UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

LUCIANA MIELI SAITO

NK cells and the profile of inflammatory cytokines in the peripheral blood of patients with advanced carcinomas

Células NK e perfil de citocinas inflamatórias no sangue periférico de pacientes com carcinomas avançados

BAURU
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Dissertação constituída por artigo apresentada à Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Mestre em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Biologia Oral, Estomatologia, Radiografia e Imaginologia.

Orientadora: Profa. Dra. Camila de Oliveira Rodini Pegoraro

BAURU

2020

Saito,Luciana Mieli
Nk cells and the profile of inflammatory cytokines in the peripheral blood of patients with advanced carcinomas. -- Bauru, 2020.
55 p. : il. ; 31 cm.

Dissertação (mestrado) -- Faculdade de Odontologia de Bauru, Universidade de São Paulo, 2020.

Orientadora: Profa. Dra. Camila de Oliveira Rodini Pegoraro

Comitê de Ética da FOB-USP
Protocolo nº: 99727018.5.0000.5417
Data: 04/12/2018

ERRATA

FOLHA DE APROVAÇÃO

DEDICATÓRIA

Esta dissertação dedico a todos os pacientes da oncologia do Hospital Estadual de Bauru e aos que aceitaram fazer parte desta pesquisa mesmo com todas as comorbidades resultantes do câncer, não mediram esforços para ajudar nesta pesquisa, o meu muito obrigado e gratidão eterna.

AGRADECIMENTOS

Á **Deus** por me permitir chegar até o final do mestrado, dando-me forças e sabedoria.

Aos meus pais, **Jorge Saito** (*in memoriam*) e **Maria Augusta M. Saito**, que aceitaram a minha escolha de momentaneamente abandonar minha carreira profissional para somente me dedicar a vida acadêmica, um sonho adormecido que se tornou realidade.

À minha queria irmã **Claudia Saito** e cunhado **Matheus Andreo** que nunca me desampararam em momento algum e me presentearam com duas anjinhas, minhas sobrinhas **Liz e Clara**, que tanta alegria trouxe para nossas vidas.

À minha **avó Lourdes** a quem expresso toda minha admiração e aos meus tios Madalena, Cecília, Angelo (*in memoriam*), Orlando e primos.

Aos meus compadres **Anthony Castilho** e **Jamile Veloso** que hoje são mais que amigos, fazem parte da minha família, também me presentearam com um anjo, meu afilhado **Octávio**.

As minhas amigas **Natasha Osajima, Myriam Gholmié, Graziela Manrique Ana Laura Moraes e Beatriz Moraes**, que sem elas muitos momentos não fariam sentido, sou grata por me acompanham nesta jornada muito antes de tudo se tornar realidade.

À minha orientadora **Profa. Dra. Camila**, obrigada por confiar em mim mesmo sabendo que estava afastada da área acadêmica, obrigada pela paciência, pelos ensinamentos e preocupação; a senhora sabe acolher e orientar a todos sem distinção, conduz a equipe com leveza e determinação. E posso dizer com propriedade que a senhora tem uma equipe, pois quando precisei todos me ajudaram, e tenha certeza que nesta dissertação tem um pouco do conhecimento de cada aluno da senhora.

Aos meus colegas que se tornaram amigos e hoje fazem parte da minha vida, **Rafael Ortiz, Nádia Amôr, Nathália Martins e Rodrigo Buzo** nunca mediram esforços para me ajudarem e me ensinarem. Com toda certeza gastaria páginas falando de cada um de vocês, mas podem ter certeza que em minhas orações sempre estarão. Nádia e Rafael preciso deixar registrado aqui, que sem a ajuda e conhecimento de vocês nessa reta final, sozinha seria difícil, mas graças a Deus trabalhamos em equipe e conseguimos finalizar.

Aos amigos da histologia **Paulinha Sanchez**, **Suellen Paini**, **Vinícius Tagliavini**, **Ever Mena**, **Jéssica Melchiades**, **Angélica Fonseca**, **Carol Bighetti**, **André Petenuci** e **Luan Macena**.

Às técnicas da histologia **Danielle Ceollin**, **Patrícia Germino** e **Dra.Tânia Cestari**, sempre dispostas a nos ajudarem.

À querida secretária **Teresa**, tão cuidadosa e prestativa.

À **Dona Divina** com toda sua energia para nos proporcionar o tão esperado café depois do almoço.

Aos técnicos do CIP **Márcia Graeff**, **Marcelo Milanda** e em especial a **Rafaela Alves** que me deu um certo "empurrão" para iniciar a pós-graduação nesta instituição, sou muito grata.

Aos professores da histologia **Prof. Dr. Gustavo Garlet**, **Prof. Dr. Gerson Francisco, Prof. Dr. Rumio Taga e Profa. Dra. Camila Rodini**, por me acolherem e colaborarem para o meu crescimento profissional.

A **toda equipe do Hospital Estadual de Bauru**, ao **Ambulatório de Quimioterapia** e aos **funcionários do LAC**, ao qual voltei a fazer parte do quadro de colaboradores, me acolheram de forma tão carinhosa e me ajudaram quando precisei, as chefes **Fabiana Pegoraro**, **Daniela Di Flora** e a técnica **Vera Moreira** que muito me ajudou com o recrutamento dos pacientes.

E não poderia deixar de agradecer a você, **meu marido Pedro**, que tão cedo foi para a morada do Senhor, e ninguém mais do que você sabia da minha vontade em realizar esse sonho da pós-graduação sempre me apoiando. Onde estiver, tenho certeza que está orgulhoso de minha conquista; consegui, venci todos os obstáculos mesmo sabendo que não estaria mais aqui para me apoiar. Dizer-lhe adeus foi o ato mais doloroso e difícil de minha vida.

À **Faculdade de Odontologia de Bauru (FOB) – USP**, representado pelo digníssimo Diretor Prof. Dr. Carlos Ferreira dos Santos.

À **Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP**, processos número 2013/07245-9 e 2015/06945-2; pelo apoio financeiro.

À **Coordenação de Aperfeiçoamento de Pessoal De Nível Superior – CAPES**, pelo apoio financeiro concedido em forma de bolsa.

Ao **Hospital Estadual de Bauru – HEB, "Dr. Arnaldo Prado Curvêllo"**, em especial ao Laboratório de Análises Clínicas e ao Ambulatório de Quimioterapia.

À **Fundação para o Desenvolvimento Médico e Hospitalar – Famesp**, por permitir a realização desta pesquisa.

“Sempre parece impossível até que seja feito.”

Nelson Mandela

RESUMO

As células natural killer (NK) são uma das células imunes mais importantes na mediação da resposta antitumoral devido à sua capacidade de reconhecer e eliminar diretamente as células neoplásicas. Devido à sua grande atividade citotóxica, a função das células NK deve ser regulada para evitar danos aos tecidos. Tal regulação é mediada por um engajamento coordenado de receptores ativadores e inibitórios, pelos quais as células tumorais podem usar para escapar da vigilância imunológica. Para tanto, nosso objetivo foi avaliar a frequência e o fenótipo das células NK circulantes em pacientes com carcinomas avançados, bem como a produção sistêmica de citocinas, quimiocinas e fatores de crescimento. O sangue periférico de 10 controles saudáveis e de 24 pacientes com carcinomas avançados foi coletado durante ou após o tratamento, quando presente. A frequência e a expressão das moléculas ativadoras (NKp46) e inibitórias (CD158b) dentre as células NK com fenótipos $CD56^{bright}CD16^{-}$ (regulatório) e $CD56^{dim}CD16^{+}$ (citotóxico) foram avaliadas por citometria de fluxo. A quantificação dos fatores secretados presentes no soro foi realizada pela plataforma multiplex Luminex. Pacientes oncológicos apresentaram menor frequência do subtipo citotóxico das NK ($CD56^{dim}$ $CD16^{+}$) em comparação com controles saudáveis. Além disso, células NK tidas como regulatórias ($CD56^{bright}CD16^{-}$) isoladas de pacientes oncológicos exibiram uma expressão significativamente mais baixa de NKp46. As quimiocinas MCP-1, IP10, eotaxin e o fator de crescimento VEGF foram os fatores secretados mais proeminentes detectados no sangue dos pacientes analisados. O teste de correlação mostrou que a IL-12p40 está positivamente correlacionada com células NK $CD56^{bright}CD16^{-}$. Também observamos uma correlação positiva entre o MCP-1 e o marcador ativador NKp46, e uma correlação negativa entre IP-10 e TNF-α e NKp46. A expressão de CD158b em NK $CD56^{dim}CD16^{+}$ foi positivamente correlacionada com EGF e negativamente correlacionada com MIP-1β. Em conjunto, esses resultados sugerem que os pacientes com câncer exibem perfil NK alterado voltado para um fenótipo menos citotóxico, o que pode contribuir para o desenvolvimento e progressão do tumor. Portanto, direcionar utilizar fatores solúveis específicos relacionados às células NK como alvo pode ser uma abordagem terapêutica relevante para o câncer.

Palavras-chave: Células natural killer; carcinoma; citocinas; fatores de crescimento; quimiocinas; coleta de amostras de sangue.

ABSTRACT

Nk cells and the profile of inflammatory cytokines in the peripheral blood of patients with advanced carcinomas

Cancer is a multifactorial disease caused by a series of mutagenic alterations in genome which allow cancer cells to evade from immune surveillance, contributing to tumor progression. One of the most crucial immune cells that mediate the antitumoral response due to their ability to immediately recognize and eliminate transformed cells are the natural killer (NK) cells. These cells can be modulated by cancer and other immune cells in tumor microenvironment though different cytokines production. Indeed, currently studies have been investigated this regulation for cancer immunotherapy. Here, we aimed to identify the cytokine levels in peripheral blood (PB) of advanced carcinoma patients related to NK cells subtypes, according to the perspective of tumor infiltrating immune cells. The study included 18 patients with advanced cancer during or after to treatment and 10 healthy donors. The frequency and the expression of activating (NKp46) and inhibitory (CD158b) molecules of $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells were assessed by flow cytometry and the multiplex Luminex platform was used to quantify the secreted factors presented in the serum. Cancer patients had a lower frequency of the cytotoxic $CD56^{dim}CD16^{+}$ NK cells subtset in comparison with healthy controls. Also, the regulatory $CD56^{bright}CD16^{-}$ NKs isolated from cancer patients exhibited a significantly lower expression of NKp46. The chemokines MCP-1, IP10, and eotaxin and the growth factor VEGF were the most prominent secreted factors detected in cancer patient´s blood. The correlation test showed that IL-12p40 is positively correlated with $CD56^{bright}CD16^{-}$ NK cells. We also observed a positive correlation between MCP-1 and the activating marker NKp46 and a negative correlation between IP-10 and TNF-α and NKp46. CD158b expression in $CD56^{dim}CD16^{+}$ was positively correlated with EGF and negatively correlated with MIP-1β.

Keywords: Natural killer cells; advanced solid tumors; cytokines; growth factors; chemokines; peripheral blood.

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LIST OF ABREVIATIONS AND ACRONYMS

ADCC	Antibody dependent cellular cytotoxicity
CCL	C-C motif chemokine ligand
CD	Cluster of differentiation
CXCL	C-X-C motif chemokine ligand
EGF	Epidermal growth factor
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HNSCC	Head and neck squamous cell carcinoma
IL	Interleukin
INF	Interferon
IP	Inducible protein
MCP	Monocyte chemoattractant protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Metalloproteinase
NK cell	Natural killer cell
PB	Peripheral blood
TGF	Tumor growth factor
Th	T helper
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TRAIL	TNF related apoptosis inducing ligand
VEGF	Vascular endothelial growth factor

SUMMARY

1 INTRODUCTION

1 INTRODUCTION

Natural killer (NK) cells are innate immune cells that control important components of the immune system. As effector lymphocytes endowed with constitutive cytolytic functions or even producing cytokines, NK cells act through activating and inhibitory receptors to ensure self-tolerance against virus and tumor development [1]. Indeed, NK cells are able to infiltrate peritumoral areas from solid tumors [2] exhibiting two main subtypes divided according their functions. $CD56^{bright}CD16^{-}$ is a poorly cytotoxic, immature and cytokine-producing NK cell, while $CD56^{dim}CD16^{+}$ is a cytolytic, mature and weakly cytokine-producing NK cell. The frequency of these cells can be variable according to site in humans, *i.e.* $CD56^{bright}$ NK cells corresponds to around 10% of NK cells in the peripheral blood; however, it is the most common NK cell in tissues and second lymphoid organs [3].

Besides NK cells, microenvironment of solid tumors is composed by stromal, other immune and vascular cells, which produce several cytokines, chemokines, growth factors and matrix metalloproteinases (MMPs) [4]. These proteins are responsible for the crosstalk between tumor cells and its microenvironment, playing an important role in cancer development, progression and metastasis [5]. Indeed, a better understanding of how cytokines are regulated by cancer cells, as well as by NK cells in the tumor microenvironment and peripheral blood is crucial to improve prognosis and therapies at the cancer field.

All cell types can be a source of the molecules of the tumor-microenvironment (TME) and they exert very distinct functions in the tumor context. For example, the anti-inflammatory cytokines TGF-β and IL-10, by inhibiting T-cell activation, can promote tumor progression [6]. IL-4, IL-15, and IL-13 are potent inducers of the Th2-related responses that also contribute to tumor scape [7]. On the other hand, IFN-γ, TNF-α, and IL-12 increase the cytotoxic activity of $CD8^{+}$ T lymphocytes and NK cells, then promote tumor elimination [8]. The angiogenic factor VEGF promotes angiogenesis, therefore, facilitates tumor spread and metastasis [9].

Considering the impact of NK cells as well as the cytokine/chemokine profile of the host for cancer outcome, the present study investigated the frequency of both subtypes of NK cells ($CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$) comparing with healthy individuals. We also evaluated the expression of the activating marker NKp46, and the inhibitory marker CD158b within both phenotypes of NK cells. Lastly, taken the advantage of a multiplex assay, we

quantified the presence of 29 soluble proteins (IL-3, IL-4, IL-5, 1L-6, IL-7, IL-8, IP-10, MCP-1 (CCL2), MIP-1α, MIP-1β, TNF-α, TNF-β, VEGF, EGF, Eotaxin, G-CSF, GM-CSF, IFN-α2, INF-γ, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IL-1ra, IL-1α, IL-1β, and IL-2) in the peripheral blood of patients with advanced solid tumors and correlated with the NKs frequencies and their activation state.

2 ARTICLE

2 ARTICLE

2.1 ARTICLE 1

Cytokine Journal

NK cells and the profile of inflammatory cytokines in the peripheral blood of patients with advanced carcinomas

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ABSTRACT

Background

Natural killer (NK) cells are one of the most crucial immune cells that mediate the antitumoral response due to their ability to immediately recognize and eliminate transformed cells. Because of their great cytotoxic activity, the function of NK cells must be robustly regulated to avoid tissue damage. Such regulation is mediated by a coordinated engagement of activating and inhibitory receptors, by which tumor cells may use to escape from immunosurveillance. NK cells are generally divided based on surface molecules, such as CD16 and CD56, and can be classified as $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells, playing distinct roles in immunity acting as regulatory and cytotoxic cells, respectively. Here, we aimed to evaluate the frequency and phenotype of circulating-NK cells in patients with advanced carcinomas, as well as their systemic cytokine/chemokine production.

Methods

Peripheral blood was collected from 24 patients with advanced solid cancer during or after treatment and from 10 healthy donors. The frequency and the expression of activating (NKp46) and inhibitory (CD158b) molecules of $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells were assessed by flow cytometry and the multiplex Luminex platform was used to quantify the secreted factors presented in the serum.

Results

Cancer patients had a lower frequency of the cytotoxic $CD56^{dim}$ $CD16^{+}$ NK cells subset in comparison with healthy controls. Also, the regulatory $CD56^{bright}$ $CD16^{-}$ NKs isolated from cancer patients exhibited a significantly lower expression of NKp46. In general, the chemokines MCP-1, IP10, and eotaxin and the growth factor VEGF were the most prominent secreted factors detected in cancer patient´s blood. Pearson correlation test showed that IL-12p40 is positively correlated with $CD56^{bright}CD16^{-}$ NK cells. We also observed a positive correlation between MCP-1 and the activating marker NKp46, as well as a negative correlation between IP-10 and TNF-α and NKp46. CD158b expression in $CD56^{dim}CD16^{+}$ was positively correlated with EGF and negatively correlated with MIP-1β.

Conclusions

Taken together, these results suggest that cancer patients present a shift towards a poorly-cytotoxic and less activated NK profile which may contribute to tumor development and progression. Also, targeting specific soluble factors may be a relevant therapeutic approach for cancer.

Keywords: Natural killer cells; advanced solid tumors; cytokines; growth factors; chemokines; peripheral blood.

1. INTRODUCTION

Natural killer (NK) cells are innate lymphoid cells that play an important role during immune responses against intracellular pathogens and damaged/transformed cells [1]. During tumorigenesis, NK cells are recruited to the tumor microenvironment (TME) by chemokine gradients, such as CXCL9, CXCL10, and CXCL11 [2] and, once infiltrating the TME, they recognize neoplastic cells mainly due to the downregulation of MHC-I-related molecules on tumor cells [3]. NK cells can eliminate tumor cells by releasing cytolytic granules, like granzyme B and perforin, which degrade the plasma membrane of the targeted-cell, or by directly activating apoptotic pathways through Fas-FasL or TNF-related apoptosis-inducing ligand (TRAIL) [4]. Therefore, NK cells are recognized as the first line of defense against cancer.

In human, two distinct subsets of NK cells have been identified based on CD56 and CD16 markers, exhibiting distinct functions. $CD56^{bright}CD16^{-}$ phenotype is frequently described as poorly cytotoxic and, although they present a late IFN-γ production after stimulation, this subset can modulate immune responses through cytokine production [5][6]. On the other hand, $CD56^{dim}CD16^{+}$ NK is a much higher cytolytic cell and an important source of cytokines and chemokines upon recognition of aberrant cells [7]. In cancer, $CD56^{bright}$ is described as pro-tumoral meanwhile, $CD56^{dim}$ is anti-tumoral. However, studies had demonstrated that $CD56^{bright}$ NK cells can exhibit potent antitumor responses upon IL-15 stimulation [8][9].

The most crucial dictators of the immune response and tumor course are soluble components such as cytokines, chemokines and growth factors [10]. Virtually, all cell types can be a source of these molecules and they exert very distinct functions in the TME. For example, the anti-inflammatory cytokines TGF-β and IL-10, by inhibiting T-cell activation, can promote tumor progression [11]. IL-4, IL-15, and IL-13 are potent inducers of the Th2-related responses that also contribute to tumor scape [12]. On the other hand, IFN-γ, TNF-α, and IL-12 increase the cytotoxic activity of $CD8^{+}$ T lymphocytes and NK cells, then promoting tumor elimination [12]. The angiogenic factor VEGF promotes angiogenesis; therefore, facilitates tumor spread and metastasis [13].

Considering the impact of NK cells as well as the cytokine/chemokine profile of the host for cancer outcome, we aimed to investigate in the present study the frequency of both subsets of NK cells ($CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$) compared with healthy individuals. We also evaluated the expression of the activating marker, NKp46, and the inhibitory marker,

CD158b, on both phenotypes. Lastly, taken the advantage of a multiplex assay, we quantified the presence of 29 soluble proteins (IL-3, IL-4, IL-5, 1L-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β, VEGF, EGF, Eotaxin, G-CSF, GM-CSF, IFN-α2, INF-γ, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IL-1ra, IL-1α, IL-1β, and IL-2) in the peripheral blood of patients with advanced solid tumors and correlated with the NKs frequencies and their activation state.

2. MATERIALS AND METHODS

2.1. Subjects

The study included 10 healthy adult volunteers and 24 cancer patients; however, only 18 patients showed enough cells to performed the subsequent analysis. Cancer patients were diagnosed with 8 different types of carcinomas (Table 1), during or after chemotherapy, radiotherapy and/or surgical treatment, selected at the Hospital Estadual de Bauru, Bauru, São Paulo, Brazil. This study was approved by the local Research Ethics Committee of Bauru School of Dentistry (CAAE 99727018.5.0000.5417), and the informed consent was obtained from all of the donors.

2.2. Peripheral blood mononuclear cell (PBMC) and serum isolation

Venous blood samples were taken from cancer patients and healthy donors and collected in two sterile tubes: the first one containing EDTA (final concentration of 1.6 mg EDTA/mL blood) and the second one was a serum gel tube, being processed within 24 hours. PBMCs were obtained by density gradient centrifugation using the Histopaque-1077 gradient (Sigma-Aldrich, St Louis, MO, USA). PBMCs were either used directly for the experiments or cryopreserved in 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA) and stored in liquid nitrogen for future analysis. The serum was obtained by centrifugation, 2200 g, 15 minutes, at room temperature (RT), and then stored at -80°C with proteases inhibitors for further analyses.

2.3. Flow cytometry, antibodies, and NK cell identification

Flow cytometry analysis was performed to identify and quantify NK cells subsets and profile in PBMC from patients with advanced solid tumors and healthy donors. Before immunostaining, the frozen PBMC were thawed and washed to eliminate the DMSO and cell

debris. Cells were then incubated with Human BD Fc Block (BD Biosciences, San Diego, CA, USA) to reduce unspecific antibody binding followed by incubation with monoclonal antibodies for 30 minutes at 4°C protected from light. The monoclonal antibodies used were from BD Biosciences: APC-conjugated mouse anti-human CD56 (clone B159), PE-conjugated mouse anti-human CD16 (clone B73.1), APC-H7-conjugated mouse anti-human CD3 (clone SK7), BV421-conjugated mouse anti-human CD335 (NKp46, clone 9E2/NKp46), and BB515-conjugated mouse anti-human CD158b (clone CH-L). The respective control isotypes were used. Samples were acquired using the Becton and Dickenson FACSAria^{TM} Fusion Cell Sorter and analyzed by BD FACSDiva^{TM} 8.0 software (BD Biosciences). Cells populations were analyzed by gating the cells according to size (FSC), granularity (SSC), and fluorescence (FL) parameters. NK cells were defined by the $CD3^-$phenotype and subpopulations profile were defined as regulatory or cytotoxic based on $CD3^-CD56^{bright}CD16^-$ and $CD3^-CD56^{dim}CD16^+$, respectively. Additionally, the expression of NKp46 was used to identify activated NK cells while CD158b was used to identify inhibited NK cells.

2.4. Milliplex® MAP-Human Cytokine/Chemokine Magnetic Bead Panel

Systemic secreted factors levels were measured in the whole population of NK cells using the Milliplex assay kit (Cat. # HCYTOMAG-60K-PX29, EMD Millipore, Billerica, MA, USA), according to the manufacturer's instructions. The analytes evaluated were: IL-3, IL-4, IL-5, 1L-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β, VEGF, EGF, Eotaxin, G-CSF, GM-CSF, IFN-α2, INF-γ, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IL-1ra, IL-1α, IL-1β and IL-2. Data were analyzed using the Luminex MAGPIX® System and Xpotent 3.1 data analysis software (EMD Millipore).

2.5. Statistics

The results were analyzed using GraphPad Prism 7.0 software (GraphPad software, Inc., CA, USA). The levels of cytokines were analyzed by unpaired Student's t-test to compare the reported mean values with standard deviations for the different variables. Pearson's correlation test was used to analyze the correlation between cytokines and NK cell subsets. Groups being compared were considered to be significantly different if $p<0.05$.

3. RESULTS

3.1 Analyses of circulating NK cells from healthy and cancer patients

The characteristics of the cancer patients are shown in Table 1. Patients were predominantly male (72.7%), aging from 18 to 69 years old, and younger than 60 years old (81.8%). According to the histological type of solid tumors, mostly were head and neck squamous cell carcinomas (36.3%), all were classified as stage III (Table 1). Unfortunately, it was not possible to identify the treatment phase which PB was collect.

NK cells were analyzed in the peripheral blood of healthy donors and cancer patients among $CD3^-$ leukocytes (**Figure 1A**). Two subpopulations of NK cells were easily identified using the CD16 and CD56 surface markers: $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^-$ (**Figure 1A**). Both healthy and cancer patients displayed similar percentages of the $CD56^{bright}CD16^-$ NK subset (**Figure 1B**). However, cancer patients had significantly lower frequencies of the subset $CD56^{dim}CD16^+$ compared to healthy individuals (**Figure 1C**).

We also analyzed the NKp46 and CD158b molecules regarding NK cells subset. The percentage of NKp46 expressing $CD56^{bright}CD16^-$ was similar among healthy and cancer patients (p=0.7564; **Figure 1D**). Despite the tendency of $CD56^{dim}CD16^+$ in showing decreased positivity for NKp46 in cancer patients, we did not observe a significant difference (p=0.0720; **Figure 1D**). Additionally, our results demonstrated a lower frequency of CD158b in $CD56^{bright}CD16^-$ population in both healthy and cancer patients (p=0.4082**; Figure 1E**). Regarding CD158b in $CD56^{dim}CD16^+$, we found an increased expression of this molecule in cancer patients compared to healthy donors, although without statistical significance (p=0.1072; **Figure 1E**).

We also analyzed the amounts of NKp46 and CD158b expression by means of fluorescence intensity (MFI). $CD56^{bright}CD16^-$ from healthy donors expressed more NKp46 than cancer patients (p=0.0020; **Figure 1F**). This pattern of NKp46 expression was also found in $CD56^{dim}CD16^+$ subset; however, without statistical significance (p=0.0591; **Figure 1F**). Finally, CD158b was increased in both NK cells subset from cancer patients compared to healthy donors although without statistical significance (p=0.8212 and p=0.1072, **Figure F**).

3.2 Cytokines in Peripheral Blood of advanced cancer patients

We performed a multiplex assay to identify differences in the systemic secretion of interleukins, chemokines, growth factors and other molecules in the blood of cancer patients. Our findings showed that peripheral blood from cancer patients presented substantial amounts

of the chemokines monocyte chemoattractant protein-1 (MCP-1/CCL2) and interferon-γ inducible protein (IP-10/CXCL10), and the angiogenic vascular endothelial growth factor (VEGF, **Figure 2A**).

Aiming to identify a specific inflammatory profile displayed by cancer patients, we clustered these molecules according to their function (**Figure 2B**) and T helper-related immune responses (**Figure 2C**). Using the mean of factors detected in the blood of cancer patients (82.93 pg/mL) as a threshold we could observe that the chemokines MCP-1 (8-fold), IP-10 (5-fold), and eotaxin (2-fold) were above the mean, (**Figure 2B**). Regarding growth factors and other cytokines, cancer patients had high levels of VEGF (2.5-fold) and granulocyte colony-stimulating factor (G-CSF) (1.5-fold), and high levels of the inflammatory interleukin-1alpha (IL-1α) (1.5-fold) cytokine (**Figure 2B**).

All cytokines related with specific T helper subsets were under the mean, except for the Th2-related cytokine, IL-4 (1.2-fold) (**Figure 2C**). However, the increased level of IL-4 observed were detected exclusively in one patient with colon cancer; therefore, we cannot assume a specific tendency of any Th-related immune profile based in the sample used in this study (**Figure 2C**).

3.3 Correlation between NKs and secreted factors of advanced cancer patients

Next, we analyzed if there was any correlation between the profile of NK cells and secreted factors presented in the peripheral blood of patients with advanced carcinomas. Pearson's correlation test was used to analyse the correlation between interleukins, chemokines, growth factors and other molecules on the peripheral blood of cancer patients and NK subset (**Figures 3 and 4**).

First, these analyses were performed related to the pro-tumoral $CD56^{bright}CD16^{-}$ NK subset (**Figure 3**). The frequency of $CD56^{bright}$ $CD16^{-}$ NK subset cells in the blood of cancer patients was positively correlated with the cytokine IL-12p40 (r=0.5975, p=0.0402; **Figure 3A**). The mean fluorescence intensity of NKp46 and CD158b from $CD56^{bright}CD16^{-}$ was positively correlated with MCP-1 (r=0.5827; p=0,0468; **Figure 3B**) and IL-6 (r=0.5751; p=0.0505; **Figure 3C**), respectively. Complementary to our previous results, MCP-1 was also positively correlated with percentage of NKp46-expressing $CD56^{bright}CD16^{-}$ cells (r=0.6016; p=0.0385; **Figure 3D**). On the other hand, IP-10 (r=-0.8471; p=0.0005; **Figure 3E**) and TNF-α (r=-0.5936; p=0.0419; **Figure 3F**) were negatively correlated with the percentage of $CD56^{bright}CD16^{-}$ $NKp46^{+}$.

Among the anti-tumoral $CD56^{dim}CD16^{+}$ subset, we only detected correlations when considering the inhibitor marker CD158b (**Figure 4**). EGF were positively correlated with both percentage (r=0.7835; p=0.0026; **Figure 4A**) and MFI (r= 0.8107; p=0.0014; **Figure 4B**) of CD158b. In addition, both percentage (r=-0.6249; p=0.0298; **Figure 4C**) and MFI (r=-0.5735; p=0.0503; **Figure 4D**) of CD158b from $CD56^{dim}CD16^{+}$ were negatively correlated with MIP-1β. Finally, we compared the expression of all evaluated cytokines, which demonstrated positive correlations between all them (Data not shown). Noteworthy, a statistically significant negative correlation was detected between EGF and TNF-α (r=-0.4790, p=0.0442; **Figure 5**).

4. DISCUSSION

Studies have shown a strong link between NK cells frequency and cancer prognosis of cancer patients, highlighting their potential role as target for development of cancer therapies [14]. We evaluated the PB from advanced cancer patients during treatment, quantifying the frequency of two NK subsets: $CD56^{bright}CD16^{-}$, a regulatory phenotype, and $CD56^{dim}CD16^{+}$, which exhibits a more cytotoxic role. We also evaluated the expression of activation (NKp46) and inhibition (CD158b) NK molecules among both NK cells subsets.

Additionally, we analyzed cytokines, chemokines and growth factors in PB from advanced cancer patients. Further, we correlated these factors with NK cells, their subsets and activated/inhibited molecules. Our findings demonstrated that cancer patients showed a lower frequency of cytotoxic NK cells $CD56^{dim}CD16^{+}$, which could be explained due to a negative regulation of the NKp46 activation marker. This negative regulation of NKp46 may be promoted by IP-10 in NK $CD56^{bright}CD16^{-}$, which decreased and/or prevented the balance of regulatory to cytotoxic NK profile. Also, EGF growth factor was positively regulated with CD158b inhibition marker on $CD56^{dim}CD16^{+}$ NK cells and, negatively correlated with TNF-α. These findings could suggest a possible tumor scape mechanism caused by immune response disbalance and EGF secretion.

In this work, the percentage of $CD56^{bright}CD16^{-}$ NK cells were similar among cancer patients and health donors. Although, $CD56^{dim}CD16^{+}$ NK cells percentage were significantly lower in cancer patients when compared to health donors. We also hypothesized if NK cells isolated from PB of advanced cancer patients could differently express activated (NKp46) and inhibited (CD158b) molecules. Besides the lower frequency of cytotoxic NK cells, cancer patients showed a tendency to have less $CD56^{dim}CD16^{+}NKp46^{+}$ NK cells, and more $CD56^{dim}CD16^{+}$CD158b NK cells. Taken together, it could infer a change from activated profile

into inhibited profile. Both NK subsets showed a tendency to low NKp46 expression; however, only $CD56^{bright}CD16^{-}$ NK subset was statistically significant. Thus, our findings suggested that advanced cancer patients could demonstrated difficulties in $CD56^{dim}CD16^{+}$ NK cells induction. It possibly occurs due the NKp46 deficiency expression on $CD56^{bright}CD16^{-}$ NK subset, since their NKp46 ligation induce NK activation [15].

Generally, solid tumors are composed by an inflammatory environment with intense angiogenesis, which eventually could influence on cancer prognosis [16]. These factors are mainly regulated by inflammatory cytokines, that may act as promoters of tumorigenesis or as important mechanisms against tumors [17]. In the present study, we found increased presence of MCP-1, IP-10 and VEGF in PB of cancer patients. He et al. (2015) demonstrated in prostate cancer that expression of CCL2 and its receptor CCR2 are positively correlated with perineural invasion [18]. Also, Ponzetta et al. (2015) showed that increased production of IP-10 both in bone marrow and PB of patients with multiple myeloma were correlated with lower CXCR3 regulation on NK cells [19]. A study with squamous cell carcinoma pointed out that approximately 50% of $CD56^{+}CD16^{-}$ NK cells expressed equal proportions of VEGF and IFN-γ, suggesting that these patients could both express pro- and anti-angiogenic cytokines abilities [20]. These findings disagree with our results, since we observed that VEGF production was higher than IFN-γ production, which suggests a pro-angiogenic profile.

In order to understand the relationship between soluble factors present in PB of cancer patients and NK subsets, as well as their activated/inhibited status, we performed a correlation analysis. Interestingly, cytokines and chemokines were mainly correlated with the activation molecule NKp46 on the $CD56^{bright}CD16^{-}$ NK subset. On the other hand, these factors were exclusively correlated with the inhibition molecule CD158b on the $CD56^{dim}CD16^{+}$ NK subset. Specifically, we observed a possible compensatory mechanism for Nkp46 expresses induction on $CD56^{bright}CD16^{-}$ NK subset, since the percentage in PB of cancer patients were positively correlated with the most expressed chemokine, MCP-1. Additionally, it was also negatively correlated with the second more expressed chemokine, IP-10. Although a study has recently demonstrated that IP-10 induced NK cells migration to solid tumors and promoted tumor regression [21], IP-10 also act recruiting regulatory T cells (Treg) [22]. As NKp46 expression was significantly lower on $CD56^{bright}CD16^{-}$ of cancer patients, our results suggest that IP-10 could development a significant role on modulation profile of activated circulating NK cells possibly via Treg recruitment/activation, promoting tumor progression. Increased expression of CD158b is correlated with failure on NK activity on cancer patients [23]. Indeed, CD158b was mostly observed on $CD56^{dim}CD16^{+}$ NK cells, positively correlated with EGF, and negatively

correlated with MIP-1β . Despite there is no study on literature directly showing EGF effects in NK activation and phenotypes, its blockade using cetuximab resulted in a higher elimination of colon and rectum tumor cells mediated by NK cells *in vitro* [24]. Costa et al. (2018) demonstrated that the anti-EGFR therapy overcomes NK cell impairment in colorectal cancer by triggering the CD16-mediated antibody dependent cellular cytotoxicity (ADCC) of mesenchymal stem cells [25]. Although MIP-1β is one of the factors produced by NK cells and has been described as important chemoattract factor for several leukocytes, including NK [26], Maghazachi et al. (1996) and Taub et al. (1995) have demonstrated that MIP-1 βalso increased the cytotoxic activity of $CD56^{+}$ NK cells *in vitro* [27] [28], which explain the opposite correlation between MIP-1 β and the inhibition marker, CD158b.

Finally, our findings also reported a negative correlation between EGF and TNF-α in cancer patients. These results are of particularly interest since EGF is involved with tumor pathogenesis and progression [29] [30], and is currently being used as antitumor target therapy in several tumors [31] [32] [33], and TNF-α has critical role in shaping antitumor immune responses [34]. EGF was the 8^{th} most detected factor in the PB of cancer patients and its known that carcinomas showed increased EGF production, which is correlated with poor prognosis [35] [36].

In summary, our findings demonstrated a general decreased frequency of NK cells on the PB of cancer patients, mainly related to the cytotoxic $CD56^{dim}CD16^{+}$ NK cells subset. Also, regulatory $CD56^{bright}CD16^{-}$ NK cells subset from cancer patients showed a lower expression of the activated marker Nkp46 compared to health donors. Our results also demonstrated a higher production of MCP-1, a potent anti-inflammatory chemokine involved with M2 macrophages recruitment and tumor development. More importantly, the higher production of IP-10 was correlated with NKp46 decrease on $CD56^{bright}CD16^{-}$ NK cells. Furthermore, we found a negative correlation between EGF and TNF-α, suggesting a possible immunosuppressive mechanism. In combination, the present results sustain new insights into the NK cell biology during tumor development that might help designing alternative therapeutic approaches.

Acknowledgements

The authors are grateful to grant #2013/07245-9, from São Paulo Research Foundation (FAPESP) for the support. The authors acknowledge the Hospital Estadual de Bauru for their

excellent assistance and providing access to the cancer patients, clinical files and biological samples.

Conflict of interests

The authors declare no conflict of interests.

REFERENCES

[1] D. Ortner, C.H. Tripp, K. Komenda, S. Dubrac, B. Zelger, M. Hermann, W. Doppler, P.Z. Tymoszuk, L. Boon, B.E. Clausen, P. Stoitzner, Langerhans cells and NK cells cooperate in the inhibition of chemical skin carcinogenesis, Oncoimmunology. 6 (2017) 1–12. https://doi.org/10.1080/2162402X.2016.1260215.

[2] K.H. Susek, M. Karvouni, E. Alici, A. Lundqvist, The Role of CXC Chemokine Receptors 1-4 on Immune Cells in the Tumor Microenvironment, Front. Immunol. 9 (2018) 2159. https://doi.org/10.3389/fimmu.2018.02159.

[3] G.P. Rédei, Missing Self Hypothesis, Encycl. Genet. Genomics, Proteomics Informatics. 11 (2008) 1225–1225. https://doi.org/10.1007/978-1-4020-6754-9_10515.

[4] C. Guillerey, N.D. Huntington, M.J. Smyth, Targeting natural killer cells in cancer immunotherapy, Nat. Immunol. 17 (2016) 1025–1036. https://doi.org/10.1038/ni.3518.

[5] A. De Maria, F. Bozzano, C. Cantoni, L. Moretta, Revisiting human natural killer cell subset function revealed cytolytic CD56 dim CD16 + NK cells as rapid producers of abundant IFN- γ on activation, (2011). https://doi.org/10.1073/pnas.1012356108/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1012356108.

[6] R. Jacobs, G. Hintzen, A. Kemper, K. Beul, S. Kempf, G. Behrens, K. Sykora, R.E. Schmidt, CD56 bright cells differ in their KIR repertoire and cytotoxic features from CD56 dim NK cells, (n.d.).

[7] C. Fauriat, E.O. Long, H. Ljunggren, Y.T. Bryceson, Regulation of human NK-cell cytokine and chemokine production by target cell recognition, 115 (2010) 2167–2176. https://doi.org/10.1182/blood-2009-08-238469.A.

[8] J.A. Wagner, M. Rosario, R. Romee, M.M. Berrien-Elliott, S.E. Schneider, J.W. Leong, R.P. Sullivan, B.A. Jewell, M. Becker-Hapak, T. Schappe, S. Abdel-Latif, A.R. Ireland, D. Jaishankar, J.A. King, R. Vij, D. Clement, J. Goodridge, K.J. Malmberg, H.C. Wong, T.A.

Fehniger, CD56bright NK cells exhibit potent antitumor responses following IL-15 priming, J. Clin. Invest. 127 (2017) 4042–4058. https://doi.org/10.1172/JCI90387.

[9] S. Dubois, K.C. Conlon, J.R. Müller, J. Hsu-Albert, N. Beltran, B.R. Bryant, T.A. Waldmann, IL15 infusion of cancer patients expands the subpopulation of cytotoxic CD56bright NK cells and increases NK-cell cytokine release capabilities, Cancer Immunol. Res. 5 (2017) 929–938. https://doi.org/10.1158/2326-6066.CIR-17-0279.

[10] G. Dranoff, Cytokines in cancer pathogenesis and cancer therapy, Nat. Rev. Cancer. 4 (2004) 11–22. https://doi.org/10.1038/nrc1252.

[11] A. Taylor, J. Verhagen, K. Blaser, C.A. Akdis, Mechanisms of immune suppression by interleukin- 10 and transforming growth factor- b: the role of T regulatory cells, (n.d.). https://doi.org/10.1111/j.1365-2567.2006.02321.x.

[12] K.L.K.Æ.M.L. Disis, Tumor antigen-specific T helper cells in cancer immunity and immunotherapy, (2005) 721–728. https://doi.org/10.1007/s00262-004-0653-2.

[13] X. Yang, Y. Zhang, K. Hosaka, P. Andersson, J. Wang, F. Tholander, Z. Cao, VEGF-B promotes cancer metastasis through a VEGF- A – independent mechanism and serves as a marker of poor prognosis for cancer patients, (2015). https://doi.org/10.1073/pnas.1503500112.

[14] E. Jun, A.Y. Song, J.W. Choi, H.H. Lee, M.Y. Kim, D.H. Ko, H.J. Kang, S.W. Kim, Y. Bryceson, S.C. Kim, H.S. Kim, Progressive impairment of NK cell cytotoxic degranulation is associated with TGF-β1 deregulation and disease progression in pancreatic cancer, Front. Immunol. 10 (2019) 1–14. https://doi.org/10.3389/fimmu.2019.01354.

[15] Y.T. Bryceson, M.E. March, H.G. Ljunggren, E.O. Long, Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion, Blood. 107 (2006) 159–166. https://doi.org/10.1182/blood-2005-04-1351.

[16] C. Costa, J. Incio, R. Soares, Angiogenesis and chronic inflammation: Cause or consequence, Angiogenesis. 10 (2007) 149–166. https://doi.org/10.1007/s10456-007-9074-0.

[17] S.I. Grivennikov, F.R. Greten, M. Karin, Immunity, Inflammation, and Cancer, Cell. 140 (2010) 883–899. https://doi.org/10.1016/j.cell.2010.01.025.

[18] Shizi He, The chemokine (CCL2-CCR2) signaling axis mediates perineural invasion, Mol. Cancer Res. 23 (2015) 1–7. https://doi.org/10.1161/CIRCULATIONAHA.110.956839.

[19] A. Ponzetta, G. Benigni, F. Antonangeli, G. Sciumè, E. Sanseviero, A. Zingoni, M.R. Ricciardi, M.T. Petrucci, A. Santoni, G. Bernardini, Multiple myeloma impairs bone marrow localization of effector natural killer cells by altering the chemokine microenvironment, Cancer Res. 75 (2015) 4766–4777. https://doi.org/10.1158/0008-5472.CAN-15-1320.

[20] A. Bruno, C. Focaccetti, A. Pagani, A.S. Imperatori, M. Spagnoletti, N. Rotolo, A.R. Cantelmo, F. Franzi, C. Capella, G. Ferlazzo, L. Mortara, A. Albini, D.M. Noonan, The proangiogenic phenotype of natural killer cells in patients with non-small cell lung cancer, Neoplasia (United States). 15 (2013) 133–142. https://doi.org/10.1593/neo.121758.

[21] E. Wennerberg, V. Kremer, R. Childs, G. Gray, CXCL10-induced migration of adoptively transferred human natural killer cells toward solid tumors causes regression of tumor growth in vivo, (2015) 225–235. https://doi.org/10.1007/s00262-014-1629-5.

[22] N. Redjimi, C. Raffin, I. Raimbaud, P. Pignon, J. Matsuzaki, K. Odunsi, D. Valmori, M. Ayyoub, CXCR3+ T regulatory cells selectively accumulate in human ovarian carcinomas to limit type I immunity, Cancer Res. 72 (2012) 4351–4360. https://doi.org/10.1158/0008-5472.CAN-12-0579.

[23] K. Mirjacic Martinovic, G. Konjević, N. Babović, M. Inić, The stage dependent changes in NK cell activity and the expression of activating and inhibitory NK cell receptors in melanoma patients, J. Surg. Res. 171 (2011) 637–649. https://doi.org/10.1016/j.jss.2010.05.012.

[24] Y.S. Rocca, M.P. Roberti, E.P. Juliá, M.B. Pampena, L. Bruno, S. Rivero, E. Huertas, F.S. Loria, A. Pairola, A. Caignard, J. Mordoh, E.M. Levy, Phenotypic and functional dysregulated blood NK cells in colorectal cancer patients can be activated by cetuximab plus IL-2 or IL-15, Front. Immunol. 7 (2016) 1–13. https://doi.org/10.3389/fimmu.2016.00413.

[25] D. Costa, R. Venè, R. Benelli, E. Romairone, S. Scabini, S. Catellani, B. Rebesco, L. Mastracci, F. Grillo, S. Minghelli, F. Loiacono, M.R. Zocchi, A. Poggi, Targeting the epidermal growth factor receptor can counteract the inhibition of natural killer cell function exerted by colorectal tumor-associated fibroblasts, Front. Immunol. 9 (2018) 1–14. https://doi.org/10.3389/fimmu.2018.01150.

[26] M.J. Robertson, Role of chemokines in the biology of natural killer cells, J. Leukoc. Biol. 71 (2002) 173–183. https://doi.org/10.1189/jlb.71.2.173.

[27] A.A. Maghazachi, A. Al-Aoukaty, T.J. Schall, CC chemokines induce the generation of killer cells from CD56+ cells, Eur. J. Immunol. 26 (1996) 315–319. https://doi.org/10.1002/eji.1830260207.

[28] Taub DD, Sayers TJ, Carter CR, Ortaldo JR. Alpha and beta chemokines induce NK cell migration and enhance NK-mediated cytolysis. J Immunol. 1995;155(8):3877-3888.

[29] N. Normanno, A. De Luca, C. Bianco, L. Strizzi, M. Mancino, M.R. Maiello, A. Carotenuto, G. De Feo, F. Caponigro, D.S. Salomon, Epidermal growth factor receptor (EGFR) signaling in cancer, Gene. 366 (2006) 2–16. https://doi.org/10.1016/j.gene.2005.10.018.

[30] Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, Carotenuto A, De Feo G, Caponigro F, Salomon DS. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene. 2006 Jan 17;366(1):2-16. doi: 10.1016/j.gene.2005.10.018. Epub 2005 Dec 27. PMID: 16377102.

[31] K. Klinghammer, T. Gauler, A. Dietz, V. Grünwald, J. Stöhlmacher, S. Knipping, M. Schroeder, O. Guntinas-Lichius, N. Frickhofen, H.W. Lindeman, R. Fietkau, B. Haxel, C. Große-Thie, G. Maschmeyer, M. Zipfel, P. Martus, M. Knoedler, U. Keilholz, Cetuximab, fluorouracil and cisplatin with or without docetaxel for patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck (CeFCiD): an open-label phase II randomised trial (AIO/IAG-KHT trial 1108), Eur. J. Cancer. 122 (2019) 53–60. https://doi.org/10.1016/j.ejca.2019.08.018.

[32] S. Evrard, G. Desolneux, C. Bellera, T. Esnaud, Y. Bécouarn, D. Collet, N. Chafai, F. Marchal, L. Cany, E. Lermite, M. Rivoire, S. Mathoulin-Pélissier, Systemic chemotherapy plus cetuximab after complete surgery in the treatment of isolated colorectal peritoneal carcinoma: COCHISE phase II clinical trial, BMC Res. Notes. 12 (2019) 1–6. https://doi.org/10.1186/s13104-019-4476-9.

[33] A. Curioni-Fontecedro, J.Y. Perentes, H. Gelpke, A. Xyrafas, H. Bouchaab, N. Mach, O. Matzinger, N. Stojcheva, M. Frueh, W. Weder, R. Cathomas, P. Gargiulo, L. Bubendorf, M. Pless, D. Betticher, S. Peters, Preoperative chemotherapy and radiotherapy concomitant to cetuximab in resectable stage IIIB NSCLC: a multicentre phase 2 trial (SAKK 16/08), Br. J. Cancer. 120 (2019) 968–974. https://doi.org/10.1038/s41416-019-0447-0.

[34] F. Balkwill, TNF-α in promotion and progression of cancer, Cancer Metastasis Rev. 25 (2006) 409–416. https://doi.org/10.1007/s10555-006-9005-3.

[35] X. Zhu, F. Zhang, W.E.I. Zhang, J. He, Y. Zhao, X. Chen, Prognostic Role of Epidermal Growth Factor Receptor in Head and Neck Cancer: A Meta-Analysis, (2013) 387–397. https://doi.org/10.1002/jso.23406.

[36] Ø. Ewska, Expression of EGF and EGFR Strongly Correlates with Metastasis of Pancreatic Ductal Carcinoma, 1404 (2008) 1399–1404.

LIST OF TABLE

Table 1. Clinicopathological data of cancer patients

Patient No.	Gender	Age (Years)	Histology	T	N	M	Stage*
1	M	20	GCT	2	1	x	III
2	M	18	GCT	1	2	0	III
3	M	51	HNSCC	N.I	N.I	N.I	N.I
4	M	42	Testicle	N.I	N.I	N.I	I
6	M	69	AdP	N.I	N.I	N.I	N.I
7	M	44	EsC	N.I	N.I	N.I	III
8	M	30	Testicle	1	x	x	N.I
10	M	27	HNSCC	N.I	N.I	N.I	N.I
11	F	34	Breast	2	1	0	II
12	M	57	HNSCC	4	x	x	IV
13	M	59	HNSCC	4	x	x	N.I
14	M	48	AdC	4	1	0	III
15	M	66	HNSCC	2	3	0	IV
16	F	34	Breast	N.I	N.I	N.I	III
17	M	57	HNSCC	N.I	N.I	N.I	III
18	M	41	EsC	3	x	N.I	N.I
19	M	67	HNSCC	4	0	0	N.I
20	F	22	Melanoma	N.I	N.I	N.I	III
21	F	37	HNSCC	2	2	N.I	III
22	F	52	AdC	N.I	N.I	N.I	N.I
23	F	53	Cervical	3	1	0	III
24	M	54	HNSCC	3	1	0	IV

*N.I. Not informed; HNSCC Head and Neck Squamous Cell Carcinoma; AdP Adenocarcinoma of prostate; AdC Adenocarcinoma of colon; GCT Germ-cell tumor; EsC Esophageal Cancer.

LIST OF FIGURES

Figure 1

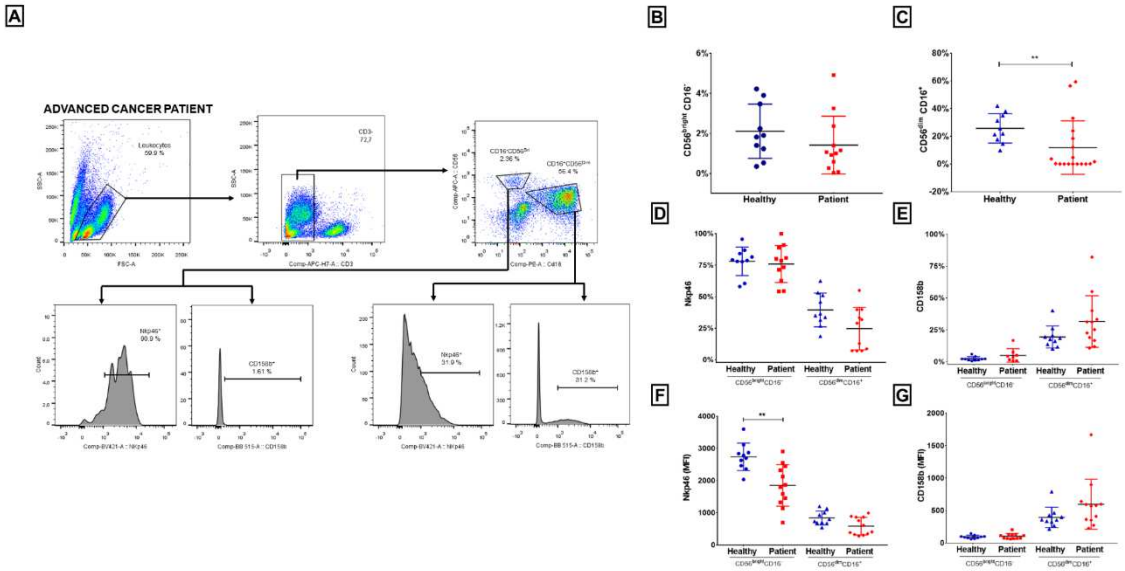


Figure 2

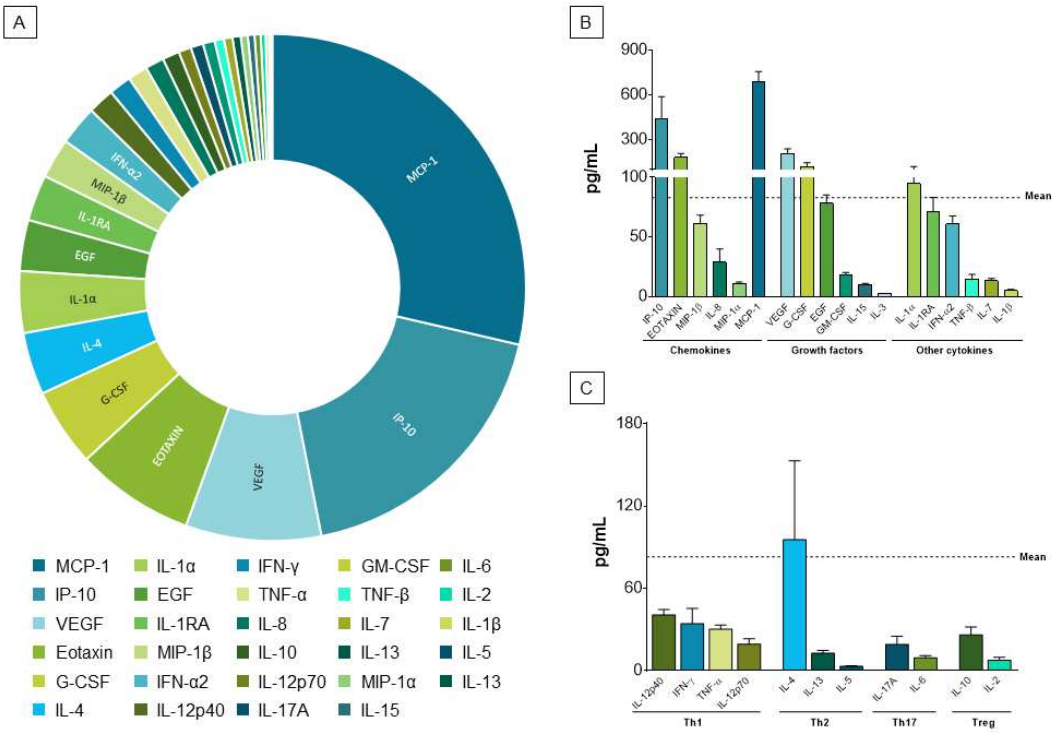

A
MCP-1
IP-10
VEGF
EOTAXIN
G-CSF
IL-4
IL-1α
EGF
IL-1RA
MIP-1β
IFN-α2
MCP-1 IL-1α IFN-γ GM-CSF IL-6
IP-10 EGF TNF-α TNF-β IL-2
VEGF IL-1RA IL-8 IL-7 IL-1β
Eotaxin MIP-1β IL-10 IL-13 IL-5
G-CSF IFN-α2 IL-12p70 MIP-1α IL-13
IL-4 IL-12p40 IL-17A IL-15
B
pg/mL
900
600
300
100
50
0
Mean
IP-10 EOTAXIN MIP-1β IL-8 MIP-1α MCP-1
VEGF G-CSF EGF GM-CSF IL-15 IL-3
IL-1α IL-1RA IFN-α2 TNF-β IL-7 IL-1β
Chemokines
Growth factors
Other cytokines
C
pg/mL
180
120
60
0
Mean
IL-12p40 IFN-γ TNF-α IL-12p70
IL-4 IL-13 IL-5
IL-17A IL-6
IL-10 IL-2
Th1
Th2
Th17
Treg

Figure 3

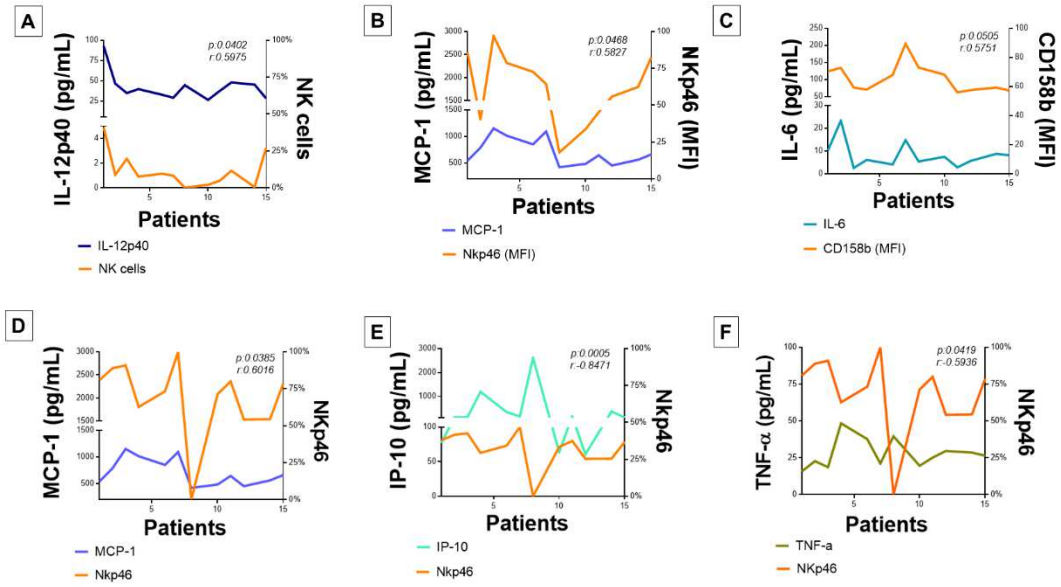

A
IL-12p40 (pg/mL)
NK cells
p:0.0402
r:0.5975
Patients
IL-12p40
NK cells
B
MCP-1 (pg/mL)
NKp46 (MFI)
p:0.0468
r:0.5827
Patients
MCP-1
Nkp46 (MFI)
C
IL-6 (pg/mL)
CD158b (MFI)
p:0.0505
r:0.5751
Patients
IL-6
CD158b (MFI)
D
MCP-1 (pg/mL)
Nkp46
p:0.0385
r:0.6016
Patients
MCP-1
Nkp46
E
IP-10 (pg/mL)
Nkp46
p:0.0005
r:-0.8471
Patients
IP-10
Nkp46
F
TNF-α (pg/mL)
Nkp46
p:0.0419
r:-0.5936
Patients
TNF-a
NKp46

Figure 4

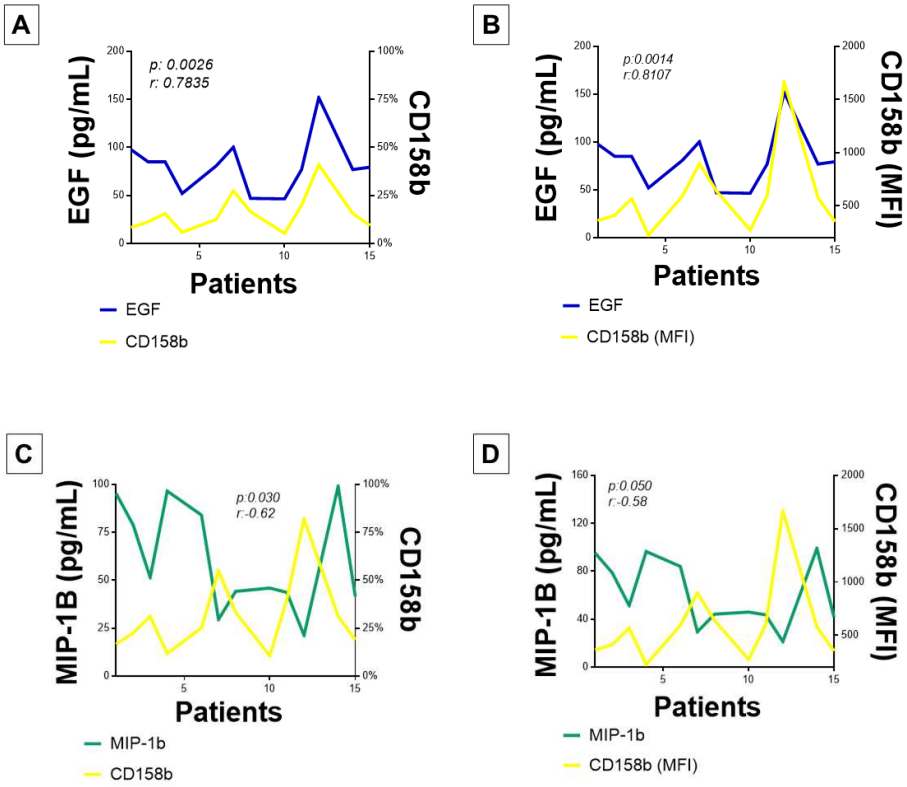
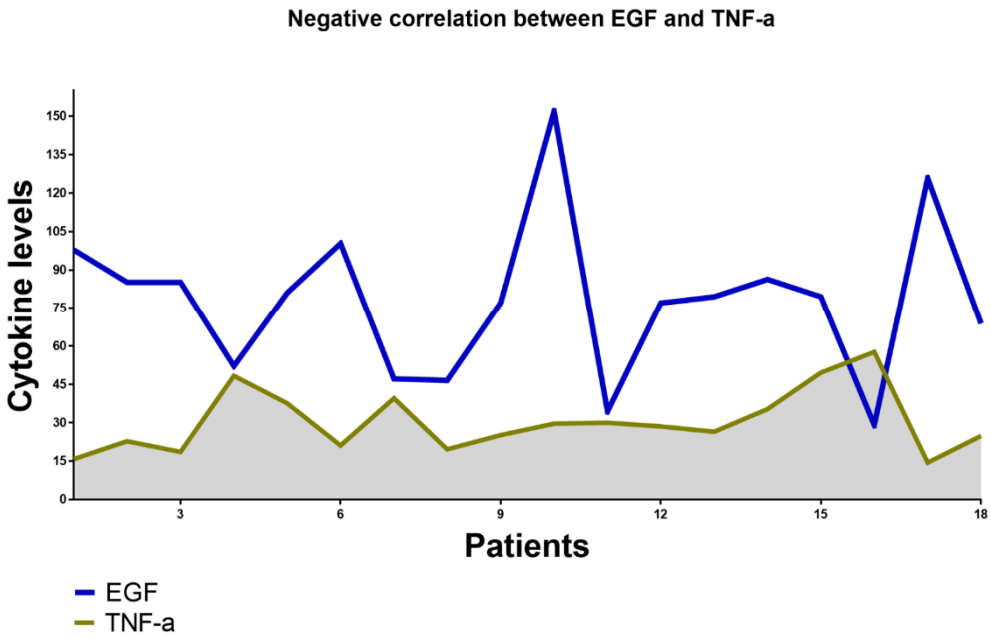

A
p: 0.0026
r: 0.7835
EGF (pg/mL)
CD158b
Patients
EGF
CD158b
B
p:0.0014
r:0.8107
EGF (pg/mL)
CD158b (MFI)
Patients
EGF
CD158b (MFI)
C
p:0.030
r:-0.62
MIP-1B (pg/mL)
CD158b
Patients
MIP-1b
CD158b
D
p:0.050
r:-0.58
MIP-1B (pg/mL)
CD158b (MFI)
Patients
MIP-1b
CD158b (MFI)

Figure 5


Negative correlation between EGF and TNF-a
Cytokine levels
150
135
120
105
90
75
60
45
30
15
0
3
6
9
12
15
18
Patients
EGF
TNF-a

LIST OF FIGURE LEGENDS

Figure 1. Analyses of circulating NK cells from healthy and cancer patients. **A)** Two subpopulations of NK cells were identified in peripheral blood of healthy donors (n=10) and cancer patients (n=18) among CD3 negative leukocytes using the CD16 and CD56 surface markers: $CD16^{-}CD56^{bright}$ and $CD16^{+}CD56^{dim}$. **B)** Both healthy and cancer patients displayed similar percentages of the $CD56^{bright}CD16^{-}$ NK subset. **C)** Cancer patients had significantly lower frequencies of $CD56^{dim}CD16^{+}$ NK cells compared to healthy individuals. **D)** Percentage of NKp46 cells expressing $CD56^{bright}CD16^{-}$ was similar among healthy and cancer patients (p= 0.7564), while a trend of decreased positivity of $CD56^{dim}CD16^{+}$ in cancer patient was found (p=0,0720). **E)** Lower frequency of CD158b in $CD56^{bright}CD16^{-}$ population in both healthy and cancer patients (p=0.4082) was found, while CD158b in $CD56^{dim}CD16^{+}$ exhibited a not statistically significant increased expression in cancer patients compared to healthy donors (p=0.1072). **F)** $CD56^{bright}CD16^{-}$ from healthy donors expressed more NKp46 (MFI) than cancer patients (p=0.0020); Despite of Nkp46 in $CD56^{dim}CD16^{+}$ which remains similar between groups (p=0,0591). **G)** A non-statistically significance of CD158b (MFI) increased was found in both NK cells subset from cancer patients compared to healthy donors, especially in $CD56^{dim}CD16^{+}$ subset (p=0.8212 and p=0.1072). MFI = mean fluorescence intensity.

Figure 2. Cytokines in peripheral blood of advanced cancer patients by multiplex assay. A) Peripheral blood from cancer patients presented substantial amounts of the chemokines monocyte chemoattractant protein-1 (MCP-1/CCL2), interferon-γ inducible protein (IP-10/CXCL10), and the angiogenic vascular endothelial growth factor. **B)** After function stratification of the investigated molecules of detected factors; Also using the mean of 82.93 pg/mL in the blood of cancer patients was defined as a threshold. The chemokines MCP-1, IP-10 and eotaxin (2 times) were 8-, 5- and 2-fold higher than the mean. Regarding growth factors and other cytokines, cancer patients had high levels of VEGF, granulocyte colony-stimulating factor (G-CSF), and of the inflammatory cytokine interleukin-1alpha (IL-1α). **C)** All cytokines related with specific T helper subsets were under the mean, except for the Th2-related cytokine IL-4.

Figure 3. Correlation between $CD56^{bright}CD16^{-}$ NK subset and secreted factors of advanced cancer patients. A) The frequency of $CD56^{bright}$ $CD16^{-}$NK cells in the blood of cancer patients was positively correlated with the cytokine IL-12p40 (r=0.5975, p=0.0402), while **B)** MFI NKp46 and (C) CD158b from $CD56^{bright}CD16^{-}$ was positively correlated with MCP-1 (r=0.5827; p=0.0468) and IL-6 (r=0.5751; p=0.0505), respectively. **D)** MCP-1 was also positively correlated with percentage of NKp46-expressing $CD56^{bright}CD16^{-}$ cells (r=0.6016; p=0.0385), while **E)** IP-10 (r=-0.8471; p=0.0005) and **F)** TNF-α (r=-0.5936; p=0.0419) were negatively correlated with the percentage of $CD56^{bright}CD16^{-}$ $NKp46^{+}$.

Figure 4. Correlation between $CD56^{dim}CD16^{+}$ NK subset and secreted factors of advanced cancer patients. EGF was positively correlated with both **A)** percentage (r=0.7835; p=0.0026) and **B)** MFI (r= 0.8107; p=0.0014) of CD158b. Also, both **C)** percentage (r=-0.6249; p=0.0298) and **D)** MFI (r=-0.5735; p=0.0503) of CD158b from $CD56^{dim}CD16^{+}$ were negatively correlated with MIP-1β.

Figure 5. Negative correlation between EGF and TNF-α in peripheral blood of advanced cancer patients. A statistically significant negative correlation was detected between EGF and TNF-α (r=-0.4790, p= 0.0442).

3 DISCUSSION

3 DISCUSSION

Studies have shown a strong link between NK cells frequency and the prognosis of cancer patients, highlighting their potential role as targets for development of cancer therapies [14]. Indeed, several studies have been evaluated the presence of NK phenotypes and their role as activated or inhibited cell to understand how it is related to carcinomas though cytokines, chemokines and grow factors secretion. Here, we evaluated the PB from advanced carcinoma patients during treatment, quantifying the frequency of two NK phenotypes: $CD56^{bright}CD16^{-}$ (regulatory) and, $CD56^{dim}CD16^{+}$ (cytotoxic). We also evaluated the expression of activation (NKp46) and inhibition (CD158b) molecules within each NK cells subset.

Additionally, we analyzed cytokines, chemokines and growth factors in the PB from advanced cancer patients. Further, we correlated these factors with NK cells, their subsets and activated/inhibited molecules. Our findings demonstrated that cancer patients showed a lower frequency of cytotoxic $CD56^{dim}CD16^{+}$ NK cells, which could be explained by a negative regulation of the NKp46 activation marker. We may hypothesize that this negative regulation of NKp46 is promoted by IP-10 in NK $CD56^{bright}CD16^{-}$, which altered the balance between NK subsets providing a shift towards a poorly cytotoxic and more regulatory NK profile. Accordingly, Accomando *et al*. (2012) suggested that the decrease of circulating NK cells in PB of head and neck squamous cell carcinoma (HNSCC) patients may be a result of the disease itself. On the other hand, Whiteside (2008) and Bauernhofer et al. (2003) reported that cancer cells manipulated the microenvironment by secreting suppressive and pro-apoptotic chemokines against cytotoxic lymphocytes and $CD56^{dim}$ NK cells subset.

In this work, the percentage of $CD56^{bright}CD16^{-}$ NK cells were similar among cancer patients and health donors. Although, $CD56^{dim}CD16^{+}$ NK cells percentage were significantly lower in cancer patients when compared to health donors. We also investigated if NK cells isolated from the PB of advanced cancer patients could differently express activation (NKp46) and inhibition (CD158b) molecules. Besides the lower frequency of cytotoxic NK cells, cancer patients showed a tendency to have less $CD56^{dim}CD16^{+}NKp46^{+}$ NK cells, and more $CD56^{dim}CD16^{+}CD158b^{+}$ NK cells. Taken together, it could infer a change from activated profile towards an inhibited NK cells profile. In the same context, both NK subsets showed a tendency to low NKp46 expression; however, only $CD56^{bright}CD16^{-}$ NK subset was statistically

significant. Thus, our findings suggest that advanced cancer patients could demonstrated difficulties in $CD56^{dim}CD16^{+}$ NK cells induction. It possibly occurs due to the NKp46 deficient expression on $CD56^{bright}CD16^{-}$ NK subset, since their NKp46 ligation induce NK activation [15]. In fact, $CD56^{bright}$ $CD16^{-}$ NK cells are considered less cytolytic NK cells, acting as an immunomodulatory cell; meanwhile, $CD56^{dim}CD16^{+}$ are characterized by their increased cytotoxic activity [16].

Furthermore, NKp46 decreased expression in $CD56^{bright}CD16^{-}$ cells was early reported in EBV infections. The authors demonstrated that when NKp46 decreases, PD-1 up-regulates, suggesting a potential regulatory mechanism responsible for the NK cell cytotoxic failure [17]. The mechanism which regulate NK phenotypes in cancer remains unclear; however, previous works demonstrated that cancer and parenchyma cells modulated tumor microenvironment, and whole organism, trough cytokines, chemokines, and growth factor secretion, which may act cooperatively rather than individually, interacting with tumor cells to influence their biological behavior [18]. Generally, solid tumors are composed by an inflammatory environment with intense angiogenesis and, which eventually could influence on cancer prognosis [19]. These factors are mainly regulated by inflammatory cytokines that may act as promoters of tumorigeneses or as important mechanisms against tumors [20]. In the present study, we found increased levels of MCP-1, IP-10 and VEGF in the PB of cancer patients. He et al. (2015) demonstrated in prostate cancer that the expression of MCP-1 and its receptor CCR2 are positively correlated with perineural invasion [21]. Also, Ponzetta et al. (2015) showed that increased production of IP-10 both in bone marrow and PB of patients with multiple myeloma were correlated with lower CXCR3 regulation on NK cells [22]. A study with squamous cell carcinoma pointed out that approximately 50% of $CD56^{+}CD16^{-}$ NK cells expressed equal proportions of VEGF and IFN-γ, suggesting that these patients could both express pro- and anti-angiogenic cytokines' abilities [23]. These findings disagree with our results, since we observed that VEGF production was higher than IFN-γ production, which suggests a pro-angiogenic profile.

In order to understand the relationship between soluble factors present in PB of cancer patients and NK subsets, as well as their activated/inhibited status, we performed a Pearson' correlation analysis. Interestingly, cytokines and chemokines were mainly correlated with the activation molecule NKp46 on $CD56^{bright}CD16^{-}$ NK subset. On the other hand, these factors were exclusively correlated with the inhibition molecule CD158b on the $CD56^{dim}CD16^{+}$ NK subset. Specifically, we observed a possible compensatory mechanism for Nkp46 expression

induction on $CD56^{bright}CD16^{-}$ NK subset, since the percentage in PB of cancer patients were positively correlated with the most expressed chemokine, MCP-1. It is known that MCP-1 contributes for tumor progression and metastasis by promoting an anti-inflammatory environment through recruitment and differentiation of pro-tumoral M2 macrophages [24]. He et al. (2015) found that MCP-1 - CCR2(chemokine receptor 2) signaling mediates perineural invasion by cancer cells, a feature associated with poor prognosis in several types of carcinomas [25]. Importantly, these cytokines could be produced by several immune cell types, being not exclusive by NK cells.

Additionally, it was also negatively correlated with the second more expressed chemokine, IP-10. Although a study has recently demonstrated that IP-10 induced NK cells migration to solid tumors and promotes tumor regression [26], IP-10 also act recruiting regulatory T cells (Treg) [27]. As NKp46 expression was significantly lower on $CD56^{bright}CD16^{-}$ of cancer patients, our results may suggest that possibly via Treg recruitment/activation, IP-10 could development a significant role on modulation profile of activated circulating NK cells, promoting tumor progression.

Increased expression of CD158b is correlated with failure on NK activity on cancer patients [28]. Indeed, we observed that CD158b was mostly found on $CD56^{dim}CD16^{+}$ NK cells, positively correlated with EGF and negatively correlated with MIP-1β (CCL4). Despite there is no study on literature directly showing EGF effects in NK activation and phenotypes, its blockage using cetuximab resulted in a higher elimination of colon and rectum tumor cells mediated by NK cells *in vitro* [29]. Costa et al. (2018) demonstrated that the anti-EGFR therapy overcomes NK cell impairment in colorectal cancer by triggering the CD16-mediated ADCC of mesenchymal stem cells [30]. MIP-1β is one of the factors produced by NK cells and has been described as important chemoattract factor for several leukocytes, including NK [31]. However, Maghazachi et al. (1996) and Taub et al. (1995) have demonstrated that MIP-1 β also increased cytotoxic activity of $CD56^{+}$ NK cells *in vitro* [32] [33], which may explain the opposite correlation between MIP-1 β and the inhibition marker CD158b found in the present study.

Also, EGF growth factor was positively regulated with CD158b inhibition marker on $CD56^{dim}CD16^{+}$ NK cells and, negatively correlated with TNF-α. This interestingly fact could suggest a possible tumor scape mechanism caused by immune response disbalance and EGF secretion.

Finally, our findings also reported a negative correlation between EGF and TNF-α in cancer patients. These results are of particularly interest since EGF is involved with tumor pathogenesis and progression [34] [35] and are currently being used as antitumor target therapy in several tumors [36] [37] [38] and TNF-α has critical role in shaping antitumor immune responses [39]. EGF was the 8th most detected factor in the PB of cancer patients and its known that carcinomas showed increased EGF production, which is correlated with poor prognosis [40] [41]. Based on that, we investigated by means of statistical analysis if EGF production could suppress immune response through TNF-α downmodulation, which promotes decreased on recruitment and activation of antitumor immune cells [42]. Actually, despite of their individual effects on cell growth, proliferation, and apoptosis, previous studies demonstrated that TNF-α [43] and EGF [44] induces EMT activation in cancer cells, by different complex signaling cross-talk mechanisms. EMT is considered as a key step for migration and metastasis in several types of human carcinomas [45] and, our findings reported that EGF seems to be higher than TNF-α on the majority of cancer patients. Taken together, it is possible to suggest that both TNF-α and EGF could be useful to tumor invasiveness by secretion in the PB of cancer patients. Also, these cytokines could be regulated in the PB through a compensatory mechanism used when both EGF or TNF-α was downregulated, contributing to EMT and tumor progression.

In summary, our findings demonstrated a decrease in NK cells on the PB of cancer patients, mainly regarding thecytotoxic CD56^{dim}CD16^{+} subset. Also, we found low expression of the activation marker Nkp46 on regulatory CD56^{bright}CD16^{-} NK cells subset compared to health donors. Our results also demonstrated a higher production of MCP-1, a potent anti-inflammatory chemokine enrolling in M2 macrophages recruitment and associated with tumor development. More importantly, the higher production of IP-10 was correlated with NKp46 decreased on CD56^{bright}CD16^{-} NK cells, it's could be associated with the low frequency of CD56^{dim}CD16^{+} NK cells. Furthermore, we found a negative correlation between EGF and TNF-α, suggesting a possible immunosuppress mechanism and promoting new insights into the NK cell biology during tumor development that might help designing alternative therapeutic approaches.

REFERENCES

REFERENCES

[1] D. Ortner, C.H. Tripp, K. Komenda, S. Dubrac, B. Zelger, M. Hermann, W. Doppler, P.Z. Tymoszuk, L. Boon, B.E. Clausen, P. Stoitzner, Langerhans cells and NK cells cooperate in the inhibition of chemical skin carcinogenesis, Oncoimmunology. 6 (2017) 1–12. https://doi.org/10.1080/2162402X.2016.1260215.

[2] K.H. Susek, M. Karvouni, E. Alici, A. Lundqvist, The Role of CXC Chemokine Receptors 1-4 on Immune Cells in the Tumor Microenvironment, Front. Immunol. 9 (2018) 2159. https://doi.org/10.3389/fimmu.2018.02159.

[3] G.P. Rédei, Missing Self Hypothesis, Encycl. Genet. Genomics, Proteomics Informatics. 11 (2008) 1225–1225. https://doi.org/10.1007/978-1-4020-6754-9_10515.

[4] C. Guillerey, N.D. Huntington, M.J. Smyth, Targeting natural killer cells in cancer immunotherapy, Nat. Immunol. 17 (2016) 1025–1036. https://doi.org/10.1038/ni.3518.

[5] A. De Maria, F. Bozzano, C. Cantoni, L. Moretta, revisiting human natural killer cell subset function revealed cytolytic CD56 dim CD16 + NK cells as rapid producers of abundant IFN- γ on activation, (2011). https://doi.org/10.1073/pnas.1012356108/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1012356108.

[6] R. Jacobs, G. Hintzen, A. Kemper, K. Beul, S. Kempf, G. Behrens, K. Sykora, R.E. Schmidt, CD56 bright cells differ in their KIR repertoire and cytotoxic features from CD56 dim NK cells, (n.d.).

[7] C. Fauriat, E.O. Long, H. Ljunggren, Y.T. Bryceson, Regulation of human NK-cell cytokine and chemokine production by target cell recognition, 115 (2010) 2167–2176. https://doi.org/10.1182/blood-2009-08-238469.A.

[8] J.A. Wagner, M. Rosario, R. Romee, M.M. Berrien-Elliott, S.E. Schneider, J.W. Leong, R.P. Sullivan, B.A. Jewell, M. Becker-Hapak, T. Schappe, S. Abdel-Latif, A.R. Ireland, D. Jaishankar, J.A. King, R. Vij, D. Clement, J. Goodridge, K.J. Malmberg, H.C. Wong, T.A. Fehniger, CD56bright NK cells exhibit potent antitumor responses following IL-15 priming, J. Clin. Invest. 127 (2017) 4042–4058. https://doi.org/10.1172/JCI90387.

[9] S. Dubois, K.C. Conlon, J.R. Müller, J. Hsu-Albert, N. Beltran, B.R. Bryant, T.A. Waldmann, IL15 infusion of cancer patients expands the subpopulation of cytotoxic CD56bright NK cells and increases NK-cell cytokine release capabilities, Cancer Immunol. Res. 5 (2017) 929–938. https://doi.org/10.1158/2326-6066.CIR-17-0279.

[10] G. Dranoff, Cytokines in cancer pathogenesis and cancer therapy, Nat. Rev. Cancer. 4 (2004) 11–22. https://doi.org/10.1038/nrc1252.

[11] A. Taylor, J. Verhagen, K. Blaser, C.A. Akdis, Mechanisms of immune suppression by interleukin- 10 and transforming growth factor- b: the role of T regulatory cells, (n.d.). https://doi.org/10.1111/j.1365-2567.2006.02321.x.

[12] K.L.K.Æ.M.L. Disis, Tumor antigen-specific T helper cells in cancer immunity and immunotherapy, (2005) 721–728. https://doi.org/10.1007/s00262-004-0653-2.

[13] X. Yang, Y. Zhang, K. Hosaka, P. Andersson, J. Wang, F. Tholander, Z. Cao, VEGF-B promotes cancer metastasis through a VEGF- A – independent mechanism and serves as a marker of poor prognosis for cancer patients, (2015). https://doi.org/10.1073/pnas.1503500112.

[14] E. Jun, A.Y. Song, J.W. Choi, H.H. Lee, M.Y. Kim, D.H. Ko, H.J. Kang, S.W. Kim, Y. Bryceson, S.C. Kim, H.S. Kim, Progressive impairment of NK cell cytotoxic degranulation is associated with TGF-β1 deregulation and disease progression in pancreatic cancer, Front. Immunol. 10 (2019) 1–14. https://doi.org/10.3389/fimmu.2019.01354.

[15] Y.T. Bryceson, M.E. March, H.G. Ljunggren, E.O. Long, Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion, Blood. 107 (2006) 159–166. https://doi.org/10.1182/blood-2005-04-1351.

[16] M.A. Cooper, T.A. Fehniger, S.C. Turner, K.S. Chen, B.A. Ghaheri, T. Ghayur, W.E. Carson, M.A. Caligiuri, Human natural killer cells : a unique innate immunoregulatory role for the CD56 bright subset Human natural killer cells : a unique innate immunoregulatory role for the CD56 bright subset, Library (Lond). 97 (2013) 3146–3151. https://doi.org/10.1182/blood.V97.10.3146.

[17] S. Wiesmayr, S.A. Webber, C. Macedo, I. Popescu, L. Smith, J. Luce, D. Metes, Decreased NKp46 and NKG2D and elevated PD-1 are associated with altered NK-cell function in pediatric transplant patients with PTLD, Eur. J. Immunol. 42 (2012) 541–550. https://doi.org/10.1002/eji.201141832.

[18] J. Brittenden, S.D. Heys, J. Ross, O. Eremin, Natural killer cells and cancer., Am. Cancer Soc. 77 (1996) 1226–1243.

[19] C. Costa, J. Incio, R. Soares, Angiogenesis and chronic inflammation: Cause or consequence, Angiogenesis. 10 (2007) 149–166. https://doi.org/10.1007/s10456-007-9074-0.

[20] S.I. Grivennikov, F.R. Greten, M. Karin, Immunity, Inflammation, and Cancer, Cell. 140 (2010) 883–899. https://doi.org/10.1016/j.cell.2010.01.025.

[21] Shizi He, The chemokine (CCL2-CCR2) signaling axis mediates perineural invasion, Mol. Cancer Res. 23 (2015) 1–7. https://doi.org/10.1161/CIRCULATIONAHA.110.956839.

[22] A. Ponzetta, G. Benigni, F. Antonangeli, G. Sciumè, E. Sanseviero, A. Zingoni, M.R. Ricciardi, M.T. Petrucci, A. Santoni, G. Bernardini, Multiple myeloma impairs bone marrow localization of effector natural killer cells by altering the chemokine microenvironment, Cancer Res. 75 (2015) 4766–4777. https://doi.org/10.1158/0008-5472.CAN-15-1320.

[23] A. Bruno, C. Focaccetti, A. Pagani, A.S. Imperatori, M. Spagnoletti, N. Rotolo, A.R. Cantelmo, F. Franzi, C. Capella, G. Ferlazzo, L. Mortara, A. Albini, D.M. Noonan, The proangiogenic phenotype of natural killer cells in patients with non-small cell lung cancer, Neoplasia (United States). 15 (2013) 133–142. https://doi.org/10.1593/neo.121758.

[24] K. Beider, H. Bitner, M. Leiba, O. Gutwein, M. Koren-michowitz, Multiple myeloma cells recruit tumor-supportive macrophages through the CXCR4 / CXCL12 axis and promote their polarization toward the M2 phenotype, 5 (2014).

[25] Shizi He, The chemokine (CCL2-CCR2) signaling axis mediates perineural invasion, Mol. Cancer Res. 23 (2015) 1–7. https://doi.org/10.1161/CIRCULATIONAHA.110.956839.

[26] N. Redjimi, C. Raffin, I. Raimbaud, P. Pignon, J. Matsuzaki, K. Odunsi, D. Valmori, M. Ayyoub, CXCR3+ T regulatory cells selectively accumulate in human ovarian carcinomas to limit type I immunity, Cancer Res. 72 (2012) 4351–4360. https://doi.org/10.1158/0008-5472.CAN-12-0579.

[27] K. Mirjacic Martinovic, G. Konjević, N. Babović, M. Inić, The stage dependent changes in NK cell activity and the expression of activating and inhibitory NK cell receptors in melanoma patients, J. Surg. Res. 171 (2011) 637–649. https://doi.org/10.1016/j.jss.2010.05.012.

[28] E. Wennerberg, V. Kremer, R. Childs, G. Gray, CXCL10-induced migration of adoptively transferred human natural killer cells toward solid tumors causes regression of tumor growth in vivo, (2015) 225–235. https://doi.org/10.1007/s00262-014-1629-5.

[29] N. Normanno, A. De Luca, C. Bianco, L. Strizzi, M. Mancino, M.R. Maiello, A. Carotenuto, G. De Feo, F. Caponigro, D.S. Salomon, Epidermal growth factor receptor (EGFR) signaling in cancer, Gene. 366 (2006) 2–16. https://doi.org/10.1016/j.gene.2005.10.018.

[30] D. Costa, R. Venè, R. Benelli, E. Romairone, S. Scabini, S. Catellani, B. Rebesco, L. Mastracci, F. Grillo, S. Minghelli, F. Loiacono, M.R. Zocchi, A. Poggi, Targeting the epidermal growth factor receptor can counteract the inhibition of natural killer cell function exerted by colorectal tumor-associated fibroblasts, Front. Immunol. 9 (2018) 1–14. https://doi.org/10.3389/fimmu.2018.01150.

[31] K. Klinghammer, T. Gauler, A. Dietz, V. Grünwald, J. Stöhlmacher, S. Knipping, M. Schroeder, O. Guntinas-Lichius, N. Frickhofen, H.W. Lindeman, R. Fietkau, B. Haxel, C. Große-Thie, G. Maschmeyer, M. Zipfel, P. Martus, M. Knoedler, U. Keilholz, Cetuximab, fluorouracil and cisplatin with or without docetaxel for patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck (CeFCiD): an open-label phase II randomised trial (AIO/IAG-KHT trial 1108), Eur. J. Cancer. 122 (2019) 53–60. https://doi.org/10.1016/j.ejca.2019.08.018.

[32] A.A. Maghazachi, A. Al-Aoukaty, T.J. Schall, CC chemokines induce the generation of killer cells from CD56+ cells, Eur. J. Immunol. 26 (1996) 315–319. https://doi.org/10.1002/eji.1830260207.

[33] Taub DD, Sayers TJ, Carter CR, Ortaldo JR. Alpha and beta chemokines induce NK cell migration and enhance NK-mediated cytolysis. J Immunol. 1995;155(8):3877-3888.

[34] Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, Carotenuto A, De Feo G, Caponigro F, Salomon DS. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene. 2006 Jan 17;366(1):2-16. doi: 10.1016/j.gene.2005.10.018. Epub 2005 Dec 27. PMID: 16377102.

[35] X. Zhu, F. Zhang, W.E.I. Zhang, J. He, Y. Zhao, X. Chen, Prognostic Role of Epidermal Growth Factor Receptor in Head and Neck Cancer: A Meta-Analysis, (2013) 387–397. https://doi.org/10.1002/jso.23406.

[36] Ø. Ewska, Expression of EGF and EGFR Strongly Correlates with Metastasis of Pancreatic Ductal Carcinoma, 1404 (2008) 1399–1404.

[37] S. Daneshmandi, S. Shahrokhi, Engineering Tumor Cells with Tumor Necrosis Factor α (TNF- α) or CD40 Ligand (CD40L) Genes Induce Anti-Tumor Immune Responses, Int. J. Pept. Res. Ther. 25 (2019) 427–436. https://doi.org/10.1007/s10989-018-9687-8.

[38] A. Curioni-Fontecedro, J.Y. Perentes, H. Gelpke, A. Xyrafas, H. Bouchaab, N. Mach, O. Matzinger, N. Stojcheva, M. Frueh, W. Weder, R. Cathomas, P. Gargiulo, L. Bubendorf, M. Pless, D. Betticher, S. Peters, Preoperative chemotherapy and radiotherapy concomitant to cetuximab in resectable stage IIIB NSCLC: a multicentre phase 2 trial (SAKK 16/08), Br. J. Cancer. 120 (2019) 968–974. https://doi.org/10.1038/s41416-019-0447-0.

[39] F. Balkwill, TNF-α in promotion and progression of cancer, Cancer Metastasis Rev. 25 (2006) 409–416. https://doi.org/10.1007/s10555-006-9005-3.

[40] M.J. Robertson, Role of chemokines in the biology of natural killer cells, J. Leukoc. Biol. 71 (2002) 173–183. https://doi.org/10.1189/jlb.71.2.173.

[41] Y.S. Rocca, M.P. Roberti, E.P. Juliá, M.B. Pampena, L. Bruno, S. Rivero, E. Huertas, F.S. Loria, A. Pairola, A. Caignard, J. Mordoh, E.M. Levy, Phenotypic and functional dysregulated blood NK cells in colorectal cancer patients can be activated by cetuximab plus IL-2 or IL-15, Front. Immunol. 7 (2016) 1–13. https://doi.org/10.3389/fimmu.2016.00413.

[42] S. Evrard, G. Desolneux, C. Bellera, T. Esnaud, Y. Bécouarn, D. Collet, N. Chafai, F. Marchal, L. Cany, E. Lermite, M. Rivoire, S. Mathoulin-Pélissier, Systemic chemotherapy plus cetuximab after complete surgery in the treatment of isolated colorectal peritoneal carcinoma: COCHISE phase II clinical trial, BMC Res. Notes. 12 (2019) 1–6. https://doi.org/10.1186/s13104-019-4476-9.

[43] H. Wang, H. Wang, B. Zhou, C. Li, F. Zhang, X. Wang, G. Zhang, X. Bu, S. Cai, J. Du, Epithelial – Mesenchymal Transition (EMT) Induced by TNF- a Requires AKT / GSK-3 b - Mediated Stabilization of Snail in Colorectal Cancer, 8 (2013). https://doi.org/10.1371/journal.pone.0056664.

[44] J. Kim, J. Kong, H. Chang, H. Kim, A. Kim, EGF induces epithelial-mesenchymal transition through phospho-Smad2 / 3-Snail signaling pathway in breast cancer cells, 7 (2016) 85021–85032.

[45] J.P. Thiery, EPITHELIAL – MESENCHYMAL TRANSITIONS IN TUMOUR PROGRESSION, 2 (2002) 442–454. https://doi.org/10.1038/nrc822.