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Clinical relevance of natural killer cells in breast cancer

Klinička važnost prirodnoubilačkih stanica u karcinomu dojke

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Abstract. Breast carcinoma is the most common malignant disease in the female and one of the leading causes of death among women worldwide. Chronic inflammation and immunosuppressive environment achieved by the interaction of tumour and infiltrating immune cells are the hallmarks of cancers, including breast cancer. Natural killer cells are one of innate immune cells in tumour bed that possess great antitumour potential due to their ability to kill tumour cells without prior sensitization. They also play an important role in priming of type I immune responses and are considered to be main source of Interferon-γ. Unfortunately, antitumour activity of natural killer cells is often suppressed by pro-inflammatory cytokines secreted in tumour microenvironment and suppression of NK cells activity often correlates with therapy response and general outcome of patient with breast carcinoma. Different types of breast cancer are associated with different subsets of natural killer cells that possess different immunoregulatory function. Identification of specific tumour associated natural killer cell subset endowed with different functional capabilities might help practitioners in therapy decisions, therapy response monitoring, as well as in predicting overall prognosis of breast cancer patients. The purpose of this review is to give an overview of natural killer cells and their subsets, their role in pathogenesis of breast cancer and to discuss NK cells as potential useful therapy and prognostic tool for breast carcinomas.

Key words: breast neoplasms; immunotherapy; killer cells, natural

Sažetak. Karcinom dojke je maligna bolest u žena i jedan je od vodećih uzroka smrti žena širom svijeta. Kronična upala i imunosupresivni okoliš koji nastaje kao posljedica interakcije tumorskih stanica i infiltrirajućih imunoloških stanica obilježja su karcinoma, uključujući karcinom dojke. Prirodnoubilačke stanice (NK stanice) jedna su od subpopulacija urođenih imunoloških stanica u tumorskom sijelu, koje posjeduju antitumorski potencijal zbog svoje sposobnosti direktnog ubijanja tumorskih stanica bez prethodne senzibilizacije. Također, igraju važnu ulogu u pripremi imunološkog odgovora tipa 1 i smatraju se glavnim izvorom interferona-γ. Nažalost, antitumorska aktivnost NK stanica često je potisnuta proupalnim citokinima koji se izlučuju u tumorskom mikrookolišu, a supresija aktivnosti NK stanica obično korelira s terapijskim odgovorom i općim lošijim ishodom kod bolesnica s karcinomom dojke. Surogati molekularnih podtipova karcinoma dojke povezani su s različitim subpopulacijama NK stanica, koje posjeduju različite imunoregulacijske funkcije. Identifikacija specifične subpopulacije NK stanica pridruženih s tumorom, koje posjeduju različite imunološke funkcije, može pomoći liječnicima u donošenju odluka o terapiji, praćenju terapijskog odgovora, kao i u predviđanju ukupne prognoze pacijenata s karcinomom dojke. Svrha je ovog preglednog članka istaknuti važnost NK stanica i njihovih subpopulacija u patogenezi karcinoma dojke te diskutirati terapijske i prognostičke mogućnosti korištenja NK stanica u osoba oboljelih od karcinoma dojke.

Ključne riječi: imunoterapija; novotvorina dojke; ubilačke stanice, prirodne

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INTRODUCTION

Breast cancer is the leading cause of death in women in developed countries. Age, gender, positive family history, heredity, hormones, dense breasts, precancerous lesions (atypical hyperplasia), ionizing radiation, physical inactivity and obesity after menopause, as well as longer exposure to estrogen (hormone replacement treatment, early menarche, late menopause) are considered to be major risk factors for the development of the disease[1, 2]. High incidence and still high mortality make breast cancer world health problem. According to data from the International Agency for Research on Cancer, from 2018, there was about 2.1 million newly diagnosed female breast cancer cases with estimated death rate of 6,6%[1, 2]. Early detection of the disease is crucial because more than 90% of breast cancer patients can be cured if the disease is detected at early stage[2, 3]. Prognostic factors include number of effected armpit lymph nodes, tumour size, hormone receptor status, and degree of tumour differentiation (grade). Breast carcinomas have ductal or lobular morphology and invasive ductal carcinoma is the most common type of breast cancer that presents with a distinct morphology and clinical behaviour in comparison to invasive lobular carcinoma[3]. Molecular classification differentiates four subtypes of breast cancers based on expression of estrogen and progesterone receptors, human epidermal growth factor receptor 2 (HER2) and Ki-67. They include luminal A (ER^+, PR^+, Ki-67 < 20; $HER2^-$), luminal B (ER^+, PR^+, Ki-67 > 20, $HER2^{+/-}$), HER2 positive (ER^- and PR^-), and triple negative (ER^-, PR^- and $HER2^-$)[4]. Prognosis and treatment strategies are based on this classification[5]. Luminal A type is associated with best prognosis, while triple negative is the most malignant type associated with poor survival and relapse is common[6]. Multifactorial therapy, including radiation, surgery, and chemotherapy is the treatment of choice nowadays that has shown the highest percentage survival in patients with breast cancer[4, 5]. At the moment surgical approach is the therapy choice for luminal A subtype while neoadjuvant chemotherapy is used for more malignant breast cancers such as HER2-positive and triple negative tumours and can be combined with immunotherapy[7, 8]. Despite the acceptable results achieved by classical therapy procedures, multidrug resistance continues to be a clinical challenge and development of new diagnostic and therapy approaches is necessary[7, 8]. Recently, the focus of research has been shifted from tumour cells itself to tumour microenvironment (TME) that has been shown to be a key triggering factor for the molecular etiopathogenetic mechanisms of tumour development and growth[9, 10]. The TME shows immunosuppressive characteristics that are partly achieved by the action of tumour cells and infiltrating immune cells[11]. The composition of tumour infiltrating immune cells can vary according to cancer type[10, 11]. Different types of infiltrating immune cells involved in both innate and adaptive immunity have diverse effects on tumour plasticity and behaviour with either protumoural or antitumoural consequences[11-13]. The innate immune cells infiltrating the tumour bed are mostly tumour associated macrophages (TAMs) and natural killer (NK) cells which exert essential roles in the development and regulation of tumour growth and dissemination potential[11, 12]. Besides their ability to kill tumour cells without prior sensitization, NK cells are also involved in the priming of type 1 T helper-cell (Th1) responses and are the major source of IFNγ *in vivo*[13]. Presence of NK cells has been correlated with clinical outcomes in some types of solid cancer and NK cells are not found in large numbers in advanced human neoplasms[14-16]. This suggests that low NK cell number could be a reason for the escape of metastatic cells from the immune system[16, 17] and frequency of NK

The tumour microenvironment is complex, and immune escape is considered an important hallmark of cancer, largely contributing to tumour progression and metastasis. NK cells serve as the main effector cells toward cancer in innate immunity and have become an important factor in the treatment of cancer.

cells may represent a new predictive biomarker of response to chemotherapy in cancer patients[14-17]. Even in tumours in which the number of NK cells is normal, their activity is often suppressed by TME. Hence, NK cells, as key effectors of the cytotoxic antitumour responses and strong inducers of the adaptive immune response, represent important target for immunotherapy of different types of tumours, including breast cancer[16, 17]. Great advantage of their use in immunotherapy is the fact that they can be efficiently expanded *in vitro* and used in autologous or allogeneic NK cell–based immunotherapies[13-15].

In this review, we will give an overview on the NK cells, their activation status in breast cancer and we will discuss present and potential use of immunotherapy that is based on natural killer cells.

NK CELLS CHARACTERISTICS

NK cells are the first line of innate immunity defence with both cytotoxicity and cytokine-producing effector functions against invading pathogens and various tumours[13, 18]. Consistent with their function as innate sentinels, NK cells are widespread throughout lymphoid and nonlymphoid tissues. In most tissues, NK cells represent a minor fraction of total lymphocytes (from 2% in mouse spleen to 10% in mouse lung and from 2% to 18% in human peripheral blood). Human NK cell turnover in blood is around 2 weeks[18, 19]. The NK cell phenotype is defined upon their expression of NCAM1 (CD56) and lack of CD3 expression. The role of adhesion molecule CD56 could mediate interactions between NK cells and target cells, whereas the cross-linking of the low-affinity Fc receptor CD16 responsible for antibody-dependent cell cytotoxicity (ADCC), the most powerful way to initiate NK cell–mediated killing[18, 19].

In humans, NK cells can be divided into $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets, which differ in their homing properties[20, 21]. Around 90% of peripheral blood and spleen NK cells are $CD56^{dim}CD16^{+}$ that expresses perforin. These $CD56^{dim}$ NK cell are cytotoxic and produce IFN-γ upon interaction with tumour cells *in vitro*[20, 21]. In contrast, most NK cells in lymph nodes and tonsils are $CD56^{bright}$-$CD16^{-}$ and lack perforin[20, 22]. Classification of NK cell receptors is based on their effects: activating, inhibitory and mixed functions[20, 22]. The anti-tumour activity of NK cells depends on the interplay of activating and inhibiting receptors repertoire which recognize changes in the expression patterns of tumour cells[23-25]. The major activating receptors expressed on human NK cells include NKG2D receptor, C-type lectin receptors (CD94/NKG2C) and the natural cytotoxicity receptors (NCRs) NKp30, NKp44, NKp46 and NKp80. Notably, NCRs mediate recognition and killing of tumour cell and cells infected with viruses[25, 26]. Namely, the activating receptor NKG2D is associate with setting of the activation threshold for NK cell activation[27, 28]. In contrast, receptors that inhibit NK cell activation are important for self-tolerance[29]. This group of receptors includes inhibitory killer immunoglobulin-like receptors (KIR), the C-type lectin inhibitory receptor CD94/NKG2A and the nectin/nectin-like binding receptors TIGIT and CD96 suppressing activation signalling processes in NK cells[29, 30]. In addition, NKG2A was revealed to be an important checkpoint controlling NK cell mediating T-cell activation[30]. This important mechanism plays a crucial role in the immune escape of certain tumours[31, 32].

Activated NK cells secrete a wide variety of cytokines such as IFN-γ, TNF-α, IL-10, IL-5, and IL-13 and chemokines such as MIP-1, IL-8, and RANTES[31-33]. IFN-γ is one of the most potent effector cytokines secreted by NK cells and has been shown to modulate FasL, and TRAIL expression and activates antitumour immunity[34]. Signalling through activating receptor NKG2D on NK cell has been shown to promote the release of IFN-γ[33, 34]. These studies point out that apart from the NK cell cytotoxic function; cytokines secreted by the NK cells also provide a significant boost to their antitumour immunity[35]. Similarly, the cytokines secreted by other immune cells or stromal cells in the tumour microenvironment can positively or negatively influence the antitumour function of NK cells[35, 36]. Functional deficiencies and phenotypic alterations of the NK cell fraction have been found in patients with autoimmune and malignant diseases[13].

NK CELLS IN BREAST CANCER

NK cells influence tumour growth and progression either by direct interaction with tumour cells or indirectly true modulation of function of other immune cells in TME[37, 38]. Although NK cells possess high anti-tumour potential, their activity is often suppressed by TME, resulting in decreased IFN-γ, perforin and granulysin production, decreased expression of activating receptors on surface of NK cells and their decreased killing potential which is associated with higher rate of tumour recidivism and with poor prognosis in general[37-39]. TME can modulate NK cells activity in several ways, mainly by high production of pro-inflammatory cytokines Transforming growth factor beta (TGF-ß), Interleukin 10 (IL-10) and Prostaglandin E2 (PGE2) that are responsible for inhibition properties of NK cells and are abundantly found in TME[40, 41]. These cytokines are produced by tumour cells, TAMs, tumour associated fibroblasts and dendritic cells (DCs)[40, 41]. Immunosuppressive influence of TME on NK cells is shown in Figure 1.

A future challenge for the implementation of NK cell-based therapy for breast cancer is to better define NK cell populations and to identify respective markers, as well as functional and regulatory pathways in each subgroup, thereby using different therapeutic strategies for the treatment of tumours infiltrated with different NK cells.

Inhibition of NK cells is supported by low concentrations of oxygen, glucose and glutamine in tumour tissue[42]. Several studies confirm that the immune profile of NK cells in TME of solid tumours is characterized by an increased expression of inhibitory receptors (CD94/NKG2A)[31, 37] and decreased gene expression of cytotoxic mediators[32-35]. Furthermore, the main groups of activating receptors such as NKG2D receptor and a family of natural killer receptors (NKp30, NKp44, NKp46 and DNAM-1) are decreased in many tumours (such as gastric cancer, colorectal carcinoma, and metastatic melanoma)[35-37].

In patients with breast carcinoma CD56 ^{bright} tumour-infiltrating NK cells are more abundant in tumour microenvironment[19, 20]. Some research groups showed positive correlation of CD56^{bright} subset frequency in tumour tissue and invasiveness of breast cancers[19, 21-23]. Accordingly, in the peripheral blood of patients with breast cancer the amount of circulating CD56^{bright} NK cells increased respectively to the grade of malignancy, suggesting tolerogenic properties on the systemic level[19, 22]. Mamesier et al.[38] have demonstrated the importance of tolerogenic NK cells in triple negative breast cancer which result in a weakened cellular immunity. Crucial receptor for the inhibitory signal after recognition of tumour cells and therefore tolerogenic properties of NK cells is NKG2A receptor whose inhibitory activity is described in breast cancer[21, 31]. Namely, higher NKG2A expression in NK cells infiltrating tumour tissue is associated with more invasive types of breast cancers[31, 32]. Moreover, activated, mature CD16^{+} NK cells although overexpress activating receptors like NCRs (NKp46, NKp30) and NKG2D have tolerogenic properties when NKG2A expression is high[23, 32].

Frazao et al.[19] observed CD16^{+}NKG2A^{+} NK cell population that is associated with higher TMS stage and lymph node invasion in patients with ductal carcinoma, subtype luminal A or B. We found increased expression of NKG2A in infiltrating NK cells of triple negative breast cancer in comparison to Luminal A and B (unpublished data). We also observed that the expression of activating receptors NKG2D, NKp44 and NKG2C is decreased in different molecular subtypes of breast cancer (luminal A, luminal B and triple negative) (unpublished data). Another mechanism that tumours may escape NK cell immunosurveillance, beside downregulation of activating and upregulation of inhibitory receptors, is the deviation of the NKG2D receptor/NKG2DL ratio[43]. Moreover, expression of non-signalling Fas receptor or TRAIL decoy receptors protects tumour cell from FasL (Fas ligand) or TRAIL (tumour necrosis factor- related apoptosis inducing ligand)-mediated apoptosis due to evading NK cell cytotoxicity[44]. Considering that function of NK cells in TME is one of the very important factors that will determine chemotherapy response and overall prognosis of patients with breast carcinoma, restore of their activity could be of choices for anti-tumour therapy.

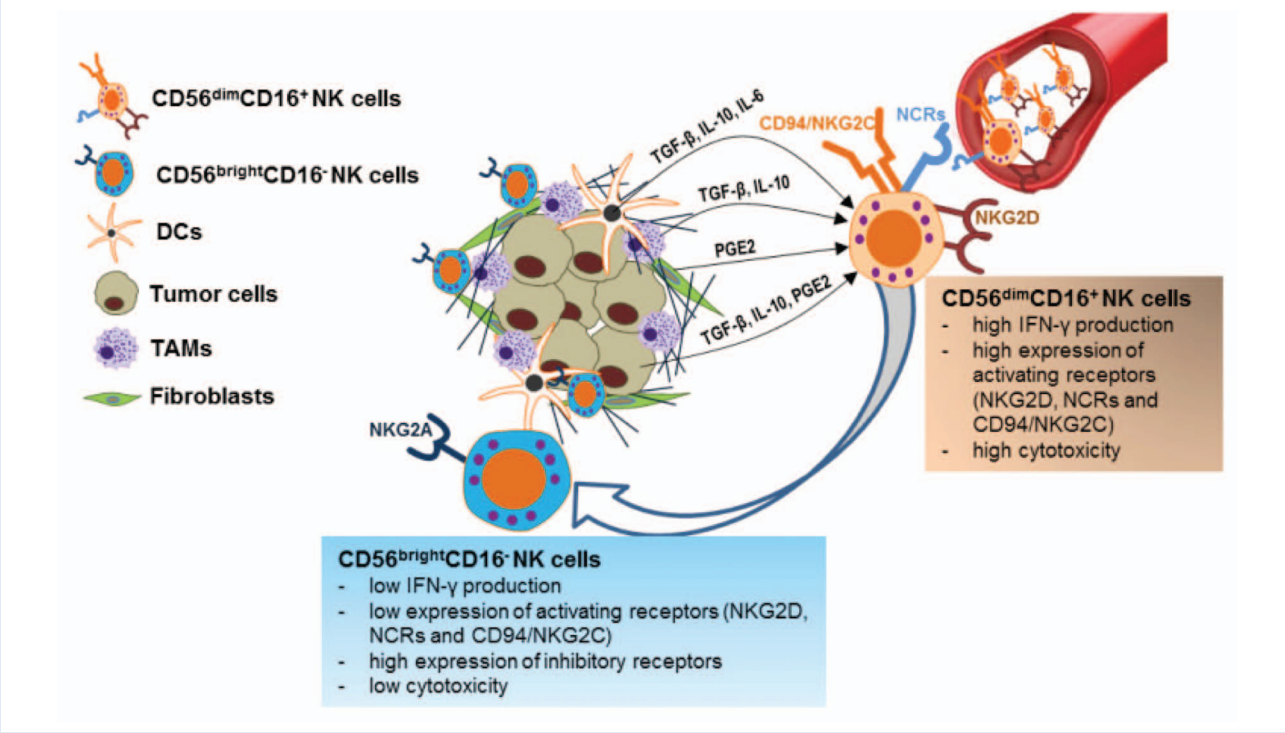

Figure 1. Mechanisms of NK cells suppression in tumour microenvironment.

Most of the NK cells in the blood stream have high cytotoxicity potential, produce high amounts of IFN-γ and expression of activating receptors ($CD56^{dim}CD16^{+}$ NK cells) on their surface is high. In the TME, under the influence of immunosuppressive cytokines, NK cells transform into the cells that possess low cytotoxic potential, produce small amounts of IFN- γ and expression of inhibitory receptors ($CD56^{bright}CD16^{-}$ NK cells) on their surface is high. The most important immunosuppressive cytokines that impair NK cells activity in TME are PGE2 (secreted mostly by fibroblasts), TGF-ß and IL-10 (both secreted mostly by DCs, tumour cells and TAMs).

THERAPY POTENTIAL OF NK CELLS

In the last years, harnessing NK cells for the therapeutic purpose is an attractive option and has received rejuvenating interest in recent times[45]. NK cells immunotherapy offers several advantages. First, the use of NK cells will bypass the need of antigen-specific T cells then they can directly kill tumour cells and can also rapidly secrete proinflammatory cytokines that can potentiate the adaptive immune response[17, 45]. In addition, NK cells are easy to isolate, manipulate and to transfer in the patient's body either as autologous (from the same patient) or allogeneic (from other healthy donors) transfer[45]. Despite expectations, transfusion of ex vivo activated and expanded autologous NK cells in different solid tumours such as breast cancer, metastatic melanoma, and gastrointestinal cancer patients did not lead to favourable anticancer response[46]. The failure of autologous NK cell adoptive therapy to produce positive clinical outcome insisted on the use of allogeneic NK cells. The advantage of use of allogeneic over autologous NK cells is due to inhibition of NK cell-mediated recognition of self-MHC molecules. Unfortunately, early phase of clinical trial using the infusion of activated allogeneic NK cells in ovarian and breast cancer patients did not show a significant expansion of transfused NK cells and therefore did not result in regression of disease[47]. In addition, contradictory results were observed with use of *ex vivo* expanded NK cells expressing chimeric antigen receptors (CAR)[47]. Those results raised the need for new therapy approaches. One of ideas was to use tumour-specific antibodies that will promote ADCC reaction as an effective way to intensify NK cell activity. This approach showed good results in the therapy of metastatic breast cancers[45, 48-50]. Considering that impaired function of NK cells in TME is result of action of secreted cytokines, mainly TGF-β, IL-10 and PEG2, that induce de-

crease in expression of activating and increase of expression of inhibitory receptors on cell surface of NK cells targeting that points is the new step in NK cells-based cancer therapy. And really, use of antagonistic mAbs against inhibitory NK cells receptors in order to promote NK cells cytotoxicity already showed promising results[45, 48]. Similarly, clinical trials in which TGF-β blockade therapy is used in patients with solid tumours demonstrated acceptable safety and preliminary evidence of antitumour activity[51]. Two independent studies showed good results of TGF-β blockade when used together with anti-PD-1/PD-L1 therapy on murine EMT6 breast mammary carcinoma and colorectal cancer[52]. At the moment, many companies are conducting clinical trials considering effects of PGE2 receptor (EP2 and EP4) antagonists in treatment of patients with cancers and results are eagerly awaited due to fact that many studies showed that EP2 is abnormally expressed in many cancers, including breast cancer[53, 54] in which it can regulate metastasis[55].

CONCLUSION

Despite a significant progress that was made over the last two decades in understanding the interrelation and complexity of causal events between a tumour microenvironment and NK cells there are still many questions yet to be answered. NK cell-based immunotherapy of solid tumours posed several challenges but combining NK cell-based immunotherapy with approaches that can target immunosuppressed tumour microenvironment may provide benefits in the treatment of solid tumours, especially those resistant to current T cell-based immunotherapy. NK cell-based immunotherapy is still being intensively tested in clinical trials that include patients with solid cancers. Although, the use of immunotherapy mediated by NK cells is not a standard part of breast cancer treatment yet, and there are many open questions to be answered, there is hope that this will change in the future considering that all data discussed in this review suggest that NK cells have great therapeutic potential and should be considered as a potential useful tool in therapy protocols for the treatment of patients with solid tumours, including breast cancer.

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