Pergamon

European Journal of Cancer, Vol. 35, No. 5, pp. 721–726, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(99)00033-7

Original Paper

Characterisation of Tumour Infiltrating Lymphocytes and Correlations with Immunological Surface Molecules in Colorectal Cancer

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Using flow cytometry, we studied the phenotype of tumour infiltrating lymphocytes (TILs) in 41 enzymatically dissociated colorectal cancers and compared this to the expression of HLA class I and II and CD80 on tumour cells. We studied the possible enzymatic damage to various surface markers after enzymatic dissociation. The reproducibility of flow cytometric determinations obtained from TILs was good (kappa value: 0.79). The median $CD4^+/CD8^+$ ratio was 2.2. Approximately 43–45% (median of cells in each tumour) of both the $CD4^+$- and the $CD8^+$-TILs expressed HLA class II; 14.2% of the $CD4^+$-TILs expressed CD25 and none of the $CD8^+$-TILs expressed CD25. $CD3^-/CD16^+/CD56^+$-TILs were very infrequent. Expression of HLA class II did not correlate with any lymphocyte surface markers. Since TILs are ''turned off'' rather than stimulated when tumour cells express HLA class II but not CD80, the lack of correlations could be due to anergy. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: colorectal cancer, tumour infiltrating lymphocytes, flow cytometry, enzymes, surface markers
Eur J Cancer, Vol. 35, No. 5, pp. 721–726, 1999

INTRODUCTION

SEVERAL ATTEMPTS have been made to clarify the immune response to colorectal cancers. Between 10 and 20% of tumours have been found to have a strong expression of HLA class II molecule and this has been associated with an improved prognosis in two studies [1, 2] whilst one study did not confirm this [3]. Expression of the costimulatory molecules CD80 and CD86 has not been found either on colorectal cancer cells [4] or on the dendritic cells infiltrating tumours [5].

Pronounced lymphocytic infiltration in colorectal cancer has been shown to be an important prognostic parameter [6, 7]. However, it has not been possible to demonstrate that predominance in the infiltrate of either one or the other T-cell subpopulation is consistently related to a better prognosis or survival [8–10]. So far, correlations between surface markers on tumour cells and the phenotype of tumour infiltrating lymphocytes (TILs) have not been reported. In relation to these findings, the aim of the present study was to clarify whether subclasses of TILs express activation markers and to clarify correlations between expression of the surface markers of both TILs and tumour cells.

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Received 22 Oct. 1998; revised 4 Jan. 1999; accepted 8 Jan. 1999.

MATERIALS AND METHODS

Patients and tissue samples

Tumour tissue was obtained from 41 patients (15 women and 26 men) with colorectal adenocarcinoma, all undergoing surgery in 1995 at either Odense University Hospital or at Svendborg Hospital. The mean age was 66 years (range 35–92 years). Histopathological examination and staging of the tumours were performed on routine fixed and paraffin-embedded sections stained with haematoxylin and eosin. 26 tumours were confined to the rectum, 9 to the sigmoideum and 6 to the remaining colon. All carcinomas were staged

Table 1. *Proportion of tumour cells expressing HLA class I and II and CD*80 *in* 41 *tumours* [4]

	Proportion of tumour cells positive				
	0–1%	1–33%	33–66%	66–99%	99–100%
HLA class I	0	1	2	3	35
HLA class II	0	25	10	6	0
CD80	37	4	0	0	0

according to Dukes [11], as modified by Turnbull (stage D, meaning distant metastasis or unresectable tumours because of parietal invasion) [12]: 5 Dukes' A, 17 Dukes' B, 10 Dukes' C and 9 Dukes' D. For the present study, a sample of viable non-necrotic tumour tissue was taken from the tumour edge immediately after removal. The tumour cells have been previously characterised by flow cytometry [4] (Table 1).

Peripheral blood lymphocytes (PBLs)

Venous blood was collected in citrate phosphate dextrose (CPD)-adenine tubes (Terumo Europe n.v., Leuven, Belgium) from healthy blood donors. Peripheral blood lymphocytes (PBL) were obtained by Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation at 1000 ***g*** for 15 min, then washed twice with RPMI 1640 (Gibco, Inchinnan, Scotland).

For use as positive controls, PBLs were stimulated for two days with phytohaemagglutinin (PHA, catalogue no. 670-0576AD, Gibco, for stock solution PHA was diluted in 10 ml sterile water). The culture medium was RPMI 1640 supplemented with glutamine (280 mg/ml), pyruvate, antibiotics (100 U penicillin/ml, 100 μg streptomycin/ml, 2.5 μg fungizone/ml) (all Gibco), 10% human heat-inactivated AB-serum and 1 ml aliquot PHA/50 ml medium.

Antibodies

The monoclonal antibodies used are summarised in Table 2. Isotype matched antibodies (Dako, Glostrup, Denmark) were used to manage background reactivity. In order to evaluate the antibodies and to estimate the optimal dilutions prior to use, all antibodies were tested at different concentrations with known positive cells as controls.

Enzymes

Collagenase type 1320 μg/ml, deoxyribonuclease type 1535 μg/ml and hyaluronidase 500 μg/ml (all Sigma, St. Louis, Missouri, U.S.A.) supplemented in RPMI 1640 (Gibco) were used to dissociate the solid tumour tissue.

Influence of the enzyme preparation on lymphocytes and surface markers

The influence of the enzyme preparations was investigated using PBLs. Freshly harvested unstimulated and PHA-stimulated PBLs were either enzymatically digested for 1 or 2 h in a rotating chamber at 37°C or left unmanipulated. The cell suspension was then washed and cryopreserved (50% human heat-inactivated AB-serum, 40% RPMI 1640, Gibco and 10% DMSO (Merck, Darmstadt, Germany) in liquid nitrogen (− 196°C) until flow cytometry.

On the day of use the cells were thawed and counted. The unfixed cells (unmanipulated, 1 and 2 h of digestion) were distributed in Falcon tubes (approx. 10^6 cells per tube) and centrifuged (200 ***g*** for 5 min). The pellet was resuspended in 5–10 μl antibody (Table 2) and incubated for 30 min in the dark at room temperature. The cell suspension was washed once more before being used in the FACScan (Becton Dickinson, Belgium). Cell acquisition was carried out within 1 h. The data were obtained and analysed by CellQuest (Becton Dickinson). Calibrite beads (Becton Dickinson) were used as an external standard. Gating on the lymphocytes in the forward/

Table 2. *Characteristics of the monoclonal antibodies used*

Antibody, anti-	Clone	Firm	Dye	Working dilutions	Control cell	Presumed cellular distribution
CD3	UCHT1	Dako	RPE-Cy5	1:1	PBL	T-lymphocytes
CD3	SK7	BD	FITC	1:2	PBL	T-lymphocytes
CD4	MT310	Dako	FITC, RPE, RPE-Cy5*	1:2, 1:2, 1:1	PBL	T_h-lymphocytes
CD8	DK25	Dako	FITC, RPE, RPE-Cy5	1:1, 1:1, 1:2	PBL	CTL
CD16	B73.1	BD	RPE	1:2	PBL	NK-lymphocytes
CD25	2A3	BD	RPE	1:2	PBL, PHA stimulated	Activated lymphocytes
CD38	AT13/5	Dako	FITC	1:1	PBL, PHA stimulated	Activated lymphocytes
CD45RA	L48	BD	FITC	1:1	PBL	Naive T-lymphocytes
CD45R0	UCHL1	Dako	FITC	1:2	PBL	Memory T-lymphocytes
CD56	MY31	BD	RPE	1:2	PBL	NK-lymphocytes
CD69	L78	BD	RPE	1:1	PBL, PHA stimulated	Activated lymphocytes
CD71	Ber-T9	Dako	FITC	1:1	PBL, PHA stimulated	Activated lymphocytes
CD80	L307.4	BD	RPE	1:1	T2	Antigen presenting cells
TcRα/β	WT31	BD	FITC	1:1	PBL	T-lymphocytes
TcRγ/δ	11F2	BD	RPE	1:1	PBL	T-lymphocytes
HLA-ABC	W6/32	Dako	FITC/RPE	1:4	PBL	Nuclear cells
HLA-DP, DQ, DR	CR3/43	Dako	FITC	1:1	PBL, PHA stimulated	Activated lymphocytes, APC

CD3 signal transducing unit for the T-cell receptor, CD4 receptor for HLA-DP, DQ, DR, CD8 receptor for HLA-ABC, CD25 receptor for IL-2, CD71 receptor for transferrin, HLA-DP, DQ, DR tissue histocompatibility antigens. BD, Becton Dickinson, Belgium; FITC, fluorescein isothiocyanate; RPE, R-phycoerythrin; RPE-Cy5, R-phycoerythrin-cyanin; PBL, peripheral blood lymphocytes; PHA, phytohaemagglutinin; CTL, cytotoxic T-lymphocytes; NK, natural killer lymphocytes; T2 is a human B and T lymphoblast hybrid cell line positive for CD80; APC, antigen presenting cells. *A generous gift from Dako.

side scatter, non-specific binding was set to 1%. Electronic compensation was calibrated using a preparation of lymphocytes incubated with FITC-conjugated anti-CD8, RPE conjugated anti-CD4 and RPE-Cy5 conjugated anti-CD3. Gating on the lymphocytes, three histograms were performed, one for each tube (unmanipulated, 1 and 2 h of digestion) and mean fluorescence intensity (MFI) was measured.

Flow cytometry of TILs

Preparation of single cell suspensions from the solid tumours were performed as previously described [13]. In brief, the tumour tissue was cut into small pieces with a pair of scissors, followed by incubation with the enzyme preparation in a rotating chamber at 37°C. After 1 h, the cell suspension was filtered, and washed twice. Remaining tumour tissue was dissociated for another hour. The cell suspensions were pooled and cryopreserved. Flow cytometry was performed as outlined above. The antibodies anti-CD4, anti-CD8, anti-CD16/56, anti-TcRα/β, anti-TcRγ/δ were used in a two- or three-colour combination with anti-CD3. The antibodies anti-CD25, anti-CD38, anti-CD45RA, anti-CD45RO, anti-CD71 and anti-HLA-DP/DQ/DR were used in a three-colour combination with anti-CD4 and anti-CD8. Gating on the lymphocytes in the forward scatter/side scatter plot, dot-plots combining the different fluorescences (FITC, RPE, RPE-Cy5) were performed and four quadrants were created (Figure 1).

Statistical evaluation

Reproducibility was studied calculating the kappa-value; 1.00 when agreement is perfect and zero when no agreement. Correlation coefficients were calculated using the Spearman's two-tailed rank correlation test. Included in the analyses were TIL phenotype and expression of HLA class I, HLA class II, CD80 on the tumour cells.

RESULTS

Enzymatic damage of lymphocytes and surface molecules

After 1 h of stirring and digestion, the MFI of anti-CD3, anti-CD45RO, anti-CD71, anti-CD80, anti-TcRα/β, anti-TcRγ/δ, anti-HLA-ABC and anti-HLA-DP/DQ/DR MAbs was unaffected, and after 2 h slightly decreased in some cases (Table 3). The MFI of anti-CD4, anti-CD8, anti-CD16/56, anti-CD25, anti-CD38, anti-CD45RA and anti-CD69 MAbs was reduced after 1 h of dissociation, and seriously decreased after 2 h. Only anti-CD69 MAb was decreased to the level of the isotype control signal after 1 h of dissociation (see Figure 2).

We next examined the possible regeneration in culture of CD4 and CD8 molecules on enzyme treated lymphocytes. During several days of culture, the MFIs gradually increased, but, only 50% of the lymphocytes survived the enzyme treatment, the cryopreservation and the succeeding culture (data not shown). Since this cell loss might have changed the original phenotype significantly, and since the surface molecules were not totally disrupted by the enzymes, we chose not to culture the TILs prior to flow cytometry.

Reproducibility of data obtained by flow cytometry

Cell suspensions from 12 tumours were measured twice for the expression of CD25, CD45RO and HLA-DP/DQ/DR on

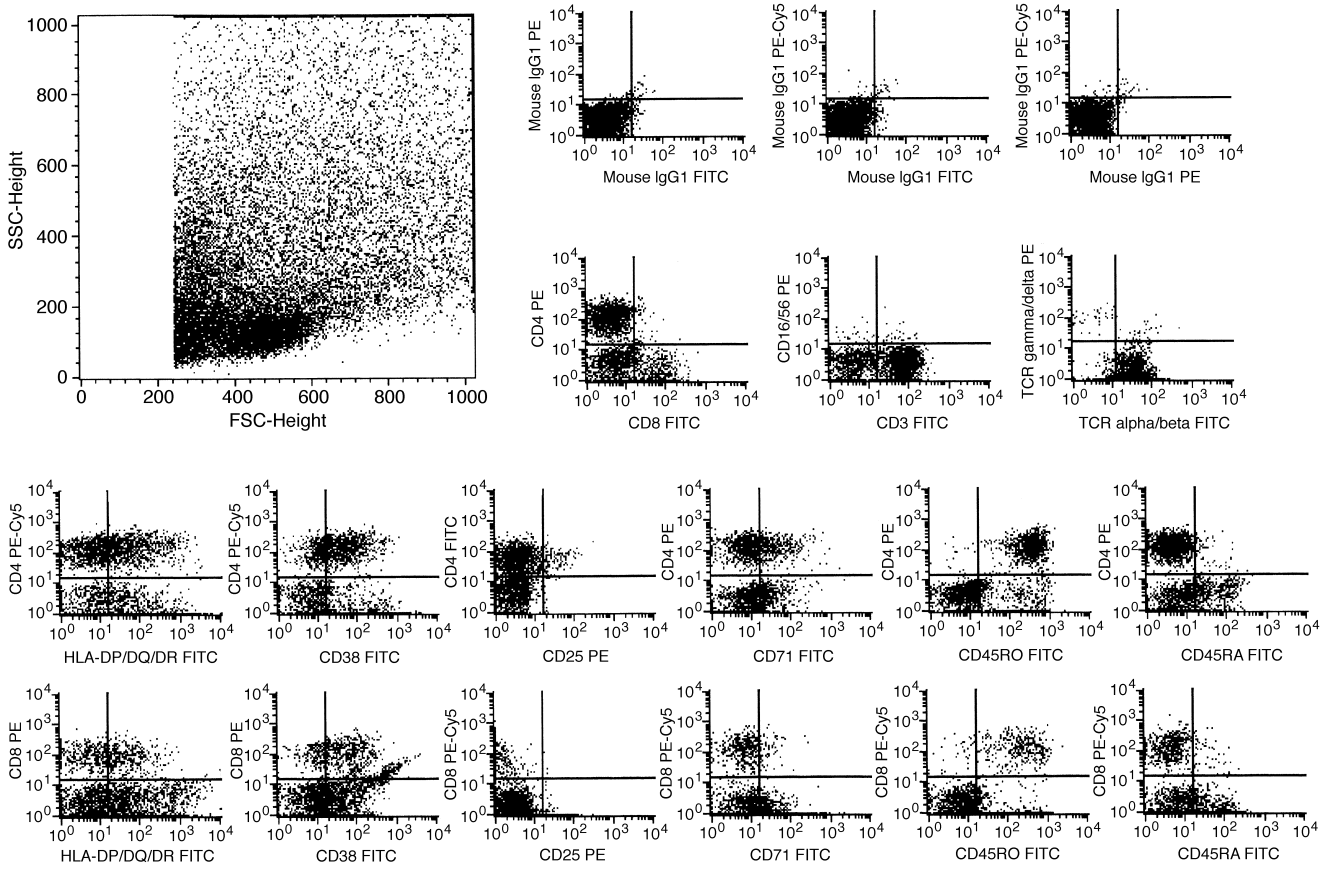

Figure 1. Forward scatter/side scatter and fluorescence dot-plots of dissociated tumour tissue. The quadrants were set according to the calibration of the FACScan.

Table 3. *Effects of the enzyme preparation on the intensity of surface marker expression by peripheral blood lymphocytes, PHA stimulated peripheral blood lymphocytes and T*2

	Enzyme treatment	
Antibody, anti	for 1 h	for 2 h
CD3	100%	80%
CD4	38%	15%
CD8	18%	13%
CD16/56	32%	33%
CD25	55%	29%
CD38	69%	23%
CD45RA	60%	48%
CD45RO	100%	94%
CD69	8%	4%
CD71	100%	70%
CD80	100%	100%
TcRα/β	100%	73%
TcRγ/δ	100%	100%
HLA-ABC	100%	100%
HLA-DP, DQ, DR	100%	76%

Numbers are (MFI after enzyme treatment/MFI before enzyme treatment)×100. PHA, phytohaemagglutinin; MFI, mean fluorescence intensity.

both $CD4^+$-TILs and $CD8^+$-TILs in different preparations and on different days. There was good agreement between two estimations of MFIs flow cytometry estimates—when dividing the estimates in five groups ('1' no positive cells; '2' < 1/3 positive; '3' > 1/3, < 2/3; '4' > 2/3; '5' all cells positive) a kappa value of 0.79 was obtained.

Phenotypes of TILs and PBLs

The phenotypes are shown in Table 4. In the forward/side scatter gate, 60.3% of the TILs and 74.9% of the PBLs were $CD3^+$. In the tumours $CD4^+$-TILs were predominant ($CD4^+/CD8^+$ ratio 2.2, range 0.8–6.8), while in the peripheral blood the $CD4^+/CD8^+$ ratio was 1.1 (range 0.3–2.1). $CD3^-/CD16^+/CD56^+$-TILs were very infrequent (median 0.6%) compared with $CD3^-/CD16^+/CD56^+$-PBLs (median 14.6%). Distribution of $TcR\alpha/\beta^+$-lymphocytes and $TcR\gamma/\delta^+$-lymphocytes in TILs and PBLs were similar. In tumours, the CD45RO phenotype was more common than the CD45RA phenotype, in contrast to blood where the distributions were similar. 14.2% of TILs and 10.3% of PBLs which expressed $CD4^+$ also expressed CD25, whilst no $CD8^+$-lymphocytes expressed CD25. Approximately 43–45% of the $CD4^+$- and the $CD8^+$-TILs expressed HLA class II antigens, whilst 10.7% of the $CD4^+$-PBLs and 28.8% of the $CD8^+$-PBLs expressed HLA class II. No TILs expressed CD80.

Correlations between flow cytometry data, pathological characteristics and survival

The correlation matrix is shown in Table 5. Within the $CD4^+$-TIL and the $CD8^+$-TIL populations, expression of the different activation markers correlated significantly with each other. Expression of HLA class I on tumour cells correlated significantly with the $CD4^+/CD8^+$ ratio, whilst expression of HLA class II did not correlate with any lymphocyte surface markers. Expression of CD71 on both $CD4^+$- and the $CD8^+$-TILs correlated with a deficiency of HLA class I at the tumour cells. CD45RA and CD45RO were not significant correlated with any markers. The Dukes' stage and intensity

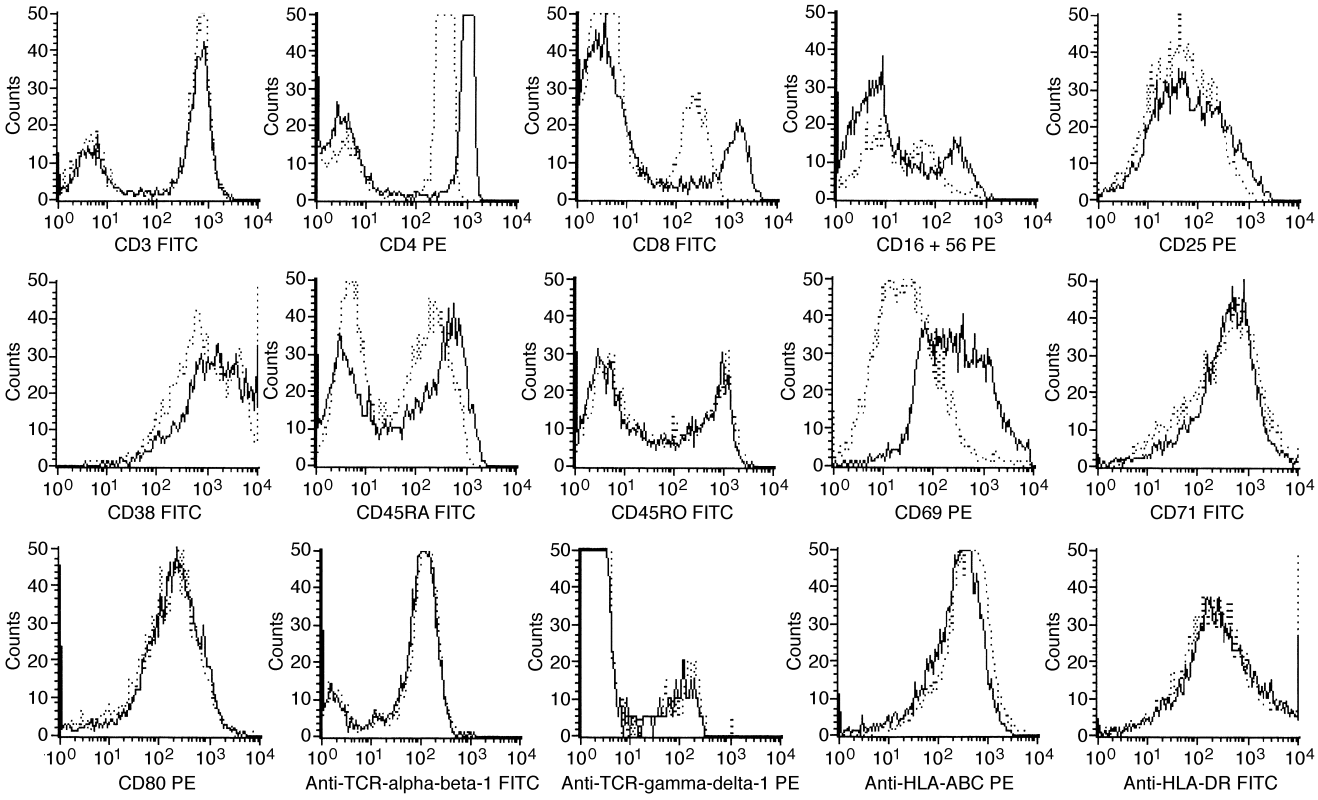

Figure 2. Histograms of the intensity of surface marker expression before and after treatment with the enzyme preparation. _____, no enzyme treatment; ..... 1 h of enzyme treatment.

Table 4. *Distribution of lymphocyte subsets among tumour infiltrating lymphocytes (TIL) in colorectal cancer* (n = 41) *and peripheral blood lymphocytes (PBL)* (n = 10)

	TIL		PBL	
	Median %	(min–max)	Median %	(min–max)
$CD3^+$	60.3	(36.8–82.5)	74.9	(52.5–90.3)
$CD3^-/CD16/56^+$	0.6	(0.0–8.3)	14.6	(3.8–42.0)
$CD3^+/CD16/56^+$	0.4	(0.0–3.5)	4.7	(0.8–9.5)
$CD3^+/TcR\alpha/\beta^+$	96.1	(89.3–99.4)	93.4	(73.2–99.1)
$CD3^+/TcR\gamma/\delta^+$	3.9	(0.6–10.7)	6.6	(0.9–26.8)
$CD3^+/CD4^+$	68.5	(43.1–86.8)	52.1	(23.6–67.1)
$CD3^+/CD8^+$	30.6	(12.8–56.1)	47.1	(32.2–75.8)
Ratio $CD4^+/CD8^+$	2.2	(0.8–6.8)	1.1	(0.3–2.1)
$CD4^+/CD45RA^+$	5.2	(1.6–37.8)	45.9	(20.4–63.2)
$CD8^+/CD45RA^+$	13.2	(0.0–79.1)	72.2	(65.6–84.7)
$CD4^+/CD45R0^+$	100.0	(91.2–100.0)	58.0	(45.5–79.4)
$CD8^+/CD45R0^+$	87.2	(58.8–100.0)	44.3	(28.7–57.4)
$CD4^+/CD25^+$	14.2	(2.4–29.2)	10.3	(0.8–16.6)
$CD8^+/CD25^+$	0.0	(0.0–1.3)	0.0	(0.0–0.0)
$CD4^+/CD38^+$	68.6	(50.6–95.0)	65.2	(49.6–81.6)
$CD4^+/CD71^+$	46.6	(14.4–73.6)	8.5	(2.9–15.9)
$CD8^+/CD71^+$	21.3	(6.1–58.9)	11.4	(6.4–17.8)
$CD80^+$	0.0	(0.0–1.0)	ND	
$CD4^+$/HLA-DR^+	43.0	(8.7–68.1)	10.7	(4.1–26.0)
$CD8^+$/HLA-DR^+	45.5	(17.5–80.3)	28.8	(6.0–72.1)

ND, not done.

of lymphoid infiltration did not correlate with any lymphocyte surface markers (data not shown).

The median follow-up was 32 months (range 27–37 months). Within this time period 13 of 41 patients died. Whilst this was correlated to the Dukes' stage, no correlation was found between mortality and the expression of surface markers of either tumour cells or TILs.

DISCUSSION

For the characterisation of TILs, previous studies have used immunohistochemistry [10, 14]. However, using multiparameter flow cytometry, we were able to describe three surface markers at the same time, giving us the opportunity to characterise $CD4^+$- and $CD8^+$-TILs further. Enzymes are commonly used when preparing single cell suspensions of tumour cells or TILs from solid tumours. Our results confirm previously published results showing that the expression of surface molecules might be damaged by the enzyme preparation [15–17]. After 1 h of enzyme preparation, expression of CD4, CD8 and CD16/56 were severely damaged, but since the MFIs were still far above isotype signal (Figure 2), we were able to describe the different phenotypes. We were not able to investigate the expression of CD69 on TILs since CD69 was destroyed very quickly (Table 3).

A number of reports have described the phenotype of lymphocytes infiltrating colorectal cancer [8, 9, 14, 18, 19]. In agreement with these papers, we found that $CD4^+$-TILs were predominant to $CD8^+$-TILs, that $CD3^-/CD16^+/CD56^+$- and $CD3^+/CD16^+/CD56^+$-TILs were very infrequent and that $TcR\alpha/\beta^+$- were more frequent than $TcR\gamma/\delta^+$-TILs (Table 4). It has been reported that colorectal cancer cells have down-regulated or even lost the expression of HLA class I molecules [3] and, since natural killer cells (NK-cells) do not recognise foreign antigens in association with HLA class I molecules, it has been speculated that NK-cells might play a major role in tumour control in these cases [20]. We found that there was no correlation between an NK-cell like phenotype and down-regulation of HLA class I on tumour cells, when analysed at the time of surgery.

We further investigated the subclasses of both $CD4^+$- and $CD8^+$-TILs and found that the TILs were activated to some extent (Table 4). This might reflect an activation of the TILs by antigens *in vivo*, but it remains unknown whether the TILs are activated specifically by tumour antigens or for other reasons. Further studies will have to elucidate this important problem. The lack of CD25 expression on CD8 + -lymphocytes indicated lack of activation due to anergy or insufficient stimulation.

It could be expected that the expression of HLA class II by tumour cells correlated with $CD4^+$- TILs. We found no correlations between expression of HLA class II at tumour cells and any specific TIL phenotype. We have found previously that the tumour cells lack expression of CD80 [4]. In the absence of the costimulatory signals delivered by CD80 and CD86, the interaction between HLA class II positive tumour cells and lymphocytes should give rise to anergy [21]. It is therefore possible that, when tumour cells express HLA class II but not CD80, TILs are turned off rather than being stimulated and this therefore would correlate with a poor prognosis. However, in the present study our follow-up is too short to elucidate this problem.

Table 5. *Correlation matrix of distribution of lymphocyte subsets among TIL and immunological surface molecules on colorectal cancer cells,* n = 41

	Ratio						
	$CD4^+/CD8^+$	$CD4^+/CD25^+$	$CD4^+/CD71^+$	$CD4^+$/HLA II^+	$CD8^+/CD71^+$	$CD8^+$/HLA II^+	$CD3^-/CD16^+/CD56^+$
$CD4^+/CD25^+$	− 0.07						
$CD4^+/CD71^+$	− 0.13	0.47†					
$CD4^+$/HLA class II^+	− 0.11	0.62‡	0.41†				
$CD8^+/CD71^+$	− 0.42†	0.35⋆	0.55‡	0.41†			
$CD8^+$/HLA class II^+	− 0.31⋆	0.50†	0.25	0.69‡	0.65‡		
$EP4^+$/HLA class I^+	0.36⋆	− 0.13	− 0.35⋆	− 0.11	− 0.41†	− 0.28	− 0.26
$EP4^+$/HLA class II^+	− 0.01	0.22	0.11	0.29	0.20	0.25	0.04
$EP4^+/CD80^+$	0.33⋆	− 0.08	− 0.10	− 0.12	0.03	− 0.00	− 0.13

Numbers are correlation coefficients. EP4 (Epithelial Antigen 4) is an epithelium cell marker. ⋆$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

In this study, we have, for the first time, studied the phenotype of TILs together with the expression of HLA and the costimulatory molecule CD80 on colorectal cancer cells. We found that tumour cells were equipped to present antigens to $CD8^+$-TILs, since HLA class I antigens were expressed. However, the tumour cells generally only expressed HLA class II antigens weakly and CD80 expression was absent. The occasional detection of activated $CD25^+$-TILs was not correlated to the tumour cell phenotype, but the ratio of $CD4^+/CD8^+$ cells was correlated to the expression of HLA class I tumour cells. The observation time in this study is too short to determine if this observation has any prognostic value but this should be followed-up in further studies.

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Acknowledgements—The authors thank Mrs. K. Kejling for excellent technical assistance. This work was supported by the Danish Cancer Society, the P. Carl Petersen's Foundation, The Novo Nordisk Foundation, the Danish Medical Association Research Fund and Odense University, Denmark.