Immunologic aspects of ovarian cancer
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HLA-E expression by gynecological cancers restrains tumor-infiltrating CD8+ T lymphocytes

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Abstract

HLA-E is a non-classical HLA class I molecule, which differs from classical HLA molecules by its non-polymorphic, conserved nature. Expression and function of HLA-E in normal tissues and solid tumors is not fully understood. We investigated HLA-E protein expression on tissue sections of 420 ovarian and cervical cancers and found equal or higher levels than normal counterpart epithelia in respectively 89.4% and 83.7% of the tumors. Expression was strongly associated with components of the antigen presentation pathway, e.g. TAP, ERAP, b2m, HLA class I and II, and for ovarian cancer with tumor infiltrating CTL. This argues against the idea that HLA-E would compensate for the loss of classical HLA in tumors. In situ detection of HLA-E interacting receptors revealed a very low infiltrate of NK cells, but up to 50% of CTL expressed the inhibiting CD94/NKG2A receptor. In cervical cancer, HLA-E expression did not alter the prognostic effect of CTL, most likely due to very high infiltrating CTL numbers in this virus-induced tumor. Overall survival of ovarian cancer patients, however, was strongly influenced by HLA-E, because the beneficial effect of high CTL infiltration was completely neutralized in the subpopulation with strong HLA-E expression. Interestingly, these results indicate that CTL infiltration in ovarian cancer is only associated with better survival when HLA-E expression is low, and that intratumoral CTL are inhibited by CD94/NKG2A receptors on CTL in the tumor microenvironment.
Introduction

HLA-E is a non-classical Major Histocompatibility Complex (MHC) class I molecule that is almost non-polymorphic, in contrast to its classical class I counterparts HLA-A, -B, and -C (1;2). There are two HLA-E subtypes, which differ by only one amino acid (3-5). This coding variation is located outside the peptide binding groove, and both HLA-E variants are indeed indistinguishable in their structure and peptide binding features (3-5). HLA-E normally presents a very limited variety of peptides, derived from signal peptide sequences of classical MHC class I. However, infections and transformation can mediate the presentation of alternative peptides (1;2). Surface expression of HLA-E is largely dependent on β2 microglobulin (β2m) as well as transporter associated with antigen processing (TAP) and tapasin (6;7).

HLA-E/peptide complexes are ligands of the CD94 receptor in conjunction with the inhibitory NKG2A or the stimulatory NKG2C molecule, which are expressed on the majority of natural killer (NK) cells and some activated CD8+ T lymphocytes (CTL) (8;9). The presentation of ‘self’ signal peptides by HLA-E enables these lymphocytes to gauge the overall MHC class I expression on the surface of target cells. Engagement of CD94/NKG2A receptors on lymphocytes reduces the reactivity of NK cells or CTL, which protects against excessive immune-mediated tissue damage, for instance during infections (10;11). On the other hand, CD94/NKG2A expression can be induced in response to cytokines such as IL-15 (12) and transforming growth factor β (TGF-β) (13). These cytokines are frequently present in the tumor microenvironment, suggesting that CD94/NKG2A inhibitory receptors play a role in immune escape by tumor cells.

With the availability of specific antibodies to detect HLA-E in native conformation (clone 3D12; ref. (6)) or as denatured protein (clone MEM-E/02; ref. (14)), we recently investigated the normal expression of HLA-E on human tissues in collaboration with the Human Proteome Resource program (HPR at www.proteinatlas.org). Expression on blood cells, endothelium, melanocytes and intestinal epithelial cells was confirmed (6;15-17). Furthermore, we observed a pattern of tissue staining that was very similar to that of classical MHC class I.

Here, we determined the expression of HLA-E in 150 cervical and 270 ovarian cancer samples and analyzed its association with clinical and immunological parameters. Our results indicate that HLA-E is frequently overexpressed in these tumor types and positively associated with expression patterns of antigen processing components, classical HLA molecules and immune cell infiltrate. In situ analysis of the interacting receptors of HLA-E, i.e. the inhibitory CD94/NKG2A and the activating CD94/NKG2C, revealed a frequent expression of the inhibitory receptor on intratumoral CD8+ T cells. NK cells, the predominant cell type expressing CD94/NKG2A and CD94/NKG2C, were hardly found in both tumor types. Importantly, the beneficial prognostic effect of infiltrating CTL in ovarian cancer was neutralized by high expression of HLA-E, indicating that HLA-E hampers activity of anti-tumor CTL in the tumor microenvironment.
Materials and methods

Patient selection
Since 1985, the Department of Gynecological Oncology at the University Medical Center Groningen (UMCG) keeps a computerized database of patients with malignant epithelial ovarian cancer treated at this hospital at any time point during the course of their disease, prospectively collecting information on clinicopathologic characteristics and follow-up. For the current study, ovarian cancer patients were selected if primary surgery was performed by a gynecological oncologist from the UMCG between May 1985 and June 2006, and if paraffin-embedded ovarian tumor tissue was available (N = 270). Follow-up was updated in July 2009. Patients were staged according to FIGO classification (61). Tumors were graded and classified according to WHO criteria by a gynecological pathologist. Adjuvant chemotherapy consisted of different platinum-based treatment regimens. Response to chemotherapy was evaluated according to WHO criteria. Specimens from seven patients (three premenopausal, four postmenopausal) who underwent prophylactic bilateral salpingo-oophorectomy were separately included as controls for the HLA-E staining of normal ovarian epithelium.

We included all patients with cervical cancer who underwent radical hysterectomy with complete pelvic lymphadenectomy in the Leiden University Medical Center (LUMC) from 1985 to 1999, for whom formalin-fixed, paraffin-embedded tissue available, and had not received radiotherapy or chemotherapy before surgery (N = 150). All patients had FIGO stages I and II disease, since higher stages are treated by primary chemoradiation. Tumors were HPV typed by PCR and sequencing. For all patients, a minimum of five years of follow up was available. Histologic specimens of normal cervixes from women who underwent hysterectomies for benign uterine diseases with no cervical abnormalities (n = 9) were used as a control group in all section stainings.

Immunohistochemistry
Previously described ovarian (20) and cervical cancer (21) tissue microarrays (TMA) were used, which were constructed as described previously (62;63). TMA sections were stained with mouse monoclonal antibodies recognizing HLA-E (clone MEM-E/02, Abcam ab2216). In brief, TMA sections were dewaxed in xylene and rehydrated using graded concentrations of ethanol to distilled water. After antigen retrieval using citrate buffer, endogenous peroxidase activity was blocked by submersion of sections in a 0.3% H₂O₂ solution for 30 minutes. Sections were incubated with the primary antibody for 60 minutes at room temperature and subsequently with DAKO Envision+ for 30 minutes. The antigen-antibody reactions were visualized with 3,3’-diaminobenzidine. Sections were counterstained with hematoxylin. Paraffin embedded sections were also stained with anti-CD56 (clone 1B6, Monosan, Uden, The Netherlands) and anti-NKp46 (polyclonal AF1850, R&D systems) and subsequently, rat-anti-mouse and goat-anti-rat as secondary and tertiary antibodies, respectively. Simultaneous detection of CD3, CD94 and NKG2A was performed by three color fluorescence staining on ten cryosections of cervical carcinomas using anti-CD3 (mouse IgG1, DAKO, clone F7.2.38), anti-
CD94 (mouse IgG2a, Abcam, clone ab61974) and anti-NKG2A (mouse IgG2b, Immunotech, clone Z199). Second step antibodies were all goat-anti-mouse isotype specific antibodies with Alexa fluorochromes Alexa Fluor 546, Alexa Fluor 647 and Alexa Fluor 488 (Molecular Probes). Images were captured with a confocal laser scanning microscope (LSM510, Zeiss) in a multitrack setting and analyzed as previously described (64).

**Scoring**

All stainings were scored independently by two observers. Observers had no prior knowledge of clinicopathological information. To obtain a high concordance rate with whole tissue slides, it was decided that minimally two cores with a minimum of 20% tumor tissue had to be present on the TMA for a sample to be entered into analysis (63). The scoring system proposed by Ruiter et al. (18) was used. The intensity of staining was scored as 0, 1, 2, or 3, indicating absent, weak, positive, or strong positive expression, respectively. The percentage of positive tumor cells was scored as 0 for 0%; 1 for 1–5%; 2 for 5–25%; 3 for 25–50%; 4 for 50–75% and 5 for 75–100%. The two scores were added up, averaged over the number of evaluable cores per tumor, and dichotomized based on the lowest quartile. This resulted in a cutoff score of 5.25 in ovarian cancer, and 5.00 in cervical cancer. We also performed analyses with previously published data on antigen processing and presentation molecules, as well as tumor infiltrating lymphocytes, using the same cutoff points as described earlier (19-22).

**Flow cytometry analyses**

Fresh ovarian and cervical cancer specimens were dissected in small fragments with surgical blades and passed through a cell strainer to obtain single cell suspensions. Patients had similar characteristics as those from the TMA cohort. After isolating living cells using centrifugation with ficoll, cells were washed twice in PBS/0.5% BSA buffer and incubated for 30 minutes with CD14-PeCy7 (clone M5E2, BD Bioscience), CD3-PB (clone UCHT1, Dako), CD56-Alexa700 (clone B159, BD Biosciences), CD4-PeTxRed (clone S3.5, CALTAG), CD8-PerCP (clone SK1, BD Biosciences), CD94-FITC (clone 131412, R&D systems), NKG2A-PE (clone z199, Beckman Coulter), and NKG2C-APC (clone 134591, R&D systems). After fixation in paraformaldehyde, cells were measured using an LSR II flow cytometer (BD Biosciences) and analyzed by FlowJo software.

**Statistics**

Associations between clinicopathological characteristics, antigen processing and presentation pathway components, and intratumoral T-lymphocytes were tested using Pearson Chi square tests using our previously published cutoff points (19-22). All continuous variables were tested for normality by plotting histograms and performing Kolmogorov-Smirnov tests. Since none of these variables was normally distributed, nonparametric tests were used to compare continuous variables. Mann-Whitney U tests were used to determine differences between CD8 scores in ovarian and cervical cancer. Survival was defined as date of surgery until death of cancer, or date of last follow-up. Survival was estimated using the Kaplan Meier method. The Log Rank test and Cox
regression analyses were used to assess survival differences between groups. To compare CD94, NKG2A, and NKG2C expression among different T lymphocyte subsets, we used the Mann Whitney U test. For all tests, p-values <0.05 were considered significant. All p-values were two-sided. All statistical analyses were performed using SPSS 16.0 software package for Windows (SPSS Inc., Chicago, IL, USA).

Results

**HLA-E expression in gynecological cancers**

Two cohorts of gynecological tumor tissue were evaluated: 270 ovarian and 150 cervical cancers. Supplementary table 1 summarizes clinicopathological characteristics and survival data of the two cohorts. Notably, ovarian cancer is generally diagnosed at a much later stage than cervical cancer. Hence, this cohort consists mainly of high stage tumors with an average disease-specific survival of only 3.5 years, whereas cervical cancer patients live on average 14 years after diagnosis.

We first determined the expression of HLA-E on nonmalignant ovarian and cervical tissue. For
HLA-E expression by gynecological cancers restrains tumor-infiltrating CD8+ T lymphocytes

In ovarian tissue, we selected seven pre- and postmenopausal samples with intact, nonmalignant ovarian epithelium. Also, we stained nine cervical sections containing normal ectocervical squamous epithelium and endocervical glands (Fig. 1). These are the structures that give rise to ovarian- and cervical cancer, respectively. The ovarian epithelium showed a weak positive staining in both pre- and postmenopausal samples, whereas ovarian stroma was negative (Fig. 1a, b). Both cervical epithelia stained negative to weak positive for HLA-E; stroma was negative (Fig. 1e, f). The endothelium of blood vessels was highly positive for HLA-E as well as resident leukocytes, in line with previous reports (15).

Next, we assessed HLA-E expression on ovarian cancer (n=270) and cervical cancer (n=150) confined in tissue micro arrays using a validated specific antibody. Examples of negative and positive staining tumors are depicted for ovarian cancer (Fig. 1c and d, respectively) and cervical cancer (Fig. 1g and h, respectively). Staining of HLA-E on tumor cells was scored for intensity and percentage surface area, as previously described (18), giving a range from 0 to 8.0. For both tumor types the median score was 6.0, with a range between 0 and 7.75. On the basis of the mean intensity score of normal epithelium (score 1 on a scale of 0-3), ovarian tumors and cervical tumors expressed equal or higher levels of HLA-E in 89% and 83% of the tumors, indicating that expression of HLA-E is mostly conserved in these tumors.

**Figure 2** Flow cytometry analysis of CD94, NKG2A and NKG2C expression on T cells. Dissociated tissues from fresh tumor samples from surgery were stained with fluorescently labeled antibodies against CD14, CD56, CD3, CD4, CD8, CD94, NKG2A and NKG2C. (A) Eight color staining of dispersed cervical tumor. Leukocytes were first gated on forward and sideward scatter plot and all CD14-negative cells to exclude non-specific staining to immunoglobulin receptors on monocytes. Natural killer cells were also excluded from the analysis by removing CD3-CD56+ cells from the selecting gate. (B) Nine tumor samples and nine age-matched PBMC samples were analyzed for percentage CD4 T cells and CD8 T cells that express CD94, NKG2A or NKG2C. Ovarian cancer contained very low numbers of CD4 T cells. Percentage CD94- and NKG2A-positive CD8 T cells in tumor tissues was significantly higher than the percentage in blood (p=0.0012 for CD94, p<0.0001 for NKG2A, Mann-Whitney U test). Other comparisons were not significantly different.
## Table 1: Relationship of HLA-E expression in ovarian and cervical cancer with immunological characteristics

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<th>Factor</th>
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<td>11 (26.2%)</td>
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*a* p-values were calculated using Pearson Chi square-test. Bold signifies p<0.05.

### Associations between HLA-E, clinicopathologic and immunologic factors

To assess whether HLA-E expression was preferentially associated with certain patient groups, we determined the relationship between HLA-E expression and well-known clinicopathologic factors. To this end, the gradual scores of HLA-E expression were dichotomized based on the lowest quartile. For ovarian cancer, there was no relationship between HLA-E expression and histology, stage, grade, or presence of residual tumor after debulking surgery (supplementary table 1). Similarly, HLA-E expression in cervical cancer was not related to histology, stage, infiltration depth,
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We previously collected data from these cohorts of tumor samples describing immune cell infiltration and HLA-related molecules (19-22). We determined associations between HLA-E and components of the antigen processing machinery, using the same cutoff values as previously described for these molecules (19-22). The proteasome subunit LMP7, peptide transporter heterodimer TAP1, and the aminopeptidase ERAP (23) were associated with increased HLA-E expression in ovarian cancer (Table 1). In cervical cancer, the TAP1 and TAP2 transporters were the only component that were associated with HLA-E expression. These results suggest that antigen processing components contribute to the protein expression of HLA-E.

Next, the association with classical HLA class I molecules (HLA-A, HLA-B/C and β2m) and HLA class II molecules (HLA-DP/DQ/DR) was analyzed (Table 1). Induction of HLA class II molecules is observed in a majority of these cancers and can be mediated by cytokines such as interferon-γ, similar to HLA-E (24-26). This revealed a very strong association with HLA-E expression, especially in ovarian cancer. This indicates that HLA-E is present in tumors with strong classical HLA expression and contrasts with the idea that HLA-E expression would compensate for loss of classical HLA molecules in cancer. In contrast, high expression of classical HLA class I promotes the stabilization of HLA-E through the delivery of leader sequences which bind to the groove of HLA-E (2;6;27).

Furthermore, expression of HLA-E was correlated with the presence of T cells. The degree of infiltration of CTL and regulatory T lymphocytes (Treg) was recently reported by our groups for these two cohorts (20;21). The number of tumor-infiltrating CTL was positively correlated to HLA-E expression in ovarian cancer, but not in cervical cancer (Table 1). We previously found that the ratio between CTL and Treg is predictive of clinical outcome in cervical cancer instead of the CTL counts as such (21). For the current study, we studied the relation between the CTL/Treg ratio and HLA-E expression, but these two parameters were not associated (p=0.343, Table 1).

Concluding, HLA-E expression in ovarian and cervical cancers is positively associated with other components of HLA-mediated antigen presentation – indicative of a well functioning processing and presentation pathway – and the influx of T cells. These associations are especially prominent in ovarian cancer.

Intra-tumoral CTLs express HLA-E engaging receptors

The receptors for HLA-E, i.e. CD94/NKG2A and CD94/NKG2C, are predominantly expressed on natural killer (NK) cells. We therefore assessed the presence of these innate immune cells in our cohort of ovarian and cervical cancers using antibodies against the NK-associated markers CD56 and CD57, and the NK-specific marker Nkp46 (28). In ovarian cancer, only 14% of the samples contained detectable NK cells, and the number of cells was very low in these tumors (less than 7/mm²). Cervical cancers also largely lacked infiltrating NK cells, and stainings with an anti-Nkp46 antibody corroborated our previous results where we scored CD3 CD57+ cells (21). Clinicopathologic factors or HLA-E expression did not differ between tumors with or without NK cells. Besides on NK cells, the inhibiting heterodimer CD94/NKG2A and the activating CD94/NKG2C are
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also expressed on a small subset of CTL (2). We hypothesized that HLA-E in cancers might serve as ligand for these receptors on intratumoral CTL. We applied 8-color flow cytometry analysis on fresh surgical samples, which were mechanically dissected to single cell suspensions (Fig. 2). Gating on CD3+CD4+ T cells and CD3+CD8+ cytotoxic T cells visualized the expression of CD94, NKG2A and NKG2C receptors on these T cell subsets (Fig. 2a). Importantly, a high frequency of the tumor-infiltrating CTL displayed the inhibiting NKG2A chain, but not the activating NKG2C chain (Fig. 2a). Nearly all NKG2A-positive CTL also co-expressed the partner CD94 (overall 98%). In contrast, CD4+ T cells were largely devoid of these HLA-E interacting receptors. The ovarian cancers contained very low numbers of CD4+ T cells, leading to seemingly high frequencies of receptor-positive subsets. Interestingly, large populations of CD4−CD8− T cells were observed in the samples of ovarian cancer and a high percentage of these cells were positive for CD94/NKG2A and these cells are currently subject of further investigation. When five cervical cancer and four ovarian cancer samples were analyzed, up to 50% of CTL were CD94/NKG2A+ with a median of 12% (Fig. 2b). The frequency of CD94/NKG2A+ CTL in age-matched normal blood was found to be around 3%, indicating that this inhibiting HLA-E binding receptor is enriched at the site of the tumor. To substantiate this finding and to analyze the localization of these CD94/NKG2A positive CTL, we performed triple stainings on cryosections of cervical cancer using fluorescently labeled antibodies to CD3, CD94, and NKG2A (Fig. 3). Most T-cells resided in stoma areas and not within tumor nests, in line with our previous findings (20, 21). Strikingly, CD94/NKG2A expression was found on only 6% of the stoma T cells, whereas 48% of intratumoral T cells displayed this inhibiting receptor (SD 9% and 32%, respectively). Together, these data implied that the frequency of tumor-interacting T cells expressing CD94/NKG2A (Fig. 3) is much higher than anticipated on basis of the total pool of T cells in the resected tumor sample (see Fig. 2b).

Expression of HLA-E neutralizes survival benefit of infiltrating CTL in ovarian cancer

We wondered whether the observed expression of HLA-E and CD94/NKG2A in the tumor site would translate into survival differences in the context of CTL infiltration. In ovarian cancer, HLA-E expression on its own did not affect survival (Table 3). We previously demonstrated (20) that high CTL counts predict improved survival in ovarian cancer (HR 0.71, Table 3 and Fig. 4a). We hypothesized that, due to the presence of CD94/NKG2A on infiltrating CTL, HLA-E high tumors might resist CTL mediated lysis. To this end, we performed survival analysis for CTL infiltration stratified by HLA-E expression. Indeed, the prognostic benefit of CD8+ T cells was strongly present in the stratum with low HLA-E expression (HR 0.53, p=0.001, Table 3, Fig. 4b). This hazard ratio was much lower than that of the whole population, without HLA-E stratification. Strikingly, patients with high HLA-E expression, representing 75% of our cohort, completely lost the benefit of infiltrating CTL (HR 0.97, p=0.816, Table 3, Fig. 4c). These data indicate that the minor subpopulation of patients with low HLA-E expression on their tumors benefits from infiltrating CTL and, moreover, that expression of HLA-E neutralizes the survival benefit of ovarian cancers with high numbers of CTL.

In cervical cancer, we observed a decreased risk of death associated with high HLA-E expression in univariate analysis. However, HLA-E expression was not an independent predictor of death in
HLA-E expression by gynecological cancers restrains tumor-infiltrating CD8+ T lymphocytes

**Figure 2** Triple fluorescence staining of cervical cancer detecting intratumoral CD94+NKG2A+ T cells. Immunofluorescent staining of T cells (CD3+ in blue) expressing NKG2A (in green) and CD94 (in red). These two pictures of different cervical tumor samples are representative of ten tumors analyzed. Arrow heads in the merged picture (lower right quadrants) designate triple positive cells within tumor nests, whereas the surrounding single blue cells (T cells without CD94/NKG2A) are located in stroma.

**Figure 3** Kaplan-Meier survival curves of ovarian cancer. Overall survival of 249 patients with ovarian cancer for whom ≥2 cores were available in months is plotted. (A) Infiltrating CD8+ T cells were counted and stratified in two groups with a cut off on the lowest tertile. Patients with a high CTL count showed a better survival than those with low CTL counts (p=0.044, log rank test). (B-C) Subsequently, HLA-E expression was added as parameter, dividing the population into HLA-E low expression (lowest quartile) (B) and high HLA-E expression (C). The beneficial effect of high CTL counts on survival was not attributable for those cancers with high HLA-E (p=0.815, log rank). Consequently, the beneficial role of high CTL infiltration of the whole cohort was the result of a small subpopulation of patients with low expression of HLA-E.
multivariate analysis (Table 3). We previously reported that infiltrating CTL frequency is not an independent predictive survival factor (p=0.879, Table 3) (21). Stratified analysis of CTL infiltration based on HLA-E expression did not affect these results. When repeating these analyses for disease free survival, similar results were obtained.

A notable difference between ovarian and cervical cancer is the number of intratumoral CTL, as cervical cancers are infiltrated with at least three times more CTL (median 95.3 ± 221.6/mm$^2$; ovarian cancer: 28.3 ± 120/mm$^2$; p<0.001), suggesting that the virus-positive cervical cancers are relatively overloaded with infiltrating CTL. When we repeated the stratified analysis in the subpopulation of cervical cancer with CTL counts comparable to ovarian cancer, HLA-E expression seemed to have the same impact as in ovarian cancer. However, the numbers of cervical cancer with such low numbers of CTL were insufficient for proper statistical analysis. We are currently further evaluating the differences between CTL numbers in several tumor types.

In conclusion, HLA-E is regularly expressed in ovarian and cervical cancer, often concurrently with classical MHC molecules. Instead of inhibiting NK cells, which are hardly present in these tumor types, the main role of HLA-E seems to be the inhibition of infiltrating CD8$^+$ CTL. This effect translates into survival differences in ovarian cancer, which contains fewer CTL and might therefore be more affected by a decrease of CTL below a certain threshold.

**Discussion**

In the current study, we determined the clinical and immunological relevance of HLA-E expression in ovarian and cervical cancer. Knowledge on the expression of HLA-E in these two cancer types was limited to small cohorts, and here we show that 89.4% of ovarian cancers and 83.7% of cervical cancers display higher levels compared to their normal epithelial counterparts. Total lack of HLA-E is rare in these tumors. Importantly, HLA-E protein expression was strongly associated with expression of classical HLA molecules (class I and class II) and components of the antigen processing machinery (immunoproteasome, peptide transporter TAP, trimming enzyme ERAP and chaperone Erp57) (Table 1). This implies that tumor expression of HLA-E is regulated in a comparable fashion to classical HLA and that its presence on tumors is not a defense mechanism against NK cell mediated lysis in classical class I-negative tumors, as sometimes suggested in literature (26;29-31). Instead, our data argue that HLA-E expression arises in the setting of an intact antigen processing apparatus and, in ovarian cancer, abundant CTL infiltration. A positive association between classical and non-classical HLA expression has recently also been reported for a large cohort of breast cancers (32), and is moreover anticipated on basis of the stabilization of HLA-E by leader peptides derived from classical HLA molecules (2;33).

Traditionally, interaction with NK cells via receptors CD94/NKG2A and CD94/NKG2C was considered the main purpose of HLA-E. The presence of infiltrating NK cells in ovarian and cervical cancers was previously reported by several groups (34-39). Detection of NK cells in tumor samples has predominantly been performed with antibodies against CD56 and CD57, whereas these molecules
can also be found on T lymphocytes. We carefully analyzed NK infiltration by inclusion of the CD3-specific T lymphocyte marker, or using the really specific molecule NKp46, which is not expressed on T lymphocytes (28). Our data reveal that NK cells hardly infiltrate ovarian and cervical cancers, conform the general impression in solid tumors (40), in contrast to leukemias, where NK cell responses have been connected to better survival (41).

In addition to NK cells, the inhibiting receptor CD94/NKG2A and activating receptor CD94/NKG2C are expressed by minor populations of CD8+ T cells. Although this subset is generally very scarce in PBMC of healthy subjects (approximately 4%) (Fig. 2) (32;42), the frequency of CD94/NKG2A expressing CD8+ T cells is much higher in tumor infiltrating lymphocytes, as shown in our study and by others (43;44).

Interestingly, the immunosuppressive cytokine TGF-β, which is regularly detected in ovarian and cervical cancer (45-47), seems to induce this inhibiting receptor on T cells (44). Several studies have shown that the inhibiting receptor CD94/NKG2A dampens the incoming activation signals of T cells by recruitment of phosphatases like SHP-1 to the signal transducing synaps, resulting in decreased effector functions (1;44;48). Strikingly, the activating receptor CD94/NKG2C was absent on tumor infiltrating T cells (Fig. 2), whereas it is expressed in other inflammatory situations (49-51). This implies that expression of the NKG2 chains is differentially and independently regulated and that NKG2A is selectively upregulated in tumors.

Protein expression of HLA-E was previously analyzed on cultured cancer cell lines and small cohorts of surgical specimen of some cancer types (16;26;52-54). HLA-E expression was correlated with increased infiltration of CD8+ CTL in glioblastoma (53), and decreased infiltration of NK cells as well as a worse progression free survival in colorectal cancer (26). In cervical cancer, HLA-E expression seemed to gradually increase from cervical intraepithelial neoplasia (CIN) I to invasive cervical cancer (54). Intriguingly, we and others (55) found no associations with tumor stage or grade.

We have to note, however, that our cervical cancer cohort represented early stage patients with
relatively highly differentiated tumors, whereas the ovarian cancer cohort consisted of mostly late stage, high grade tumors. The expression pattern and frequency of HLA-E was quite similar in our two studied cancer types as well as its positive association with antigen presenting molecules. The effect on survival was however clearly different. High HLA-E expression in ovarian cancer appeared to neutralize the beneficial effect of CTL infiltration. These results are in line with the in vitro data by Malmberg et al. (56), who demonstrated that HLA-E on freshly isolated ovarian cancer cells was upregulated by IFN-γ treatment, resulting in a CD94/NKG2A-mediated resistance to CTL lysis. However, in cervical cancer HLA-E did not influence the prognostic effects of CTL nor the CTL/Treg ratio. This difference might be explained by the significantly higher numbers of infiltrating CTL in cervical cancer. At least three times more intratumoral CTL can be found in this tumor type (20;21;57), which is most likely the result of the presence of viral antigens from HPV and an active inflammatory response.

In conclusion, our results suggest that HLA-E expression in ovarian and cervical cancer is the result of a smoldering inflammatory response. This concept, described by Coletta et al. (58), entails the presence of an inflammatory milieu which can either promote tumor progression or antitumor activity. The inhibiting impact of HLA-E in cervical cancer is limited due to beneficial signs of inflammation such as high CTL infiltrate, strong viral antigens and stimulating HLA ligands (MICA and classical HLA). In ovarian cancer, the presence of HLA-E is able to neutralize the protective role of the relatively scarce intratumoral CTL (19-21;59;60).

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Supplementary information

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