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ORIGINAL ARTICLE

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High expression of adhesion molecules/activation markers with little interleukin-2, interferon γ, and tumor necrosis factor β gene activation in fresh tumor-infiltrating lymphocytes from lung adenocarcinoma

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Abstract Little is known about the activation level of tumor-infiltrating lymphocytes (TIL) in human lung adenocarcinoma. We investigated the activation of fresh TIL at cellular and molecular levels and compared it with autologous and healthy normal peripheral blood lymphocytes (PBL) for baseline level. TIL were extracted from 12 primary lung adenocarcinomas by mechanical disruption without enzyme use and isolated by double-density Ficoll gradients. Flow-cytometry analysis of TIL subset distribution revealed that the majority was composed of T lymphocytes, and double labeling with α-CD3 and adhesion/activation markers revealed T cell subsets expressing CD49a, CD49b, CD54, and CD15, each of which was almost absent in autologous T peripheral blood lymphocytes (T-PBL). Moreover, the proportions of T-TIL expressing CD58, CD65, or CD25 were increased severalfold compared to T-PBL. Lymphokine gene activation in TIL was assessed by mRNA reverse transcriptase/polymerase chain reaction (RT-PCR) and primers for interleukin(IL)-2, IL-4, interferon (IFN) γ, granulocyte/macrophage-colony-stimulating factor (GM-CSF), and tumor necrosis factor (TNF) β. Semiquantitative comparisons between patients' TIL and PBL and healthy normal and activated PBL were performed by computerized image analysis. RT-PCR gel band products were quantified in relative units as a function of their size and intensity. TIL expressed detectable lymphokine mRNA but seemed poorly activated with respect to the total number of lymphokine genes and the amount of mRNA compared with α-CD3-activated healthy PBL. IL-2, IFNγ, and TNFβ did not appear to be expressed at higher levels in TIL than in autologous or healthy normal PBL. However, two-thirds of the patients had TIL distinguishable from autologous PBL by specific expression of GM-CSF and from healthy normal PBL by expression of IL-4. These results show that lung adenocarcinoma TIL populations had little lymphokine gene activation despite the presence of several T cell subsets expressing different adhesion/activation markers. The lack or deficient combination of lymphokine production may be a factor that prevented efficient activation of TIL in these tumors.

Key words Cell adhesion molecules · Lung adenocarcinoma · Lymphokines · Polymerase chain reaction · Tumor-infiltrating lymphocytes

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Introduction

Tumor tissue generally contains a mixed population of leukocyte subsets [31, 51] that are presumed to infiltrate the tumor as part of an inflammatory process induced by the tumor's development. However, the presence of leukocytes in the tumor does not correlate with tumor cell destruction [47, 48, 51]. The activation state of these infiltrating leukocytes in the tumor microenvironment is poorly understood, mainly because of the difficulty of obtaining sufficient amounts of TIL to perform cellular or molecular assays [50]. In lung adenocarcinoma, data characterizing

Table 1 Antibodies used in this study. Sources: Immunotech, Westbrook, Me.; T cell Diagnostics, Cambridge, Mass.; *BD* Becton Dickinson, San Jose, Calif. *S-Le-Le*^{x} Sialosyl-Lewis-Lewis^x, *NA* not applicable, *PE* phycoerythrin, *FITC* fluorescein isothiocyanate, *GAM* goat anti-(mouse Ig), *TCR* T cell receptor, *LPS* lipopolysaccaride

Antibody	Specificity	Ligands[a]	Source
CD29	β1	NA	Immunotech
CD49a	VLA-1	Collagen IV, I and laminin	T Cell Diagnostics
CD49b	VLA-2	Collagen I, IV and laminin	Immunotech
CD54	ICAM-1	LFA-1 (CD11a)	Immunotech
CD58	LFA-3	CD2	Immunotech
CD15	Lewis^x	CD15	Immunotech
CD65	S-Le-Le^x	?, E-selectin	Immunotech
Leu8	L-Selectin	GLYCAM-1	BD
CD25	IL-2 Rα	IL-2	Immunotech
CD3-PE	TCR CD3 complex	NA	BD
CD4-FITC	TCR CD4 complex	MHC Class II Ag	BD
CD8-FITC	TCR CD8 complex	MHC Class I Ag	BD
αβ-FITC	TCR αβ chains	Ag	BD
γδ-PE	TCR γδ chains	Ag	BD
CD14-FITC	Monocyte LPS receptor	LPS	Immunotech
IgG1 mouse	None	NA	Immunotech
IgG1-PE mouse	None	NA	Immunotech
GAM-FITC	Mouse IgG+IgM	NA	Immunotech

[a] See [16, 28, 32, 35, 41, 44]

fresh TIL are limited. The TIL are known to be predominantly a CD3$^+$ T cell population consisting of a mixture of CD4$^+$ and CD8$^+$ cells [31, 53]. Although there are previous reports of fresh TIL expressing the activation markers CD25 and HLA-DR [31, 45, 53], little is known about TIL expression of adhesion/activation markers. Cell adhesion molecules (CAM) like CD49a, CD49b, CD54, CD58, and CD15 are expressed on activated T cells [6, 13, 14, 36, 41]. Activation or increased expression of CAM is necessary in the leukocyte process of tissue infiltration and adhesion to the targeted tumor cells [8, 22, 26, 38, 41]. In this study, we hypothesized that fresh T tumor-infiltrating lymphocytes (T-TIL) are distinguishable from autologous T peripheral blood lymphocytes (T-PBL) by the expression of certain cell-surface adhesion/activation markers.

The paradox that fresh TIL might express an activated phenotype with apparently no antitumor effect in lung adenocarcinoma led us to assess concurrently lymphokine gene mRNA expression as an index of current activation. The development of an active immune response is dependent on the production of a combination of lymphokines from the involved lymphocytes [2]. Because it is technically difficult to assess lymphokine production in the tumor microenvironment, the assessment of lymphokine gene mRNA in fresh TIL seemed a suitable approach to monitor functional activation. Expression of lymphokine gene mRNA in fresh TIL is poorly documented [51], partially because of the standard enzymatic technique of TIL extraction that favors mRNA degradation. In this study, we rapidly extracted TIL by mechanical tumor disruption without enzyme use and then isolated them on a Ficoll gradient. We then phenotyped the fresh T-TIL with CD49a, CD49b, CD54, CD58, and CD15 adhesion/activation markers and other relevant adhesion molecules like CD29, L-selectin, and CD65. In addition, we used CD25 as a T-cell marker of activation [7]. Molecular analysis of TIL activation status in the tumor microenvironment was done by detection of lymphokine mRNA expression with reverse transcriptase/polymerase chain reaction (RT-PCR) and quantification of the gel band PCR products with image analysis. This technique allowed us to compare interleukin-2 (IL-2), IL-4, granulocyte/macrophage-colony-stimulating factor (GM-CSF), interferon γ (IFNγ), and tumor necrosis factor β (TNFβ) mRNA expression semiquantitatively between TIL and baseline control populations of autologous and healthy PBL [24]. In this report, we present evidence that lung adenocarcinoma TIL contain phenotypically activated T cell subsets but have little lymphokine gene activation.

Materials and methods

Human tumor samples

We selected tissue resected from 12 patients whose non-small-cell primary adenocarcinoma of the lung had not been treated previously. Aseptically excised tumors were examined immediately by a pathologist, and a sample of about 1 g pure tumor tissue was selected for analysis. Necrotic tissue was discarded. The tissue was immediately placed on ice and rapidly processed.

TIL isolation

All manipulations were done under sterile conditions, and special precautions were taken with pathogenic materials. All plastic dishes and pipettes were coated with protein-rich AIM-V medium (Gibco Laboratories, Grand Island, N.Y.) immediately before use, and processing was done rapidly on ice to reduce loss of TIL by adherence. The tumors were rinsed in cold AIM-V medium to remove blood, minced into 2- to 4-mm fragments, and passed consecutively through 380-, 230-, and 140-μm mesh. We separated the TIL population from the tumor cells by loading the mixed cell suspension on two superimposed layers of 100% and 75% Ficoll-Histopaque 1077 (Sigma Chemical Co., St. Louis, Mo.) and centrifuging at 900 *g* for 20 min at 20 °C [50]. The enriched tumor-infiltrating mononuclear cell fraction was col-

lected from the interface between 100% and 75% Ficoll, washed once with cold AIM-V, and centrifuged at 650 *g* for 10 min at 4 °C. TIL were then counted by Trypan blue exclusion (Sigma), divided into aliquots of 3×10^6 cells/1.5-ml microcentrifuge tube, spun, and frozen as a pellet at −70 °C.

PBL isolation

Peripheral blood was collected from patients before surgery and from healthy persons, and the PBL were isolated by standard techniques as described elsewhere [33]. Viable cells were then counted by Trypan blue exclusion, resuspended in AIM-V medium for phenotyping or divided into aliquots of 3×10^6 cells/1.5-ml microcentrifuge tube, spun, and frozen as a pellet at −70 °C.

Phenotyping with monoclonal antibodies and flow cytometry

The cells suspended in AIM-V medium were distributed in V-bottom plates at 1×10^5 cells/well in a 100-µl volume. All labeling procedures were carried out at 4 °C. The antibodies used in this study are described in Table 1. In accordance with the supplier's technical instructions, 5 µl or 20 µl of an anti-CAM antibody was added to the cells before they were incubated for 30 min. The cells were then washed twice with AIM-V and, because the CAM antibodies were not conjugated, the cells were incubated with (0.1 µg/10^5 cells) a goat anti-(mouse Ig) conjugated with fluorescein isothiocyanate (GAM-FITC) for 30 min in darkness. The cells were washed twice with AIM-V, and double labeling was achieved by a third incubation of the anti-CAM-FITC-labeled cells with 20 µl anti-CD3 phycoerythrin (PE) conjugate for 30 min in darkness. A nonconjugated mouse IgG1 subjected to labeling with the GAM-FITC antibodies was used as control for the CAM-FITC labeling, and then the PE labeling control was added using a mouse IgG1-PE conjugate. Finally, the cells were washed twice with AIM-V and a third time with phosphate-buffered saline (PBS) and then fixed in PBS with 1% paraformaldehyde (Sigma). For the directly conjugated antibodies described in Table 1, the cells were labeled according to the manufacturer's standard procedure. The tubes were kept at 4 °C in darkness and analyzed by flow cytometry within 2 days.

Flow cytometry was done using a FACScan cell sorter operated with FACScan Research software version A (Becton Dickinson, San Jose, Calif.). Forward- and side-scatter lights were used to select only viable cells, and 5000 cells were counted in each sample. FITC immunofluorescence was detected at 530 nm, and PE was detected at 585 nm. The data were analyzed with CONSORT-30 software (Becton Dickinson). Four-dimensional analysis of T cells was performed by gating on the lymphocyte population using forward- and side-scatter lights combined with gating on the doubly fluorescent red and green cells to obtain the $CD3^+CAM^+$ lymphocytes. Proportions of $CD3^+CAM^+$ cells were expressed as percentages of the total CD3 lymphocyte population, which was assessed as a separate control.

Activation of PBL with α-CD3

PBL from the blood of a healthy individual were purified as above and activated by cross-linking the CD3 structure with α-CD3 (AMAC, Westbrook, Me.) cross-linked via immobilized goat anti-(mouse IgG) [10]. Tissue-culture flasks (25 cm^2) (NUNC, Kamstrup, Denmark) were coated with 3 ml 20 µg/ml solution of goat anti-(mouse IgG) (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) in TRIS/HCl, pH 9.5, for 4 h at room temperature. Immediately before use, the flasks were washed three times with PBS. Each flask was then filled with 10 ml AIM-V mixture supplemented with 10% human serum, fresh 2-mM glutamine, and penicillin/streptomycin (50 IU/ml and 50 µg/ml respectively) and containing 1.2×10^6 cells/ml with 1 µg/ml α-CD3. These flasks were incubated for 48 h at 37 °C in 95% humidity and 5% CO_2. After incubation, the cells were gently scraped off the bottom of the flask, washed in PBS, and centrifuged at 400 *g* for 10 min. They were then counted by Trypan blue exclusion, divided into aliquots of 3×10^6 cells/1.5-ml microcentrifuge tube, spun, and frozen as a pellet at −70 °C.

Reverse transcriptase/polymerase chain reaction

The mRNA was directly isolated from the frozen pellets using oligo (dT) MicroFastTrack kit (InVitrogen Corporation, San Diego, Calif.) according to the instructions provided. RT and PCR were carried out in a Perkin-Elmer Cetus GeneAmp PCR System 9600 using the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, Conn.). The 6-µl mRNA solution was mixed with 34 µl RT master mix containing the following reagents at the recommended final concentrations: 5 mM $MgCl_2$, 1 × PCR buffer (50 mM KCl, 10 mM TRIS-HCl, pH 8.3), 1 mM each dNTP, 40 U RNase inhibitor, 2.5 µM random hexamers, and 10 U RT. The RT mixture was incubated at 22 °C for 10 min, 42 °C for 45 min, 98 °C for 1 min, and 4 °C for 5 min. The resulting cDNA solution was then divided into aliquots. Aliquots of 4.6 µl (equivalent to 345 000 cells each) were amplified with one of the cytokine-specific primer pairs: IL-2, IL-4, IFN γ, GM-CSF, or TNFβ (Clontech Laboratories, Palo Alto, Calif.). A 2-µl sample of the remaining cDNA solution was mixed with 48 µl water, and 2 µl of the resulting 1:25 dilution (equivalent to 6000 cells) was amplified with our glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA primers. PCR reagents were mixed in a final volume of 30 µl to obtain the final concentration of 2 mM $MgCl_2$, 1 × PCR buffer, 200 µM each dNTP, 0.75 U AmpliTaq DNA polymerase, 0.4 µM Clontech's primer, and 0.18 µM GAPDH mRNA primers. The PCR reaction was performed under conditions specially adapted to the GeneAmp PCR System 9600: 94 °C for 1 min, 35 cycles of 94 °C for 15 s, 60 °C for 12 s, and 72 °C for 80 s, and then 72 °C for 7 min. RNA and DNA cross-contamination was continuously monitored by the use of water instead of the mRNA or cDNA template in the RT and PCR reaction. Positive DNA control for each cytokine (Clontech) and GAPDH was also amplified and run parallel in agarose gel to indicate the position of the specific amplified product.

PCR product analysis

Half of each PCR product (15 µl) was electrophoresed in TAE buffer in a composite gel of 3% NuSieve GTG and 1% SeaPlaque agarose (FMC Bioproducts, Rockland, Me.) containing 0.5 µg/ml ethidium bromide (Sigma). A 1-µg sample of *Hae*III-digested ϕ × 174 DNA (Boehringer Mannheim, Indianapolis, Ind.) was run in parallel to serve as molecular mass marker. Bands were visualized under ultraviolet light, and the fluorescence intensity was captured with a camera using Polaroid 667 coater black-and-white instant film (Polaroid Corporation, Cambridge, Mass.). Quantitative comparison of the PCR product bands was done by computerized image analysis. Our system was composed of a color/black-and-white camera (MicroImage Video Systems, Boyertown, Pa.), a Targa color-frame grabber and software (Truevision Inc., Indianapolis, Ind.), and a Trinitron high-resolution color monitor (Sony Corp., Tokyo, Japan), all linked to an IBM-compatible 486/33-MHz DX turbo computer (Fortune Systems, Houston, Tex.) and operated with JAVA video-analysis software version 1.4 (Jandel Scientific, Corte Madera, Calif.). Hardware and software components were integrated into a functional system by Meyers Instruments (Houston, Tex.). Photos of PCR product electrophoresis were loaded as digital images at the highest resolution field of the camera, and optimal incandescent lighting conditions were created in a black box to protect against stray light. The image analysis system intensity was calibrated from black (0) to white (255) on a gray scale of 256 channels/pixel, and the distance measurements were calibrated in millimeters. The size and intensity of each band were assessed, and the results were integrated in the following function to be quantified in relative units (RU) that were proportional to the relative amount of PCR products: relative amount of PCR products = band area × band-specific intensity, where band-specific intensity was calculated as I_{av} of the band area $-I_{av}$ of the photo background and I_{av} was the average intensity per pixel detected in the area of interest, according to the previously calibrated 256-channel gray scale.

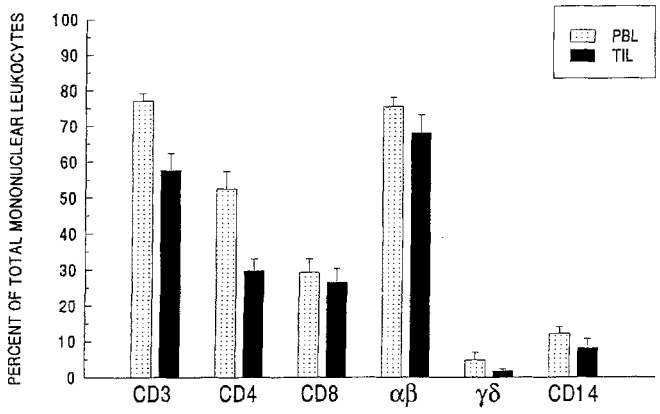

Fig. 1 Histograms showing the average and SEM of the percentage of the total mononuclear leukocytes for different T cell subsets and monocytes present in patients' tumor-infiltrating lymphocyte (*TIL*) and peripheral blood lymphocyte (*PBL*) populations

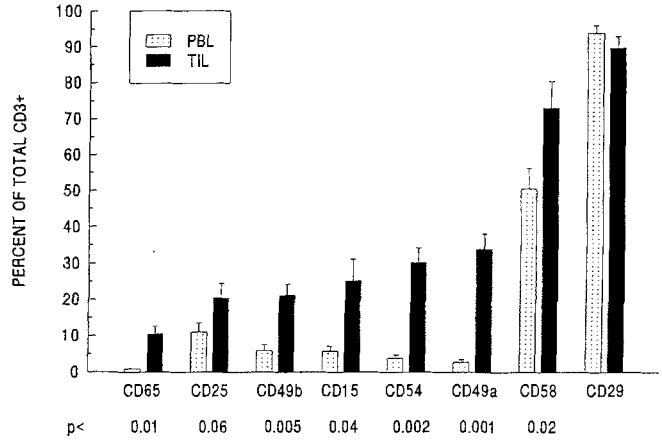

Fig. 2 Proportion of CD3+ T cells expressing different adhesion/activation markers in patients' PBL and TIL ($n = 12$)

The statistical significance of the differences obtained between groups was determined by computerized paired *t*-test analysis.

Results

Cellular phenotype

We consistently recovered (3–5) × 10^6 TIL/g lung tumor tissue with our isolation method. The contaminating percentage of intact lung tumor cells still alive was negligible with this technique. TIL constituted 95% of the live cells in these preparations. The mononuclear-subset distribution of the TIL appeared similar to that of the patients' PBL but with a slight reduction in CD3+ and CD4+ T cells (Fig. 1). The main infiltrating mononuclear leukocytes were αβ+ T lymphocytes (70 ± 6%); there were also natural killer cells and B cells (about 20%) and monocytes (about 10%). The γδ+ T cells were virtually absent from the TIL population: 1.62 ± 0.49% of the infiltrating CD3+ cells. The following results of T-TIL expression of adhesion/activation markers are presented as percentages of CD3+CAM+ cells in the total CD3+ population.

Integrins β1

CD49a and CD49b or VLA-1 and VLA-2 are expressed on activated T cells and are normally present only in trace amounts in resting T-PBL [13]. We found a major population of CD3+CD49a+ and CD3+CD49b+ cells in the TIL (Fig. 2). The CD3+ subset expressing VLA-1 or VLA-2 had a mean fluorescence intensity (MFI) five times that of CD3+CD49a- or CD49b- cells (Fig. 3). A few patients' CD3+ PBL expressed CD49a or CD49b but with a much weaker MFI. CD29, the common β chain for the VLA integrins [13], is a sum marker for the VLA integrins on T cells. High CD29 expression has been described as a marker of memory T cells, whereas low CD29 expression represents more naive T cells [13, 30, 37]. We consistently found that over 90% of the CD3+ cells in patients' TIL and PBL expressed CD29. The proportion of cells with high or low CD29 expression were the same in PBL whereas cells with high CD29 were predominent in TIL (Figs. 2,3).

CAM of the Ig gene superfamily

CD54 or ICAM-1 is expressed on a variety of cells including activated T and B lymphocytes [41]. CD54 binds to LFA-1 and is involved in antigen-independent T-cell adhesion and T-cell cosignal activation [22, 41]. T-TIL contained a distinct CD3+CD54+ subset with an MFI of about 25 for all patients tested. The expression of CD54 in T-PBL was detected in trace amounts (Figs. 2, 3). CD58 or LFA-3 is expressed on a majority of T cells and is involved in the CD2-LFA-3 T-cell activation pathway by binding to CD2 [15, 22]. CD58 has been shown to have bimodal expression in CD3+ cells by defining a negative population and a positive population that is believed to be composed of memory T cells [36]. Activation of CD3+CD58- naive T cells with mitogen causes expression of CD58 on these cells [36]. In the CD3+ TIL, CD58 defined a dual-type population in which most of the cells were in the CD3+CD58+ subset. CD3+ TIL consistently expressed CD58 in a higher proportion than did T-PBL (Figs. 2, 3).

Carbohydrate-related adhesion molecules

CD15, the Lewis^x carbohydrate antigen, is a cell-surface differentiation marker on neutrophils and monocytes [21, 40], and it has recently been reported to be associated with T cell activation [6]. We detected a distinct population of CD3+CD15+ cells (MFI = 47) in the TIL populations but only a very small percentage of CD3+CD15+ cells in the patients' PBL (Figs. 2, 3). CD65 is a type-II chain fucoganglioside that is expressed on granulocytes and mono-

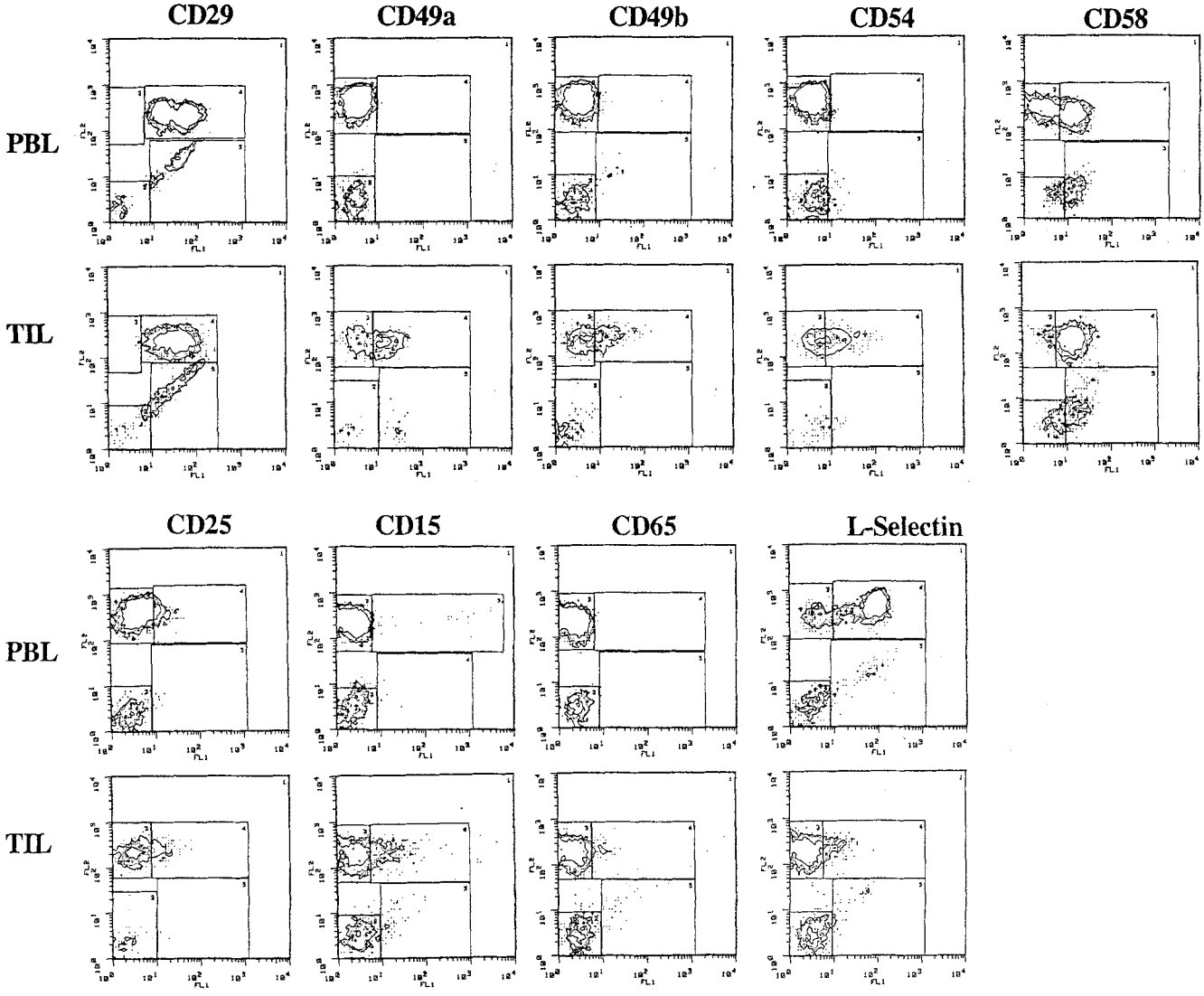

Fig. 3 Flow-cytometry contour plots (for 2, 3, 5, and 10 cells/channel) of different cell adhesion molecule (CAM) markers assayed on PBL and TIL populations of one representative patient. *Horizontal axis* four-decade scale for green fluorescence on a logarithmic plot; *vertical axis* four-decade scale for red fluorescence on a logarithmic plot. On the basis of $CD3^+$ control samples, contour plots were divided into boxes representing unstained cells ($CD3^-CAM^-$, *lower left*), cells stained only with FITC ($CD3^-CAM^+$, *lower right*), cells stained only with phycoerythrin (PE; $CD3^+CAM^-$, *upper left*), and cells stained with both PE and fluorescein isothiocyanate ($CD3^+CAM^+$, *upper right*)

cytes [20]. Sialylated, fucosylated glucoconjugates were recently reported as ligand-adhesion molecules for E-, P-, and L-selectin [28, 43]. Similar to CD15, a well-defined subset of T cells expressing CD65 (MFI = 60) was detected in the TIL whereas $CD3^+CD65^+$ cells were virtually undetectable in the patients' PBL (Figs. 2, 3).

L-Selectin, previously described as Leu8 or LAM-1, is one of a three-member family of selectins and is only expressed on leukocytes [42]. L-Selectin binds to carbohydrate ligands and has been shown to mediate the initial rolling contact of leukocytes with endothelium that is necessary for extravasation [39]. L-Selectin has a trimodal expression in T-PBL with negative, low ($CD4^+$), and high ($CD8^+$) subsets of expression [17, 29]. T cells have a rapid loss of L-selectin expression during activation [17, 42]. Accordingly, we found a marked decrease in L-selectin expression in the T-TIL population. It was expressed only on $18 \pm 2\%$ of the $CD3^+$ TIL (data not shown), and with a low intensity (MFI = 18) (Fig. 3). However, we detected a trimodal expression of L-selectin: negative, low (MFI = 20), and high (MFI = 80) in the $CD3^+$ PBL (Fig. 3). L-Selectin was present on $53 \pm 9\%$ of T-PBL (data not shown).

Activation marker CD25

The IL-2 receptor α chain is not expressed on resting T-PBL but it is a T cell activation marker of recent stimulation [27, 49]. Accordingly, we found the proportion of $CD3^+$ TIL expressing CD25 to be consistently twice the proportion of autologous T-PBL (Fig. 2).

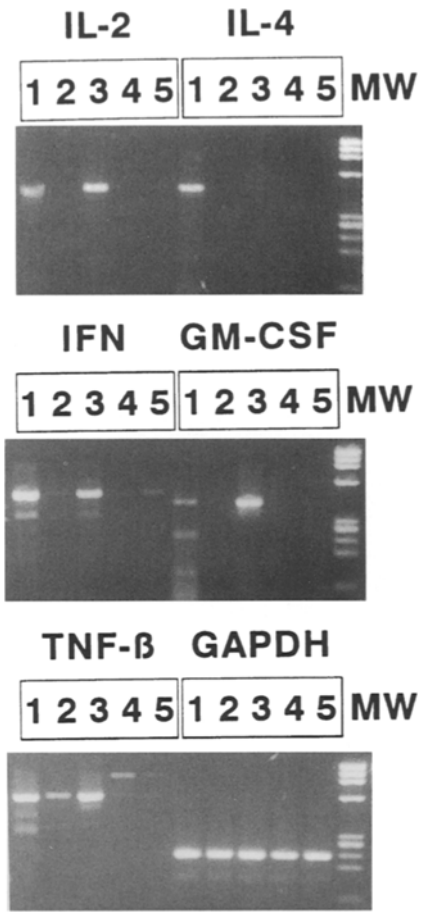

Fig. 4 Typical reverse transcriptase/polymerase chain reaction (RT-PCR) product gel electrophoresis for a representative patient. RT-PCR was performed with different primers on mRNA extracted from 3×10^6 cells as described in Materials and methods. For each different primer, the results shown are as follows: positive control primer template (*lane 1*), normal and CD3-activated healthy PBL (*lanes 2, 3*), and patients' PBL and TIL (*lanes 4, 5*). *GAPDH* glyceraldehyde phosphate dehydrogenase mRNA, *IL-2* interleukin-2, *TNF-β* tumor necrosis factor β

Lymphokine gene activation

TIL and PBL cytokine mRNA expression

To compare semiquantitatively the different PBL and TIL populations, the entire RT-PCR was always performed starting with the same number of cells (3×10^6) and under identical conditions. To relate the cytokine gene activation level of the patients' PBL and TIL populations with a strongly activated mononuclear leukocyte population, we compared them with healthy PBL activated by cross-linking of the T cell receptor CD3 structure with immobilized α-CD3 [10]. The results were expressed as $\log_{10}$ [amount of PCR product (RU)], measured to emphasize the magnitude of the difference observed between the diverse PBL and TIL populations. We tested the linear sensitivity of our film to capture the fluorescent signal emitted by the ethidium bromide gel bands under ultraviolet light and the ability of our image analysis system to achieve PCR product measurements of these bands. We found that the calculated relative value (RU) was always proportional to the amount of PCR products ($r \geq 0.99$) for several lymphokine primer sets. The band was very thin and gray for a value of 1 $\log_{10}$ unit (10 RU), a little larger with a whiter appearance for a value of 2 $\log_{10}$ units (100 RU), and very large and bright white for a value of 3 $\log_{10}$ units (1000 RU) (Fig. 4). These α-CD3-activated PBL populations produced PCR bands of $\log_{10}$ units for the cytokine genes IL-2, IFNγ, GM-CSF, and TNFβ, and about 1.5 $\log_{10}$ units for IL-4 (controls, Fig. 5). The PCR results obtained with these activated PBL are known to correlate with cellular secretion of the cytokines [10]. Similar results were obtained with three different healthy donors.

In the patients' TIL or PBL, IL-2, IFNγ, and TNFβ mRNA was detected in small amounts, 1 – 1.5 $\log_{10}$ units, or was not detectable (Fig. 5). In most cases, TIL populations did not express mRNA at levels significantly above those of autologous or healthy PBL (Fig. 5). However, the TIL of two-thirds of the patients showed specific activation of the GM-CSF gene, whereas autologous PBL did not express detectable GM-CSF mRNA. GM-CSF gene activation in TIL was 1 – 1.5 $\log_{10}$ units above the levels in healthy normal PBL but still significantly below the levels in healthy α-CD3-activated PBL (Fig. 5). In addition, the TIL of two-thirds of the patients expressed IL-4 mRNA at levels significantly above those of healthy normal PBL.

Control of reproducibility

To monitor the reproducibility of our RT-PCR reaction throughout our study, we included two external reference samples of mRNA with all patient samples and for each cytokine primer set tested: one extracted from normal and the other from α-CD3-activated healthy PBL populations (Figs. 4, 5). These control results were reproduced with high consistency (variation in amounts of PCR products less than 5%) more than 100 times in our laboratory. In addition, we included a GAPDH primer set in each sample population to verify the presence of mRNA and PCR reaction. We detected GAPDH mRNA consistently in all samples and noted a slight increase in the α-CD3-activated PBL population (Fig. 4).

The PCR reaction has been described as an exponential DNA amplification followed by a plateau effect [3]. After a certain number of PCR cycles and/or DNA concentration, the total amount of DNA amplified, plotted as a function of different starting DNA concentrations, shows that it accumulates as a logarithmic function [5]. We found that, for all cytokine primers used, we could produce and detect the PCR product accumulation as a logarithmic function of the mRNA cell number equivalent with correlation coefficients of at least 0.95 (data not shown). We also verified that our RT-PCR conditions were sufficiently sensitive to assess cytokine gene expression in the different PBL and TIL populations. We tested each cytokine primer used in our RT-PCR after 35 amplification cycles, using serial dilutions

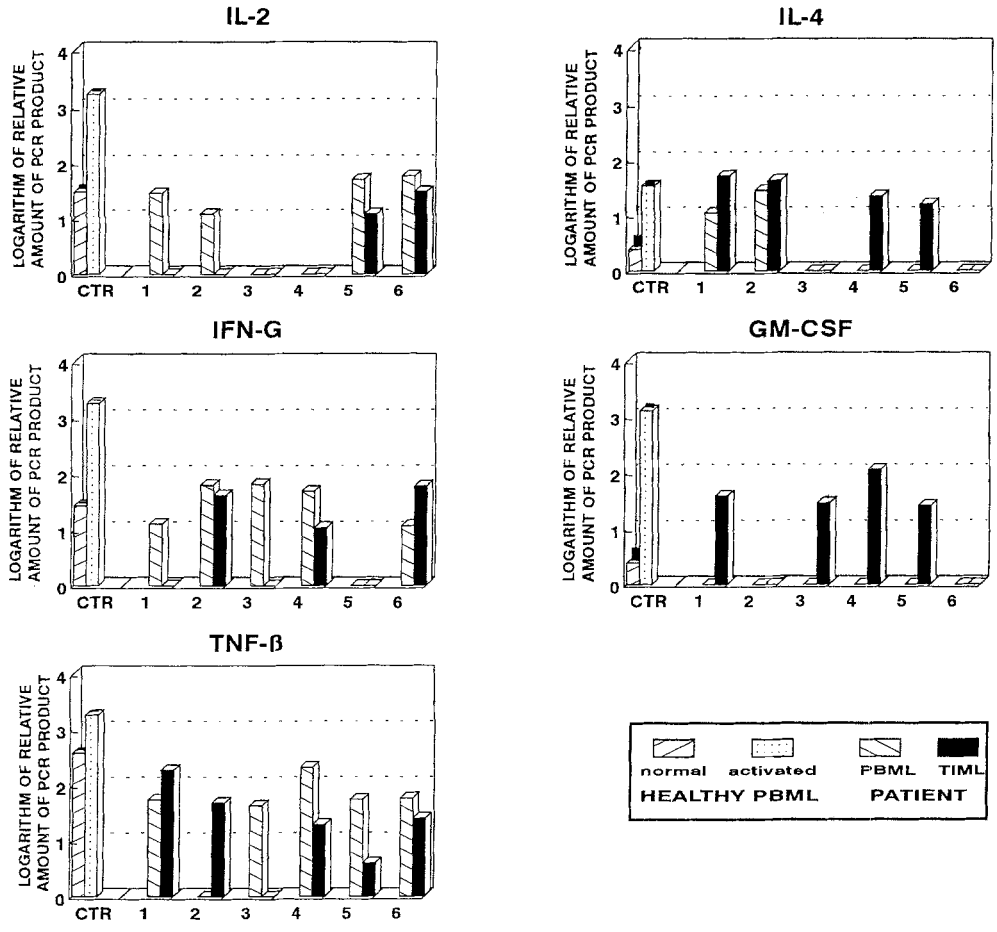

Fig. 5 Lymphokine profile with the logarithmic PCR product values for each lung adenocarcinoma patient obtained after mRNA RT-PCR done with the corresponding primers. In each graph the average logarithm of the product yields (relative units) for three normal and three activated healthy PBL populations (*CTR*) are presented with SEM bars. All RT-PCR were performed with autologous PBL and TIL of six different patients; all samples contained the same amounts of cell-number-equivalent mRNA and were treated under identical conditions described in Material and methods

of mRNA extracted from the α-CD3-activated PBL population. The RT-PCR product yields (RU) plotted as a function of mRNA cell equivalent indicated that, for this strongly activated population, the amount of products detected with the IL-2, IFNγ, GM-CSF, and TNFβ primers was still underestimated because of the plateau effect. Even at maximum mRNA concentrations, we detected little product for IL-4. However, PBL and TIL populations appeared in the reaction's exponential range for all the primer sets used. Thus, our RT-PCR conditions were sensitive enough to discriminate between our α-CD3-activated PBL reference population and the less-activated PBL and TIL populations.

Discussion

These results show for the first time that lung adenocarcinoma T-TIL can be distinguished from autologous T-PBL on the basis of the presence of T-cell subsets expressing adhesion/activation molecules. The T-TIL appeared as a more heterogeneous population containing several subsets defined mainly by the expression of the activation markers CD49a, CD54, CD15, CD49b, and CD25 in addition to a threefold decrease in the proportion of cells expressing L-selectin. Although TIL were not assayed for increased binding to the respective ligands of these adhesion molecules, our findings are in accordance with the fact that adhesion molecules are expressed on activated T cells and used to migrate through tissue layers and would thus be expected to be found on T-TIL extracted from tumor tissue. These adhesion/activation molecules were expressed at low levels or not at all on patients' autologous T-PBL or T-PBL from healthy individuals (data not shown). The presence of these various CD3+CAM+ subsets in TIL was consistent among all patients, as indicated by the small SEM of each group. The absence of enzymatic treatment in the TIL extraction, which reduced the risk of sensitive surface-antigen denaturation, in combination with rapid processing in cold conditions and the use of protein-coated plastic dishes to minimize adherence and loss contributed to the reproducibility of these data. Initial experiments done at room temperature with uncoated dishes caused reduction of the number of TIL recovered per gram of tissue by up to 90%.

Previous studies have shown that the majority of lymphocytes infiltrating non-small-cell lung tumors were T cells and that they expressed the activation markers CD25 and HLA-DR [31, 45, 53]. However, no cytotoxicity against autologous tumor was observed in vivo or in vitro unless the TIL were cultured with IL-2 for a few days [52, 53]. Our study aimed to extend the phenotypic characterization of lung TIL and test for functional activity in vivo. In

accordance with previous work, we found a significant proportion of T-TIL, extracted from tumor samples without necrosis that had the phenotype of activated T cells. However, most of these adhesion/activation markers like CD49a, CD49b, CD54, CD58, and CD29 are also known as memory T cell markers [1, 13, 30, 36, 37, 41]; therefore, phenotyping these T-TIL did not distinguish an activated T cell from an anergic memory T cell. Because activated T cells produce lymphokines, we assessed these fresh TIL for lymphokine mRNA. Our data showed for the first time that fresh TIL from lung adenocarcinoma did not express significantly more IL-2, IFNγ, and TNFβ mRNA than did autologous or healthy PBL.

There is a possibility that tumor cells contaminated some of our TIL preparations. However, the extensive tumor tissue grinding necessary to release the TIL left very few tumor cells (< 5%) intact and alive when detected. As in another study [53], our TIL extracted from lung carcinoma were 95% pure. Moreover, the free mRNA possibly released from broken tumor cells is sensitive to degradation and is unlikely to separate with the mononuclear leukocytes in the Ficoll gradient. Even if tumor-cell mRNA contaminated some TIL mRNA preparations, it would not alter the end result, suggesting that, despite having T cell subsets with an activated phenotype, the levels of IL-2, IFNγ, and TNFβ produced by TIL were apparently insufficient to support cytokine activation of lymphocyte tumorotoxicity [11, 19, 25] in the microenvironment of these progressing tumors. We found similar results in a study on fresh glioma TIL in which we found phenotypically activated T cell subsets but no significant IL-2, IFNγ, or TNFβ mRNA expression above PBL baseline levels (Roussel et al., submitted). Another study that used in situ hybridization to determine TIL lymphokine mRNA expression reported that, in most ovarian carcinomas and invasive ductal breast carcinomas, TIL did not express mRNA for IL-2, IFNγ, or TNFα [46]. However, in some mucin-producing breast carcinomas, localized TIL populations expressed cytokine mRNA [46]. This last study suggested the possibility that local concentrations of TIL subsets develop some activation upon exposure to local stimuli in a heterogeneous tumor. Our study did not discriminate leukocyte subset localization, but it supports the existence of several subsets according to adhesion/activation marker expression. Accordingly, local activation of TIL subsets might have been responsible for the detection of activated IL-4 and GM-CSF genes. Two-thirds of the patients had expression of GM-CSF and/or IL-4 mRNA significantly above healthy PBL baseline levels. Similarly, GM-CSF and IL-4 genes were activated in glioma TIL (Roussel et al., submitted). However, GM-CSF has been reported to be a suppressor of T-TIL in animal models of lung tumor by generating the presence of GM-CSF-dependent granulocyte suppressor cells [54]. IL-4 has also been reported to reduce IL-2-induced large granular lymphocyte killing activity in vitro [4, 9, 18]. IL-4 could exert a similar effect on TIL subsets in the tumor microenvironment. It has been suggested that the deficiency of T-TIL in killing lung tumor in vivo might be due to the absence of IL-2 in the tumor microenvironment as well as the release of tumor-derived suppressing factors [53, 54]. Lymphokines are known to be important regulators in activation, proliferation and effector-cell function of leukocytes in tissue [2, 23]. Therefore, insufficient amounts or deficient combinations of lymphokines produced by TIL in tumors may be factors that prevented optimal activation and amplification of the immune response.

Other mechanisms have been proposed to explain TIL inefficiency. Pisani et al. have suggested that even TIL or lymphokine-activated killer cells cultured with IL-2 do not easily kill non-small-cell lung tumors [31]. They suggested that the lack of killing was due not only to TIL deficiency but to some inherent resistance of the tumor cells. Accordingly, aberrant expression of cell adhesion molecules by tumor cells may jeopardize adhesion and cause a lack of costimuli encountered by TIL in their attempt to kill tumor cells [22, 26, 38, 41]. This possibility is supported by a study done on expression of cell adhesion molecules by lung adenocarcinomas that were found to have a loss of integrin β1 and markedly reduced expression of CD54 and CD58 in comparison with autologous normal pneumocytes [34]. In addition, aberrance or loss of MHC class I antigen expression by tumors [12] may also jeopardize T cell tumor antigenic recognition and cause a lack of T cell activation. Among possible mechanisms for tumor escape from immunodestruction, our work proposes that the lack of IL-2, IFNγ, and TNFβ and the possible production of GM-CSF and IL-4 by TIL in these tumors may be factors that contribute to the TIL energy.

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