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ORIGINAL ARTICLE

Lung carcinomas do not induce T-cell apoptosis via the Fas/Fas ligand pathway but down-regulate CD3 epsilon expression

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Abstract
Background Non-small cell lung carcinoma (NSCLC) patients have impaired cellular immune responses. It has been hypothesized that tumor cells expressing Fas Ligand (FasL) induce in T lymphocytes: (a) apoptosis (tumor counterattack) and (b) down-regulation of CD3ζ expression. However, the hypothesis of tumor counterattack is still controversial.
Methods We analyzed FasL expression on NSCLC cell lines and on tumor cells from lung adenocarcinoma patients by flow cytometry and immunocytochemistry. FasL mRNA expression was detected in NSCLC cell lines using RT-PCR, and functional FasL was evaluated on Fas-expressing Jurkat T-cells by annexin-V-FITC staining and by $SubG_1$ peak detection. Also, the proapoptotic effect of microvesicles released from NSCLC cell lines in Jurkat T-cells was studied. Alterations in the expression levels of CD3ζ, CD3ε, and CD28 [measured as mean fluorescence intensity (MFI)] were determined in Jurkat T-cells after co-culture with NSCLC cell lines or tumor-derived microvesicles. Furthermore, the expression levels of CD3ζ and CD3ε in CD4+T and CD8+T lymphocytes from lung adenocarcinoma patients was studied.
Results Our results indicate that NSCLC cells neither FasL expressed nor induced apoptosis in Jurkat T-cells. Tumor-derived microvesicles did not induce apoptosis in Jurkat T-cells. In contrast, NSCLC cell lines down-regulated CD3ε but not CD3ζ chain expression in Jurkat T-cells; this effect was induced by soluble factors but not by microvesicles. In lung adenocarcinoma patients, significant decreases of MFI values for CD3ε, but not CD3ζ, were found in CD4+T and CD8+T cells from pleural effusion compared to peripheral blood and in peripheral blood of patients compared to healthy donors.
Conclusions Our data do not support the tumor counterattack hypothesis for NSCLC. Nonetheless, down-regulation of CD3ε in T-cells induced by NSCLC cells might lead to T-cell dysfunction.

Keywords Fas Ligand · Tumor-derived microvesicles · Tumor counterattack · CD3 epsilon chain · CD3 zeta chain · Non-small cell lung carcinoma

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Introduction

Lung cancer is one of the most important malignancies worldwide in terms of incidence and prevalence as well as a leading cause of cancer death. Non-small cell lung carcinomas (NSCLC) account for ~85% of all cases of lung cancer [36]. Using the immune system to fight cancer has been a goal of cancer oncologists and immunologists. Cytotoxic CD8+T lymphocytes play a major role in the anti-tumor immune response. Nevertheless, NSCLC have developed mechanisms to subvert the immune system and suppress the anti-tumor CD8+T lymphocytes reactivity [3, 26]. We have reported that the effector subset of CD8+T lymphocytes from pleural effusions of lung adenocarcinoma patients is reduced compared to peripheral blood, a phenomenon that is not observed in tuberculous pleural effusions [26]. We also found an increase in the proportion of Fas-positive CD8+T

cells in pleural effusion and peripheral blood [26]. Decrease in the proportion of effector CD8+T lymphocytes in pleural effusions might be a consequence of evasion mechanisms of lung tumors such as: (a) tumor counterattack or (b) blocking of the terminal differentiation process of CD8+T cells, possibly mediated by a down-regulation of CD3ζ expression.

Tumor counterattack hypothesis postulates that tumor cells evade their elimination by constitutively expressing membrane-bound Fas ligand (FasL/CD95L) [43]. Interaction of FasL with Fas receptor can trigger apoptosis in Fas-positive cells [30]. Several authors have reported that different types of cancer, including lung carcinomas [13, 24], express functional FasL [8, 12]; for a review, see [43]. Recently, some studies report that in distinct types of cancers tumor-derived microvesicles express FasL [1, 11, 14, 23]. So, FasL-positive NSCLC might be responsible for the reduction of effector CD8+T cells given that a high percentage of Fas-positive CD8+T cells is found in pleural effusions of lung adenocarcinoma patients [26].

The tumor counterattack hypothesis, however, is controversial given the contradictory reports regarding both pro- and anti-inflammatory properties of FasL [19, 28]; also, the specificity of some of the anti-FasL mAbs used in previous studies has also been questioned [34, 37]. In addition, methodological problems in cytotoxicity assays and a lack of suitable controls have not allowed confirmation of the validity of this biological phenomenon [6].

Another mechanism suggested for tumor evasion is diminished expression of CD3, particularly the CD3ζ chain [2], which leads to suppression of T-cell activity. Some studies have reported in patients with cancer a loss or decrease of CD3ζ expression in tumor-infiltrating and peripheral blood T lymphocytes; for a review see [2]. Sikora et al. report a deficiency of CD3ζ in T-cells from malignant pleural effusions caused by distinct types of cancer [31, 33]. One mechanism for CD3ζ down-regulation is the activation of caspases mediated by the FasL–Fas receptor interaction between tumor cells and T lymphocytes, respectively [10]. Nevertheless, other mechanisms for CD3ζ decrease have also been reported [2].

In the present study, FasL expression on NSCLC cells was analyzed. Apoptosis of Fas-expressing Jurkat T-cells induced by NSCLC cell lines was detected. In addition, alterations in the expression levels of CD3ζ, CD3ε, or CD28 in Jurkat T-cells were determined. Besides, CD3ζ and CD3ε expressions in CD4+T and CD8+T lymphocytes from lung adenocarcinoma patients were studied. Our results indicate that NSCLC cells neither expressed FasL nor induced apoptosis in Jurkat T-cells. In contrast, tumor cells down-regulated CD3ε and CD28 but not CD3ζ chain expressions in Jurkat T-cells. In patients with NSCLC, only the expression level of CD3ε was reduced in CD4+T and CD8+T lymphocytes.

Our data do not support that NSCLC counterattack T lymphocytes. However, down-regulation of CD3ε and CD28 expressions in T lymphocytes induced by NSCLC might be responsible for T-cell anergy in lung adenocarcinoma patients.

Materials and methods

Population studied

A total of 17 patients with pleural effusion caused by primary lung adenocarcinoma were studied. Diagnosis established by histological examination of pleural biopsy or cytological observation of malignant cells in pleural effusion was according to WHO criteria [15]. Malignancy stage was evaluated according to the UICC TNM classification [35] and was as follows: four patients were in stage IIIb and 13 patients were in stage IV. None of the patients received any type of anti-cancer therapy before the study. Median age of the group was 62 years (range 41–74 years).

To rule out the possibility that apoptosis induced by pleural effusion might be related to the anatomical compartment rather than to the underlying pathology; 13 patients with pleural effusion from distinct origins (five pneumonias, five congestive heart failures, and three COPD) were also included in this study, called non-malignant group; median age was 68 (range 37–75 years). As control, 11 healthy volunteers were included; median age was 50 years (range 41–72 years).

A written informed consent was obtained from all participants included in this study. The Committee of Science and Bioethics of the Instituto Nacional de Enfermedades Respiratorias approved of the protocol for the collection of biological samples.

Isolation of mononuclear cells and tumor cells

Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood on Lymphoprep (Axis-Shield, Oslo, Norway). Pleural effusion was obtained by thoracocentesis used for routine diagnostic procedures. From malignant pleural effusions, mononuclear cell-enriched fraction (PEMC) and tumor cell-enriched fractions were obtained as previously reported [26].

Pleural fluid samples from lung adenocarcinoma group and non-malignant group were collected. Also plasma from lung adenocarcinoma group and control group were collected.

Cell lines

Twelve human NSCLC cell lines were used. The squamous carcinoma cell lines SK-MES-1 and Calu-1 and the

adenocarcinoma cell lines A-427, A-549, and SK-LU-1 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Adenocarcinoma cell lines 3A5A, 1.3.11, 1.3.15, and 3B1A were established by us [17]. AJG-1, OSJV-1, and RCC1-20 cell lines were established from malignant pleural effusion of three patients with diagnosis of lung adenocarcinoma. Each cell line was obtained and characterized morphologically and by immunocytochemical staining as previously reported [17]. The normal lung fibroblast MRC-5, cervix carcinoma HeLa, and T-cell Jurkat cell lines were obtained from the ATCC. All cell lines were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml of streptomycin, referred to as complete medium. With the exception of Jurkat T-cells, all cell lines grew forming monolayers.

Determination of FasL expression on cell lines and tumor cells from lung adenocarcinoma patients

Expression of FasL was evaluated on NSCLC cell lines and tumor cell-enriched populations obtained from pleural effusions using flow cytometry; HeLa cell line was used as a positive control for FasL expression [12]. Three clones of anti-FasL mAbs were used: the R-phycoerythrin (PE)-labeled clones 100413 (R&D, Minneapolis, MN, USA) and Alf-2.1 (Caltag, Burlingame, CA, USA), and biotin-labeled clone NOK-1 (PharMingen, San Diego, CA, USA). The Alf-2.1 and NOK-1 mAbs show no cross-reactivity with unrelated molecules and recognize FasL specifically [34].

After non-enzymatic treatment (EDTA-MOPS) for cell line detachment, 1×10^6 cells/ml resuspended in PBS (containing 1% bovine serum albumin and 0.1% NaN_3) were incubated at 4°C for 30 min with the respective mAb or PE-labeled isotype control (R&D). In the case of biotin-labeled NOK-1 mAb, cells were washed and incubated with streptoavidin-PE (PharMingen) at RT for 30 min. Cells were fixed with 1% paraformaldehyde and further analyzed on a FACScalibur flow cytometer using Cell Quest Software (Becton Dickinson, San Jose, CA, USA), and 10,000 events were acquired.

As the tumor cell-enriched fraction obtained from pleural effusion of lung cancer patients may contain leukocytes and as it has been reported that leukocytes express FasL [28, 38], cell fractions were also labeled with PE-Cy5-labeled anti-CD45 mAb (PharMingen). Tumor cells were selected according to a forward scatter (FSC) versus side scatter (SSC) in a dot plot graph. Using a CD45 versus SSC graph, a second gate was selected to identify only tumor cells (as a CD45-negative population) and from them the proportion of FasL-positive tumor cells was detected.

Determination of FasL expression by immunocytochemistry

Non-small cell lung carcinomas cell lines were grown on poly-L-lysine-covered glass chamber slides (BD Falcon, Bedford, MA, USA), fixed with 70% ethanol, and stained for immunocytochemistry using clone G-247 anti-FasL mAb (PharMingen) as reported previously by Strater et al. [37]. Stained cells were counterstained with Mayer's hematoxylin.

Isolation of tumor-derived microvesicles

Supernatants from NSCLC cell lines cultured in T-80 flasks were harvested after 72 h of confluent tumor cell cultures with the following final cell densities: 4×10^5 cells/ml for AJG-1, 3A5A, and SK-MES-1 cell lines; 5×10^5 cells/ml for SK-LU-1, OSJV-1, and Calu-1; 1.5×10^6 cells/ml for A-549, A-427, and HeLa cell lines, using a total volume of 25 ml for each cell culture. Also, supernatants from Jurkat T-cells (2×10^7 total cells) stimulated with PHA for 24 h or non-stimulated were collected [21].

Microvesicles from NSCLC cell lines and from Jurkat T-cells (non-stimulated and PHA-stimulated) were obtained by ultracentrifugation as reported previously [21, 23]. Briefly, the supernatants were centrifuged at 800*g* for 15 min to remove cellular debris and further clarified by centrifugation at 2,000*g* at 4°C for 20 min. Then, supernatant was centrifuged at 100,000*g* at 4°C for 3 h. After centrifugation, supernatant was collected and the pellet containing microvesicles was resuspended in 500 μl of sterile PBS. Quantification of total proteins contained in microvesicles was determined by Lowry assay (Pierce Biotechnology, Rockford, IL, USA). The mean of protein recovery for non-stimulated Jurkat T-cells and NSCLC cell lines was 21 μg/ml (range 15–30 μg/ml) and for stimulated Jurkat T-cells was 50 μg/ml.

RT-PCR for FasL

After lysis of NSCLC cell lines with Trizol reagent (Invitrogen, Paisley, UK), total RNA was isolated according to the manufacturer's instructions. After testing that there was no DNA contamination in RNA samples, cDNA synthesis was performed by reverse transcription [16]. Oligonucleotide primers were: for FasL sense 5′-ACACCTATGGA ATTGTCCTGC-3′ and anti-sense 5′-GACCAGAGAGA GCTCAGATACG-3′, 308 bp; for β-actin sense 5′-GGG TCAGAAGGATTCCTATG-3′ and anti-sense 5′-GGTCT CAAACATGATCTGGG-3′, 237 bp. PCR was performed in a 25 μl total volume using 3 μl cDNA, 2.5 μl 10× PCR buffer, 1 mM $MgCl_2$, 0.25 μl of Taq polymerase (1.5 U), 10 pmol of corresponding 5′ and 3′ amplification primers.

The PCR reaction was integrated by one initial denaturation step at 94°C for 5 min, and 30 cycling conditions at 94°C for 50 s, 60°C for 50 s, and 72°C for 50 s, with one final extension cycle at 72°C for 7 min.

NSCLC cell lines and Jurkat T-cells co-cultures

Tumor cell lines (5×10^5 cells/ml) were seeded in a 24-well tissue culture plate for 4–6 h to allow cell-adhesion to plastic. Non-adherent cells were eliminated by aspiration and Jurkat T-cells (5×10^4 cells/ml) were added for an incubation period of 24 h. Jurkat T-cells at total cell density were used as a co-culture control, and Jurkat T-cells in the presence of recombinant human (rh) FasL (50 ng/ml, Alexis, San Diego, CA, USA) were used for induction of apoptosis and down-regulation of CD3ζ, CD3ε, and CD28 molecules [10, 18].

In some experiments, indirect co-cultures were done using a Transwell system with a 0.4 μm membrane (Costar, Cambridge, MA, USA) to form a double chamber with a semipermeable membrane. The lower chamber contained the tumor cell line, whereas the upper chamber contained only Jurkat T-cells, using a total volume of 1.2 ml.

In any case, at the end of co-culture, Jurkat T-cells were collected by gentle mechanical shaking, and assayed for: (a) viability (detected by trypan blue staining); (b) apoptosis (determined by $subG_1$ peak and by annexin-V staining); and (c) level of CD3ζ, CD3ε, and CD28 expressions [measured by flow cytometry as mean fluorescence intensity (MFI)].

$SubG_1$ peak detection

Cellular DNA of permeabilized Jurkat T-cells was stained with propidium iodide and quantified by flow cytometry as we have previously reported [17]. A total of 10,000 events were acquired, ModFIT LT software (Becton Dickinson) was used for analysis of the $SubG_1$ peak.

Cytotoxic assay for detection of apoptosis in Jurkat T-cells

We employed an improved method for detection of apoptosis and for distinguishing apoptotic Jurkat T-cells from detached/apoptotic lung carcinoma cells, which was adapted from Fischer and Mackensen [7]. Briefly, before co-culture, each tumor cell line was stained with the PKH-26 fluorescent dye (PKH-26 Red fluorescent cell linker kit, Sigma Chemical, St. Louis, MD, USA), which is incorporated into lipid regions of cell membrane.

Tumor cells, harvested by trypsination, were adjusted to 1×10^6 cells/ml in RPMI-1640 medium without FCS. The cell pellet was resuspended in the diluent provided with the kit. PKH-26 dye (final concentration 2 μM) was added to cell suspension and, after incubation at RT for 5 min, the reaction was stopped by adding an equal volume of FCS. Finally, cells were washed using complete medium and resuspended at the appropriate concentration. Cell viability was >94%. After staining, labeled-tumor cell line was seeded in 24-well tissue plates and co-cultures were done as described above.

After co-culture, harvested Jurkat T-cells were washed with PBS. Cell pellet was resuspended in 100 μl of staining solution containing 1 μg/ml Annexin-V-FITC (Santa Cruz, Santa Cruz CA, USA). After incubation at RT for 15 min, 300 μl of HEPES buffer was added and cells were immediately analyzed by flow cytometry. Jurkat T-cells were identified according to a SSC versus PKH-26 dye in a dot plot graph and gated from PKH-26-negative cells. A total of 10,000 events were acquired.

Jurkat T-cells treated with plasma or pleural effusion samples from lung adenocarcinoma patients, or tumor-derived microvesicles

Plasma or pleural effusions samples from lung adenocarcinoma patients were tested for proapoptotic activity in Jurkat T-cells as described previously [14]. Briefly, Jurkat T-cells were plated at 1×10^6 cells/ml per well in 48-well plates in medium and incubated with plasma or pleural effusion samples (0.5 ml/1 ml medium). Total incubation period was 4 days; each day, metabolized medium was removed and the initial cell concentration was seeded using fresh medium, plasma, or pleural effusion dilutions. After incubation, apoptosis was analyzed (measured by Annexin V/propidium iodide staining [17]).

In addition, Jurkat T-cells were incubated with tumor-derived microvesicles (diluted 1:10 and 1:25 with complete medium) or with stimulated Jurkat T-cells microvesicles (diluted 1:40) for 24 h. After incubation, proapoptotic activity and the CD3ε, CD3ζ, and CD28 expressions were analyzed.

Determination of CD3ε, CD3ζ, and CD28 expression levels in Jurkat T-cells

Jurkat T-cells (10^5 cells) harvested from the different co-cultures (direct, indirect, or treated with microvesicles) were tested for CD3ε, CD3ζ, and CD28 expressions using the following mAbs: anti-CD3ε FITC (UCHT1 clone, Sigma Chemical), anti-CD3ζ PE (2H2D9 clone, Coulter, Marseille, France), and anti-CD28 PE-Cy5 (CD28.2 clone, PharMingen). Briefly, after incubation with anti-CD3ε and anti-CD28 mAbs at 4°C for 30 min, cells were fixed with 1% paraformaldehyde. After membrane-staining, cells were permeabilized with FACS Permeabilizing Solution (BD PharMingen) at RT for 10 min. Permeabilized cells were incubated with anti-CD3ζ mAb or isotype-matched

control antibody at RT for 30 min. Cells were fixed with 1% paraformaldehyde. After triple staining, MFI value for each CD molecule was determined in 10,000 cells acquired.

Determination of the level of CD3ε and CD3ζ expression in CD4+T and CD8+T cells from cancer patients

For estimating the MFI value of CD3ε and CD3ζ in T-cell subpopulations, PBMC or PEMC cells were stained with the following combinations of mAbs: anti-CD4 Quantum Red or anti-CD8 Quantum Red (Sigma Chemical) plus anti-CD3ε FITC and anti-CD3ζ PE. After cell surface staining, T-cell subpopulations were stained with anti-CD3ζ mAb as described above for Jurkat T-cells. From a FSC versus SSC dot-plot graph, 10,000 gated lymphocytes were analyzed. A second gate was selected to identify the CD4+T or CD8+T cell subpopulations on a Quantum Red (CD4+ or CD8+) versus SSC dot plot graph. MFI values for CD3ε and CD3ζ were measured in CD4+T or CD8+T cells.

Statistical analysis

All values are expressed as mean ± SE. For statistical comparison of the MFI values of CD3ζ, CD3ε, or CD28 in Jurkat T-cells under all experimental conditions, two-way analysis of variance (ANOVA) was used followed by Dunnett's multiple comparison test.

Paired Student's *t*-test was employed for comparison of data from pleural effusion versus peripheral blood in adenocarcinoma patients. For comparison between peripheral blood from healthy donors and peripheral blood from adenocarcinoma patients, unpaired Student's *t*-test was used. Differences between experimental groups were considered significant at $P < 0.05$.

Results

FasL expression on NSCLC cells

Expression of FasL was determined on NSCLC cell lines and on tumor cells from lung adenocarcinoma patients using flow cytometry. With respect to tumor cell lines, HeLa cells (positive control) expressed a low level of FasL (see Fig. 1a), which agrees with a previous report [12]. None of the lung carcinoma cell lines expressed membrane-bound FasL, even when this molecule was assessed with three different clones of antibodies recognizing FasL (see Materials and methods). Histograms obtained from five representative cell lines (A-427, SK-LU-1, OSJV-1, A-549, and RCC1-20 cell lines) are shown in Fig. 1a.

Several reports indicate that FasL is expressed intracellularly [1, 14]. To analyze the possibility that FasL is expressed in the cytoplasm of NSCLC cell lines, two methodological strategies were used: (1) Immunocytochemical staining as reported by Strater et al. [37] and (2) flow cytometric analysis of lung carcinoma cell lines treated with brefeldin-A (10 μg/ml) for 4 h.

In the immunocytochemical staining, tonsillar tissue sections (as positive controls of G247mAb reactivity) and

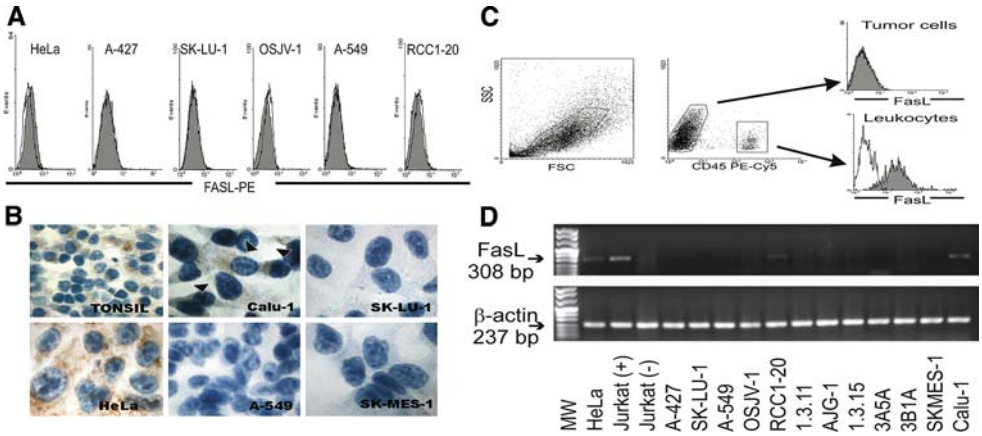

Fig. 1 FasL expression on NSCLC. **a** Histograms obtained by flow cytometry from HeLa cells (positive control) and five representative lung carcinoma cell lines. Cells were stained with 100413 clone anti-FasL mAb (*dark area*) or with an isotype-matched IgG (*solid line*). Similar results were obtained using the NOK-1 and Alf-2.1 mAbs (not shown). **b** Immunocytochemistry of FasL in tonsillar tissue and HeLa cells (positive controls). A granular pattern was observed in HeLa cells. Most of NSCLC cell lines were negative for FasL expression. RCC1-20 (not shown) and Calu-1 cell lines showed a low expression of FasL with a homogeneous pattern. Representative cell lines are shown (original magnification, ×1,000). **c** A representative result from tumor cell-enriched fraction obtained from pleural effusion is shown; tumor cells were gated from the CD45-negative population to exclude leukocytes, which were FasL-positive cells. Cells were stained with 100413 clone anti-FasL mAb (*dark area*) or with an isotype-matched IgG (*solid line*). **d** FasL mRNA expression in NSCLC cell lines. HeLa cells and stimulated-Jurkat T-cells (+) were used as positive controls. From a panel of 12 NSCLC cell lines, only RCC1-20 and Calu-1 cells expressed FasL mRNA

the HeLa cell line were employed. FasL was detected intracellularly in some lymphoid cells from tonsillar tissue. In HeLa cell line, FasL was observed into the cytoplasm showing a granular pattern. From the NSCLC cell lines, a weak FasL expression with a homogeneous pattern confined to nuclear periphery was observed in two cell lines, Calu-1 and RCC1-20, see Fig. 1b. With respect to treatment with brefeldin A and flow cytometric analysis, FasL expression was detected in HeLa cell line but not in the NSCLC cell lines (data not shown).

Regarding lung adenocarcinoma patients, 76% of tumor cell-enriched fractions were FasL-negative, detected by flow cytometry. However, ~90% of leukocytes present in some of the tumor cell-enriched fractions were FasL-positive, see Fig. 1c.

FasL mRNA expression in lung carcinoma cell lines

Jurkat T-cells stimulated with anti-CD3 mAb (UCHT-1 clone) and HeLa cells were used as positive controls for detection of FasL mRNA, see Fig. 1d. In contrast, non-stimulated-Jurkat T-cells did not express FasL transcript; these data agree with previous reports [4, 12]. From a panel of 12 NSCLC cell lines studied, only the RCC1-20 and Calu-1 cell lines showed a low expression of FasL mRNA, see Fig. 1d.

Induction of apoptosis in Fas-sensitive cells by NSCLC cell lines

To rule out the possibility that functional FasL could be released from lung carcinoma cell lines (even when we did not detect FasL in most of the cell lines) or that apoptosis might be induced by other TNF-related apoptosis-inducing ligands, such as TRAIL, a biological assay using Jurkat T-cell as target cell was done.

In our experimental conditions, rhFasL induced apoptosis in almost the whole population of Jurkat T-cells as detected by subG$_1$ peak or the Annexin-V staining, see Fig. 2. In general, lung carcinoma cell lines did not alter Jurkat T-cell viability (detected by trypan blue-staining). In contrast, NSCLC cell lines induced a decrease in Jurkat T-cell proliferation rate (detected by alamar-blue, data not shown). Also, a reduction in cell size and granularity was observed, see Fig. 2a. These phenomena were not mediated by apoptosis (detected by phosphatidilserine exposure, Fig. 2b, or subG$_1$ peak, Fig. 2c). Data obtained from controls and representative lung carcinoma cell lines A-427, A-549, SK-LU-1, and Calu-1 are shown in Fig. 2. NSCLC cell lines did not induce apoptosis even after 48 h of incubation (data not shown).

Lack of apoptosis in Jurkat T-cells treated with plasma or pleural effusion samples from lung adenocarcinoma patients

Pleural effusion and plasma samples from lung adenocarcinoma patients were tested for apoptotic activity in Jurkat T-cells. Plasma from healthy donors or from cancer patients did not affect the viability of Jurkat T-cells compared to Jurkat T-cells in medium (control). With respect to pleural effusion, a slight but significant reduction in the viability of Jurkat T-cells treated with malignant pleural effusion

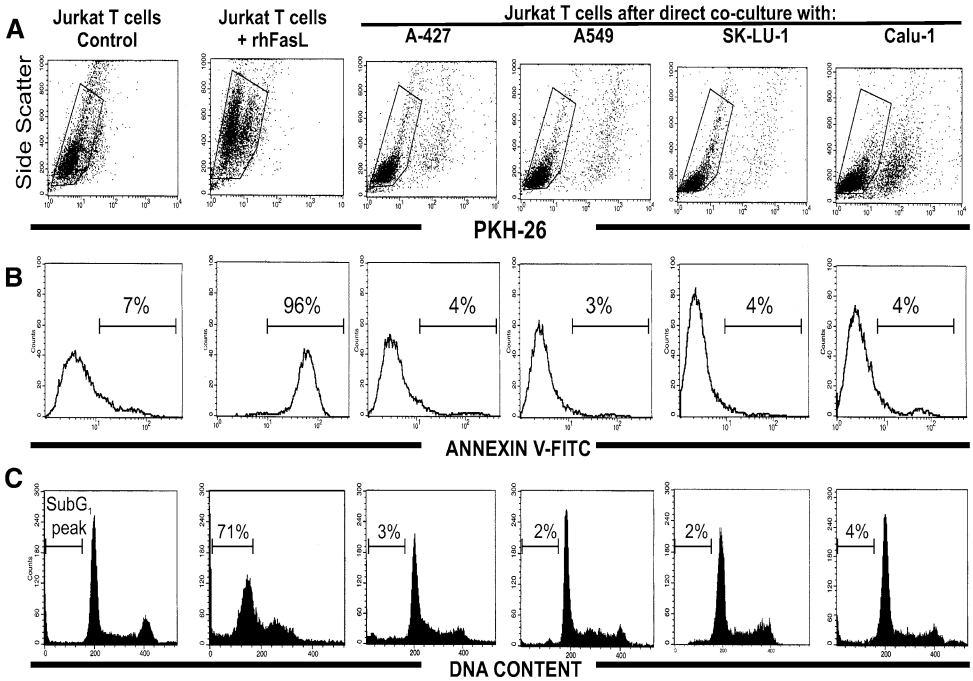

Fig. 2 Absence of apoptosis in Fas-sensitive Jurkat T-cells after co-culture with NSCLC cell lines. **a** Cellular distribution of Jurkat T-cells in a PKH-26 dye versus side scatter dot plot graph. **b** Annexin V-FITC histograms showing non-apoptotic Jurkat T-cells gated from the PKH-26-negative cells. **c** Absence of apoptosis in Jurkat T-cells detected by propidium iodide staining and further subG$_1$ peak detection. rhFasL (positive control) induced apoptosis in Jurkat T-cells

samples was observed compared to untreated Jurkat T-cells. Non-malignant effusions samples did not affect cell viability, see Fig. 3a. This effect was a consequence of the increase of necrotic cells (Annexin V+/PI+ and Annexin V−/PI+ subsets), data not shown. To rule out the possibility that in our experimental conditions we might be detecting apoptosis in late stages, a kinetic of apoptosis was done. Even so, early apoptotic cells were not detected; nevertheless, necrosis was observed after 48 h of treatment, and after this time an increase in the percentage of necrotic cells was detected, see Fig. 3b.

Lack of apoptosis in Jurkat T-cells treated with tumor-derived microvesicles from NSCLC cell lines

Recent studies have suggested that FasL and TRAIL may be released via microvesicles secreted by tumor cells [1, 11, 14]. Hence, we studied whether microvesicles derived from eight NSCLC cell lines induce apoptosis in Jurkat T-cells. Microvesicles from PHA-stimulated Jurkat T-cells and HeLa cells (positive controls) reduced Jurkat T-cell viability as a consequence of an increase in the percentage of apoptotic cells. As a control of our experimental procedure, Jurkat T-cells were treated with the microvesicle-free supernatant; under this experimental condition, no apoptosis in Jurkat T-cells was observed, see Fig. 4. Microvesicles derived from normal lung fibroblasts MRC-5 (employed as control of non-transformed cells) induced a slight reduction in Jurkat T-cell viability. A similar percentage of cell viability was observed in Jurkat T-cells treated with microvesicles derived from each NSCLC cell line with a slight increase in the percentage of early apoptotic cells (<10%), see Fig. 4.

Levels of CD3ζ, CD3ε, and CD28 expression in Jurkat T-cells co-cultured with lung carcinoma cells or with tumor-derived microvesicles

It has been reported that tumor cells down-regulate CD3ζ expression in Jurkat T-cells in vitro [10]. As control, addition of rhFasL significantly reduced the expression of CD3ζ, CD3ε, and CD28 molecules in Jurkat T-cells (see Table 1); our data agree with previous reports [9, 18]. Normal lung fibroblasts MRC-5 in co-culture with Jurkat T-cells did not reduce MFI values of CD3ζ, CD3ε, and CD28, see Table 1. CD3ζ expression in Jurkat T-cell was

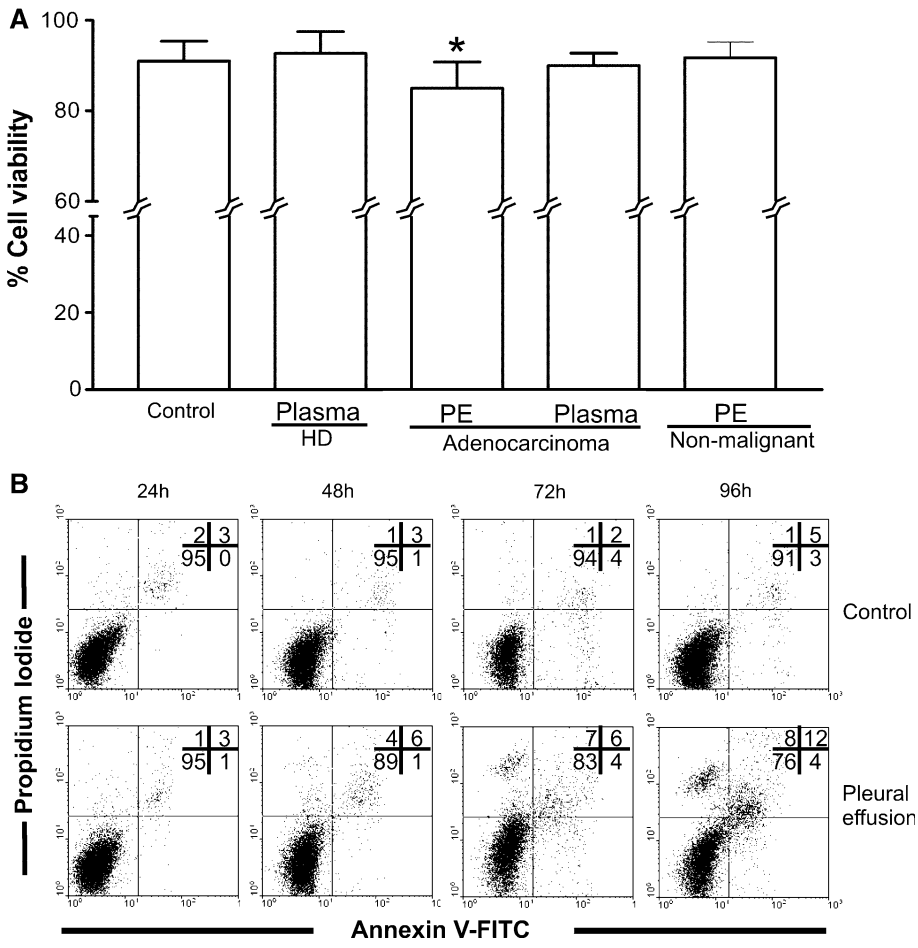

Fig. 3 Lack of apoptosis in Jurkat T-cells after treatment with plasma or pleural effusion samples from lung adenocarcinoma patients. **a** Viability of Jurkat T-cells co-cultured with plasma or pleural effusion samples (diluted 1:3 with complete RPMI-1640 medium) from adenocarcinoma patients after 96 h of incubation, determined by Annexin V-FITC/propidium iodide staining. A small reduction in viability was observed in Jurkat T-cells treated with malignant pleural effusion (*PE*), $*P < 0.05$ with respect to control. **b** Kinetic of apoptosis in Jurkat T-cells treated with pleural effusion sample from a representative lung adenocarcinoma patient

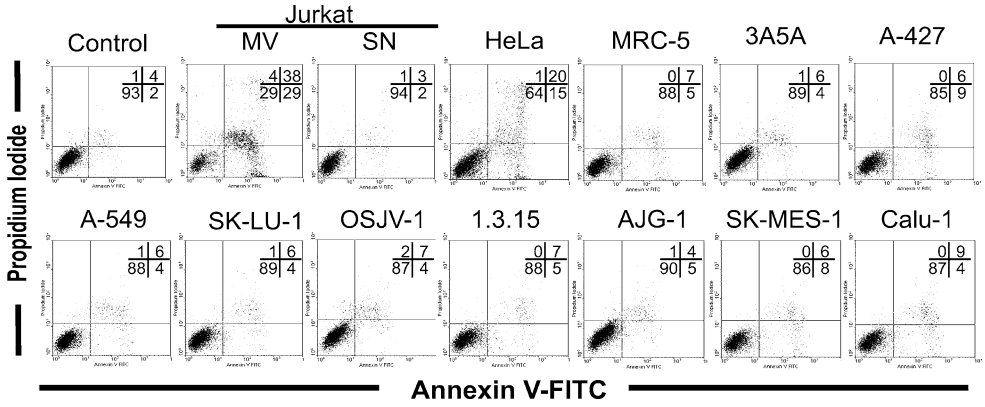

Fig. 4 Absence of apoptosis in Jurkat T-cells co-cultured with microvesicles (1–2 μg/ml) from eight NSCLC cell lines and detected by Annexin V-FITC/propidium iodide staining. As positive controls microvesicles (*MV*) from PHA-stimulated Jurkat T-cells and from HeLa cell lines were used. Microvesicle-free supernatant (*SN*) from Jurkat T-cells did not induced apoptosis

Table 1 Mean fluorescence intensity values of CD3ε, CD3ζ chains, and CD28 in Jurkat T-cells

Co-culture with	MFI ± SD		
	CD3ε	CD3ζ	CD28
Medium	69 ± 3	22 ± 2	82 ± 3
rhFasL	41 ± 2*	7 ± 2*	71 ± 2**
MRC-5	66 ± 2	26 ± 7	49 ± 12*
A-427	33 ± 4*	25 ± 7	53 ± 17**
A-549	47 ± 3*	23 ± 1	46 ± 2*
SKLU-1	47 ± 8*	23 ± 3	55 ± 4**
OSJV-1	46 ± 12*	15 ± 4	43 ± 3*
1.3.11	37 ± 5*	28 ± 8	74 ± 14
1.3.15	42 ± 3*	19 ± 4	73 ± 26
3B1A	54 ± 10**	19 ± 3	55 ± 9**
Calu-1	58 ± 4	22 ± 5	56 ± 9**

* $P < 0.001$ with respect to medium

** $P < 0.05$ with respect to medium

not affected by the NSCLC cell lines after direct co-culture. However, CD3ε expression was significantly reduced by 7/8 lung carcinoma cell lines, see Table 1. Moreover, CD28 expression was significantly decreased by 6/8 lung carcinoma cell lines in Jurkat T-cells. These effects were not exclusively mediated by cell–cell contact, as Jurkat T-cells in an indirect co-culture with NSCLC cell lines also showed a significant reduction of MFI values for CD3ε and CD28. Data from A-549, A-427, and OSJV-1 cell lines are shown in Fig. 5a.

Recently, some studies have reported that tumor-derived microvesicles containing FasL reduce CD3ζ expression [1, 14]. We tested whether microvesicles derived from NSCLC cell lines down-regulate CD3ε, CD3ζ, or CD28 expressions. Microvesicles derived from stimulated-Jurkat T-cells down-regulated CD3ζ expression; besides, we found a reduced expression of CD3ε chain, see Fig. 5b. With respect to CD28, a slight increase in MFI value was observed. MRC-5-derived microvesicles did not modify any of the molecules studied. Similarly, microvesicles from most of the NSCLC cell lines did not modify the expression of CD3ε, CD3ζ, and CD28 molecules (data not shown).

Our results suggest that CD3ε and CD28 down-regulation induced by NSCLC cell lines is not mediated by cell–cell interaction or tumor derived-microvesicles, but depends of soluble factors released by tumor cells. See Fig. 5b for representative AJG-1 and OSJV-1 cell lines.

Levels of CD3ζ and CD3ε expression in T-cells from lung adenocarcinoma patients

To evaluate whether the reduced expression of CD3 in Jurkat T-cells, induced by NSCLC cell lines and detected by us, also occurs in vivo, MFI values of CD3ζ and CD3ε were determined in CD4+T and CD8+T cells from peripheral blood and pleural effusion of lung adenocarcinoma patients.

Respect to the CD3ζ chain expression in CD4+T and CD8+T cells from peripheral blood, MFI values from lung cancer group did not differ from MFI values of healthy group, see Fig. 5c. In lung cancer patients, CD4+T cells from pleural effusion showed a non-significant decrease of CD3ζ expression, whereas CD8+T cells showed an increase of CD3ζ expression compared to the respective T-cell subpopulation from peripheral blood.

With regard to the CD3ε chain expression in CD4+T and CD8+T cells from peripheral blood, MFI values from lung cancer patients were significantly reduced compared to MFI values from healthy donors. In lung cancer patients, CD4+T and CD8+T cells from pleural effusion showed a significant decrease in MFI values for CD3ε regarding the respective T-cells subpopulations from peripheral blood, see Fig. 5c.

In summary, NSCLC cell lines did not express FasL and neither induced apoptosis in Jurkat T-cells, used as target cells in a biological assay. In contrast, NSCLC cell lines down-regulated CD3ε and CD28 expressions in Jurkat T-cells. Also, CD3ε down-regulation was observed in T lymphocytes from peripheral blood and pleural effusion of lung adenocarcinoma patients.

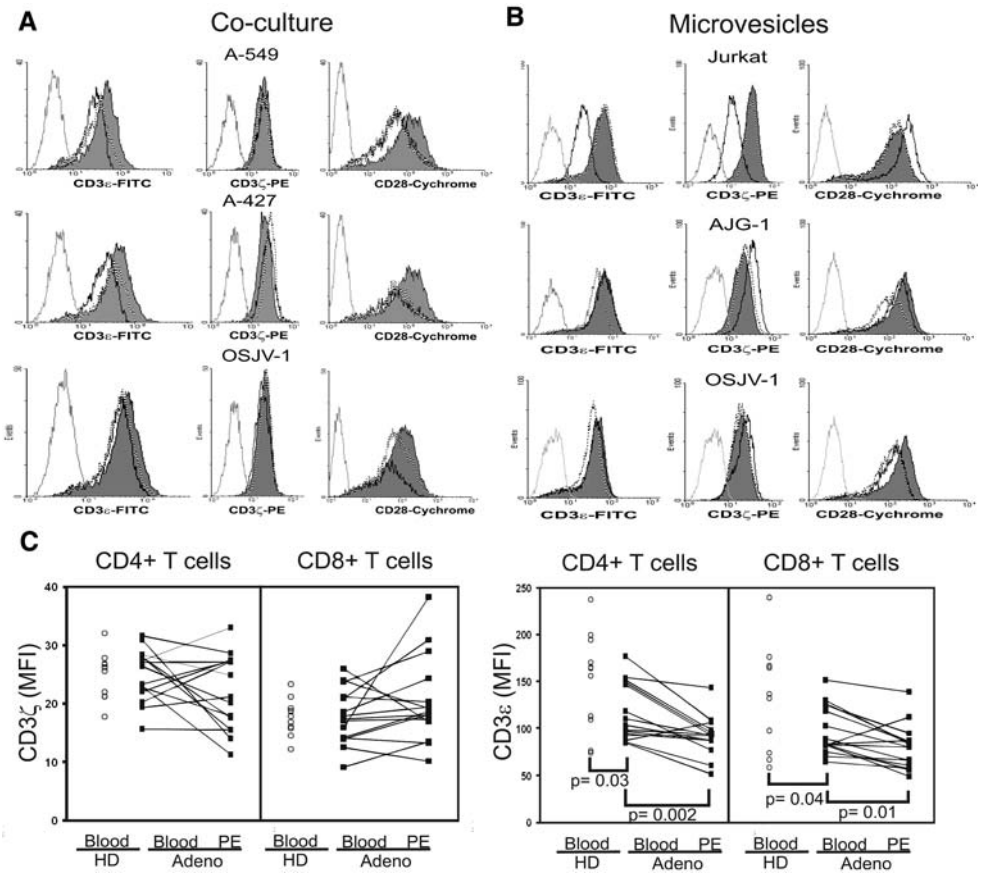

Fig. 5 Expression of CD3ε, CD3ζ, and CD28 in Jurkat T-cells and in CD4+T and CD8+T lymphocytes from lung adenocarcinoma patients. **a** Down-regulation of CD3ε and CD28 but not CD3ζ expressions in Jurkat T-cells after direct or indirect co-culture with representative NSCLC cell lines: A-549, A-427, and OSJV-1. Control: *gray area*; direct co-culture: *solid line*; indirect co-culture: *discontinuous line*; isotype-matched control: *gray line*. Results from three independent experiments. **b** Microvesicles released by NSCLC cell lines did not reduce CD3ε, CD3ζ, and CD28 expressions. In contrast, microvesicles-free supernatants down-regulated CD3ε and CD28 but not CD3ζ expressions in Jurkat T-cells. Results from two representative NSCLC cell lines (AJG-1 and OSJV-1) are shown. Microvesicles from PHA-stimulated Jurkat T-cells were employed as positive control for CD3ε and CD3ζ down-regulation. Control: *gray area*; microvesicle treatment: *solid line*; treatment with microvesicle-free supernatant: *discontinuous line*; isotype-matched control: *gray line*. Results obtained from three independent experiments. **c** MFI values for CD3ζ and CD3ε expression in CD4+T and CD8+T cells from peripheral blood and pleural effusion (*PE*) of healthy donors (*HD*) and lung adenocarcinoma patients (*Adeno*)

Discussion

Tumor counterattack via Fas/FasL interaction has been proposed as a mechanism for tumor evasion. Several studies have shown that functional FasL is expressed in distinct types of solid tumors [43]. Some authors have previously reported that lung cancer cells express membrane-bound FasL using the C-33 anti-FasL mAb [13, 24]. However, Strater et al. [37] and Smith et al. [34] have reported that this mAb shows cross-reactivity with unrelated molecules. In a preliminary study using the C-33 anti-FasL mAb, we found that a particular percentage of cells from each NSCLC cell line were FasL-positive (data not shown). Accordingly, the choice of the antibody for FasL detection is a critical step. For these reasons, in this report, three different mAbs that specifically recognize FasL (NOK-1, Alf-2.1, and G-247) were used [34, 37]. Most of NSCLC cell lines (84%) did not express membrane-bound or intracellular FasL, detected by flow cytometry and immunocytochemistry. The lack of FasL protein correlated with the absence of the FasL transcript.

It has been proposed that a possible biological role for tumor cells expressing FasL might be to facilitate the metastastatic process [41, 43]. So, FasL-positive tumor cells should be found in metastatic tumors of different origins. In this regard, Sikora et al. [32] have reported that some tumor cells from malignant pleural effusions of distinct origins express FasL. Nevertheless, leukocyte contamination could be responsible for FasL detection in tumor samples as was determined in our study, in which, leukocytes were discarded as a CD45-positive population, leading to identification of tumor cells from adenocarcinoma pleural effusions as a FasL-negative population. Our data agree with the study of Toomey et al. [38] who report that

tumor-infiltrating lymphocytes (TILs) and macrophages but not NSCLC cells express FasL in tissue specimens. Additionally, Viard-Leveugle et al. [41] report a diminished or minimal FasL expression in tumor cells from biopsies of NSCLC compared to normal lung tissue.

On the other hand, a biological assay has been used to study whether FasL-positive tumor cell induces apoptosis in a Fas-sensitive cell line [6, 10, 24]. Using this assay, Niehans et al. [24] reported that two NSCLC cell lines (H2009 and H522) induce apoptosis in Jurkat T-cells using trypan blue staining. Nevertheless, detached tumor cells might be responsible for detection of apoptotic cells. In other methodologies, such as the JAM assay, similar issues have arisen [6]. We improved the reported method for detection of apoptosis in Jurkat T-cells; using this methodology, apoptosis in Jurkat T-cells induced by NSCLC cell lines was not detected.

Our results indicated that FasL is not expressed on NSCLC cell lines (determined by flow cytometry, immunocytochemistry, and the biological assay). Recently, several authors have reported that FasL is mostly contained in microvesicles released by tumor cells of distinct origin, such as melanoma [1], oral [14], ovarian [23], and colorectal cancer [11]; furthermore, several authors have reported that microvesicles present in plasma and ascitic fluids from cancer patients induce apoptosis (>30%) in Jurkat T-cells [11, 14, 39]. So, we carried out experiments using microvesicles derived from tumor cells culture media; also, plasma and pleural effusion samples from lung adenocarcinoma patients were used. However, we demonstrated that neither microvesicles from NSCLC cell lines nor plasma or pleural effusion samples induced apoptosis in Jurkat T-cells. These data are in accordance with the absence of FasL protein and of transcript observed in most NSCLC cell lines.

Even when two NSCLC cell lines, Calu-1 and RCC1-20, expressed intracellular FasL, microvesicles derived from these cell lines did not alter Jurkat T-cell viability. Possible explanations for this behavior are: (1) Microvesicles released by these cell lines express a very low amount of FasL molecules, leading to undetectable apoptosis of Jurkat T-cells by our experimental conditions or the methodology applied in this study and (2) FasL is contained in the inner side of microvesicles derived from Calu-1 and RCC1-20 cell lines and membrane expression is required for inducing apoptosis, as has been reported in ovarian cancer [23].

The biological assay using FasL-sensitive Jurkat T-cells as targets cells has been used to evaluate functional FasL; nevertheless, Jurkat T-cells are also sensitive to TRAIL-induced apoptosis [11, 21]. As we did not detect apoptosis in Jurkat T-cells in co-culture with NSCLC cell lines or with tumor-derived microvesicles, it is unlikely that other TNF-related apoptosis inducing ligands are expressed by tumor cells. Currently, we are studying the expression of some of these molecules in NSCLC. Given that lung carcinoma cells neither express FasL nor induced apoptosis in Jurkat T-cells, our data do not support the tumor-counterattack hypothesis.

We have previously reported an increase in the percentage of Fas-expressing CD8+T cells, particularly in the naïve subset, in lung adenocarcinoma patients [26], and as in this study we discarded the tumor counterattack as a evasion mechanism in NSCLC; perhaps activation-induced cell death (AICD) is involved in immune tolerance to NSCLC. It has been hypothesized that AICD is responsible for apoptosis of TILs observed in cancer patients [27, 28]. Chronically stimulated T-cells undergo AICD mediated by the Fas/FasL interactions, causing them to kill themselves and each other [19]. In this regard, we have previously reported that CD8+T cells, from pleural effusion with a memory-like phenotype, may be associated with chronic antigen stimulation, which might render this memory CD8+T cell subset susceptible to AICD when it reaches the effector-cell stage. Further studies are required to assess whether this mechanism is relevant in NSCLC.

In another aspect, it has been reported that in an early step of apoptosis mediated by Fas/FasL interaction, T lymphocytes show a reduction in CD3ζ expression [9, 10]. Down-regulation of the CD3ζ chain has been proposed to be a mechanism that mediates T-cell immunosuppression in different types of cancer [2, 42]. Several studies have reported that tumor cells co-cultured with T-cells induce in the latter a reduction or loss of CD3ζ expression [10, 42]. In addition, Sikora et al. [31, 33] report down-regulation of CD3ζ in T lymphocytes from malignant pleural effusions.

Using the same mAb (2H2D9 clone) employed by Sikora et al. [31], we found no change in the level of CD3ζ expression in Jurkat T-cells after co-culture with lung carcinoma cell lines or in T-cells from lung cancer patients ex vivo. Some authors have reported that, in many kinds of cancer, including NSCLC [44], CD3ζ is not down-regulated in TILs [5, 25]. Recently, Broderick et al. [3] found that memory T-cells isolated from biopsies of lung carcinoma patients express CD3ζ at levels similar to healthy donors. Our data agree with these reports; lack of down-regulation of CD3ζ correlates with our observation that apoptosis was not induced in Jurkat T-cells.

Although we found no down-regulation of the CD3ζ chain in T lymphocytes, co-culture with NSCLC cell lines reduced the level of CD3ε expression in Jurkat T-cells. Down-regulation of CD3ε was also observed in T lymphocytes from peripheral blood and pleural effusion of lung adenocarcinoma patients. Riccobon et al. [29] reported that TILs from patients with renal cancer down-regulate CD3ε and this phenomenon occurs to a lesser degree in near-tumor-tissue lymphocytes and PBMC. Also, CD3ε

down-regulation has been reported in T-cells from peripheral blood of cancer patients [22, 42].

Microvesicles-derived from NSCLC cell lines did not alter CD3ζ and CD3ε expressions. Recently, Valenti et al. [39] have suggested that tumor-derived microvesicles from tumors of several origins have a role as immunosuppressive agents, promoting differentiation of monocytes to myeloid-derived suppressor cells; even when we have observed immunosuppresion in Jurkat T-cells in direct or indirect co-culture with tumor cells (data not shown), this phenomenon was not dependent of down-regulation of epsilon and zeta chains in the CD3 complex induced by tumor-derived microvesicles.

Based on our results, we suggest that soluble factors released by tumor cells induce CD3ε down-regulation. Further studies are required to identify the molecules responsible of this phenomenon.

No previous studies mention whether tumor cells reduce the level of CD28 expression in the Jurkat T-cell experimental model. However, CD28 down-regulation has been observed in T-cells from peripheral blood in patients with hairy cell leukemia [40] or hepatocellular carcinoma [20]; in the latter report, CD3ζ down-regulation was also observed. We found that lung carcinoma cell lines reduced the level of CD28 expression in Jurkat T-cells.

All of these results suggest that the diminished expression of CD3ε might induce T-cell anergy, leading to hyporesponsiveness of T-cells observed in NSCLC patients. Given the importance of the CD3 complex, reduction of CD3ε expression might alter key TCR-dependent biochemical events.

In conclusion, tumor counterattack mediated by FasL is not an important evasion mechanism in NSCLC. However, NSCLC may affect CD3 complex stoichiometry necessary for induction of efficient T-cell signaling, leading to a dysfunction of the T-cell-mediated immune response.

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