Analysis of NK Cells in Peripheral Blood and Tumor Infiltrating Lymphocytes in Cervical Cancer Patients

Artículo original

Análisis de la población de células asesinas naturales (NK) en sangre periférica y en linfocitos infiltrantes de tumor en pacientes con cáncer de cuello uterino

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Abstract

Objective: To understand the biologic and clinical importance of intratumoral natural killer cells CD16+CD56+CD3 and NKT CD16+CD56+CD3 cells in immune surveillance against cervical cancer. Methods: To understand the significance of NK (CD16+CD56+CD3-) and NKT (CD16+CD56+CD3-) in immune surveillance against cervical cancer, we analysed 39 peripheral blood and 30 biopsy samples from cervical cancer patients, and 40 peripheral blood and 5 biopsy samples from healthy women with normal cytology. The frequencies of NK and NKT and HLA-I expression in keratinocytes were analysed by flow cytometry. Results: In peripheral blood, a higher frequency of NK was observed in the patient group compared with the controls (p=0.002). However, this increase was not reflected in TILs (p=0.095). A significant reduction of HLA-I expression was observed in the patient group compared to the control group (p=0.019). A low number of NK infiltrated was observed in tumors with HLA-I down regulation, but it was not significant (p=0.374). A low number of NK infiltration was associated with shorter survival, but it was not significant (p=0.275). Conclusions: Our results show that although an increase in NK population was observed in peripheral blood, this increase was not reflected in TILs. It is possible that this inefficient migration of NK’s into the tumor milieu could be related to the expression of immunosuppressive cytokines, in particular IL-10.

Key words: Lymphocytes, tumor-infiltrating, killer cells, natural, uterine cervical neoplasms
Resumen

Objetivo: Entender la importancia biológica y clínica de las células intratumorales natural killer (NK) CD16+CD56+CD3- y de las células natural killer T (NKT) CD16+CD56+CD3- en la inmunovigilancia del cáncer de cuello uterino (CCU). Métodos: Para comprender el papel de las NK (CD16+CD56+CD3-) y de las células natural killer T (NKT) (CD16+CD56+CD3-) en la inmunovigilancia del CCU, se analizaron 39 muestras de sangre periférica (SP) y 30 biopsias de pacientes con CCU, así como de 40 muestras de SP y 5 biopsias de cuello uterino de mujeres con citología normal. Las frecuencias de NK y NKT y la expresión de HLA-I se analizaron por citometría de flujo. Resultados: Se observó una mayor frecuencia de NK en SP en el grupo de pacientes comparado con el grupo control (p = 0,002). Sin embargo, este aumento no se reflejó en TIL (p = 0,095). Una reducción significativa de HLA-I se observó en el grupo de pacientes (p = 0,019). Esta disminución se asoció a una disminución en el número de NK, pero no fue significativa (p = 0,374). Un bajo número de NK se asoció con una menor supervivencia, pero no fue significativo (p = 0,275). Conclusiones: Nuestros resultados muestran que aunque en SP se observa un incremento de NK, este no se refleja en los TIL. Es posible que este tráfico ineficiente de células NK hacia el tumor esté alterado por la expresión de citocinas inmunosupresoras, en particular IL-10.

Palabras clave: Linfocitos infiltrantes de tumor, células asesinas naturales, neoplasias de cuello uterino

Introduction

Cervical cancer is the second most common female cancer in women worldwide (1). In Colombia, there is a high incidence rate of 21.5 per 100,000 per year, and a mortality rate of 10 per 100,000 per year (2). Infection with high-risk Human Papilloma Virus (HR-HPV) types such as HPV 16, 18, 31, 45 and 58 is the main etiological factor associated with the development of high grade intraepithelial squamous lesions (HSIL) and cervical cancer (3,4). Although a very high percentage of women are positive for HPV-16, most HPV infections are sub-clinical in immunocompetent patients or manifest intermitently as self-limiting warty lesions. Only a small, but medically important fraction, of the lesions will progress to HSIL, and if left untreated, to invasive and metastatic carcinomas.

Numerous lines of evidence suggest an important role of immune responses in the control of HPV infection and progression to cancer. It has been reported a higher incidence of persistent HPV infections and more rapid progression of lesions in immunosuppressed patients compared to immunocompetent individuals (5,6). Additionally, the successful immune response to genital HPV infection is characterized by a strong CD4+ and CD8+ T cell infiltration, associated with spontaneous regression of lesion and protection against further infection(7,8). Moreover, the tumor-infiltrating lymphocytes have been considered to be a good prognostic factor in some tumors, including cervical cancer (9,10).

Tumor escape from host immunosurveillance is frequently attributed to the release of immunosuppressive cytokines or to the alteration of antigen-presenting molecules on neoplastic cells (11). The complete loss of human leukocyte antigen (HLA) class I results in resistance to cytotoxic T lymphocyte (CTL)-mediated lysis, but it in turn renders tumor cells susceptible to natural killer (NK) cell-mediated apoptosis.

The NK cells are large granular lymphocytes which constitute from 5-20% of peripheral blood lymphocytes (PBL). They are important cytolytic and cytokine-producing effector cells of the innate immune system and posses the ability to lyses tumor cells without the need for presentation of tumor-specific antigens (12). NK cells can be divided into two subsets, on the basis of their cell surface density of CD56 (12). The majority of NK cells are CD56dim NK circulating (90%). They have a greater cytolytic activity and express a high level of CD16 (13). The remaining 10% are CD56bright NK. They produce significantly greater levels of cytoquines (INF γ, TNFα, TNFβ, IL-10, IL-13, GMCSF and they predominate in lymph nodes and sites of inflammation (14). Although the number of intratumoral NK cells constitutes a
small percentage of the infiltrating lymphocytes, they may be enough to have local antitumor effects. Accumulation of intratumoral NK cells in solid tumors, including gastric and esophageal squamous cell carcinoma, was correlated with improved survival rates (15,16).

In cervical cancer, the CD8 T cell - infiltrates has been reported to be a significant prognostic factor, but the biologic and clinical importance of intratumoral NK cell infiltration is not yet clear. To better understand the biological and clinical significance of NK cells involved in the immune surveillance against cervical cancer, in this study, we analysed the subpopulation of NK (CD16+CD56+CD3-) and NKT (CD16+CD56+CD3+) cells from peripheral blood and intratumoral tissue of cervical cancer patients.

Materials and methods

Forty patients with invasive cervical cancer staged IB I to IV, according to the International Federation of Gynecologist and Obstetricians (FIGO), attending the outpatient gynecology clinic at the Instituto Nacional de Cancerología (INC), in Bogotá, Colombia, from February to June 2006, were enrolled in the study. A complete clinical history was obtained for each patient at the time of the procedure. Patients were not included if they had undergone any cancer treatment before the study or, if they showed prior or concurrent second malignancies, if they had apparent cervicitis, or if they were pregnant. Forty women with normal cytology, who were being treated at the Liga Colombiana de Lucha contra el Cáncer (Cancer Society), and 5 women who had undergone hysterectomy for benign uterine conditions, made up the control group. This study was approved by the INC Medical Ethic’s Committee. Informed consent was obtained from each cancer and control patient involved in the study.

**Buffy coat preparation from whole blood**

Peripheral blood from patients and controls was obtained and transferred into tubes containing ethylenediaminetetraacetic acid (EDTA). Whole blood was centrifuged at 200 x g for 10 minutes at room temperature. The concentrated leukocyte band (Buffy coat) was removed. Cell suspension was washed twice in 10%-FBS (Fetal Bovine Serum) RPMI and resuspended in PBS (Phosphate Buffered Saline) for subsequent immunofluorescence labelling.

**Isolation of tumor-infiltrating lymphocytes (TILs) and intraepithelial lymphocytes (IELs) from cervix**

Cervical biopsies were obtained from 30 patients with cervical cancer and 5 women who had had a hysterectomy for benign uterine conditions. Fragments of cervical tissue were carefully washed with RPMI 1640 to remove contaminating blood. Then, adipose and necrotic tissues were removed with a surgical knife. The samples were weighted and cut in fragments of 1-2 mm³ and incubated at 37°C for 1-2 h in RPMI medium containing 0.5 mg/ml collagenase II (Sigma) at 10 ml per gram of tissue. After that, the cell suspensions were passed through a nylon sieve with 100 µm pore size (Sigma). Finally, Fetal Bovine Serum (FBS) was added to a final concentration of 10% to block proteolytic activity. The average yield was 48 x 10⁶ cells/g tissue and the viability was 90% as estimated by the trypan-blue exclusion method. Cell suspension was washed twice in 10%-FBS RPMI and resuspended in PBS.

The following monoclonal antibodies (mAbs) were used: anti-CD3 FITC, anti-CD16+/anti-CD56 PE (SK7, B73.1, MY31), anti-CD45 PE (HI30), anti-CD45 PerCP (2D1), anti-HLA class I PE-Cy5 (W6/32), anti-mouse IgG (H+L) FITC (FI), anti-cytokeratins 5, 6, 8, 17, and 19 (MNF116), and the respective isotypes as negative controls. All antibodies were purchased from BD Biosciences.

**Cell lines and culture**

Three cervical carcinoma cell lines: C33a derived from cervical cancer biopsies, SiHa derived from a patient with a grade II squamous cell carcinoma of the cervix and HeLa derived from cervical adenocarcinoma biopsies were cultured in RPMI 1640
supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/ml streptomycin and 100 IU/ml penicillin. All cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

**Cell Staining and Flow Cytometry**

The Buffy coat suspension and tumor infiltrating cells from cervical tissue were resuspended in PBS to a final concentration of 1 x 10⁶. The cells were incubated with the optimal dilutions of each mAb (CD3, CD16, CD56, and CD45) for 30 min at 4°C in the dark. Afterwards, the cells were fixed with 2% paraformaldehyde (PFA), between 20,000 to 50,000 events were acquired in list mode on an FACSCalibur Flow Cytometer™ (BD Immunocytometry Systems, San Jose, CA) using CellQuest software; the lymphocytes were gated according to their normal forward and side scatter properties and also by CD45 fluorescence. For each sample, a negative control with isotype-matched mAbs was used to determine positive and negative cell populations. Cells were analysed using WinMDI 2.8 and CellQuest-Pro software. NK cells were divided into two subsets, on the basis of the surface density of CD56. CD3-CD16brightCD56dim (CD56dim NK) was defined using a geometric mean fluorescence intensity (MIF) between 90-250, while the CD3-CD16dim CD56bright (CD56 bright NK) was defined using a MIF between 250 - 550.

**Analysis of HLA - I protein expression using flow cytometry**

To determine the HLA class I expression, the cells (5 x 10⁶) were fixed with 250µl of Cytofix / Citoperm (Pharmingen) for 20 min at 4 °C and washed twice with Perm / Wash 1X (Pharmingen). Then, the cells were incubated for 30 min at 4°C with the cocktail of antibodies directed against cytokeratins (5, 6, 8, 17 and 19) and washed with Perm / Wash 1X. After one wash with PBS, the cells were incubated for 20 min at 4°C with anti-mouse FITC and after another wash with the same buffer; they were stained for 30 min at 4°C with anti-HLA class I PE-Cy5 and finally fixed with 2% PFA. As a control of HLA down regulation the Caski, SiHa and C33a cells were used. For this, the cells were fixed and permeabilized, and then they were stained with anti-HLA, as described previously. Data acquisition was performed with FACSCalibur flow cytometer, and HLA class I expression was analysed using CellQuest software.

**IL-10 detection by Immunohistochemistry**

The analysis of IL10 expression was performed on 21 frozen cervical biopsies. Briefly, 4 µm thick cryostat sections were cut and placed on glass slides. After 15 minutes air drying, the sections were fixed for 10 min in ice-cold acetone and stored at -20°C until their use. Nonspecific binding sites were blocked by incubating slides with 20% AB serum/ phosphate-buffered saline for at least 15 minutes at room temperature. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 15 minutes. Then, the slides were incubated for 30 min. at room temperature with the corresponding dilution of anti-IL-10 mAb (E10). Incubation with PBS was used as a negative control. After three washes with 1X PBS, biotinylated goat anti-mouse immunoglobulins (DAKO) were applied and incubated at room temperature for 30 min. Then, the slides were incubated with an avidin-biotin-peroxidase conjugate for 30 min. The immunocomplexes were developed with 0.05% DAB and 0.01% hydrogen peroxide in PBS for 5–10 min at room temperature. Subsequently, the slides were counterstained with hematoxylin, dehydrated in alcohol, and cleared in xylene before mounting. The staining intensity of HLA class I expression in the tumor cells was scored as described by Feenstra, with some modifications (17): Negative, no staining of tumor cells was observed; 1 to weak or focal HLA class I expression staining compared to staining of the surrounding stromal cells ; 2 For > 75 % expression; Positive, normal HLA class I expression, when the staining of tumor cells showed the same intensity of surrounding stromal cells.

**Statistical analysis**

Statistical analysis was performed using SPSS version 18. The Shapiro-Wilk test was used to determine if continuous variables were normally distributed. Because the number of TILs does not follow a normal distribution pattern, the mean was used to discriminate between low and high
number of TILs (18). The distribution of the frequencies of NK cells and NKT were plotted using box and whisker diagrams. The Mann-Whitney test was used to compare the frequencies of NK and NKT cells from peripheral blood and TILs between cancer and control groups. The level of HLA class I expression was determined as MFI. To compare the MIF of HLA class I expression between cancer and control groups, the Mann-Whitney test was used. Chi-square test, or where appropriate Fisher’s exact test, was used to assess differences in NK cell frequencies in different stages of cervical cancer with HLA class I and IL-10 expression. Survival rates were calculated by the Kaplan–Meier method and the differences between the survival curves were determined by the log-rank test. Overall survival (OS) was defined as the interval from beginning of the treatment to death or last visit date. Statistical significance was defined as a $P$ value less than 0.05.

**Results**

The clinical characteristics of the study population are listed in Table 1. A total of 40 patients with invasive cervical cancer staged IB I to IV was enrolled in this study. Age range was between 28 to 60 years and the mean age of the patients was 46.2 years, SD± 10. 18/40 (45%) patients received combined chemo-radiotherapy (CHT-RT), 6/40 (5%) patients received radiotherapy alone, and one patient (2.5%) was treated with surgery/RT. 14/40 (35%) patients did not accept any therapy. After starting treatment, one patient abandoned treatment. The mean follow-up time was 22.28 months, with a time range from 1 to 57 months.

![Figure 1](image-url)

**Figure 1.** (a) Frequencies of NK and NKT cells in peripheral blood of cervical cancer patients and control group. The frequencies of lymphocytes from a cervical cancer patient were determined over a gated CD45 positive with low light-scatter properties (lymphogate). (b, c) Representative flow cytometry results of NK and NKT cells from cervical cancer patient and normal subjects are showed respectively. (d) Cumulative results from 39 cervical cancer patients and 40 normal subjects show an increase of NK cells in the cancer group ($P = 0.002$). (e) while there are not differences in the frequencies of NKT cells between the cancer and control group ($P = 0.70$). (f) Proportion of circulating NK cells in cancer and control group. The length of each box corresponds to the interquartile range. The bottom and top edges of the box mark the 25th and the 75th percentiles, respectively. The centre horizontal line is drawn at the sample means.
An increase of NK cells in the peripheral blood of cervical cancer patients

The frequencies of tumor-infiltrating lymphocytes from cervical cancer patients were determined over a gate CD45 positive with low light-scatter properties (lymphogate) (Fig. 1a). NK cell population was identified by CD16+ and CD56+ coexpression without CD3 expression, whereas NK cell population was identified by the simultaneous expression of CD16+ CD56+, and CD3+. Representative density plots from a cancer patient as well as from a normal donor are shown in Figure 1b and 1c. In this study, a higher frequency of NK cells was observed in the patient group compared to the control group (median: 19.05% vs 13.15% \( p = 0.002 \)) (Fig. 1d). Similarly, significant differences in absolute counts of NK cells between cancer group and control group were observed: 376, Interquartile range (IR): 227.1 – 517.1 vs 256, IR: 174.6 - 337.5 respectively \( p <0.05 \). In contrast to NK cells population, the frequencies and absolute counts (68, IR:37.8 – 91.4 vs 69, IR: 32.1 – 95.8 respectively) of NKT cells in the cancer group were similar to those observed in the control group and no significant differences were observed \( (p >0.05) \) (Fig. 1e). In this study, the majority of NK cells circulating observed in patient group was of CD56dim NK cell phenotype; whereas, in the control group, there was a similar proportion between these two subpopulations (Fig. 1f).

Low numbers of NK cells were observed in tumors from cervical cancer patients

Analysis of NK and NKT cells was performed using three-color FACS analysis on freshly isolated TILs from 30 patients with cervical cancer and IELs from 5 control group members. A representative density plot of NK and NKT cells from a patient is shown in Figure. 2a.; although in peripheral blood an increase in NK cell population was observed in patient group, this increase was not reflected in TILs. Low frequencies of NK and NKT cells were observed in cervical cancer patients compared to those in the control group (Mann-Whitney test, \( p = 0.095 \) and \( p > 0.05 \) respectively). In patient group, the means were 0.53% and 1.41%, respectively; whereas in the control group, the means were 0.86% and 1.3%, respectively (Figure 2 b and c). Figure 3 shows the analysis of NK cell numbers infiltrating the tumor in relation to the stage. A greater number of NK cells infiltrated was observed in more advanced stages; however, there was no statistically significant difference between groups \( (p = 0.153) \).

Lower HLA class I expression was not associated with higher NK cell infiltration

The HLA class I expression was analysed over a gate of 20,000 keratinocytes as described in the Materials and Methods section. Figure 4 shows the...
HLA class I expression from normal and tumoral keratinocytes. The histogram shows the geometric mean of fluorescence intensity (MFI) of HLA class I expression. In general, a reduced HLA class I expression was observed in keratinocytes from cervical cancer biopsies (MFI = 330), as well as in the three cervical cancer cell lines analysed: SiHa, HeLa and C33a (MFI = 143, 156 and 100 respectively). The MIF of HLA class I expression from normal keratinocytes was 864 (figure 4a). Figure 4b shows the cumulative analysis of HLA class I expression in tumor tissue from 30 cervical cancer patients and normal cervical tissues from 5 control subjects. A significant reduction of HLA class I expression was observed in the patient group compared to the control group (759 vs 989 respectively, \( p = 0.019 \)).

NK cells were described as non-MHC-restricted in their recognition process because of their ability to kill target cells that either lacked MHC or expressed allogeneic MHC molecules (19); when we analysed the association between HLA class I down regulation and the number of tumor infiltrating NK cells, a low number of NK-cell infiltrated was observed in tumor of patients with HLA class I down regulation. However, there were no statistical differences (\( p = 0.374 \)). Finally, in order to determine if IL-10 expression is associated with the low NK cell infiltration, the IL10 expression was analysed in 16 tissue samples by IH. 12/16 (75%) samples analysed were IL-10 positive, from these samples, 8 (66.7%) showed a low number of NK cells infiltrated; however, there were no statistically significant differences (\( p=0.182 \)).

**Analysis of survival and infiltration of NK cells**

We analysed the role of NK cell infiltration on OS. Univariate analysis of OS is shown in figure 5. Although patients that showed a low number of NK cell infiltration had a shorter survival in comparison with those that had a higher number of NK cells, this difference was not significant (\( P=0.275 \)).

![Figure 3](image3.png)

**Figure 3.** NK cell infiltration in tumor lesions according to stage. A greater number of NK cells infiltrated was observed in more advanced stages, but this was not significant (\( p = 0.153 \)).

![Figure 4](image4.png)

**Figure 4.** HLA class I expression from normal and tumoral keratinocytes. (a) The histogram illustrates the geometric mean fluorescence intensity of HLA class I expression of three cervical cancer lines (SiHa, HeLa and C33a), normal keratinocytes and representative tumoral cells of a cervical cancer patient. The dotted magenta line shows the HLA class I expression and the green line shows the isotype control. (b) Cumulative results from 30 tumor samples and 5 normal cervixes are shown in box and whisker plot graphs. The mean HLA class I expression frequencies in cervical cancer were significantly higher than in normal control (\( P = 0.019 \)). The length of each box corresponds to the interquartile range. The bottom and top edges of the box mark the 25th and the 75th percentiles, respectively. The center horizontal line is drawn at the sample means.
Discussion

Natural Killer cells constitute a small portion of the tumor infiltrating lymphocytes, which exhibit spontaneous cytotoxic activity against tumor cells and represent the first line of defence in tumor surveillance (20). It has been reported that NK cells play an important role in the regulation of tumor proliferation and that NK cells can be a useful marker in terms of survival of patients in early stages of cancer (21). In cervical cancer, the distribution of lymphocyte subpopulations in tumor milieu, particularly the CD8 T lymphocytes (10,22) has an important implication in disease progression and as a prognosis factor. However, the clinical importance of intratumoral NK cell infiltration is not fully understood.

A critically important function of NK cells responsible for surveillance against cancer is the capacity to extravasate and localize in tissue (23). In this study, a higher frequency of peripheral blood CD56dimCD16 bright NK cells was observed in cervical cancer patients compared to control group. However, a low infiltration of NK cells in the tumor samples was observed. These results are in accordance with studies in established human tumors that have shown that there are often only a few infiltrating NK cells which are unlikely to greatly contribute to the elimination of tumor cells (24-26). Moreover, it has been suggested that despite low NK cell numbers in tumors, due to their inefficient homing into malignant tissues (24,27), this situation may be overcome by cytokine-mediated activation in immunotherapeutical regimens (28).

Development of SIL and cervical cancer is preferentially associated with a shift from Th1 type to a Th2 type cytokine response characterized by increased level of IL-4 and IL-10 and decreased amount of IL-12 and IFN-γ (11,29,30). Moreover, it has been established that IL-10 down regulates the IL-12 production, thereby preventing the migration and activation of Natural Killer Cells and CTLs (31,32). When we analysed the IL-10 expression, a high percentage of samples were IL-10 positive (75%), and from these samples 66.7% had a low number of NK cells infiltrated, but, it was not significant. This result could suggest that inefficient migration of NK cells into the tumor milieu could be related to the expression of immunosuppressive cytokines, in particular IL-10, which down regulates the IL-12 production and thereby prevents the Natural Killer Cells’ migration (31). This study is limited by the small number of patients and other studies will be needed to confirm whether IL-10 could be one of the factors responsible for the inefficient migration of NK cells into tumor milieu.

Loss of MHC class I expression in cervical cancer results in the inability of tumor cells to directly present tumor antigens to cytotoxic T lymphocytes (33). However, loss of MHC class I expression also might render tumor cells susceptible to NK cell lysis (24). In this study, even if we observed a low HLA class I expression, the number of infiltrating CD16+CD56dim NK cells was low. We can assume that the low migration and infiltration of NK cells in tumors could be associated with insufficient concentrations of chemotactic chemokines (IL-8, MIP-1α, MIP-1β, RANTES, IP-10, MCP-2, MCP3, IFNγ, IL-12 and TNFα) as well as a low expression of chemokine receptors such as CCR1, CCR2,
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CCR4, CCR5, CCR6, CCR7, CXCR3 in those cells (34-36). In addition, it has been shown that a higher percentage of CD56 bright NK cells migrate, in response to CXCL10 and CXCL11 in vitro, when compared with CD56 dim NK cells (35). On the other hand, previous studies have reported that patients with epithelial cancer show a higher onset of spontaneous apoptosis of NK cell subsets in peripheral circulation. Only CD56 dim NK cells were sensitive to the higher onset of spontaneous apoptosis in cancer patients, whereas CD56 bright NK cells were more resistant to programmed cell death (37,38).

Alternatively, HLA-G is a non-classical MHC molecule and its expression contributes to evasion of immunosurveillance in tumor cells(39). It has been reported that an increasing HLA-G expression allows HLA class I deficient cells to escape from NK-mediated lysis through interaction with killer inhibitory receptors on NK cells (39). In a study carried out by our group, the expression of HLA-G was observed in approximately 40% of invasive cervical cancer cases (unpublished data). These results suggest that tumor cells can protect themselves from NK cells lysis by expressing HLA-G molecules.

Finally, there have been some studies detailing the correlation between prognosis and local immune infiltration. It has been reported that a high NK cell counts is associated with a better prognosis in colorectal and gastric carcinoma patients than those who had low numbers of NK infiltration (16,40); In this study, it was observed that those patients who had a low number of NK cell infiltration had a shorter survival time than those who had a higher number of NK cells, but it was not significant (P=0.275). In another report, a positive correlation between NK cell counts and survival of patients with stage I pulmonary adenocarcinoma, but not with disease in stage II and III, has been reported. These results can be explained by the difference of population analysed, the majority of our study population was in advanced stages IIB and IIIB.

In gastric carcinoma, as in pulmonary adenocarcinoma, a high level of NK cell infiltration was associated with the clinical stage; a higher infiltration was observed in early stage (IA, IB) (21,41).

In this study, we did not observe an association between clinical stage and infiltration of NK cells. It can be explained by differences in the population analysed. In Ishigami and Takanami reports, they observed differences only in patients with a high NK cell infiltration and no differences were observed with a low level of NK infiltration. In our study, the majority of the population had low infiltration.

In conclusion, our results show that although in peripheral blood an increase in NK cell population was observed in patient group, this increase was not reflected in TILs. It is possible that this inefficient migration of NK cells into the tumor milieu could be related to the expression of immunosuppressive cytokines, in particular IL-10; however other studies will be needed to confirm this.

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