Human Leukocyte Antigen Class I, MHC Class I Chain-Related Molecule A, and CD8$^+$/Regulatory T-Cell Ratio: Which Variable Determines Survival of Cervical Cancer Patients?

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Abstract

Purpose: To investigate the effect of intraepithelial tumor-infiltrating lymphocytes (ieTIL) and their ligands expressed by cervical tumor cells on the outcome of cervical cancer patients.

Experimental Design: The prognostic value of ieTILs was investigated in 115 cases of cervical cancer. T-cell subsets, CD57$^+$ cells, and regulatory T cells (Treg) were enumerated. The associations of these different ieTIL subtypes with human leukocyte antigen (HLA) class I and MHC class I chain-related molecule A (MICA) expression were determined in relation to clinical variables and patient survival.

Results: Survival analysis showed that a high number of intraepithelial Treg (FoxP3$^+$), a low CD8$^+$/regulatory T-cell ratio, and a weak HLA-A expression were all associated with worse survival ($P = 0.034$, 0.025, and 0.033, respectively, log-rank test). Further stratification of patient groups based on HLA-A-MICA expression and HLA-A-MICA-CD8$^+$/Treg ratio revealed an even poorer survival ($P = 0.005$). In a multivariate Cox analysis, low CD8$^+$/Treg ratio ($P = 0.047$), weak HLA-A-MICA expression ($P = 0.003$), and weak HLA-A-MICA expression combined with low CD8$^+$/Treg ratio ($P = 0.002$) were all found to be independent unfavorable prognostic predictors in cervical carcinoma (hazard ratios, 2.7, 4.0, and 4.9, respectively).

Conclusion: Weak HLA-A-MICA expression combined with low CD8$^+$/Treg ratio reveals a patient group with the poorest survival in cervical cancer. As a single variable, low CD8$^+$/Treg ratio was a significant independent unfavorable prognostic factor.

Cervical cancer is the second most frequent cancer in women worldwide (1). The development of cervical cancer is a multistep process initiated by persistent infection with high-risk human papillomavirus (HPV; ref. 2). After high-risk HPV infection, in a limited number of patients, cervical lesions progress via cervical intraepithelial neoplasia I to cervical intraepithelial neoplasia III to cervical cancer. Cervical carcinoma cells constitutively express the HPV-encoded E6 and E7 oncoproteins. Therefore, peptides derived from these viral antigens can be generated and presented in the context of human leukocyte antigen (HLA) class I to cytotoxic T cells. The presence of substantial numbers of lymphocytes in cervical carcinomas as previously reported by our group and others suggests the presence of a cellular antitumor response (3–6). Indeed, HPV-specific CD4$^+$ and CD8$^+$ T cells can be isolated from cervical tumors and CD8$^+$ T-cell clones are able to kill cervical cancer cells *in vitro* (7–10). However, this does not seem to be sufficient to eradicate the tumor. During tumor development, cancer cells have acquired several strategies to avoid eradication by cytotoxic T cells and natural killer (NK) cells. Among these mechanisms are down-regulation of HLA class I, down-regulation of MHC class I chain-related molecule A (MICA; a cytotoxic T-cell and NK cell ligand), production of immunosuppressive cytokines such as transforming growth factor-β, and the induction of immunosuppressive FoxP3$^+$ regulatory T cells (Treg; refs. 11, 12). Previously, we reported that loss of expression of HLA class I (13–15) and high expression of plasminogen activator inhibitor-1 (a surrogate marker of active transforming growth factor-β1) by cervical cancer cells are both associated with decreased survival (16). The role of Treg on survival in cervical cancer has not been investigated yet. In several human cancers, high numbers of tumor-infiltrating lymphocytes (TIL) were associated with improved overall survival of patients (4, 17–20). In contrast, other studies suggest that tumor cells actively attract inflammatory cells and that high numbers of TIL are associated with decreased overall survival of patients (21). Thus, clinical outcome may not be dependent on the total number of infiltrating cells but may depend on the type of immune cells present (e.g., cytotoxic CD8$^+$ T cells, CD4$^+$ T cells, CD57$^+$ cells,

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Received 10/5/07; revised 11/27/07; accepted 12/4/07.

Grant support: Netherlands Organization for Scientific Research Zon/Mw 917.56.311 (S.H. van der Burg).

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doi:10.1158/1078-0432.CCR-07-4554

and Treg), their cell number, their functional status, and the presence of appropriate ligands on the tumor cells. Previous reports have described a high prognostic significance of intraepithelial TIL (ieTIL) in comparison with stromal TIL (18, 22). For ovarian carcinomas, it was shown recently that a high intraepithelial CD8+/Treg ratio is critical for survival (18). In the present study, we identified and enumerated the number of intraepithelial CD8+ T cells, CD4+ T cells, Treg, and CD57+ cells and measured the expression of the cytotoxic CD8+ T-cell, NKT cell, and NK cell ligands HLA class I and MICA in 115 cervical carcinomas. We investigated the correlation between the presence of the different ieTIL subsets, expression of their ligands, and association with clinicopathologic variables. Finally, we determined whether the variables studied were independent predictor of clinical outcome in cervical cancer patients.

Materials and Methods

Patient characteristics and material. All patients with cervical cancer who underwent radical hysterectomy (type III) with lymphadenectomy in our hospital from 1985 to 1999 had enough archival formalin-fixed, paraffin-embedded tissue available, were inhabitants of the Netherlands, and had not received radiotherapy or chemotherapy before surgery were included in the study (N = 115). All patients with International Federation of Gynecologists and Obstetricians stages I and II were surgically treated in our hospital. The characteristics of the study population are presented in Table 1. Tumors were HPV typed by PCR and sequencing, as described previously (23). Overall survival was termed the interval between diagnosis and death from cervical cancer or the interval between diagnosis and the last observation for surviving patients. Data were censored at the last follow-up for patients who were alive at the time of the analysis in July 2005. Histologic specimens of normal cervices from women who underwent hysterectomies for benign uterine diseases with no cervical abnormalities (n = 9; median age, 46 y; range, 31-60) were used as the control group in all experiments. The use of clinical material was approved by the institutional review board according to the guidelines of the Dutch Federation of Medical Research Associations.

Table 1. Clinicopathologic variables

Characteristic	Category	N = 115 (%)
FIGO stage	Ib1	55 (48)
	Ib2/II	60 (52)
Histopathology	SCC	89 (77)
	ADC/ADSC	26 (23)
Lymph nodes	Negative	85 (74)
	Positive	28 (24)
Tumor size (mm)	≥40	66 (57)
	≥40	42 (37)
Vasoinvasion	Negative	71 (62)
	Positive	38 (33)
Infiltration depth (mm)	<15	64 (56)
	≥15	50 (43)
HPV type	HPV 16	59 (51)
	HPV 18	25 (22)
	Other	16 (14)
Recurrent disease	Yes	24 (21)
	No	81 (70)

Abbreviations: FIGO, International Federation of Gynecologists and Obstetricians; SCC, squamous cell carcinoma; ADC, adenocarcinoma; ADSC, adenosquamous carcinoma.

Quadruple fluorescent and enzymatic immunostaining of TILs and their ligands. A newly developed technique for simultaneous immunohistochemical staining of four different epitopes was applied to 4-μm formalin-fixed, paraffin-embedded tissue sections (24). In brief, deparaffinized and citrate antigen retrieval–treated sections were stained by a silver staining kit (Aurion) with the anti-cytokeratin AE1/AE3 antibody (IgG1; DAKO). Next, a mixture of the antibodies ab828 (rabbit polyclonal, anti-CD3; Abcam), HNK-1 (mouse monoclonal IgM, anti-CD57; developed in our laboratory), and 4B11 (mouse monoclonal IgG2b, anti-CD8; Novocastra) was added to each slide. TILs were visualized by a combination of fluorescent antibody conjugates (goat anti-rabbit IgG-Alexa Fluor 546, goat anti-mouse IgM-Alexa Fluor 488, and goat anti-mouse IgG2b-Alexa Fluor 647; Molecular Probes). Images were captured with a confocal laser scanning microscope (LSM510, Zeiss) in a multitrack setting. All images were 1,024 × 1,024 pixels, 8-bit depth, and an average of two successive scans at scan speed 6. A PH2 Plan-NEOFluar 25×/0.80 Imm Korr objective or a Plan Apochromat 63×/1.40 oil objective (both Zeiss) was used. Fifteen images were scanned per slide. For each case, one successive negative control slide was included. Intraepithelial infiltrating lymphocyte cell counts were represented as the number of cells per mm^2 of silver-stained tumor area as measured by the LSM program.

The HLA class I staining and quantification using the mouse monoclonal antibodies HCA2 and HC10 (anti-HLA-A and anti-HLA-B/C, respectively; Dr. J. Neefjes, Netherlands Cancer Institute, Amsterdam, the Netherlands) and the primary rabbit polyclonal anti-β2M (A 072; DAKO) was previously described (15). Standard diaminobenzidine (Sigma) immunohistochemical staining was done on a tissue array containing three cores from all patients (15). In addition, 4-μm sections of the same tissue array were stained with the rabbit polyclonal MICA antibody (provided by Dr. L. Durrant; ref. 25). The goat anti-rabbit IgG-Alexa Fluor 546 (Molecular Probes) was used to visualize this molecule. Three cores were scored per patient according to the Ruiter system, in which percentages and intensities of cells are taken into account to determine groups with different expression levels (26).

For the detection of Treg, the anti-FoxP3 antibody (IgG1, clone 236A/E7; Abcam) was applied to whole 4-μm paraffin sections from all patients. Positive cells were counted in the tumor fields in 15 randomly selected, high-power (400×) fields.

Statistical analysis. Two-sided χ^2 and Fisher's exact tests were applied where appropriate. Patient groups were based on the median (50th percentile) of the numbers of infiltrating immune cells per mm^2 as none of the data for the TIL subtypes followed a normal distribution pattern. In addition, we calculated intraepithelial $CD8^+/CD4^+$ ($CD3^+CD8^+$ T-cell count divided by $CD3^+CD8^-$ T-cell count), $CD8^+$/Treg ($CD3^+CD8^+$ T-cell count divided by $FoxP3^+$ T-cell count), and $CD4^+$/Treg ($CD3^+CD8^-$ T-cell count divided by $FoxP3^+$ T-cell count) ratios. Spearman's rank correlation analysis was done to test the associations between the different types of infiltrating immune cells. The two-sided χ^2 test was used for determining associations of ligand expression with clinical variables and number of TILs. Statistical analyses were done with the Statistical Package for the Social Sciences software package 14 (SPSS). Cumulative survival rate was calculated by the Kaplan-Meier method and analyzed by the log-rank test. Univariate and multivariate Cox proportional models were used to determine the hazard ratio (HR) that represents the relative risk of death among patients in the different groups. Two-sided P values of <0.05 were judged to be significant.

Results

Patients. The clinicopathologic characteristics of the total group of 115 patients are shown in Table 1. The mean age of the patients was 48.5 years, with the youngest 24 years and the

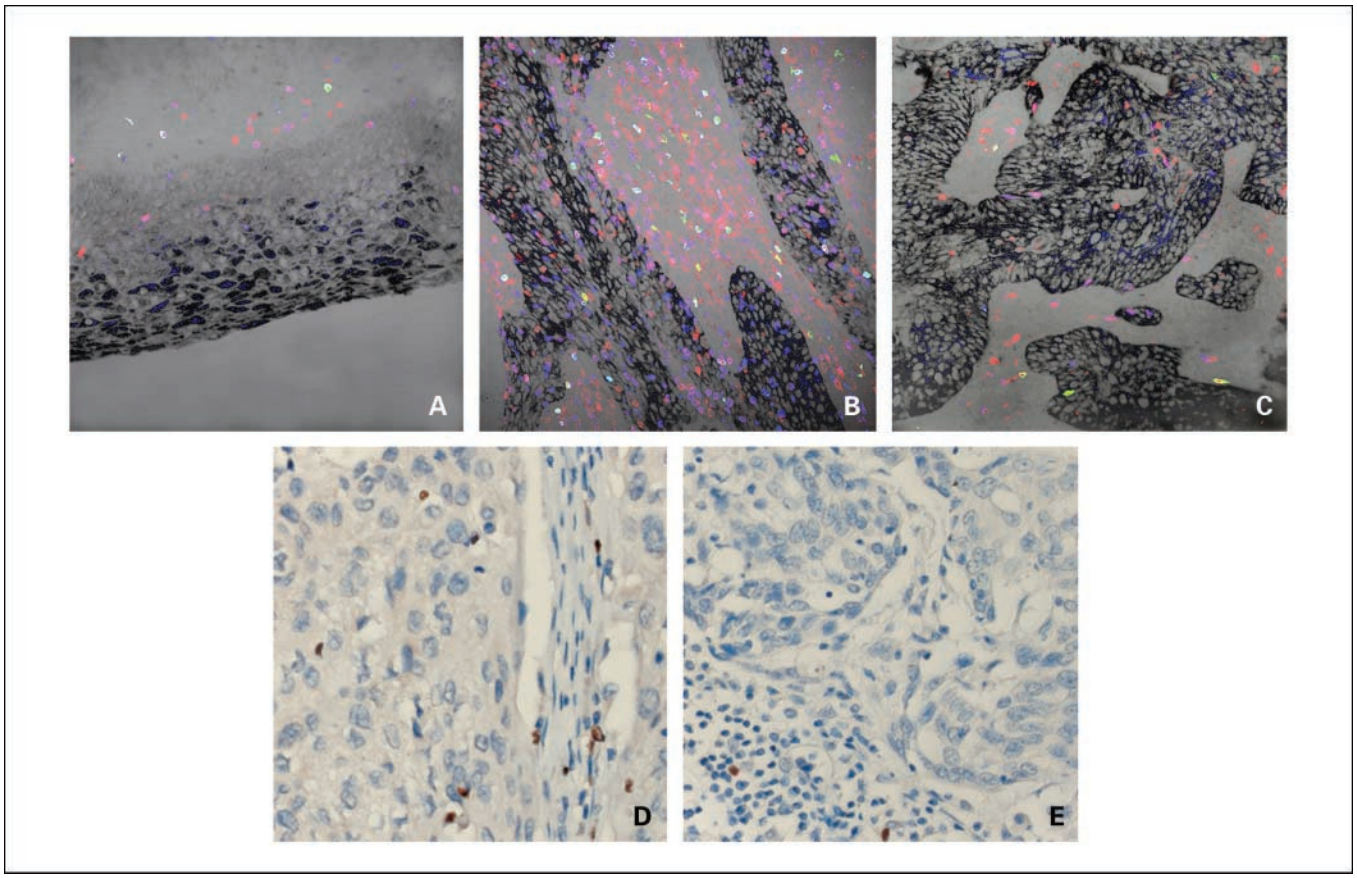

Fig. 1. Quadruple and enzymatic immunohistochemical staining of TIL. *A,* normal cervical epithelium. Black, silver-stained keratinocytes. The different TILs enumerated are $CD3^+CD8^+CD57^-$ T cells (*purple*), $CD3^+CD8^-$ T cells (*red*), $CD3^+CD8^+CD57^+$ cells (*white*), $CD8^+CD57^+$ cells (*blue green*), $CD3^+CD57^+$ cells (*yellow*), and $CD57^+$ cells (*green*). *B,* cervical cancer sample with a robust intraepithelial and stromal infiltrate pattern. *C,* cervical cancer sample with low numbers of TIL. *D,* cervical cancer sample with a high number of $FoxP3^+$ Treg (*brown nuclear staining*). *E,* cervical cancer sample with low numbers of $FoxP3^+$ Treg.

oldest 87 years at the time of surgery. Fifty-one patients received postoperative radiotherapy because of either tumor-positive lymph nodes or a combination of two of the following variables: depth of infiltration ≥15 mm, tumor size ≥40 mm, and presence of vasoinvasion. At the end of the 5-year follow-up period, 23 patients had died of disease, 85 were alive, 5 patients had a recurrence, and 2 died of causes unrelated to the primary disease but showed no evidence of disease.

Subtypes of ieTIL in cervical cancer. First, we identified and measured the number of intraepithelial $CD8^+$ T cells, $CD4^+$ T cells ($CD3^+CD8^-$ T cells), Treg ($FoxP3^+$), and $CD57^+$ cells (comprising subpopulations of T, NKT, and NK cells) by quadruple and standard immunostaining (Fig. 1). TILs were abundant within the tumor cell nests (ieTIL) and in the tumor stroma. In general, tumors were infiltrated by all the different TIL subtypes studied. Substantial variations in the number of ieTIL among individual tumors were observed (Table 2). The ieTIL subtypes designated in Table 2 will be used further in the text. In normal cervical tissue, lymphocytes were detected mainly in the stroma under the epithelial cells and not in the epithelium itself (Fig. 1). The majority of ieTIL consisted of $CD8^+$ T cells (66%) followed by $CD4^+$ T cells (23%), NKT1 cells (7%), Treg (2%), NKT2 cells (2%), NK1 (<1%), and NK2 cells (<1%). Using the Spearman's rank test, all T cells correlated with each other with a correlation coefficient (r) ranging from 0.298 to 0.691 ($P < 0.002$) and with NKT subsets with a r of 0.289 to 0.676 ($P < 0.004$). In addition, a significant correlation between NKT subsets ($r = 0.150$; $P < 0.001$) and NK subsets ($r = 0.316$; $P = 0.001$) was found.

HLA class I and MICA expression. Next, we investigated the expression of the $CD8^+$ T-cell and NK cell ligands HLA class I and MICA on the tumor cell surface. HLA-A expression (defined by HCA2 staining) was absent in 33%, weak in 20%, and normal in 47% of the cases. HLA-B/C expression (defined by HC10 staining) was absent in 22%, weak in 27%, and normal in 39% of the cases. Total HLA class I expression was classified as either complete loss (absent in 19%; Fig. 2A), weak expression (when the expression of either HLA-A or HLA-B/C was decreased or absent in comparison with stromal cells, 41%; Fig. 2B), or normal expression (39%; Fig. 2C). All cases evaluated expressed MICA, although MICA expression was weak in 64% of the cases (Fig. 2D and E). Normal cervical epithelium samples showed high expression of MICA (data not shown). Expression of HLA-A, HLA-B/C, or total HLA class I did not correlate with MICA expression (data not shown).

Association between ieTIL subtype, HLA class I, and MICA expression. Next, the association between the numbers of different ieTIL subtypes, HLA class I, and MICA expression was determined. Because the number of ieTIL does not follow a normal distribution pattern, the median was used to discriminate between low and high number of ieTIL. A significant association between $CD8^+$ T cells and HLA-A expression was

observed, with the HLA-A weak expression group showing lower numbers of $CD8^+$ cells compared with the HLA-A–positive and HLA-A–negative expression groups ($P = 0.025$). No apparent differences with respect to low or high cell number of $CD8^+$ T cells, $CD4^+$ T cells, Treg, and $CD57^+$ cells between tumors with complete loss, weak expression, or normal expression of MICA, HLA-B/C, or total HLA class I expression were found. As Treg exert their effect on $CD4^+$ T cells and $CD8^+$ T cells, and $CD4^+$ T cells exert their effect on $CD8^+$ T cells, $CD8^+$/Treg, $CD4^+$/Treg, and $CD4^+/CD8^+$ ratios were included in the analyses. The median was used as a cutoff value to divide high and low ratios (Table 2). In addition, high $CD8^+$/Treg ratio and high $CD4^+$/Treg ratio were both only associated with HLA-A expression, with weak HLA-A expressors showing lower $CD8^+$/Treg and $CD4^+$/Treg ratios ($P = 0.002$ and 0.014, respectively).

Association of ieTIL subtypes, HLA class I, and MICA with clinicopathologic variables. Subsequently, the correlation between ieTIL subtype, HLA class I, and MICA expression on the tumor cells and clinicopathologic characteristics of the patients was evaluated. $CD8^+$ T-cell numbers only showed an association with infiltration depth, with high numbers of $CD8^+$ cells present in deeper infiltrating tumors ($P = 0.016$). An increased number of $CD4^+$ T cells was found in squamous tumors when compared with adeno and adenosquamous tumors ($P = 0.047$). Treg were more often present in squamous tumors ($P = 0.001$). High or low numbers of the $CD57^+$ subpopulations showed no significant associations with clinicopathologic variables. A low $CD4^+$/Treg ratio was found to be associated with positive lymph node status ($P = 0.017$), whereas a low $CD8^+$/Treg ratio was associated with larger tumors ($P = 0.007$). There was no significant association between $CD4^+/CD8^+$ ratio, HLA class I, and MICA expression with clinicopathologic variables.

Five-year overall survival analyses. The Kaplan-Meier method and the log-rank test were used to analyze the correlation between HLA class I expression, ieTIL subtype, MICA expression, and survival of the patients. Only weak HLA-A expression ($P = 0.033$; Fig. 3A), high number of Treg ($P = 0.034$; Fig. 3B), and low $CD8^+$/Treg ratio ($P = 0.025$; Fig. 3C) were associated with decreased 5-year overall survival of cervical cancer patients. No significant association between HLA-B/C, total HLA class I expression, other ieTIL subtypes, or MICA expression and 5-year survival was found (data not shown).

Although MICA by itself did not associate with survival, MICA is an important activating ligand for effector cells and therefore MICA may play a role on tumor cells that express HLA-A. For this reason, patients were grouped based on their HLA and MICA expression. Indeed, patient that had both a weak HLA-A and a weak MICA expression was shown to have the poorest 5-year overall survival ($P = 0.005$; Fig. 3D). The latter patient group was further stratified based on low and high $CD8^+$/Treg ratio, as we do not expect that suppression of effector cells would be needed when the tumor cell lacks their ligands. The combination of factors leading to the poorest survival of the patients was weak HLA-A expression, weak MICA expression, and a low $CD8^+$/Treg ratio ($P = 0.005$; Fig. 3E).

A univariate Cox proportional hazards model analysis was done to determine the HR of each of the variables analyzed (Table 3). In agreement with previously published data, lymph node status ($P = 0.0001$), tumor size ($P = 0.003$), vasoinvasion ($P = 0.017$), and infiltration depth ($P = 0.002$) were strongly associated with poor survival (27). Only the number of Treg, the $CD8^+$/Treg ratio, and the HLA-A expression affected survival of patients ($P = 0.048$, 0.032, and 0.042, respectively). For combined HLA-A-MICA expression and HLA-A-MICA-$CD8^+$/Treg ratio, the patient group with the worst survival based on the log-rank test was compared with the other patients. Patients with combined weak HLA-A and weak MICA expression had a HR of death by cervical cancer of 4.56 ($P = 0.001$; 95% confidence interval, 1.93-10.81), whereas patients further stratified for low $CD8^+$/Treg ratio had a HR of 5.44 ($P = 0.0002$; 95% confidence interval, 2.24-13.21).

To determine whether the variables that significantly correlated with survival in the univariate Cox analysis were independent prognostic factors, these factors were used as covariates in a multivariate Cox analysis and measured against the strongest clinicopathologic variables: lymph node status, infiltration

Table 2. ieTIL subtypes in normal cervix and cervical cancers

Statistics	$CD3^+CD8^+CD57^-$	$FoxP3^+$	$CD3^+CD8^+/FoxP3^+$	$CD3^+CD8^-$	$CD3^+CD8^+/CD3^+CD8^-$	$CD3^+CD8^+CD57^+$	$CD3^+CD57^+$	$CD8^+CD57^+$	$CD57^+$
	$CD8^+$*	Treg	$CD8^+$/Treg ratio	$CD4^+$	$CD8^+/CD4^+$ ratio	NKT1	NKT2	NK1	NK2
Normal epithelium									
Mean	60.1	0.9	138.2	23.4	3.2	3.1	1.6	0.4	0.2
SE	12.0	0.3	39.8	3.6	0.9	1.1	0.8	0.2	0.2
Median †	48.5	0.8	97.2	25.0	2.6	2.0	0.7	0.2	0.0 ‡
SD	33.8	0.8	112.5	10.3	2.4	3.0	2.2	0.5	0.4
Minimum	19.4	0.0	10.7	9.1	0.7	0.0	0.0	0.0	0.0
Maximum	111.3	2.0	328.0	40.0	8.0	8.5	6.2	1.3	1.1
Cervical cancer									
Mean	187.4	5.9	79.9	65.9	3.8	19.1	5.2	0.3	1.0
SE	21.9	0.7	15.2	6.7	0.5	2.7	1.0	0.1	0.2
Median †	95.3	3.9	26.1	39.0	2.4	7.1	2.5	0.0 ‡	0.0 ‡
SD	221.6	6.6	146.5	67.8	5.1	27.4	10.0	0.6	1.6
Minimum	1.5	0.2	1.7	4.7	0.2	0.0	0.0	0.0	0.0
Maximum	1,186.7	45.2	986.0	382.8	38.8	150.2	65.6	2.7	9.0

* These designations of the different ieTILs will be used in the text.
† Used as a cutoff value.
‡ The majority of cases lacked these cells.

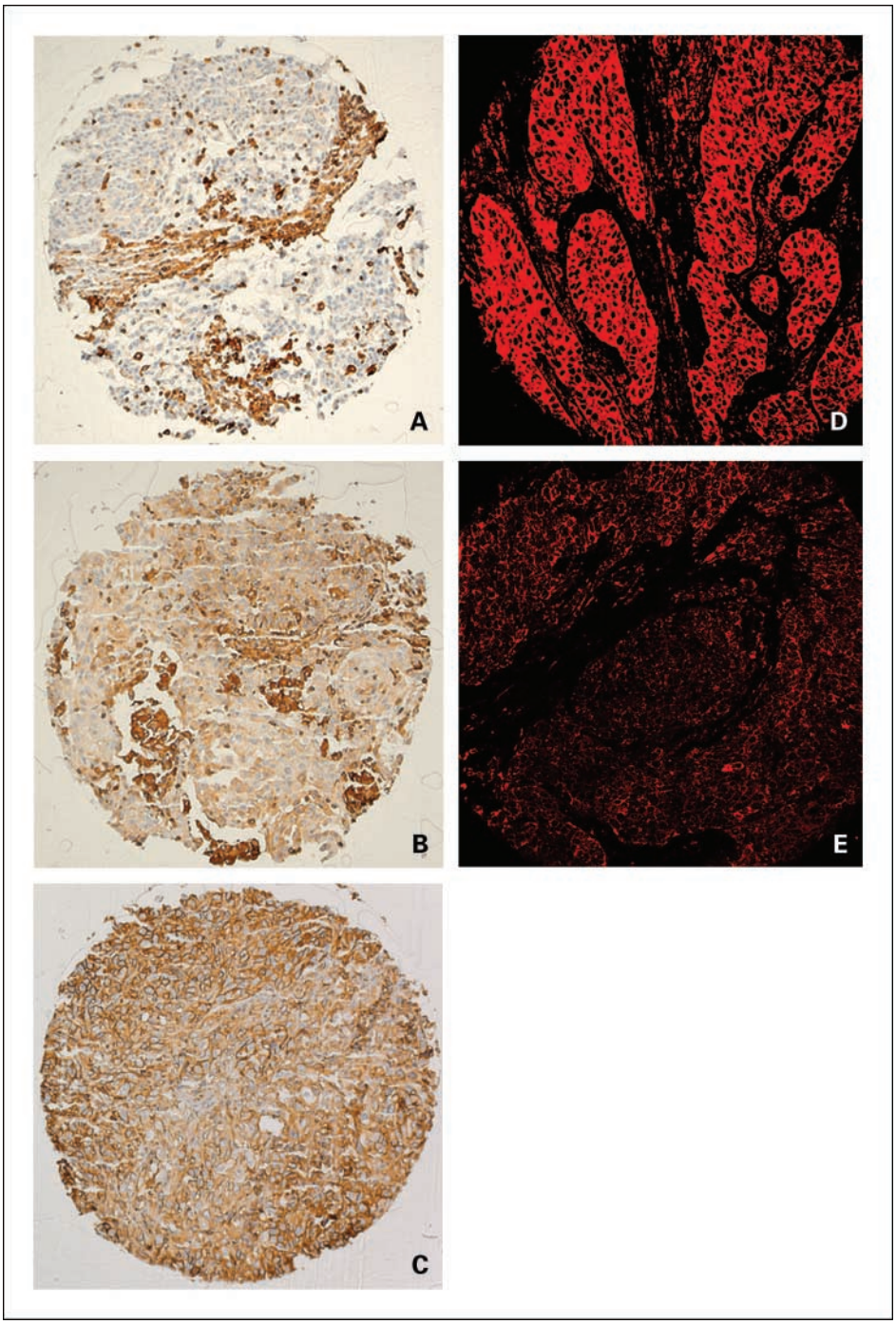

Fig. 2. Immunohistochemical staining of ligands in cervical cancer samples. *A,* HLA-A – negative tumor. Notice the normal staining in the stroma. *B,* weak HLA-A staining. *C,* normal pattern of HLA-A staining. *D,* fluorescent immunostaining showing normal MICA expression. *E,* weak MICA staining.

depth, tumor size, and vasoinvasion (Table 3). From all the statistically significant single variables tested, $CD8^+$/Treg ratio proved to be an independent prognostic factor in cervical carcinoma with a HR of 2.71 ($P = 0.047$). From the combined variables, both HLA-A-MICA and HLA-A-MICA-$CD8^+$/Treg ratio were found to be independent prognostic variables with a HR of 4.04 ($P = 0.003$) and 4.91 ($P = 0.002$), respectively.

Discussion

We and others have previously reported that cervical carcinomas can be infiltrated by lymphocytes (3–6). Notably, the infiltration by $CD3^+$ lymphocytes was described to affect clinical outcome (4). In the present study, detailed analysis of various subtypes of ieTIL and their ligands in a large series of patients with cervical cancer reveals that it is not the infiltration with $CD3^+$ lymphocytes per se but that both the expression of ligands (HLA-A and MICA) on cervical cancer cells and the $CD8^+$/Treg ratio influence survival. The $CD8^+$/Treg ratio proved to be the only single variable independent prognostic factor in cervical carcinoma by multivariate statistical analysis in addition to known strong clinical prognostic variables, such as lymph node status.

In addition to the commonly studied T-cell subtypes, we have determined the number of $CD57^+$ cells (which comprise

T, NKT, and NK cell subsets; refs. 28, 29) because it has been shown that these cells may affect cancer development and prognosis (30–32). Although (subsets of) $CD57^+$ was detected in the tissue specimens, we did not detect a significant correlation between the number of (subsets of) $CD57^+$ cells and clinical variables (including survival) of the patients.

To better understand the complex dialogue between ieTIL and cervical cancer cells, we did an assessment of two TIL ligands, HLA class I and MICA, expressed by the tumor cells. We previously reported that the number of tumor-infiltrating $CD8^+$ T cells was lower in tumors that showed HLA class I down-regulation (13). Our present findings support an important role particularly of HLA-A in the elimination of HPV-positive cervical cancer cells. In contrast to patients with HLA-A–positive tumors, patients with tumors that had weak HLA-A expression displayed a poorer survival. This highlights the importance of appropriate effector cell ligand expression by tumor cells. This is supported by the observed association between increased risk of HPV infection, and progression to cervical cancer, and particular HLA alleles (33, 34). We did not find an association between the down-regulation of HLA-B and/or HLA-C and survival. This may be due to the fact that we used the HC10 antibody, which does not allow us to distinguish between loss of HLA-B and/or HLA-C at the tumor cell surface and as such precludes a similar analysis as done for HLA-A. Notably, the relatively small group of patients displaying complete loss of HLA expression in their tumors showed a better survival than patients left with weakly HLA-positive tumors. Although we do not have a clear explanation for this observation, we must assume that complete loss of HLA expression renders tumor cells susceptible to NK cell–mediated killing. Although the number of tumor-infiltrating NK cells is not very high, they are present in vast numbers in the blood and lymph system. Because the presence of metastases in lymph nodes is an important prognostic variable in cervical cancer, the prevention of metastasizing HLA-negative tumor cells by NK cells thus may improve clinical outcome.

Interestingly, higher numbers of $CD8^+$ T cells and as a result also a higher $CD8^+$/Treg ratio were observed in tumors displaying a complete loss of HLA when compared with tumors with weak expression (loss of either HLA-A or HLA-B/C) of HLA. Similar observations have been reported in colorectal cancer (35) and in testicular tumors (36). It has been hypothesized that these $CD8^+$ T cells are likely to be activated through nonclassic HLA restriction elements that are also expressed on tumor cells (37) and that are recognized by $CD8^+$ $TCR\alpha\beta^+$ T cells (38).

MICA interacts with the NKG2D receptor, which is expressed both on $CD8^+$ T cells and NK cells. Engagement of NKG2D provides a costimulatory signal that enhances the effector function of these immune cells (39). Although the expression of MICA did not correlate with the number of ieTILs, weak MICA expression in combination with weak HLA-A expression was associated with poor survival of the patients. Down-regulated expression of MICA, in addition to weak expression of HLA-A, may surpass the threshold for the infiltrating $CD8^+$ T cells to exert their tumoricidal function. This concept was underscored by the observation that even patients with a low

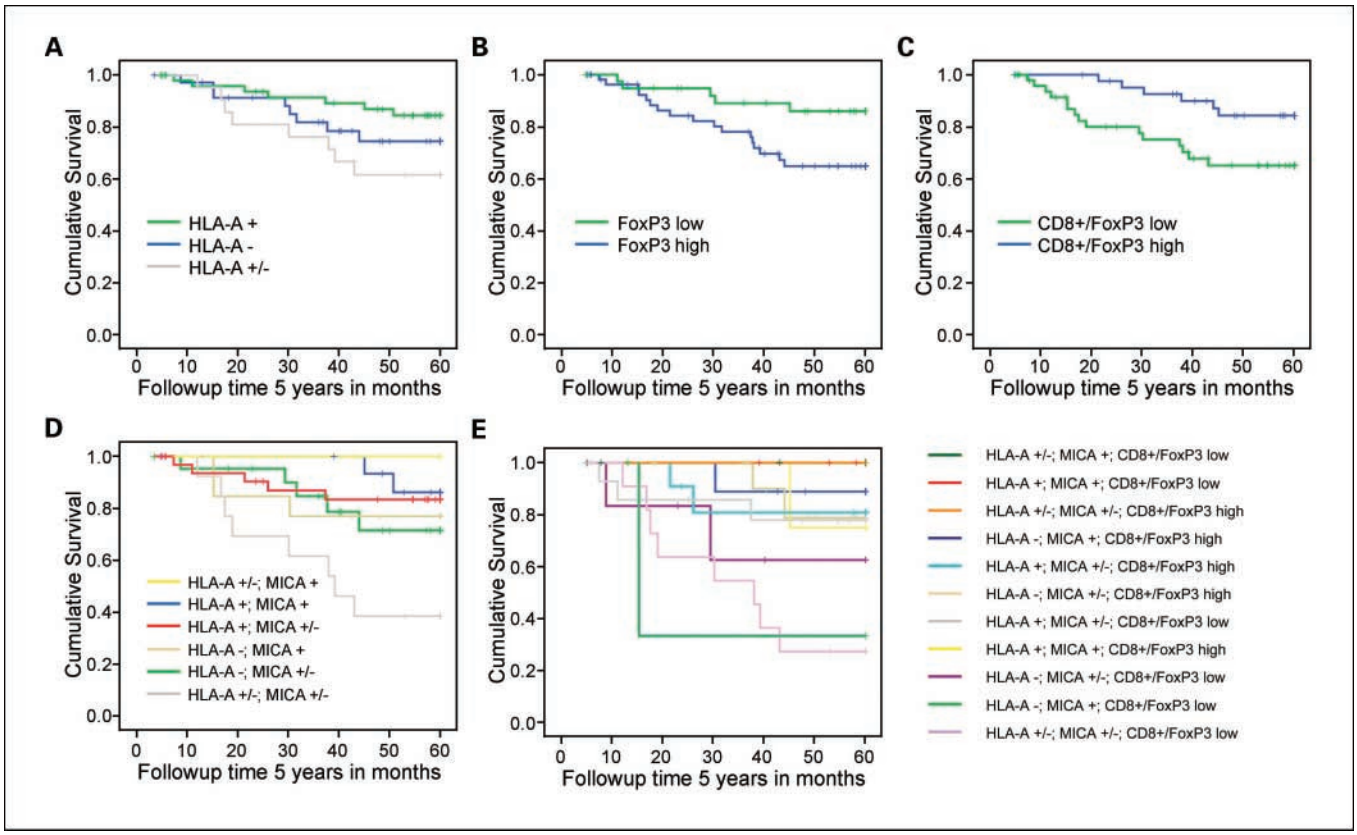

Fig. 3. Kaplan-Meier curves and log-rank test results of variables significantly associated with overall 5-y survival. *A,* Treg. *B,* $CD8^+$/Treg ratio. *C,* HLA-A expression. *D,* HLA-A expression stratified for MICA expression. *E,* HLA-A and MICA expression stratified for $CD8^+$/Treg ratio.

Table 3. Univariate and multivariate Cox analysis of ieTIL subtypes, HLA-A, MICA, and clinicopathologic variables

Variable		HR (95% CI)	P
Univariate Cox analysis			
Lymph node status	Positive vs negative	6.44 (2.79-14.82)	0.001
Tumor size (mm)	≥40 vs <40	7.77 (2.85-21.16)	0.001
Vasoinvasion	Positive vs negative	3.11 (1.36-7.11)	0.007
Infiltration depth (mm)	≥15 vs <15	4.56 (1.80-11.60)	0.001
Treg	High vs low number	2.76 (1.01-7.55)	0.048
$CD8^+$/Treg ratio	Low vs high ratio	2.82 (1.09-7.27)	0.032
HLA-A	Weak vs strong expression	2.87 (1.04-7.95)	0.042
Multivariate Cox analysis: single variables			
Lymph nodes	Positive vs negative	3.38 (1.16-9.83)	0.025
Tumor size (mm)	≥40 vs <40	5.39 (1.89-67.05)	0.002
Vasoinvasion	Positive vs negative	1.14 (0.42-3.08)	0.800
Infiltration depth (mm)	≥15 vs <15	2.69 (0.76-9.36)	0.126
$CD8^+$/Treg ratio	Low vs high ratio	2.71 (1.01-7.26)	0.047
Treg*	High vs low number	2.25 (0.67-7.50)	0.187
HLA-A*	Weak vs strong expression	2.70 (0.90-8.12)	0.077
Multivariate Cox analysis: HLA-A + MICA			
Lymph nodes		3.48 (1.15-10.52)	0.027
Tumor size		6.70 (2.38-18.91)	0.0003
Vasoinvasion		1.36 (0.52-3.59)	0.534
Infiltration depth		1.97 (0.64-6.08)	0.240
HLA-A + MICA	Weak expression vs rest	4.04 (1.61-10.13)	0.003
Multivariate Cox analysis: HLA-A + MICA + $CD8^+$/Treg			
Lymph nodes		2.15 (0.67-6.90)	0.199
Tumor size		6.21 (2.13-18.12)	0.001
Vasoinvasion		1.32 (0.48-3.62)	0.588
Infiltration depth		2.97 (0.80-11.04)	0.105
HLA-A + MICA + $CD8^+$/Treg	Weak expression/low ratio vs rest	4.91 (1.82-13.22)	0.002

Abbreviation: 95% CI, 95% confidence interval.
*Variables were analyzed in separate multivariate analyses as not to influence each other's HR.

$CD8^+$/Treg ratio had an excellent survival when HLA-A and MICA were expressed at normal levels. Apart from expression of the appropriate ligands on the tumor cell population, the nature of the inflammatory infiltrate has been shown to associate with clinical outcome of cancer patients. Tumor infiltration by $CD8^+$ T cells was associated with improved survival in ovarian, colorectal, and endometrial carcinoma and in melanoma (17–20). In cervical cancer, a potential positive effect of infiltrating $CD8^+$ T cells was found in patients with either bulky low-stage cervical tumors or high-stage (International Federation of Gynecologists and Obstetricians II) carcinomas (40, 41). In our study, we only observed an association between $CD8^+$ T cells and survival when these variables were analyzed in the context of Treg. Similar to our observations, a high intraepithelial $CD8^+$/Treg ratio corresponded to a 70% reduction in the risk of death (HR, 0.31; 95% confidence interval, 0.17-0.58) in patients with ovarian cancer (18).

Although not found in our study on cervical cancer, other investigators have reported that a high intraepithelial $CD8^+$/$CD4^+$ T-cell ratio resulted in improved survival in ovarian and colon cancer, suggesting that coinfiltration by tumor-specific $CD4^+$ T cells is detrimental in these types of cancer (18, 42). Our results indicate that only the subpopulation of $CD4^+$ cells that express the regulatory T-cell marker FoxP3 is involved in modulating CTL function at the tumor site. Direct negative effect of intraepithelial Treg on survival was also found for ovarian cancer (43).

In conclusion, we have shown that weak HLA-A-MICA expression combined with low $CD8^+$/Treg ratio reveals a patient group with the poorest survival in cervical cancer. Furthermore, this is the first study showing the effect of Treg on survival of cervical cancer patients. As a single variable, low $CD8^+$/Treg ratio was a significant independent unfavorable prognostic factor. Our findings provide support for therapies that aim to reduce the number or the effect of Treg. We predict that this type of therapy will positively influence the clinical outcome of these patients with cervical cancer.

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Clin Cancer Res 2008;14:2028-2035.