

*Featured Article***Characterization of Effusion-Infiltrating T Cells: Benign versus Malignant Effusions**

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**ABSTRACT**

**Purpose:** While naïve T cells circulate between peripheral blood and lymph nodes, memory effector T cells acquire certain surface molecules that enable them to travel to peripheral tissues and exert their effector function. We analyzed whether deficient numbers of effector-type T cells within the malignant effusion might contribute to tumor escape from immunosurveillance.

**Experimental Design:** We analyzed the expression of a broad range of adhesion molecules and chemokine receptors (CD62L, CD56, CCR4, CCR5, CCR7, CXCR3, CLA, and integrin  $\alpha4\beta7$ ) on tumor-associated lymphocytes in effusions and peripheral blood lymphocytes of patients with malignant ascites ( $n = 11$ ) or malignant pleural effusion ( $n = 16$ ). A tumor-associated lymphocyte:peripheral blood lymphocyte ratio was calculated as an indicator for homing of lymphocytes into the effusions and was compared with patients with nonmalignant ascites ( $n = 17$ ).

**Results:** Patients with malignancies show an increased enrichment of T cells expressing the phenotype of “naïve” (CD62L+ and CD45RA+CCR7+), “central memory” (CD45RA-CCR7+), and type 2-polarized (CCR4+) T cells within their effusions. In contrast, enrichment of “effector”-type (CD45RA-CCR7- or CD45RA+CCR7-) and presumably type 1-polarized T cells (CCR5+) at the tumor site is deficient. The same is true for natural killer cells and potentially cytotoxic CD56+ T cells.

**Conclusions:** Here we show for the first time that patients with malignant effusions show a deficient enrichment of T cells expressing the phenotype of type-1-polarized effector T cells at the tumor site. This mechanism is likely to contribute to the escape of tumor cells from immunosurveillance.

**INTRODUCTION**

During the past decade accumulating evidence has indicated that the immune system can influence the initiation and development of cancer, and it is widely believed that T cells represent the most potent antitumor effector cells (1–3). Malignant tumors, however, possess an arsenal of mechanisms to evade immunosurveillance, absence of unique tumor antigens, down-regulation of target antigens or MHC molecules, insufficient expression of costimulatory and/or adhesion molecules, or secretion of immunosuppressive cytokines (4). In addition, it might be that the absence of “danger” signals in the tumor milieu prevents effector T cells from homing into the malignant tissue. Therefore, it seems to be of major importance to study mechanisms that allow T cells to travel to the tumor site and exert their effector function.

Malignant effusions are a relatively easily accessible source of tumor-associated T cells (TAL) and, thus, represent a suitable model for the study of interactions between tumor cells and the host immune system. Surprisingly, only a few studies have thus far examined the *ex vivo* phenotype (5–7) or function (8–10) of these cells, and no study has thus far investigated which homing receptors might characterize human TAL within malignant effusions.

A multistep process mediated by the interplay of adhesion molecules and chemokines that involves rolling, firm adhesion, and diapedesis results in the extravasation of immune effector cells into peripheral tissues (11). Adhesion molecules and chemokine receptors can be up-regulated or lost as cells differentiate, allowing leukocytes to coordinate their migratory routes with their immunological differentiation state. Thus, tissue-homing effector T cells express inflammatory chemokine receptors like CCR5 or CXCR3. In contrast, naïve T cells, that in search for antigen circulate between secondary lymphoid organs and the peripheral blood, express lymph node homing receptors like CD62L or CCR7 (12, 13). Here, we have analyzed a broad variety of chemokine receptors and adhesion molecules on lymphocytes infiltrating malignant or nonmalignant effusions.

In addition, the expression of different chemokine receptors on T cells might help to differentiate between type 1 or type 2 polarized T cells. Thus, CCR5 or CXCR3 seem to be expressed mainly by type-1 polarized T cells, which produce cytokines that support a specific T cell-mediated immune response (14). CCR4, on the other hand, represents a marker for T cells producing type 2 cytokines (14), a cytokine pattern that is thought to have a negative effect on T cell-mediated control of tumors. Performing an analysis of the expression of these chemokine receptors on CD4<sup>+</sup> or CD8<sup>+</sup> T cells, we intended to gain information on the cytokine pattern presumably produced by effusion infiltrating T cells.

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**Table 1** Diagnoses of patients with malignant ascites ( $n = 11$ ) and patients with malignant pleural effusions ( $n = 16$ )

Numbers of patients with the respective tumor type are shown in brackets.

Malignant ascites	Malignant pleural effusion
Gastric carcinoma (5)	Lung cancer (5)
Colorectal carcinomas (2)	Gastric carcinoma (3)
Cholangiocellular carcinoma (1)	Breast cancer (2)
Breast cancer (1)	Ovarian carcinoma (2)
Hepatocellular carcinoma (1)	Mesothelioma (1)
Neuro-endocrine carcinoma (1)	Sarcoma (1)
	Pancreatic carcinoma (1)
	Bone metastasis of unknown origin (1)

## PATIENTS AND METHODS

**Patients.** A total of 44 consecutive patients were included into the study. Among these were 17 patients with nonmalignant cirrhotic ascites, 11 patients with malignant ascites, and 16 patients with malignant pleural effusions (Table 1). All of the patients with nonmalignant peritoneal effusions had an alcohol-induced cirrhosis of the liver.

**Isolation of Mononuclear Cells from Peripheral Blood, Peritoneal, and Pleural Effusions.** Peripheral blood mononuclear cells were isolated from heparinized blood by Biocoll (Biochrom, Berlin, Germany) density gradient centrifugation and washed twice in PBS (Gibco, Paisley, United Kingdom). Specimens of peritoneal and pleural exudate were obtained from patients and were immediately centrifuged for 5 min at 1390 rpm. Cell pellets were then washed and were resuspended in 30 ml of PBS. The cell suspension was layered on discontinuous gradients containing 10 ml each of 100% and 75% Biocoll in 50-ml plastic tubes. After centrifuging at 1700 rpm for 30 min lymphocyte-enriched mononuclear cells were collected from the 100% interface, and tumor and mesothelial cells from the 75% interface.

**Flow Cytometry.** Peripheral blood lymphocytes of each patient were tested on the same day as the lymphocytes infiltrating the effusion. Cell fluorescence was measured using a FACSCalibur cytometer (Becton Dickinson, Heidelberg, Germany). Data analysis was performed using CELLQuest software (Becton Dickinson). Lymphocytes were generally stained with FITC-, PerCP-, or APC-conjugated monoclonal antibodies to CD3, CD4, CD8, CD25, and CD56 (Becton Dickinson, San Jose, CA) to determine lymphocyte subpopulations.

To investigate the expression of chemokine receptors and adhesion molecules we performed costaining with phycoerythrin-conjugated monoclonal antibodies to CCR4, CCR5, CXCR3, CD62L, CLA,  $\alpha 4\beta 7$  integrin, CD11a, and CD44 (Becton Dickinson). IgG isotype controls were used in all of the experiments. A total of  $1 \times 10^6$  peripheral blood mononuclear cells were washed in PBS, were resuspended in 100  $\mu$ l PBS containing 2% human serum, and were stained using the appropriate antibodies according to the manufacturer's instruction. After incubation on ice for 30 min, cells were washed, resuspended in 500  $\mu$ l PBS, and were analyzed using a morphological lymphocyte gate.

Costaining for CCR7 expression was performed by first

applying 6  $\mu$ l of a purified mouse antihuman CCR7 IgM antibody. After incubation for 30 min on ice and one wash, 1  $\mu$ l of a biotin-conjugated antimouse IgM (both Ab Pharmingen, San Diego, CA) were added to the cells. After another 30-min incubation period and one wash, 2  $\mu$ l streptavidin-phycoerythrin (Immunotech, Marseilles, France) were added, and cells were incubated for 25 min on ice.

To costain the intracellular  $\zeta$  chain, the patient's peripheral blood mononuclear cells were first fixed in 1% paraformaldehyde in PBS for 10 min at room temperature. After two washes, cells were permeabilized for 10 min in 100  $\mu$ l PBS containing 12.5  $\mu$ g digitonin. Cells were then stained with 10  $\mu$ l mouse-anti  $\zeta$  mAb (2H2D9; Immunotech) or mouse IgG isotype control for 10 min on ice.

To perform costaining with anti-granzyme A antibody, peripheral blood mononuclear cells were fixed using 2 ml of a 1:10 diluted fluorescence-activated cell sorter lysing solution (BD Biosciences) for 10 min at room temperature. After one wash, cells were permeabilized using 500  $\mu$ l of a 1:10 diluted permeabilizing solution (BD Biosciences) for 10 min at room temperature. After one more wash, cells were stained applying 5  $\mu$ l anti-Granzyme A antibody (BD Biosciences) for 15 min at room temperature.

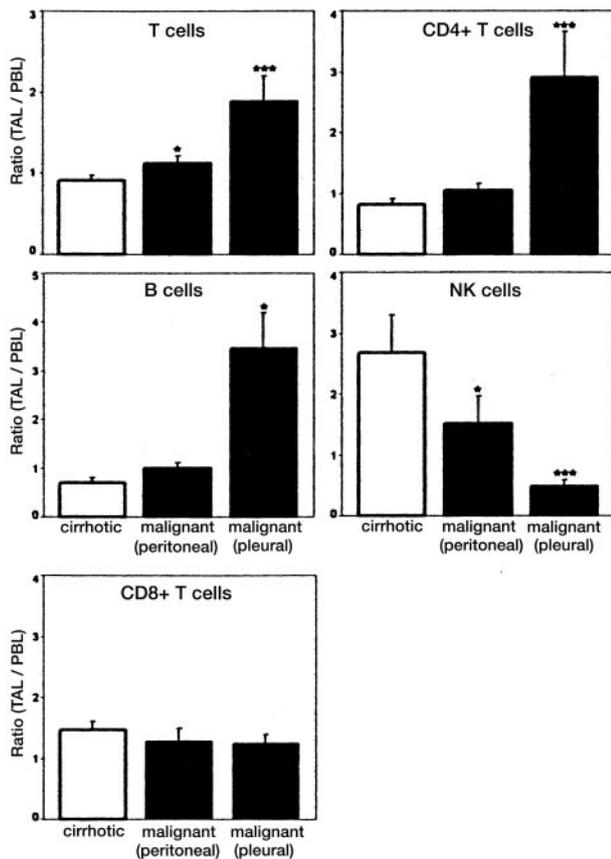
**Statistical Analysis.** The significance of observed differences was calculated using the Mann-Whitney  $U$  test. All of the differences with a  $P < 0.05$  were considered significant.

## RESULTS

**Cytological Analysis of Benign and Malignant Effusions.** Cytospin samples were prepared from all of the effusions, and cells were cytologically analyzed after staining with May-Grünwald-Giemsa. Effusions were only diagnosed as malignant when they clearly showed the presence of malignant cells. The features of the malignant cells were determined by the underlying tumor type, mostly arising from adenocarcinoma. All of the effusions, whether they were of benign or malignant origin, contained mesothelial and histiocytic cells. In addition to lymphocytes, which were present in all of the samples, almost all of the effusions contained some inflammatory cells such as neutrophils and eosinophils. Hemorrhagic effusions were excluded from the study to prevent contamination with intravascular lymphocytes.

**The Effusion:Peripheral Blood Ratio of Effector-Type Memory T Cells Is Reduced in Patients with Malignant Effusions.** In a first step, we asked whether a defective enrichment of effector-type T in the tumor environment might contribute to the escape of these malignancies from immunosurveillance. Therefore, an effusion:peripheral blood ratio of the percentages of different T-cell subtypes was calculated as an indicator for the enrichment of these cells around the tumor site.

Patients with malignant pleural effusion showed an intrapleural enrichment of CD3<sup>+</sup> T cells, which in the case of patients with pleural effusion was clearly caused by CD4<sup>+</sup> but not CD8<sup>+</sup> T cells. The same patient group also evidenced an enrichment of B cells within their effusion. In contrast, in both groups of patients with malignancies, the effusion:peripheral blood ratio of natural killer (NK) cells was reduced significantly compared with patients with benign disease (Fig. 1). Interest-



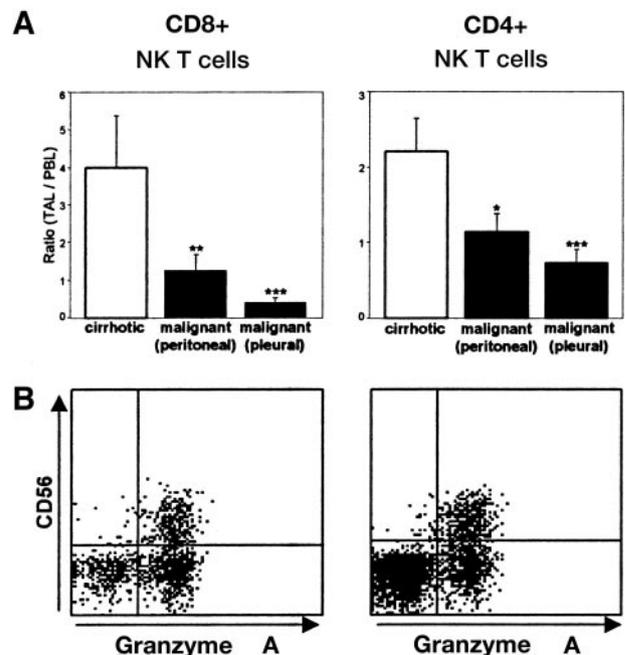
**Fig. 1** Ratio of lymphocyte subpopulations within the effusion and in the peripheral blood of patients with nonmalignant cirrhotic ascites ( $n = 17$ ), malignant ascites ( $n = 11$ ), and malignant pleural effusions ( $n = 16$ ). Percentages of T cells (CD3<sup>+</sup>), CD4<sup>+</sup> T cells (CD3<sup>+</sup>CD4<sup>+</sup>), CD8<sup>+</sup> T cells (CD3<sup>+</sup>CD8<sup>+</sup>), B cells (CD3<sup>+</sup>CD19<sup>+</sup>), and natural killer (NK) cells (CD3<sup>+</sup>CD56<sup>+</sup>) were determined using four-color flow cytometry and gating on morphologically defined lymphocytes. In each patient an effusion:peripheral blood ratio was calculated for all lymphocyte subsets. Bars represent mean values of this ratio and SE of means. \* indicate significant differences in cancer patients when compared with patients with benign effusions (\*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ). TAL, tumor-associated T cell; PBL, peripheral blood lymphocyte.

ingly, in both patient groups with malignancies, enrichment of CD4<sup>+</sup> or CD8<sup>+</sup> T cells expressing the NK cell marker CD56 at the tumor site was even more diminished (Fig. 2A). This finding may be of clinical importance, because we found these CD4<sup>+</sup> and CD8<sup>+</sup> NK T cells to contain high intracellular concentrations of granzyme A supporting their role as effector cells with a strong cytolytic capacity (Fig. 2B).

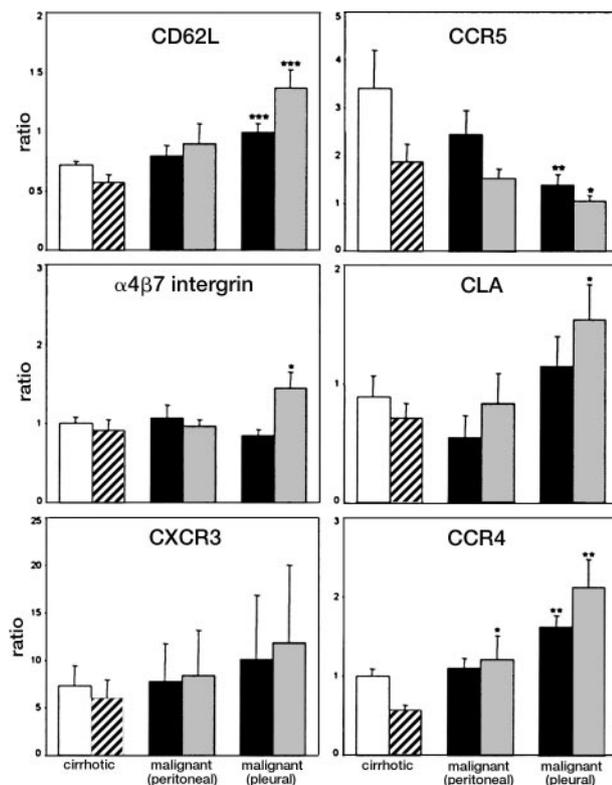
In accordance with the finding of a reduced homing of NK cells and NK T cells into their effusions, patients with malignant pleural effusions showed a diminished effusion:peripheral blood ratio of CCR5<sup>+</sup> effector-type CD8<sup>+</sup> T cells within their effusion. The same was true for CCR5<sup>+</sup> T-helper (Th) cells, presumably being Th1 type (Fig. 3). In contrast, enrichment of CD62L<sup>+</sup> noneffector-type CD4<sup>+</sup> and CD8<sup>+</sup> T cells within the malignant pleural effusion was strongly enhanced when compared with nonmalignant peritoneal effusions. Expression of  $\alpha_4\beta_7$  integrin plays an important role in tissue-specific homing

of memory T cells to Peyer's patches and to the intestinal lamina propria (15). We did not observe any differences between the effusion:peripheral blood ratio of T cells expressing this molecule in our patients with malignant or nonmalignant ascites. Patients with malignant pleural effusions, however, showed an increased enrichment of CD8<sup>+</sup> T cells expressing this marker within their effusion (Fig. 3). The same patients showed an increased ratio of effusion-infiltrating *versus* peripheral T cells expressing CLA, a marker usually expressed by skin-homing T cells.

T-cell mediated control of tumors is thought to be promoted by type 1 cytokine responses [*i.e.*, interleukin (IL)-2, IFN- $\gamma$ , and tumor necrosis factor  $\alpha$ ] and impaired by type 2 cytokine responses (*i.e.*, IL-4, IL-5, IL-6, IL-10, and IL-13). To elucidate the cytokine pattern presumably produced by the TAL of our patients, we analyzed the expression of CXCR3 and CCR4 on these cells. The effusion:peripheral blood ratio of potentially type 1 polarized CXCR3<sup>+</sup> T cells was comparable in patients with malignant and nonmalignant effusions. In contrast, we observed an increased enrichment of CCR4<sup>+</sup> T cells,



**Fig. 2** A, ratio of CD4<sup>+</sup> or CD8<sup>+</sup> natural killer (NK) T cells within the effusion and in the peripheral blood of patients with nonmalignant cirrhotic ascites ( $n = 17$ ), malignant ascites ( $n = 11$ ), and malignant pleural effusions ( $n = 16$ ). NK T cells were defined as CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> T cells expressing the NK cell marker CD56. Percentages of these cells were determined using four-color flow cytometry and gating on morphologically defined lymphocytes. In each patient an effusion:peripheral blood ratio was calculated for all lymphocyte subsets. Bars represent mean values of this ratio and SE of means. \* indicate significant differences in cancer patients when compared with patients with benign effusions (\*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ). B, intracellular concentration of granzyme A in CD56<sup>+</sup> NK T cells. Concentration of intracellular granzyme A was determined using flow cytometry and gating on morphologically defined lymphocytes, CD3<sup>+</sup>, and CD4<sup>+</sup> or CD8<sup>+</sup> cells. TAL, tumor-associated T cell; PBL, peripheral blood lymphocyte.



**Fig. 3** Ratio of T cells expressing different adhesion molecules within effusions and in the peripheral blood. T cells expressing the given marker were analyzed in patients with nonmalignant cirrhotic ascites ( $n = 17$ ), malignant ascites ( $n = 11$ ), and malignant pleural effusions ( $n = 15$ ). Percentages of CD4<sup>+</sup> (□ and ■) or CD8<sup>+</sup> (▨ and ▩) T cells expressing the given homing receptor were determined using four-color flow cytometry and gating on morphologically defined lymphocytes, CD3<sup>+</sup> T cells, and CD4<sup>+</sup> or CD8<sup>+</sup> cells. In each patient an effusion: peripheral blood ratio was calculated for all lymphocyte subsets. Bars represent mean values of this ratio and SE of means. \* indicate significant differences in cancer patients when compared with patients with nonmalignant effusions (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). □, CD4<sup>+</sup> T cells, cirrhotic patients. ▨, CD8<sup>+</sup> T cells, cirrhotic patients. ■, CD4<sup>+</sup> T cells, cancer patients. ▩, CD8<sup>+</sup> T cells, cancer patients.

presumably being type 2 polarized, within the effusions of both patient groups with malignancies (Fig. 3).

Finally, we examined the effusion:peripheral blood ratio of different memory/effector T-cell subtypes, defined by their expression of CD45RA and CCR7. In both patient groups we observed an enhanced enrichment of CCR7+CD45RA+ “naïve” and CD45RA-CCR7+ “central memory” T cells within the malignant effusions. In marked contrast, the effusion: blood ratio of “memory effector” type CD8<sup>+</sup> T cells was reduced in patients with malignant pleural effusions, and enrichment of terminally differentiated CD45RA+CCR7- CD4<sup>+</sup> and CD8<sup>+</sup> T cells within the malignant effusions was dramatically impaired in patients with malignant peritoneal and pleural effusions (Fig. 4).

We also analyzed differences in the percentages of lymphocyte subpopulations within the effusion of the three groups. Differences between groups were most pronounced in patients with malignant pleural effusions when compared with patients

with benign ascites. These patients showed elevated percentages of tumor-associated CD4<sup>+</sup> T cells and reduced levels of CD8<sup>+</sup> T cells within their pleural effusion (Table 2). However, both patients with malignant ascites and patients with malignant pleural effusions showed significantly lower levels of NK cells than patients with benign peritoneal effusions. In addition, patients with malignant pleural effusions evidenced drastically reduced percentages of potentially highly cytotoxic CD56<sup>+</sup> NK T cells when compared with effusion-infiltrating lymphocytes of patients with nonmalignant ascites.

Patients with malignant pleural effusions had higher percentages of presumably Th2-type CCR4<sup>+</sup> CD4<sup>+</sup> T cells and more noneffector type CD62L<sup>+</sup> CD8<sup>+</sup> T cells than patients with nonmalignant effusions (Table 3). In addition, the same patients showed elevated percentages of effusion-infiltrating CD45RA+CCR7+ naïve CD8<sup>+</sup> and CD45RA-CCR7+ central memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells within their effusion (Table 4). In marked contrast, patients with malignant pleural effusions evidenced dramatically reduced levels of terminally differentiated CD45RA+CCR7- CD4<sup>+</sup> T cells, and both patient groups with cancer had markedly lower levels of effector-type CD45RA+CCR7- CD8<sup>+</sup> T cells than patients with nonmalignant peritoneal effusions.

**Malignant Effusions Do Not Contain Higher Percentages of CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells.** CD4<sup>+</sup> T cells coexpressing the IL-2 receptor  $\alpha$  chain (CD25) have been suggested recently to represent suppressor T lymphocytes (16). We assessed the number of CD25<sup>+</sup> Th cells infiltrating the effusions of patients with malignant and nonmalignant disease. There were no significant differences in the percentages of effusion-associated CD4<sup>+</sup> T cells expressing CD25 between the three groups (data not shown).

**T cells associated with malignant and nonmalignant effusions show a reduced expression of the T-cell receptor (TCR)  $\zeta$  chain when compared with peripheral T cells.** A decreased or absent expression of the signal-transducing  $\zeta$  chain in CD4<sup>+</sup> or CD8<sup>+</sup> T cells as well as in NK cells has been described in patients with various malignancies, and it has been suggested that this phenomenon might be responsible for functional alterations of tumor-infiltrating lymphocytes (TIL) and TAL in these patients (17). We examined the intracellular expression of the TCR  $\zeta$  chain in the peripheral and the effusion-associated T cells of our patients. Nearly 100% of all CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the peripheral blood of all three patient groups expressed the TCR  $\zeta$  chain (Fig. 5). The vast majority of T cells infiltrating the malignant or nonmalignant effusions also expressed this signal-transducing molecule. However, in our patients with cirrhosis of the liver, slightly fewer effusion-associated CD4<sup>+</sup> and CD8<sup>+</sup> T cells were positive for the TCR  $\zeta$  chain (Fig. 5).

## DISCUSSION

Human solid tumors are often infiltrated by significant numbers of mononuclear cells, including T lymphocytes. These TIL have been the subject of great interest because of reports of an association between the presence of TIL and a favorable prognosis (1, 3). TIL can be used as a source of tumor-specific T cells which, upon *in vitro* culture in the presence of IL-2, exert

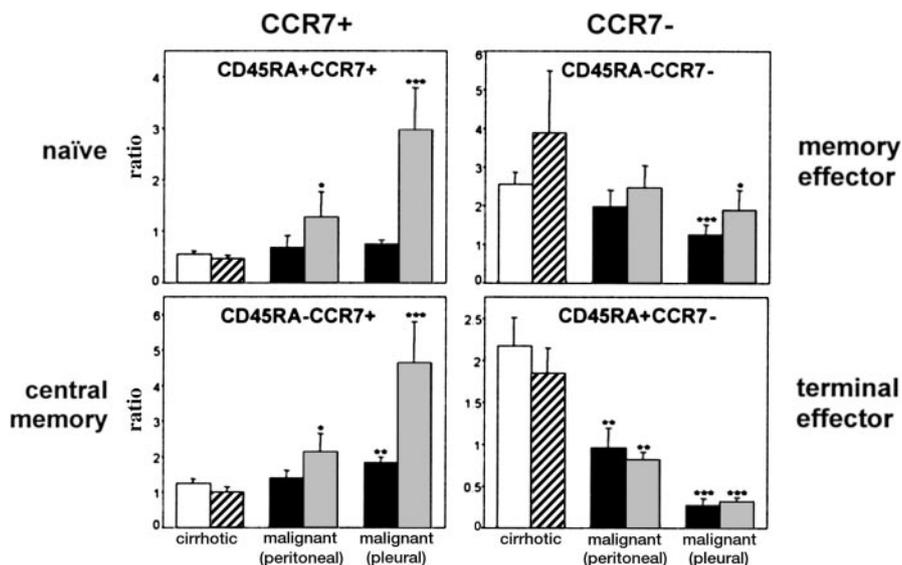


Fig. 4 Ratio of naïve:memory T-cell subsets within effusions and in the peripheral blood of patients with nonmalignant ascites ( $n = 17$ ), malignant ascites ( $n = 11$ ), and malignant pleural effusions ( $n = 15$ ). Percentages of CD4+ (□ and ■) or CD8+ T cells (▨ and ▩) expressing the given combination of markers were determined using four-color flow cytometry and gating on morphologically defined lymphocytes, CD3+ and CD4 or CD8. Costaining with CD45RA and CCR7 was applied to determine the composition of different memory T-cell subsets. In each patient an effusion:peripheral blood ratio was calculated for all lymphocyte subsets. Bars represent mean values of this ratio and SE of means. \* indicate significant differences in cancer patients when compared with patients with benign effusions (\*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$ ). □, CD4+ T cells, cirrhotic patients. ▨, CD8+ T cells, cirrhotic patients. ■, CD4+ T cells, cancer patients. ▩, CD8+ T cells, cancer patients.

Table 2 Percentages of lymphocyte subpopulations within the effusions

PBMC<sup>a</sup> of patients with nonmalignant ( $n = 17$ ) and malignant ( $n = 11$ ) ascites or pleural effusion ( $n = 16$ ) were stained using conjugated monoclonal antibodies against CD3, CD4, CD8, CD56, and CD19 and were analyzed using four-color flow cytometry. Data represent the mean percentage of cells expressing the given markers and SE in brackets.  $P$  values indicate significant differences in patients with malignant effusions when compared with patients with nonmalignant effusions.

	Nonmalignant	Malignant (peritoneal)	Malignant (pleural)
CD3+ Total T cells	68.9 (4.2)	70.4 (5.9)	79.6 (3.6)
CD3+CD4+ T cells	40.4 (4.2)	42.7 (5.5)	56.6 (4.7) <sup>b</sup>
CD3+CD8+ T cells	30.0 (2.5)	24.8 (4.0)	24.1 (3.4) <sup>b</sup>
CD3+CD8+CD56+ NK T cells	7.1 (1.7)	4.4 (1.1)	2.0 (0.6) <sup>c</sup>
CD3+CD4+CD56+ NK T cells	2.1 (0.4)	2.1 (0.8)	0.7 (0.2) <sup>c</sup>
CD3-CD56+ NK cells	24.9 (3.9)	13.3 (3.7) <sup>b</sup>	3.6 (1.1) <sup>c</sup>
CD3-CD19+ B cells	2.9 (0.8)	10.1 (5.1) <sup>b</sup>	6.0 (1.2) <sup>b</sup>

<sup>a</sup> PBMC, peripheral blood mononuclear cell; NK, natural killer.  
<sup>b</sup>  $P < 0.05$ .  
<sup>c</sup>  $P < 0.001$ .

antitumor activity. However, freshly *ex vivo* isolated TIL and TAL are often functionally impaired and do not fully respond in proliferation or cytotoxicity assays (8–10). One reason for this could be a deficient enrichment of antigen-specific effector/memory-type T cells within the local tumor environment.

As observed in previous studies, we found an enrichment of CD4+ T cells within the malignant pleural effusions (9, 18)

but not within malignant ascites (19). This increase in CD4+ T cells was caused by an increased effusion:peripheral blood ratio of CCR4+ Th cells. Moreover, we observed an increased enrichment of CD8+ T cells expressing CCR4 within the effusions

Table 3 Percentages of T cells expressing different homing receptors within the effusions

Effusion-infiltrating lymphocytes of patients with nonmalignant ( $n = 17$ ) and malignant ( $n = 11$ ) ascites or pleural effusion ( $n = 15$ ) were stained using conjugated monoclonal antibodies against CD3, CD4, and CD8, and the given homing receptor. Events were analyzed using flow cytometry a combination of a morphological lymphocyte gate and a gate for CD3+CD4+ or CD3+CD8+ cells. Results represent the mean percentage of CD4+ or CD8+ T cells expressing the given marker and SE in brackets.  $P$  values indicate significant differences in patients with malignant effusions when compared to patients with nonmalignant effusions.

		Nonmalignant	Malignant (peritoneal)	Malignant (pleural)
CD62L	CD4+	55.9 (3.9)	51.5 (5.3)	66.7 (5.5)
	CD8+	31.0 (3.2)	33.9 (4.3)	58.3 (7.0) <sup>a</sup>
CCR5	CD4+	37.6 (2.8)	45.2 (5.8)	28.7 (3.8)
	CD8+	51.7 (4.4)	62.5 (6.5)	50.5 (5.0)
α4β7	CD4+	31.3 (2.1)	36.0 (6.0)	32.3 (2.1)
	CD8+	40.4 (4.0)	43.1 (3.2)	48.8 (3.9)
CLA	CD4+	6.0 (1.0)	2.4 (0.5) <sup>b</sup>	7.7 (1.3)
	CD8+	3.8 (1.3)	1.3 (0.2)	8.5 (2.4)
CXCR3	CD4+	43.8 (3.7)	35.5 (10.1)	44.4 (8.3)
	CD8+	46.1 (5.4)	56.2 (9.8)	64.0 (7.5)
CCR4	CD4+	32.5 (2.4)	38.6 (5.0)	46.8 (4.1) <sup>a</sup>
	CD8+	8.4 (1.5)	5.6 (1.0)	20.8 (7.8)

<sup>a</sup>  $P < 0.01$ .  
<sup>b</sup>  $P < 0.05$ .

Table 4 Percentages of naïve/memory T-cell subtypes within the effusions

PBMC<sup>a</sup> of patients with nonmalignant ( $n = 17$ ) and malignant ( $n = 11$ ) ascites, or pleural effusion ( $n = 15$ ) were stained using conjugated monoclonal antibodies against CD3, CD4, CD8, CD45RA, and CCR7. A FACS analysis was performed using a combination of a morphological lymphocyte gate and a gate for CD3+CD4+ or CD3+CD8+ cells. Results represent the mean percentage of CD4+ or CD8+ T cells expressing the combination CD45RA and CCR7 defining the memory/effector subsets. Data represent mean values and SE in brackets. *P* values indicate significant differences in patients with malignant effusions when compared to patients with nonmalignant effusions.

		Nonmalignant	Malignant (peritoneal)	Malignant (pleural)
CD45RA+CCR7+	CD4+	24.8 (3.5)	20.6 (4.3)	25.4 (3.5)
Naïve	CD8+	16.5 (2.5)	22.2 (4.6)	37.2 (4.3) <sup>b</sup>
CD45RA-CCR7+	CD4+	42.9 (3.8)	43.1 (4.5)	56.4 (3.0) <sup>c</sup>
Central memory	CD8+	9.8 (2.3)	8.9 (1.8)	26.9 (4.1) <sup>b</sup>
CD45RA-CCR7-	CD4+	20.2 (2.7)	27.7 (4.7)	15.1 (2.9)
Memory effector	CD8+	19.9 (2.9)	31.6 (6.4)	17.0 (2.7)
CD45RA+CCR7-	CD4+	12.0 (2.0)	8.5 (2.3)	3.0 (8.2) <sup>b</sup>
Terminal effector	CD8+	53.9 (4.8)	37.3 (5.6) <sup>d</sup>	18.9 (3.9) <sup>b</sup>

<sup>a</sup> PBMC, peripheral blood mononuclear cell; FACS, fluorescence-activated cell sorter.

<sup>b</sup>  $P < 0.001$ .

<sup>c</sup>  $P < 0.01$ .

<sup>d</sup>  $P < 0.05$ .

of both patients with malignant pleural effusions and patients with malignant ascites.

CCR4 has been described to be expressed preferentially by Th2-type CD4<sup>+</sup> T cells (14, 20–22), and increased numbers of CCR4<sup>+</sup> Th cells have been observed in patients with Th2-dominated diseases (20, 23, 24). Our finding of an enrichment of Th2-type T cells within malignant effusions is supported by a previous study reporting an increased production of Th2-type cytokines by CD4<sup>+</sup> T cells derived from malignant effusions when compared with TAL from nonmalignant effusions (25). In addition, it has been shown that malignant effusions contain higher levels of Th2 promoting cytokines (18, 26) and lower levels of Th1 promoting cytokines (27, 28) than nonmalignant effusions. This prevalence of a Th2-type milieu favors a B cell-mediated response, a concept that seems to be supported by our finding of an increased homing of B cells into malignant pleural effusions. T cell-mediated control of tumors, however, is thought to be impaired by type 2 cytokine responses.

CXCR-3 and CCR5 are chemokine receptors preferably expressed by antigen-experienced effector type 1-polarized CD8<sup>+</sup> T cells (14, 29, 30) and Th1-type effector CD4<sup>+</sup> T cells (14, 20–22, 31–33). Whereas T cells within secondary lymphoid organs are negative for both markers, the vast majority of antigen-experienced T cells infiltrating nonlymphoid tissues usually express these chemokine receptors (34, 35). Thus, in humans a predominance of CXCR3- and CCR5-expressing T cells has been described in inflammatory infiltrates (35–38), and T cells expressing these markers are more likely to mediate an effective T cell-based antitumor response. We did not observe any differences between the effusion:peripheral blood ratios of CXCR3+ T cells in patients with malignant effusions compared with patients with nonmalignant disease. However, the enrichment of CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressing CCR5 within malignant pleural effusions was significantly impaired when compared with nonmalignant effusions.

Adhesion molecules and chemokine receptors can be up-regulated or lost as cells differentiate, allowing leukocytes to coordinate their migratory routes with their immunological dif-

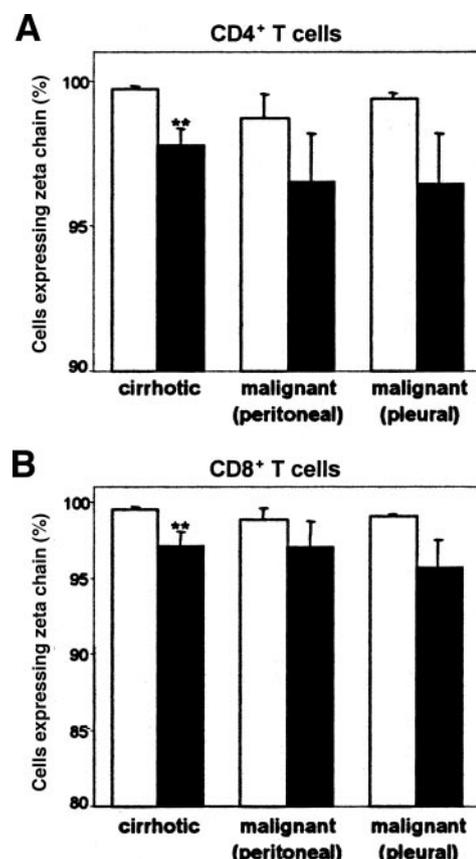


Fig. 5 Percentage of CD4<sup>+</sup> or CD8<sup>+</sup> T cells expressing the T-cell receptor  $\zeta$  chain in the peripheral blood ( $\square$ ) and within effusions ( $\blacksquare$ ). T cells in patients with nonmalignant cirrhotic ascites ( $n = 14$ ), malignant ascites ( $n = 9$ ), and malignant pleural effusions ( $n = 7$ ) were analyzed using four-color flow cytometry and gating on morphologically defined lymphocytes, CD3+ T cells, and CD4+ or CD8+ cells. Bars represent mean values and SE of means. \* indicate significant differences between  $\zeta$  chain expression in T cells within the effusion when compared with  $\zeta$  chain expression in peripheral T cells (\*\*,  $P < 0.01$ ).  $\blacksquare$ , peripheral blood lymphocytes;  $\square$ , effusion-associated lymphocytes.

ferentiation state. Thus, tissue-homing effector T cells express inflammatory chemokine receptors like CCR5 or CXCR3. In contrast, naïve T cells, which, in search for antigen circulate between secondary lymphoid organs and the peripheral blood, express lymph node homing receptors (13). It has been suggested recently that the pattern of expression of the lymph node homing receptor CCR7 and CD45RA divides human CD4<sup>+</sup> and CD8<sup>+</sup> T cells into distinct subsets (39). Both the CD45RA+CCR7+ naïve as well as the CD45RA-CCR7+ central memory fractions circulate between the peripheral blood and lymphoid tissue. In accordance with this, both cell types to a large extent also express lymph node homing molecule L-selectin (CD62L). CCR7+ or CD62L+ cells, however, do not infiltrate tissue and do not possess any significant effector function (12). In contrast, it has been shown that peripheral CD8<sup>+</sup> T cells expressing effector function against viral (40–44) or tumor antigens (45, 46) are almost uniformly negative for CD62L and CCR7.

We observed an augmented enrichment of CD62L+ non-effector-type CD4<sup>+</sup> and CD8<sup>+</sup> T cells within malignant pleural effusions. In accordance, we found an increased enrichment of naïve CD45RA+CCR7+ CTL within malignant peritoneal and pleural effusions. The effusion:peripheral blood ratio of CD45RA-CCR7+ memory effector T cells, however, was significantly lower in patients with malignant pleural effusions than in patients with nonmalignant peritoneal effusions. Moreover, numbers of terminally differentiated effusion-associated CD4<sup>+</sup> and CD8<sup>+</sup> T cells were dramatically reduced in both patients with malignant pleural effusions and in patients with malignant ascites.

NK T cells are phenotypically and functionally diverse (47). Initially, NK T cells were described as cells that express an invariant TCR V<sub>α</sub>14 in mouse and V<sub>α</sub>24 in humans (48). The existence of NK T cells expressing a variety of TCRs has been demonstrated recently (49). In humans, CD56<sup>+</sup> T cells represent one of these NK T-cell subpopulations.

CD56<sup>+</sup> T cells have long been thought to mediate only NK-like MHC-unrestricted cytotoxicity, *e.g.*, against tumor cell targets (50–52). Only recently, it has been demonstrated that CD56<sup>+</sup> NK T cells at the same time provide very potent MHC-restricted cytotoxicity (53–55), and it has been suggested that specific T cells with this kind of dual function are potentially of great clinical importance as they have a backup mechanism that may go into action when tumor cells escape specific killing by down-regulating their HLA-class I molecules (53, 54).

It has been proposed that peripheral blood CD56<sup>+</sup> NK T cells represent the currently circulating effector lymphocytes (56), specifically, *e.g.*, for viral (57, 58) or tumor antigens (58), and it has been shown that these effector cells are elevated in the peripheral blood of cancer patients (59). In both of our patient groups with malignancies, however, we not only observed a strongly deficient enrichment of CD56+ NK cells but also of CD8<sup>+</sup> T cells expressing CD56 at the tumor site.

Moreover, the same was true for CD4<sup>+</sup> T cells expressing this NK cell marker. In this context, it is noteworthy that CD56 has been shown to be expressed by peripheral (60, 61) and tissue-infiltrating (62) CD4<sup>+</sup> T cells with a strong cytotoxic potential and a Th1 cytokine profile. This finding is in agree-

ment with our observation of high concentrations of intracellular granzyme A within these CD4<sup>+</sup> T cells, a protein that is found exclusively in the cytoplasmic granules of cytolytic cells (63).

Could a deficient homing of effector-type T cells into malignant effusion be a reason for the herein described reduced numbers of these cells in the tumor environment? Activated in secondary lymphatic organs, T cells are armed with a number of potent effector mechanisms, including the capacity to produce immunoregulatory cytokines and induce apoptosis of target cells. These antigen-experienced T cells are equipped with chemokine receptors and adhesion molecules that allow them to efficiently relocate to the precise site of occurrence of antigenic material in the periphery (12). It has been suggested that an unspecific inflammatory response toward a malignant tumor might help to attract antigen-specific T cells. Thus, it has been shown that in the hepatic tissue of patients with alcoholic liver disease, increased levels of chemokines like monocyte chemoattractant protein-1, and macrophage inflammatory protein-1 $\alpha$  and -1 $\beta$  attract immune cells and cause chronic inflammation (64). In contrast, it has been suggested recently that many cancers might be characterized by dysregulated production of chemokines, and abnormal chemokine receptor signaling and expression (65). Therefore, the lack of a strong inflammatory “danger signal” at the tumor site might have led to an impaired homing of effector T cells into the local tumor environment in our patients. This impaired homing mechanism might, in turn, explain the main finding of this study, the reduced numbers of effector-type T cells infiltrating malignant effusions.

Other factors may contribute to the lack of an effective antitumor response in patients with cancer. Several reports have demonstrated a decreased expression of the  $\zeta$  chain in TIL (10), TAL (9, 66), and even in the peripheral blood lymphocytes of patients with solid tumors (17), and a reduced expression of  $\zeta$  has been suggested to predict a poorer prognosis (17). The TCR-associated  $\zeta$  chain is responsible for transduction of signals delivered via the receptors, and, therefore, its expression is important for activation of T cells. However, the finding of a down-regulation of  $\zeta$  in cancer patients is not undisputed. Thus, a number of studies could not confirm changes in the expression of the signal-transducing  $\zeta$  chain in lymphocytes of patients with solid tumors (67, 68). In agreement with these studies, we observed a normal expression of the  $\zeta$  chain in T cells in the peripheral blood of patients with solid tumors. When we compared the number of  $\zeta$  chain-expressing T cells in the peripheral blood to the number of effusion-associated T cells expressing this molecule, we found a slightly lower expression in T cells infiltrating cirrhotic ascites. This finding seems to support other studies showing that a reduction in  $\zeta$  chain expression is not specific for T cells in cancer patients but can also occur under different pathological conditions, *i.e.*, systemic infections (69).

In conclusion, we showed in this study that patients with malignant effusions evidenced a deficient enrichment of effector-type and presumably type 1-polarized T cells in the tumor environment. In contrast, the same patients demonstrated an increased enrichment of naïve and type 2-polarized T cells within their effusions. Future studies should investigate which local factors are responsible for impaired numbers of effector-type T cells in the tumor environment.

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