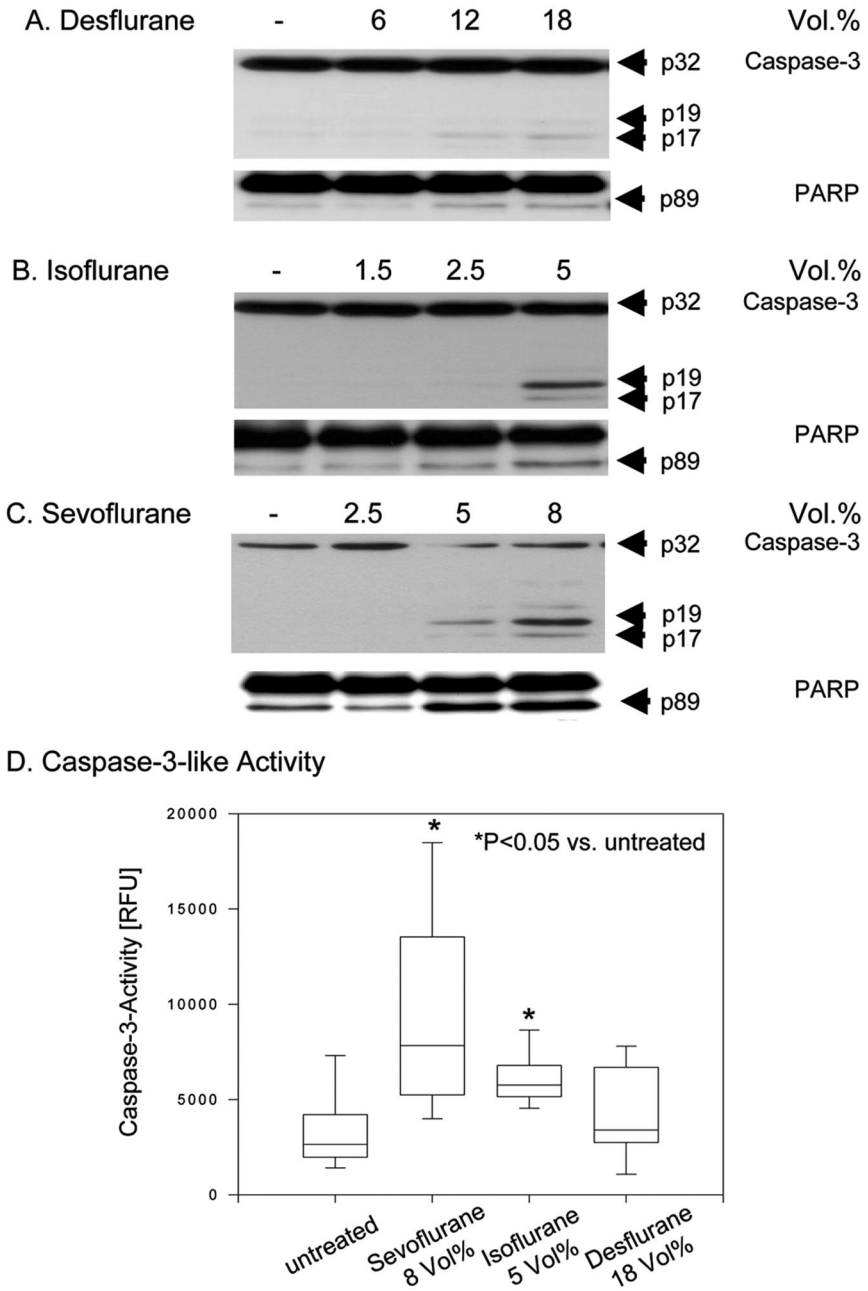

Fig. 3. Caspase-3 processing, poly-ADP-ribose-polymerase (PARP) cleavage, and caspase-3–like activity in response to various concentrations of desflurane, isoflurane, and sevoflurane. (*A–C*) Anti–caspase 3 (*upper panel*) and anti-PARP (*lower panel*) Western blot analysis of the cytosols of Jurkat T lymphocytes exposed to the indicated doses of anesthetics. p32: inactive caspase-3 proform; p19/p17: processed, active caspase-3 fragments. The data shown are representative of six independent experiments. (*D*) Fluorogenic caspase-3 assay (DEVDase assay) of the above cytosols. The results are given in arbitrary fluorescent units (RFUs). Data are presented as the median and 25–75% and 95% confidence intervals of 10 independent experiments. * $P < 0.05$ *versus* untreated cells.

23% [17–29%] of the "Fas-associating protein with death domain"–deficient T cells [fig. 6C *vs.* D], and 8% [5–12%] *vs.* 27% [19–33%] of the caspase-8–deficient T cells [fig. 6E *vs.* F] expressed phosphatidylserine on their surface). Therefore, sevoflurane-induced apoptosis is not mediated *via* the death receptor signaling pathway.

Sevoflurane-mediated Apoptosis Involves the Mitochondrial Pathway

Loss of mitochondrial transmembrane potential and release of mitochondrial intermembrane proteins are important effectors of apoptosis. The incubation of Jurkat T lymphocytes with the fluorochrome $DiOC_6(3)$ allows the determination of the mitochondrial transmembrane potential. Flow cytometric analysis of $DiOC_6(3)$-stained Jurkat T cells demonstrated that only 6% (3–10%, n = 10; $P < 0.05$) of untreated cells (fig. 7A) *versus* 18% (9–23%, n = 10; $P < 0.05$) of sevoflurane-treated cells had a significantly lower mitochondrial transmembrane potential. Because sevoflurane significantly decreased the mitochondrial transmembrane potential in Jurkat T lymphocytes, we hypothesized that cytochrome c would be released from the mitochondrial intermembrane space. As expected, MG132-treated Jurkat T cells exhibited cytochrome c release from mitochondria into the cytosol, with a resultant decrease in the mitochondrial content (fig. 7B, upper panel). In addition,

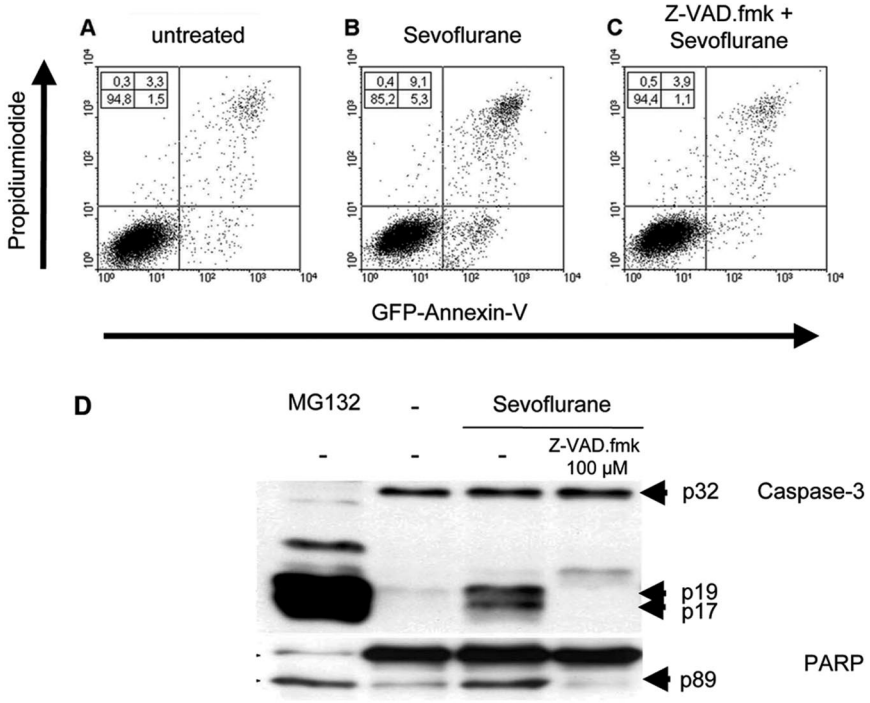

Fig. 4. Effect of the general caspase inhibitor Z-VAD.fmk on sevoflurane-induced apoptosis in Jurkat T lymphocytes. (*A–C*) Flow cytometric analysis of green fluorescent protein (GFP)-annexin V and propidium iodide staining of Jurkat T lymphocytes after sevoflurane exposure (8 vol% for 24 h) in the absence or presence of Z-VAD.fmk (100 μM). (*Insets*) Percentage of cells for each quadrant. (*D*) Anti–caspase-3 (*upper panel*) and anti–poly-ADP-ribose-polymerase (PARP; *lower panel*) Western blot of MG132 (1 μM)– and sevoflurane-treated Jurkat T cells in the absence or presence of Z-VAD.fmk. The data shown are representative of six independent experiments.

semiquantitative analysis *via* enzyme-linked immunosorbent assay revealed a significant decrease in mitochondrial cytochrome c content after MG132 treatment (fig. 7C). Interestingly, sevoflurane (8 vol% for 24 h) exposure of T cells resulted also in a smaller, although significant decrease of cytochrome c content in the mitochondrial fraction, with a concomitant increase in the cytosol as shown by both immunoblotting (fig. 7B, upper panel) and enzyme-linked immunosorbent assay (fig. 7C). Equal loading of the mitochondrial fraction was assured by immunodetection of the mitochondrial marker cyclooxygenase 1 (fig. 7B, bottom panel). These data clearly indicate that sevoflurane uses the mitochondrial signaling pathway for apoptosis induction in T cells.

Discussion

The inflammatory stress response and the postoperative immunosuppression after anesthesia and major surgery are characterized by peripheral T cell lymphopenia and leukocytosis.[6,13,27–29] It has recently been shown

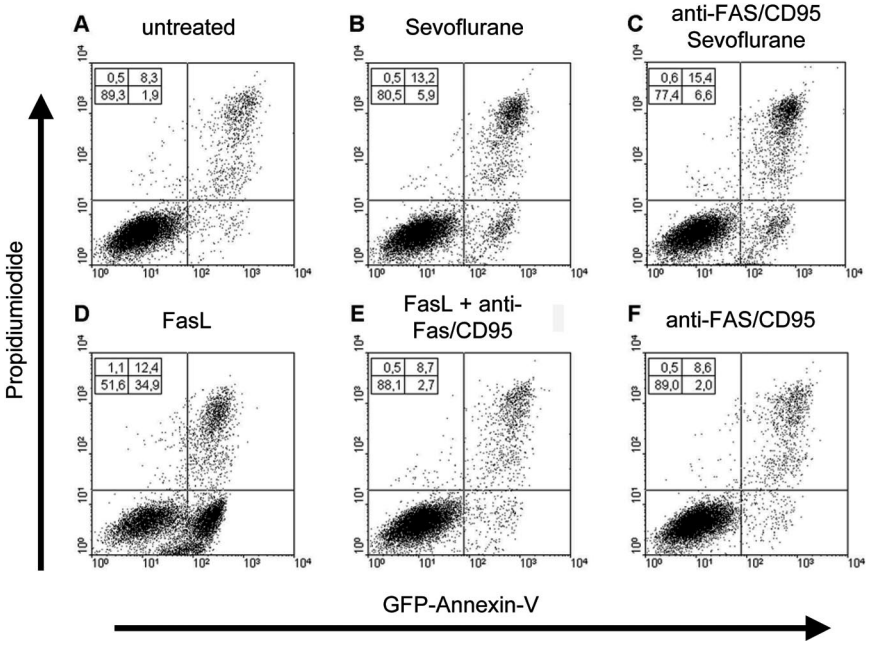

Fig. 5. Impact of neutralizing anti-Fas/CD95 antibody on sevoflurane-induced apoptosis. (*A*) Flow cytometric analysis of untreated Jurkat T lymphocytes stained with green fluorescent protein (GFP)-annexin V and propidium iodide after exposure to sevoflurane (8 vol%; *B* and *C*) or 100 μl/ml FasL (*D* and *E*) in the absence or presence of pretreatment with 1 μg/ml anti-Fas/CD95 neutralizing antibody. *F* shows the anti-Fas antibody alone. (*Insets*) Percentage of cells of each quadrant. The data shown are representative of six independent experiments.

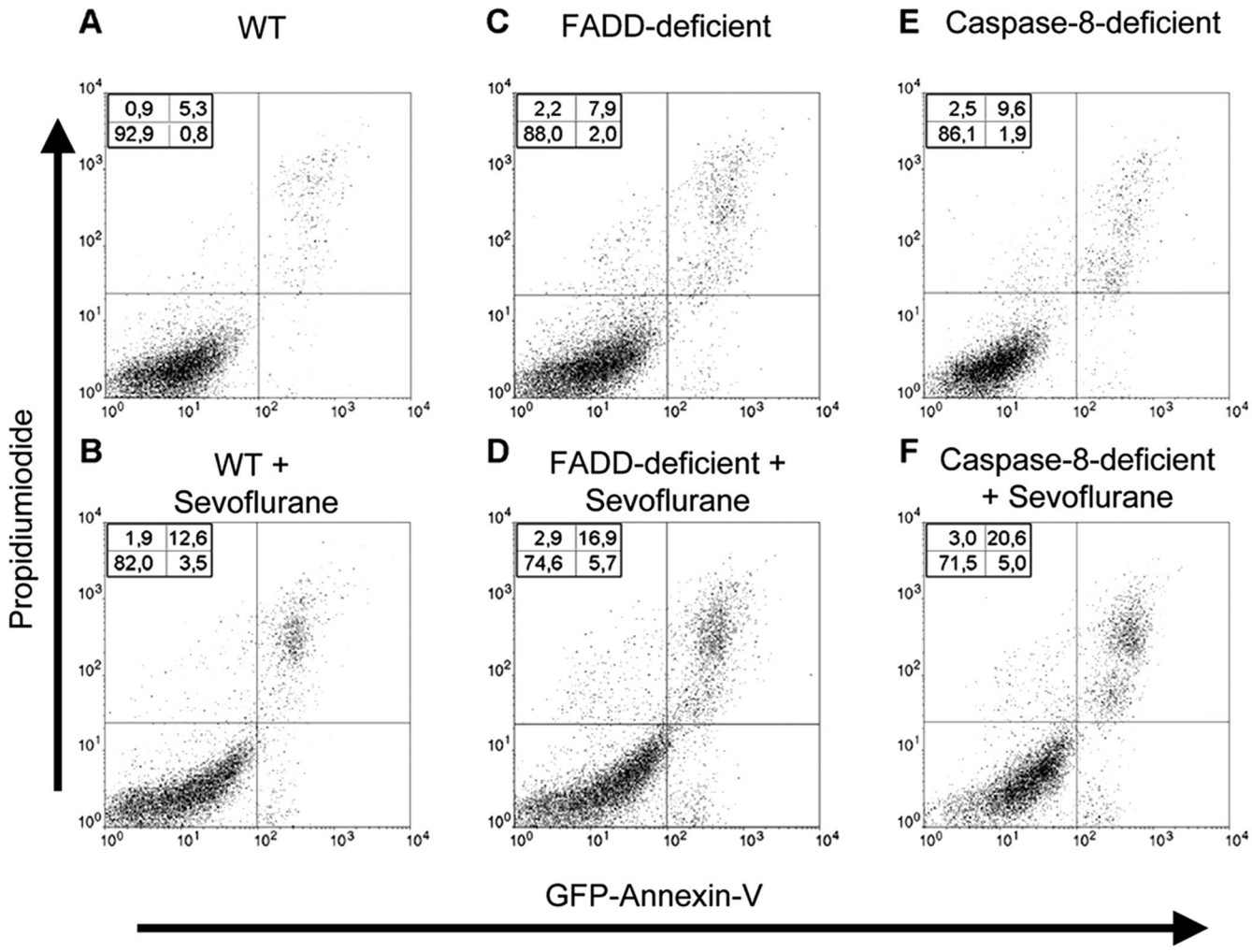

Fig. 6. Sensitivity of wild-type (WT), "Fas-associating protein with death domain" (FADD)–, or caspase-8–deficient Jurkat T lymphocytes to sevoflurane-induced apoptosis. Flow cytometric analysis of WT, FADD–, or caspase-8–deficient Jurkat T lymphocytes stained with green fluorescent protein (GFP)-annexin V and propidium iodide after exposure to sevoflurane (8 vol%; *B*, *D*, and *F*). (*A*, *C*, and *E*) Untreated cells. (*Insets*) Percentage of cells of each quadrant. The data shown are representative of six independent experiments.

that general anesthetics can modulate the inflammatory stress response.[1,6] This may result in an increased susceptibility to infectious complications, systemic inflammatory response syndrome, and sepsis.[30] Programmed cell death is an essential part of life in multicellular organisms and represents the predominant process responsible for the termination of surgical injury–induced inflammation and immunologic homeostasis.[13,27] However, evidence regarding immunomodulatory effects of volatile anesthetics due to apoptosis is conflicting. This may result from differences in study design as well as the inherent limitations of *in vitro* models. The work presented here supports the hypothesis that volatile anesthetics, in particular sevoflurane, may act as triggers of apoptosis in primary $CD3^+$ and established Jurkat T lymphocytes. This effect may contribute to perioperative T-cell apoptosis after major surgery performed during general anesthesia and may play a crucial role in the defense against nosocomial infections.[18]

Our data demonstrate that apoptosis induced by sevoflurane and, to a lesser extent, by isoflurane was caspase dependent and mitochondria mediated. We think that these effects were consistent and physiologically significant for the following reasons: (1) Both agents acted in a specific manner because another volatile anesthetic, desflurane, did not exert any apoptotic effect. (2) They provoked caspase-3 processing and apoptosis in a dose-dependent, pharmacologically relevant manner. (3) Caspase-3 processing was accompanied by increased caspase-3 activity and the cleavage PARP, a major caspase-3 substrate. (4) Apoptosis and caspase-3 processing was caspase dependent, *i.e.*, blocked by the general caspase inhibitor Z-VAD.fmk. (5) Sevoflurane-induced apoptosis was independent of the death receptor signaling pathway but involved mitochondrial signaling, *i.e.*, the decrease in the mitochondrial membrane potential and the release of cytochrome from the intermembrane space into the cytosol.

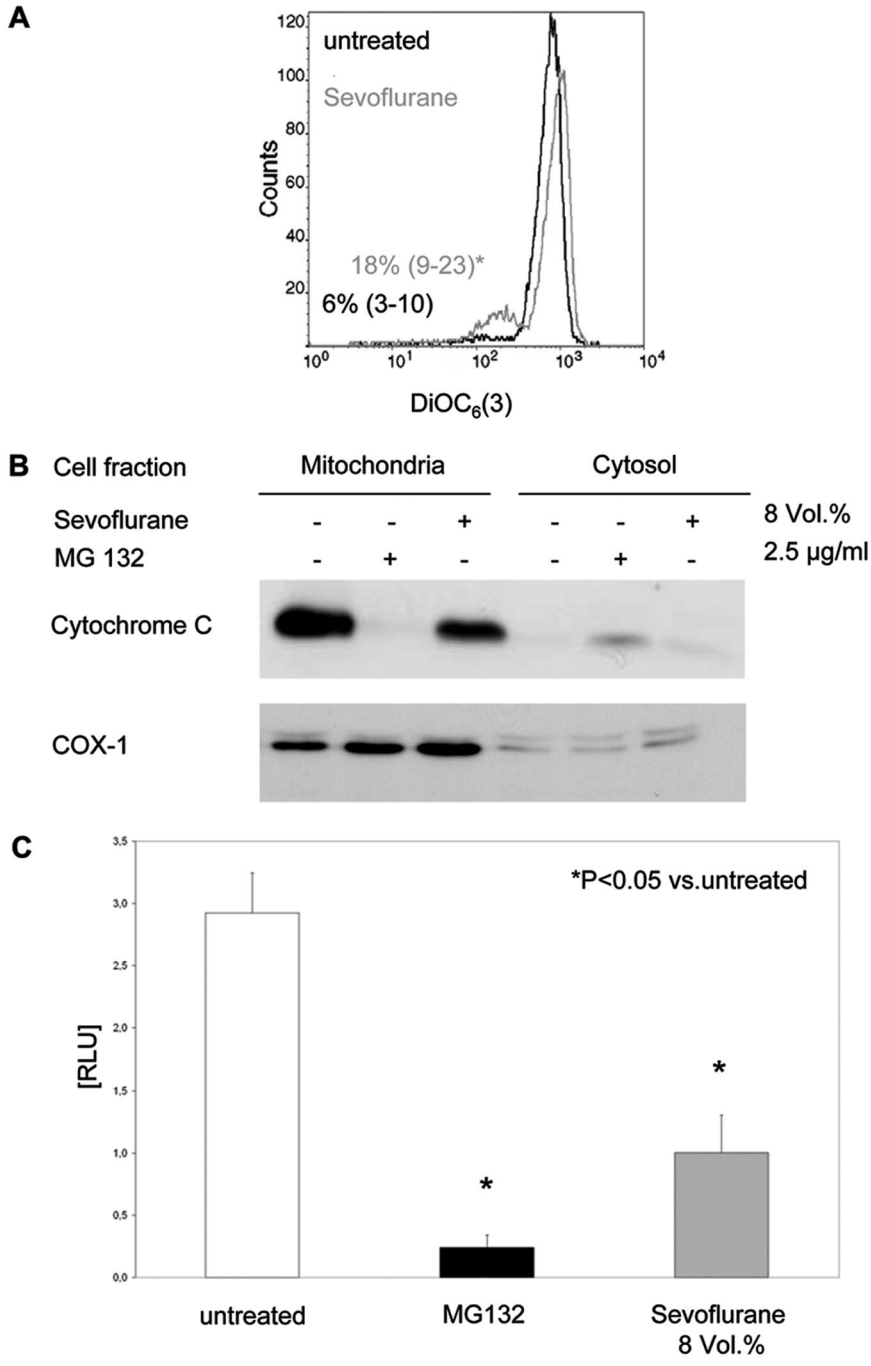

Fig. 7. Involvement of the mitochondrial pathway in sevoflurane-induced apoptosis. (*A*) Flow cytometric analysis of Jurkat T lymphocytes stained with $DiOC_6(3)$ for the assessment of the mitochondrial membrane potential after sevoflurane exposure (8 vol%). (*B*) Anti–cytochrome c Western blot of mitochondrial and cytosolic fractions of Jurkat T lymphocytes exposed to 8 vol% sevoflurane or 2.5 μg/ml MG132. Anti–cyclooxygenase-1 (COX-1) immunoblot of mitochondrial fractions as a loading control. (*C*) Anti–cytochrome c enzyme-linked immunosorbent assay of the mitochondrial fraction. The results are given in arbitrary light units (RLUs). Data represent the mean and SD of 10 independent experiments. * $P < 0.05$ *versus* untreated cells.

Several studies have already reported anesthetic-induced, caspase-dependent apoptotic cell death. For example, treatment of various immune cells with propofol resulted in growth inhibition, formation of apoptotic bodies, DNA laddering and fragmentation, and activation of caspases 3, 6, 8, and 9.[19,20] Morphine and various opioids potently induce opioid receptor–dependent apoptosis of human T cells, macrophages, microglia, and neurons.[31–35] This could in part be responsible for recurrent infections often seen with patients who are addicted to opiates. In addition, phenobarbital and diazepam cause apoptotic neurodegeneration in the developing rat brain at plasma concentrations relevant for seizure control in humans.[36] Anesthesia-related drugs, such as neuromuscular blocking agents, in particular pancuronium, seem to regulate apoptosis by increased expression and activation of the Fas/CD95 death signaling pathway.[21] A previous report also demonstrated that sevoflurane and isoflurane induced apoptosis in normal peripheral blood mononuclear cells *in vitro*.[24] However, compared with this latter study, our analysis here differed in the experimental setup and the obtained results. First, we studied $CD3^+$ T lymphocytes instead of peripheral blood mononuclear cells. Second, we administered

volatile anesthetics by gaseous application, not by liquid injection. The former application may lead to an artificial initial peak of the volatile anesthetic concentration in the medium, a situation that is not comparable to clinical application.[24] With this protocol, the authors observed a higher percentage of apoptotic cells in the isoflurane as compared with the sevoflurane groups and concluded that the toxicity of isoflurane was higher than that of sevoflurane at equimolar aqueous concentrations.[24] This is in marked contrast to our results, where sevoflurane showed a higher apoptosis rate in T cells than isoflurane did.

The following lines of evidence suggest that our *in vitro* findings of sevoflurane- and isoflurane-mediated apoptosis of T cells may be of biologic relevance and therefore crucial in a clinical setting. First, the apoptotic effect of these volatile anesthetics could be observed in human primary $CD3^+$ T lymphocytes that had been obtained from healthy donors. Second, the caspase-3 activation occurred at *in vitro* concentrations comparable to those attained in the plasma of patients during administration of sevoflurane for general anesthesia.[5,37,38] Finally, it was previously shown that exposure of T cells to sevoflurane caused a diminished production of interleukin 3, a cytokine that plays a functional role in T-cell survival, growth differentiation, and leukocyte adhesion.[5,39,40] Likewise, sevoflurane was reported to inhibit the activation of the transcription factor activator protein 1 (AP-1) in human T lymphocytes *in vitro*.[5] AP-1 is known to be down-regulated after withdrawal of interleukin 2, interleukin 3, or granulocyte-monocyte colony-stimulating factor, and this is associated with decreased cell cycle progression and apoptotic cell death.[24,41,42] Therefore, the inhibition of AP-1 and interleukin 3 by sevoflurane observed could be responsible for the induction of apoptosis seen in our study.

Characteristically, cytotoxic drug–induced apoptosis involves permeabilization of mitochondrial outer membranes and provokes the release of mitochondrial components, such as cytochrome c ("intrinsic" pathway).[43] Therefore, it was not surprising that sevoflurane activated this pathway rather than death receptor signaling, to induce apoptosis. The release of cytochrome c, the sole water-soluble component of the electron transfer chain, leads to the failure of maintaining mitochondrial membrane potential and adenosine triphosphate synthesis. This would increase the production of reactive oxygen species with subsequent lipid peroxidation. We found that sevoflurane, at concentrations that induced apoptosis, provoked reactive oxygen species production (data not shown). These findings are consistent with the view that impaired functionality and induction of altered mitochondrial metabolism is an early key step of initiator caspase activation.[12] Alterations in mitochondrial energy metabolism after surgical trauma and general anesthesia are in accord with our *in vitro* findings of organelle-specific initiation of cell death by volatile anesthetics.[44] We therefore conclude that mitochondria are central mediators of sevoflurane-induced apoptosis.

Taken together, our data suggest that sevoflurane and isoflurane are specific inducers of caspase-dependent, mitochondria-mediated apoptosis in human T cells. Therefore, our data provide a potential molecular mechanism for the apoptotic effects associated with general anesthesia.

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