REVIEW

Lib Oncol. 2021;49(1):20–28

doi: 10.20471/LO.2021.49.01.03

IMMUNITY AND CANCER: ROLE OF TUMOR-INFILTRATING LYMPHOCYTES IN TRIPLE-NEGATIVE BREAST CANCER

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Summary

Tumor stimulates specific innate and acquired immune mechanisms. Main carriers of body's immune response to tumor are T lymphocytes and main mechanism is killing of tumor cells by cytotoxic T lymphocytes CD8 +. In some cases, immune system can also have a protumor role, which is a paradox, given that it is known that the inflammatory state promotes tumor growth. One of the major characteristics of tumors is the evading of immune response, in particular by mechanisms of inhibition of active antitumor immune response via two major physiological inhibitory signals, CTLA-4 and PD1 / PDL1. Blockade of these checkpoints, that are T cell inhibitory mechanisms, has recently yielded best results in an immunotherapy approach to cancer treatment. Immune infiltrate in the tumor, as evidence of existence of an active intrinsic response of the organism, is heterogeneous, and composition often differs between different tumors and tumor cells, and mainly divides into two main cell lines: lymphoid and myeloid. On type of cell lines in the immune infiltrate and their activation and orientation depends the clinical response in different tumors. It is well known that immune infiltrate, especially tumor-infiltrating lymphocytes (TILs), can be predictive of response to therapy and have a prognostic role. In some solid tumors they are a good sign, while in some they signal worse prognosis. Numerous studies have evaluated role of lymphocytic infiltrate in breast cancer (BC) and, based on this knowledge, first consensus on standardization of TILs evaluation in solid tumors has been established on the BC model. Prognostic role of TILs in triple-negative breast cancer has received the most attention.

KEYWORDS: *antitumor immune response, tumor-infiltrating lymphocytes, immunotherapy, triple-negative breast cancer*

IMMUNITY AND CANCER

Tumor antigens

Despite being produced by the cells of the host organism, the tumor cells for the immune system represent a foreign body and the immune system responds to them. Tumor immunogenicity, ie the ability to elicit an immune response of the organism, is generated by tumor antigens (1). There are several categories of tumor antigens. Most of these are protein neo-antigens resulting from sporadically mutated genes, (so-called passenger mutations), and less often due to mutations in oncogenes or tumor suppressor genes involved

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in oncogenesis (so-called driver mutations). Another type are oncoviral antigens, that is, products of oncogenic viruses, which can elicit a specific T cell response. The third category includes overexpressed cellular proteins. These are the products of genes that are silenced in normal cells and released in tumor cells or proteins present in normal cells but overproduced in the tumor. These are, for example, cancer - testis antigens (CTA), such as MAGE, an oncogenic variant of the epidermal growth factor HER2 / Neu, and differentiation antigens such as CD on lymphoma and leukemia cells. Tumor antigens also include various alterated glycolipids and glycoproteins, as well as oncofetal antigens alpha feto protein (AFP) and carcinoembryonic antigen (CEA) (1,2).

Immune response

The physiological role of the immune system is immunosurveillance (1,3), the recognition and destruction of clones of transformed cells before they grow into a tumor and the killing of tumors. Evidence that this surveillance system is needed is also the fact that an increased incidence of a number of tumors appears in an immunocompromised organism, but also that is imperfect - the fact that also in an immunocompetent organism some tumors escape immune control and develop. Tumor stimulates specific innate and acquired immune response mechanisms. The main carriers of the body's immune response (1,4) to tumor are T lymphocytes and the main mechanism is the killing of tumor cells by cytotoxic T lymphocytes CD8 + (CTL). CTLs conduct immune surveillance by recognizing and killing potentially malignant cells expressing peptides of tumor antigen origin, presented in interaction with major histocompatibility class I (MHC I) molecules. Thus, in order for CD8 + CTL to function, the presentation of tumor antigens by antigen presenting cells (APC), usually dendritic cells, is required in MHC I. Also, APC express costimulatory molecules, and those and simultaneously activated helper T lymphocytes send major signals for differentiation of naive CD8 + T lymphocytes into competent CTLs. CD4 + helper T lymphocytes contribute to the antitumor immune response in many ways: Th1 cells enhance the CD8 + T cell response and activate macrophages by secreting TNF and IFN γ, which enhances MHC I. In addition to T cells, the host organism often produces antibodies against tumor antigens, but the significance of them is not completely clear. Natural killer (NK) cells are capable of killing different types of tumor cells. Tumor cells become susceptible to NK cell killing when MHC I expression is reduced or the expression of ligands that bind to activating NK cell receptors is increased, and cytokines such as IL-2 and IL-15 stimulate NK cells. Depending on the state of activity, macrophages can inhibit or promote tumor growth. Classic M1 activated macrophages kill various types of tumor cells. They are activated by the described IFN γ production process by Th1 helper CD4 + lymphocytes, and it is not clear how they are activated by tumors (1,4,5).

Protumor role of immunity

In some cases, the immune system may also have a protumor role. It has long been known that chronic inflammation is a risk factor for the development of many tumors (1,6) although the exact mechanism of this cascade is unknown. Among immune cells, cells of innate immunity are considered the key culprits for protumor effects. Tumor-associated macrophages (TAM) of the alternatively activated M2 phenotype, as well as some other cells, are the source of vascular endothelial growth factor (VEGF), which promotes angiogenesis as well as extracellular space-modifying enzymes, thereby supporting tumor growth and spread. By accumulating free radicals, cells of innate immunity contribute to DNA damage and mutations, which lead to malignant alteration, and by secreting soluble factors such as NFκB support the progression and survival of tumor cells. Alternatively activated M2 macrophages and myeloid-derived immunosuppressive cells, MDSC, indirectly support tumor growth by suppressing effective antitumor immunity. In response to the tumor, dendritic cells can condition the differentiation of CD4 + into anti-inflammatory Th2 cells and T regulatory cells, which suppress the tumor-killing immune response and support the development of M2 and other protumor cell lines. The described protumor effects of the anti-inflammatory mechanisms of the immune system are somewhat paradoxical, since it is known, as described above, that the inflammatory state promotes tumor growth, and therefore finding a balance between the described mechanisms is a challenge for potential therapeutic interventions (1,6).

Immunoediting and tumor escape

One of the major characteristics of tumors is evading the immune response. This suggests that the immune control of the host organism over the tumor is only one of the possible variants, that is, the phase in the interaction with the tumor, and that the interaction of the tumor and the host is best described through the process of immunoediting. Immunoediting (7) involves the interaction of a tumor and host organism, which takes place in three stages. The first phase is the elimination phase, a stage where the mechanisms of immune control are adequately functional and eliminate potentially malignant altering cells and tumors. In the equilibrium phase, the coexistence of the host and the tumor is achieved, that is, the host immune system does not eradicate the tumor cells although they do not progress but are dormant. The third phase is the tumor escape phase from immune surveillance. The mechanisms of avoidance of immune surveillance by tumors are generally categorized as either active inhibition of the antitumor immune response or loss of antigens that stimulate these immune responses (1). Inhibition of an active antitumor immune response is accomplished in several ways: by the interaction of inhibitory molecules on the T cell surface and APC, which otherwise have the function of preventing autoimmune reactions, with ligands, or cofactor molecules on the tumor surface, then suppressing the antitumor response by secreted molecules, such as TGF β, by producing regulatory T cells that are actually immunosuppressive in nature and by accumulating the described immature MDSC, a heterogeneous group of cells, such as dendritic, monocytes, and neutrophils that suppress innate immunity and T-cell-mediated antitumor immune mechanisms, either by secretion of immunosuppressive cytokines or by stimulating the accumulation of regulatory T cells. The best known inhibitory signaling pathways of T cells and APC, which naturally serve mainly to avoid and control autoimmune responses, and in the case of tumor escape are the most commonly abused pathways to immune surveillance are the signaling pathways via CTLA - 4 and PD - 1 (1,7,8). Beside of the described mechanisms of inhibition of the active antitumor immune response, the avoidance of immune control of the tumor also allows the loss of expression of tumor antigens, ie the decrease in immunogenicity, which is usually accompanied by the decreased activity of antigen presenting molecules (1).

Immunotherapy approaches

Immunotherapy is defined as any attempt to use any component of the immune system to enhance an intrinsic host response to a tumor (9). There are two main principles in the immunotherapy approach: both innate and acquired immunity are involved in the fight against tumors, by mechanisms similar to those used to fight external pathogens, and tumor oncogenesis and progression occur through the selection and outgrowth of tumor cells with reduced immunogenicity, and with the creation of an immunosuppressive microenvironment (10). Therefore, therapeutic strategies have focused on stimulating both immune mechanisms, to induce tumor cell death. These strategies include therapy with various cytokines, growth factors, and immunomodulatory agents, impact on the tumor microenvironment, passive immunotherapy with monoclonal antibodies, vaccination with tumor antigens, adoptive cell therapy with antitumor T cells, and blockade of checkpoints (T cell inhibitory pathways). Blockade of checkpoints, or T cell inhibitory mechanisms, has recently yielded the best results in an immunotherapy approach to cancer treatment and gave some hope that acting on the immune system in the fight against tumors would surely bring about solutions that could not have been hoped for in the era of classic nonselective cytotoxic therapy. Checkpoint inhibitors have been developed and proven in preclinical and more recently phase I, II, and III clinical trials and approved in the treatment of numerous solid tumors and hemoblastoses (11). They act on the principle of inhibiting inhibition, mainly in two ways: by interfering with the inhibitory CTLA-4 pathway and interfering with the PD-1 / PDL-1 inhibitory pathway. By binding to a T cell receptor in one case, a costimulatory molecule in the second case, or a ligand on a tumor cell in the third case, inhibitors, which are monoclonal antibodies in structure, harbor inhibitory molecules and thus release T cell mechanisms of previous inhibition and allow active antitumor response, which was missing (10). Pembrolizumab (12) and atezolizumab (13) have shown the best results in the treatment of breast cancer to date,

and their efficacy is most significant in the treatment of TNBC.

TUMOR – INFILTRATING LYMPHOCYTES

The origin, composition and role of immune infiltrate in tumor

Tumor is a complex system, composed of two main components: tumor cells and stromal compartment. The stromal compartment is composed of normal host cells and tissue, such as fibroblasts and vasculature cells, nerves, and extracellular matrix molecules, which support the biochemical and structural environment that ensures tumor survival (10). Immune cells, or immune infiltrate, are one of the major host cell lines in this stromal compartment. The immune infiltrate in a tumor is heterogeneous, and the composition often varies among different tumors and tumor sites (14). It is mainly divided into two main cell lineages: lymphoid and myeloid. Research evidence to date indicates that myeloid leukocyte cells, such as TAM, dendritic cells (DC) and MDSC, are primarily responsible, through the factors they produce, for the creation of microenvironment in the direction of an immunostimulatory antitumor or tumor-supporting, and antitumor T cells, by migration into such environments can consequently be activated or suppressed. Conversely, T cells themselves regulate macrophage recruitment in the direction of functional M1 or protumor M2, indicating the importance of intercellular interaction (1). Likewise, the type of cell lines in the immune infiltrate determine the clinical response in different tumors (10). Acquired immunity mediated by T and B lymphocytes is known to play a key role in effective antitumor response, and infiltration by cytotoxic CD8 + cells has been shown to be associated with better response to therapy and survival (15). The presence of CD4 + regulatory lymphocytes can be prognostically both good and bad (10), and of the other cellular subpopulations, IFNγ-secreting Th1 cells have been shown to be associated with a good prognosis (16) and Th2 with a decrease in antitumor response (17). The presence of Th17 cells, the producer of the proinflammatory cytokines IL - 17, has variable effects depending on the cytokine environment in which they are located, which may also depend on the type of tumor and the organ site (1,10). The precise role of B lymphocytic infiltrate is still not completely clear. In conclusion, the presence of CD8 +, Th1, NK, M1, and DC1 is associated with a good antitumor response, and the presence of Th2, M2, DC2, MDSC, and FOXP3 + regulatory T lymphocytes, which secrete IL - 10 and TGFβ, has immunosuppressive effects (1,10) CTLA-4, PD1 and PDL1 are expressed on immune infiltrate cells, especially cytotoxic CD8 + lymphocytes, as well as on tumor cells, which, as previously described, are of particular importance today as targets for immunotherapy (18).

Tumor – infiltrating lymphocytes in solid tumors

As mentioned above, the composition of the immune infiltrate may vary, depending on the tumor and the organ site. Also, depending on the composition of the immune infiltrate in individual tumors, different clinical responses and different levels of prognostic value of the immune infiltrate have been reported (10,14,19,20). Prognostic role of the presence of tumor lymphocytes (TILs) in melanoma has long been known, and today melanoma is one of the most successfully treated tumors with immunotherapy (21) A number of studies evaluating TILs in melanoma in different ways, using different evaluation methods and techniques (HE, IHC), in primary site and metastatic lesions, and most studies have shown an association between the presence of lymphocytic infiltrate and good prognosis and good response to therapy (22 129). Similarly, the prognostic potential of TILs in colorectal cancer has also long been known. Particularly interesting is the ability to predict tumor MSI (microsatellite instability) by simply evaluating HE TILs in the tumor, with greater or less precision, which is a marker of dMMR (MissMatchRepair deficiency), one of the two major pathogenetic pathways of colorectal cancer, and that is of prognostic and predictive value (23). In addition, TILs have been shown to be an independent prognostic factor in all types of colorectal cancer, independent of MSI status, as well as predictor of response to neoadjuvant therapy in rectal cancer (24). Concerning other tumors of the digestive system, studies have been conducted on the role of TILs in gastric, pancreatic and hepatocellular carcinoma (22), but at the present time without concrete positive results and

with the need for larger prospective studies. In the group of gynecologic tumors, interest in TILs in endometrial cancer is mainly associated with the possibility of predicting MSI /dMMR status, similar to colorectal cancer (22), and encouraging results have been observed in studies of the role of TILs in ovarian cancer, although obvious differences in the prognostic role of TILs have been observed, depending on the type of cellular infiltrate as well as in which compartment they were evaluated (25). In the group of urogenital tumors, the results differ from seemingly immunogenic bladder cancer, which showed not only prognostic but also predictive role of TILs (22), via renal cancer, in which some studies clearly highlighted negative association of TILs and prognosis (22), which has been shown to exist due to immunosuppressive cells in the infiltrate (such as T regulatory cells), to clearly non-immunogenic prostate cancer, whose response to immunotherapy remains to be investigated (22). Non-small cell lung cancer is considered to be a highly immunogenic tumor, given the multitude of mutations detected and the resulting genomic instability and neo-antigen formation, which is why the mechanisms of immune response have been of great clinical interest and have been well investigated. TILs have been shown to be associated with good prognosis, and lung cancer is also one of the tumors with excellent results in immunotherapy treatment (26,27). The role of TILs has been investigated in a heterogeneous group of head and neck tumors as well as in brain tumors (22), and it seems that in the future a more accurate, simple and standardized methodology, first developed with breast cancer and subsequently reproduced in the other mentioned tumor sites, will be used to evaluate the clinical value of TILs also in other solid tumors.

Tumor – infiltrating lymphocytes in breast cancer

Numerous studies have evaluated the value of lymphocytic infiltrate in breast cancer (BC) and, based on this knowledge, the first consensus on the standardization of TILs evaluation in solid tumors has been established on the BC model (28). Various evaluation methods have been used in TILs studies in BC, from HE, IHC to molecular methods of gene expression (29-31). Most relevant studies are retrospective TILs analyzes on samples from large prospective cohort studies conducted in either a population of patients with early or locally advanced, luminal, HER2-positive or triple negative breast cancer (TNBC), who have investigated the effect of some chemotherapy approach, in adjuvant or neoadjuvant setting, on tumor tissue specimens obtained by core biopsy or complete specimens obtained operatively, as well as in paired specimens of primary tumor and metastatic site, in case of advanced disease (29,32-37). Studies have shown very diverse results, and these differences are mainly explained by differences in the methodology and technology applied, the method of evaluation and the selection of infiltrate sections for evaluation, and the different sample sizes on which the evaluation was conducted, which affects the statistical significance of the results obtained. Nevertheless, the retrospective nature of most of the studies, which provided the first and crucial information thus far, proved to be non-inferior in this case and the results obtained may be considered relevant (38). Most BCs show some degree of lymphocytic infiltrate. The presence of a higher proportion of TILs was observed in more aggressive cancers, with negative endocrine receptors, high grade, basaloid characteristics, and BRCA mutants. Lymphocyte-predominant type of BC (LPBC) is defined as one in which 50-60% of tumor is infiltrated by lymphocytes. It is more common among tumors of TNBC (20%) or HER2-positive immunophenotype (16%), compared to luminal, ER-positive (6%) (39) The stromal TILs were mainly evaluated by the HE method, which proved to be of the greatest importance, while the intratumoral TILs played a minor role (22,28), which may also be due to limitations of the HE method itself, which is why the role and significance of the intratumoral TILs are further evaluated by IHC. It has been found that evaluation of TILs on core biopsy specimens closely correlates with post-operative specimen analysis and that both specimens can be used equally (22). Studies using the HE method of evaluating TILs in the results correlated highly with studies using the IHC method as well as mRNA profiling (22,28-31). The presence of lymphocytic infiltrate was also observed in DCIS specimens, most commonly those with HER2 positive, and the percentage appears to be approximately between that in normal breast tissue (where it is low) and that in invasive carcinoma (where it is high) (40). Compared to the

primary site, lymphocytic infiltrate was less prevalent in metastatic lesions, which is consistent with the theory of immunoediting and tumor escape from immune control (22,37). In prognostic terms, TILs are associated with better disease-free survival (DFS) and overall survival (OS) most reliably in TNBC and HER2-positive BC, and no statistically significant benefit was observed in luminal tumors (especially in luminal A variant) (41,42). Furthermore, when they were prognostically favorable indicator, TILs showed that for every 10% of infiltrate intensification, the risk of disease recurrence and death decreased by about 10-20% (34), indicating the need for TILs expression as continuous variables (22,28,35). Given that both chemotherapy and endocrine therapy exert their effects to some extent by immunomodulatory mechanisms, it is not surprising that results suggesting predictive role for TILs present in the pretherapeutic bioptic sample for response to neoadjuvant anthracycline therapy, where they have been found to be independent predictors of pCR (35), or an adjuvant combination of anthracyclines and taxanes (32), as well as aromatase inhibitor therapy (43), and have been shown to be a good predictor of response to trastuzumab therapy (33). Thus, the described results undoubtedly demonstrate the predictive and prognostic role of TILs in TNBC and HER2-positive BC, and the clinical value of evaluating TILs in BC is related to the development of predictive risk models, (given the continuity of the variable), therapeutic decision making for use of adjuvant and neoadjuvant chemotherapy, first and further lines of treatment, and with the highly interesting benefit of immunotherapy today.

Tumor – infiltrating lymphocytes in triple-negative breast cancer

TNBC is the most aggressive BC subtype in addition to HER2, a tumor loaded with many mutations and thus genomic instability, the result of which is a multitude of tumor neo-antigens, making TNBC the most immunogenic BC. This explains the highest prevalence of lymphocytic infiltrate in TNBC, as well as the highest incidence of LPBC among this type of BC (44,30). Research into the role of TILs in BC has so far yielded the most significant results in the TNBC population. A series of studies and meta-analyzes have demonstrated the possible association of TILs with prognosis in TNBC (29,33,34,41,42,45). Results relate to the impact of TILs on improved disease-free survival (DFS), overall survival (OS), survival without distant dissemination (DDFS), reduction of disease recurrence locally and remotely (45). This favorable prognostic impact of TILs has been shown to be independent of age, lymph node status, tumor size and histologic grade, peritumor vascular invasion, or Ki67 proliferation index (40). Further subdivision of TNBC into core - basal type (CBP) and 5NP revealed no significant difference in the clinical value of TILs expression. Some studies have shown that there is no statistically significant effect on OS in 5NPs, however relevant data for the 5NP population is scarce (46,45). Despite the different and in some studies conflicting results of the prognostic value of IHC analyzed separately for different subpopulations of TILs (CD8 +, FOXP3 +), pooled analyzes show that there is really no enough strong data to support the existence of this difference in clinical value (45). Likewise, according to some studies, the prognostic value of TILs in TNBC seem to be more significant if analyzed without the influence of chemotherapy: HR (hazard ratio) for OS in TIL-rich tumors in the presence of chemotherapy was found to be lower in compared to that without chemotherapy (46), but several studies and pooled multivariate analyzes have shown that TILs are a good independent predictive indicator of response to chemotherapy (31,35,47,48,49). As mentioned above, the presence of TILs in a pre-therapeutic biopsy specimen may predict good response to therapy and the achievement of pCR (50,51), and the presence of TILs in the residual tumor tissue, following treatment, is also a prognostic indicator of better metastasis-free survival and overall survival (52,53). Finally, enhanced expression of PD1 and PDL1 was demonstrated on activated immune cells, much more than on tumor cells, in TIL-rich TNBC (54,55). This signaling pathway is known to be the target of today's immunotherapy approaches with checkpoint inhibitors, such as already mentioned pembrolizumab and atezolizumab, although with different ways of evaluation of PDL1 positivity (from PDL1 positive immune cells exclusively, for atezolizumab, to combined positive score, of immune and tumor cells, carrying PDL1, among all cells, for pembrolizumab). Role of PDL1 positivity was crucial in these trials, and only the PDL1 positive tumors responded, yet significantly

more and better in those trials where immunotherapy was combined with chemotherapy (12,13,56). Therefore, TILs in TNBC, as an indirect indicator of PDL1 expression, is seriously considered for use as a marker in routine clinical practice for its ease and cost-effectiveness of detection.

CONCLUSION

Therapeutic potential of the immune system-tumor interaction is truly great. The use of this potential has shifted from initial unselective attempts to exploit the immune system, to more *natural* interventions and release of an intrinsic immune response, such as blocking immune inhibitory checkpoints. The success of such an approach appears to be more likely in the presence of indicators of intrinsic immune response activity, which is an immune infiltrate in the tumor. Understanding the origin, composition and function of the immune infiltrate in a tumor is the basis for more accurate assessment and prediction of the success of immunotherapy in the fight against cancer.

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Sažetak

IMUNITET I RAK: ULOGA TUMOR-INFILTRIRAJUĆIH LIMFOCITA KOD TROSTRUKO NEGATIVNOG KARCINOMA DOJKE

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Tumor stimulira specifične urođene i stečene imunološke mehanizme. Glavni nositelji imunološkog odgovora tijela na tumor su T limfociti, a glavni mehanizam je ubijanje tumorskih stanica citotoksičnim T limfocitima CD8 +. U nekim slučajevima imunološki sustav također može imati protumorsku ulogu, što je paradoks, s obzirom na to da je poznato da upalno stanje potiče rast tumora. Jedna od glavnih karakteristika tumora je izbjegavanje imunološkog odgovora, posebno mehanizmima inhibicije aktivnog antitumorskog imunološkog odgovora putem dva glavna fiziološka inhibitorna signala, CTLA-4 i PD1 / PDL1. Blokada ovih kontrolnih točaka, koji su mehanizmi inhibicije T stanica, nedavno je dala najbolje rezultate u imunoterapijskom pristupu liječenju karcinoma. Imuni infiltrat u tumoru, kao dokaz postojanja aktivnog unutarnjeg odgovora organizma, je heterogen, a sastav se često razlikuje između različitih tumora i tumorskih stanica i uglavnom se dijeli na dvije glavne stanične linije: limfoidnu i mijeloidnu. O vrsti staničnih linija u imunološkom infiltratu i njihovoj aktivaciji i orijentaciji ovisi klinički odgovor kod različitih tumora. Dobro je poznato da imuni infiltrat, posebno limfociti koji infiltriraju tumor (TIL), mogu predvidjeti odgovor na terapiju i imati prognostičku ulogu. Kod nekih solidnih tumora oni su dobar znak, dok kod nekih signaliziraju lošiju prognozu. Brojne studije procjenjivale su ulogu limfocitnog infiltrata u raku dojke (BC), a na temelju tog znanja uspostavljen je prvi koncenzus o standardizaciji procjene TIL u solidnim tumorima na BC modelu. Prognostičkoj ulozi TIL u trostruko negativnom raku dojke posvećeno je najviše pažnje.

KLJUČNE RIJEČI: *antitumorski imunološki odgovor, tumor infiltrirajući limfociti, imunoterapija, trostruko negativni rak dojke*