International Conference on Cancer Research 2019

Website: www.CancerConf.org

June 10-11, London, UK

Breast Cancer Multi-Therapy and Immune System Activation, Checkpoint Modulators, Signal Inhibitors and T Cells Reprogramming

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Abstract

Cancer is one of the leading causes of death worldwide according to data from the U.S. National Cancer Institute, with approximately 14 million new cases and 8.2 million of cancer-related deaths in 2018. More than 60% of the new annual cases in the world occur in Africa, Asia, Central America, and South America, with 70% of cancer deaths in these regions. Recently they have been used for breast cancer, novel approaches among which several molecules that block signaling pathways and also reactivate the immune system by inhibiting the activities of two lymphocytes T receptor inhibitors; CTLA-4 and PD-1 in triple negative breast cancer. Although there is evidence, which supports the blocking of these inhibitory molecules, reactive the response of T cells does not always result, surely because in addition to need to reactivate the response Th1 is necessary co-activation of killer cells Natural (NK), the latter are the main actors of the anticancer response. Therefore, combining therapies with the coordinated activation of each cell of the immune response involved will probably produce better results. Through a better understanding of the interactions of the Th1 response and the action of the inhibitors of PD-1 and CTLA-4 and the intratumoral microenvironment, we believe that it would improve breast cancer therapy. In this article, recent advances in the treatment of cancer aimed at blocking signaling pathways and the use of monoclonal antibodies directed to receptors were reviewed. Likewise, it is proposed to combine therapies with antibodies that block PD-1 and CTLA-4 with the activation of the Th1 and NK response, in situ or with extracorporeal activation of autologous cells.

Keywords:

Breast cancer,

Immunotherapy,

EGFR,

PD-1,

CTLA-4,

Signal transduction inhibitors.

1. Introduction

In 2018, the National Cancer Institute declared 1,735,350 new cases of cancer in the United States, with 609,640 cancer deaths.[1] The most common types of cancer from 2016-2018 were breast cancer, lung cancer, prostate cancer, and colorectal cancer, followed by bladder cancer, melanoma, non-Hodgkin lymphoma, thyroid cancer, kidney cancer, and renal pelvis. The GLOBOCAN Annual Report shows that from 2002 to 2011 the mortality rate for most cancers decreased by approximately 1.8 percent per year among men, and 1.4 percent per year among women.[2] Therefore, although the mortality of most cancers in some countries has declined, the global incidence continues to grow and, according to current projections, cancer deaths will increase from 7.6 million in 2008 to 13 million in 2030. However, the development of new approaches for previous therapies and the success of new therapies, including immunotherapy, is expected to gradually reduce cancer mortality rates for the next decades. Breast cancer is the most common cancer in women, with 266,120 new cases in American women, and an estimated 40,920 deaths for 2018. Approximately one in six women diagnosed with breast cancer will die in the coming years. It is the main cause of cancer-related death in women in developing countries, and it is the second-leading cause in women in developed countries.[3] Mortality rates

are influenced by the occurrence of the disease, and the availability of screening programs and appropriate treatment. Despite lower breast cancer incidence, breast cancer mortality rates are higher in many developing countries because of advanced disease at presentation, suboptimal access to treatment, more aggressive biological subtypes, and younger age at diagnosis. In recent years a number of major medical advances have improved the treatment of primary breast cancer.[4] In the last decade, a great step forward has been taken with the discovery of molecular markers in breast cancer, originating "targeted therapies against cancer". Targeted therapies are drugs or other substances that block or inhibit specific molecules involved in cancer growth, progression and spread, specifically targeted to receptors and signaling pathways.

2. The immune system and cancer

The immune system has three main functions: to distinguish between the self and the exogenous, or in other words the immune system must be able to distinguish what is nonself (foreign) from what is self, to contain external invading agents (pathogens, molecules, etc.) and to destroy abnormal cells such as cancer cells. It is known that many tumors escape immune detection due to several factors such as a) lack of recognition of the HLA antigen, b) altered co-receptors that prevent their recognition and c) T cell anergy or natural killer (NK) cells that allow or promote the development of cancer through "immunoediting". Thus, many of the new anti-cancer strategies aim to redirect immune protection against these abnormal cells .[5] It should be noted that the adoptive immunotherapy treatment based on antibodies has not developed as dramatically as expected, due to the lack of specific markers in the tumors. However, inhibitors of several signaling pathways or antibodies that block receptors, such as blocking antigen 4 activity of cytotoxic T lymphocytes (CTLA-4) and programmed death protein 1 (PD-1) are being used to activate the antitumor response of T cells, with good results.[6] Therefore, for a more efficient antitumor response, it will probably be necessary to increase the degree of activation of the immune system, which could be achieved through the stimulation of cytotoxic T lymphocytes (CTL) and natural killer cells (NK), stimulated with cytokines such as: interleukin (IL)-2, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and (IL)-12, plus the presentation of extracorporeal antigen.[7] This review highlights recent therapeutic approaches and their effectiveness for one of the most common cancers. Immunotherapy plus the use of specific inhibitors, the recent experience with new approaches to chemotherapy, and finally an evaluation of the effectiveness of therapies directed against cancer. Likewise, we make a treatment proposal, which contemplates reactivating the immune system: 1) Treating the explanted tumor cells of the patient *in vitro* with IFN-γ, IL-2 and TNF-α, confronting them with autologous T cells ($CD4^+$ and $CD8^+$) and NK cells ($CD56^{bright}$, $CD16^{dim}$), to subsequently reincorporate the reactivated T cells to the patient; and , 2) Treating the cells of the tumor explant with Th1 cytokines (IL-2 IFN-γ and TNF-α) IL-15, IL-22, and IL-23 and then encapsulate these in nanotubes or a material of the type used in hormone implants (pellets), to prevent the escape of tumor cells. Afterward, the encapsulated tumor will be implanted in the patient, to reactivate the immune response.

3. Anti-EGFR antibody therapy in breast cancer

The greatest impact in the treatment against breast cancer arose with the discovery of the over-expression of the HER-2 surface protein in some tumors. This transmembrane protein is a member of the human epidermal growth factor receptor family (HER/EGFR/ERBB). As an oncogene, it plays a crucial role in the development and progression of some aggressive breast cancers, such as invasive lobular carcinoma (ILC) or ductal carcinoma *in situ* (DCIS). In recent years, this protein has become an important biomarker of breast cancer, and about 30% of patients respond to therapy directed against this molecule.[8] The humanized monoclonal antibody (HuMab) trastuzumab (Herceptin®) recognizes the HER-2 receptor. The binding of trastuzumab to HER-2 promotes the increase of the p27kip1 protein that, in turn, stops cell proliferation.[9,10] Conceivably, treatment with trastuzumab is more effective if combined with surgery and chemotherapy.[11] Another HuMab used in breast cancer treatment is pertuzumab (Perjeta®) that inhibits dimerization of HER-2 and HER-3; it is used in combination with trastuzumab as neoadjuvant therapy for breast cancer at an early stage and, recently, also as adjuvant therapy to treat tumors in advanced stages (**Figure 1** and **Table 1**).[12] Unfortunately, a group of breast cancer tumors does not respond to treatment with HuMab anti-HER-2, and it comprises almost 70% of the cases. These tumors are classified into two categories according to their phenotype: 1) Tumors that do not express HER protein-2 ($HER\text{-}2^-$), but express hormone receptors (HR^+), such as estrogen receptor positive (ER^+), and progesterone receptor positive (PR^+); and 2) tumors that do not express HER-2, or hormone receptors, called triple negative (TNBC).[13,14] It should be noted that TNBCs are very aggressive and the number of treatment options limited, therefore, many new therapies are currently being investigated.[15] Additionally, in these tumors, heterogeneity makes specific biomarkers that may serve as target molecules for targeted therapies difficult to identify., Preclinical studies of these tumors have also identified several potential targets such as Src kinase, tyrosine-protein kinase Met (MET) and Poly [ADP-ribose] polymerase 1 and 2 (PARP-1/2).[16] Therapeutic agents such as bevacizumab (Avastin®), ranibizumab (Lucentis®) and aflibercept (Zaltrap®) (directed against vascular endothelial growth factor [VEGF]), have become elected treatments for retinal neovascular disorders, but their use in breast cancer treatment in combination with chemotherapy is increasing.[17] Some authorized combinations consist only on the use of standard chemotherapy and antibodies such as bevacizumab, even though they do not produce the expected results, mainly in metastatic breast cancer (MBC). Other neoadjuvant drugs such as gemcitabine, capecitabine, docetaxel, doxorubicin, and

cyclophosphamide have also been tested in several combinations with bevacizumab.[18] Unfortunately, these combinations have shown not too many benefits in TNBC patients.[19,20] Breast tumors that do not overexpress HER-2 could be treated by combining antibodies against EGFR, more inhibitors for other target molecules (such as PARP-1) and chemotherapy. Additionally, stimulation of antitumor immune activators such as cell-mediated cytotoxicity (ADCC) before or after surgery could prove to be a useful treatment. To obtain an effective anti-tumor immune response, the state of lymphocyte activation during treatment must be determined, since the success of the treatment depends on it.[20] The binding of several antibodies directed to specific molecules in tumor cells that act as target molecules of the immunotherapy, as well as their combinations according to the type of tumor are depicted in **Figure 1** and listed in **Table 1**.

Table 1. Molecular targeted therapy in Breast Cancer treatment.

	Approved agents	Molecular targets	Mechanism of action	Ref.
Monoclonal antibody	***Trastuzumab*** (Herceptin®)	HER2, HER3	RAS/Raf/MAPK Inhibition	9, 10, 21, 22, 70
	Pertuzumab (Perjeta®)	EGFR y HER4		12, 13, 21, 70
	Bevacizumab (Avastin®)	VEGF	VEGFR PI3K, PLCγ prevents neovascularization	17, 18
	Ranibizumab (Lucentis®)			17
	Aflibercept (Zaltrap®)			17, 19
	Cetuximab (Erbitux®)	EGFR	PI3K, RAS, STAT signaling inhibition	21, 24-26
	Panitumumab (Vectibix®)			27
Antibody-drug conjugate	***T-DM1***	HER2	Cytotoxic agent vinca alkaloid RAS/Raf/MAPK	70
	Vinorelbine- (Navelbine®)			
	Trastuzumab (Herceptin®)			22
Inhibitors conjugate	**Fulvestrant-Palbociclib** (Ibrance®)	HR+(HER2-)	CDK) 4/6 inhibitor	22, 71
	Palbociclib-Letrozole (Femara®)	(ER) Estrogen receptor positive	Cell cycle arrest	71
Signal transduction inhibitors				
Inhibitors	**Iniparib** (Sanofi® BSI 201)	BRCA1 or BRCA2 mutation.	Not repair their DNA	30, 40
	Talazoparib (BMN-673)			30
	Niraparib (Tesaro®)			34
	Olaparib (Lynparza® AZD-2281			31, 45

PARP-1/2	y TOPARP-A)	PARP-1 selective inhibitor		
	Rucaparib (AG014699, PF-01367338)			35
	Veliparib (ABT-888)			36
Preclinical studies phase 1 of Inhibitors PARP-1/2	CEP-9722	PARP-1/2 inhibitor	Not repair their DNA	37
	MK-4827			32
	MK-2206			38
	NMS-P118			39
Tyrosine Kinase Inhibitors	**Dasatinib** (Sprycel®)	Multiple tyrosine kinases (TK)	Bcr/Abl, Src, c-Kit and Eph receptor family	41
	Saracatinib (AZD0530)	Src protein	Src inhibitor Bcr/Abl	42
	Imatinib (Gleevec®)	Geminina y c-Abl nuclear	Inhibitor Geminina y c-Abl nuclear	43
	Nilotinib (Tasigna®)	Abl tyrosine kinases	Bcr/Abl	72
	Lapatinib (Tykerb®)	TK receptor		11, 44
	Palbociclib (Ibrance®) **Ribociclib** (Kisqali®)	Inhibitor kinases (CDK4,6)	Cell cycle	22, 71
	MK-2206	AKT inhibitors	Cell cycle arrest	38
	Vemurafenib (Zelboraf®)	inhibits the enzyme B-Raf. Serine/threonine-specific protein kinase	BRAF serine/threonine kinase inhibitor	60, 61
	Cobimetinib (Cotellic®)	MEK1 protein kinase	Inhibits the activity of ERK2 transcriptional	60, 61
Immunomodulators	**Nivolumab** (Opdivo®)	PD-1	Blocks ligands PD-L1 and PD-L2	49, 57, 59
	Pembrolizumab (Keytruda®)			55
	Durvalumab (Imfinzi®)	PD-L1	Block activation of PD1	56
	Atezolizumab (Tecentriq®)			57
	Avelumab			58

	(Bavencio®)			
	Ipilimumab (Yervoy®)	CTLA-4	B7-1/2	55
	Tremelimumab			56
TKIs	Afatinib Erlotinib Gefitinib Dacomitinib Neratinib Sorafenib Alectinib	EGFR, HER2 y ErbB-4, VEGFR	PI3K/Akt/	42 60, 61
	Crizotinib Ceritinib	competitive inhibitor Akt	ALK-fusión	

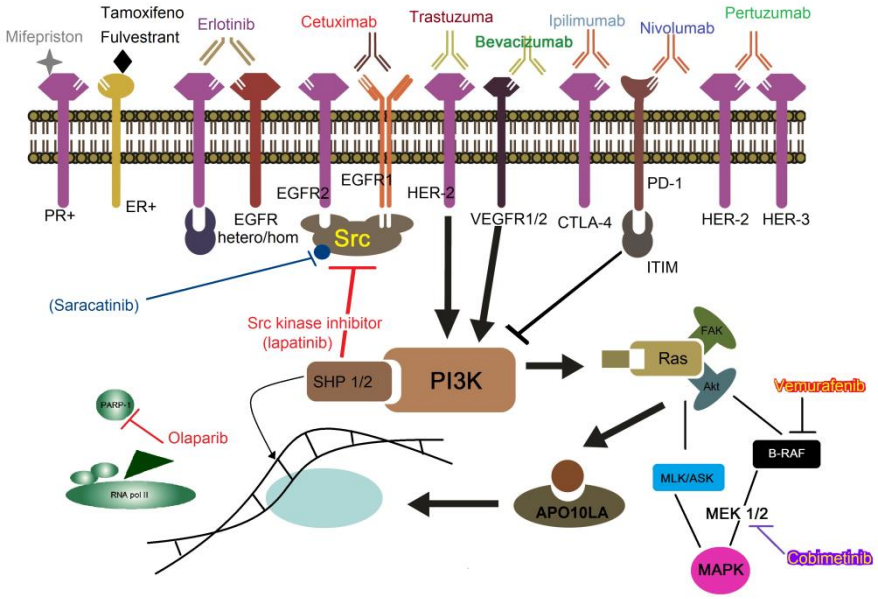

Figure 1. Tumoral receptors targeted for immune therapy. From right to left, HER-2 and HER-3 receptors which have a strong tendency to dimerize in cancer, *Pertuzumab* (green). Antibodies colored in blue are *Nivolumab* (anti-PD-1) and *Ipilimumab* (anti-CTLA-4),. *Bervacizumab* (dark green). HER-2 receptor is blocked by *Trastuzumab* (brown),. *Cetuximab* (red) is an antibody directed to EGFR that inhibits signal transduction for cell growth. Other mAbs similar are: *Rituximab*, *Bevacizumab* and *Ranibizumab*). Erlotinib (purple) is a drug that prevents dimerization of EGFR receptors from heterodimer to homodimer. Tamoxifen and Fulvestrant (black) can inactivate estrogen action by their competitive antagonism,. Mifepristone (gray) is an antagonist of the progesterone receptor, to be used in PR-dependent breast cancers. Lapatinib (red) is an inhibitor of tyrosine kinase from EGFR (ErbB1) and HER2 (ErbB2) receptors. Other inhibitors such as Saracatinib, Bosutinib or Dasatinib have also been used to avoid signaling on MAPK and PI3K pathway through Src or dimerization of Bcr-Abl tyrosine-kinase. Olaparib, is a molecule that inhibits PARP1. Cobimetinib is a reversible inhibitor of MEK that blocks MAPK's (yellow with purple) and Vemurafenib (red-yellow) inhibitors used for breast cancer metastatic.

For instance, the treatment of breast cancer HER-2^{+} with antibodies such as trastuzumab and pertuzumab has shown good results, while cetuximab, discussed later, is used successfully in TNCB.[21] On the other hand, the combination of vinorelbine-trastuzumab, palbociclib and fulvestrant or palbociclib and letrozole seems to be a successful therapy in MBC. Fulvestrant degrades the receptors while palbociclib for the cell cycle.[22] Regarding hormone-dependent breast tumors (HR^{+}), their treatment is tamoxifen and hormonal suppression, while the use of antibodies against the estrogen receptor α (ER-α) or another natural molecule. Like to Genistein, Isoliquiritigenin, Kurarinone, Sceptrin, Silibinin or Soranjidiol is not recommended despite the possibility of blocking ER-α by means of a humanized monoclonal antibody (similar to that of clone SP1 used for immunohistochemistry). However, it is not possible to deny the good results

obtained in the treatment with selective estrogen receptors pharmacological blockers or with aromatase inhibitors that prevent the conversion of androgens to estrogens such as tamoxifen, anastrozole, Letrozole or Exemestane.[23]

4. Other monoclonal antibodies in breast cancer treatment

Recent findings indicate that the treatment of patients with basal inflammatory type breast cancer or TNCB with anti-EGFR antibodies improves their prognosis.[24] Although predictive biomarkers in breast cancer have not yet been established, it is highly possible that EGFR expression in the three types of breast is not related to the main oncogenic factor responsible of inducing the proliferative alteration, but none the less, playing an important role in the disease and its control. Cetuximab (Erbitux®) is a chimeric monoclonal antibody (mAb) approved for the treatment of human metastatic colorectal cancer (mCRC), squamous cell carcinoma of the head and neck (HNSCC) and advanced non-small cell lung cancer (NSCLC). This mAb inhibits EGFR activation by blocking its ligand binding or hindering its interaction with other proteins (**Figure 1**).[25] Although the first clinical trials with cetuximab were costly, a new anti-EGFR mAb has been developed at a lower cost. Noticeably, their blockade with cetuximab contributes to the suppression of several signaling pathways such as JAK/STAT, Raf/MEK/ERK, and PI3K/Akt, which seems to be sufficient to inhibit the aggressive behavior in the TNCB.[26] Panitumumab (Vectibix®) is a huMab used primarily in the treatment of metastatic colorectal cancer and acts in the same way as cetruximab. Vectibix is in phase III clinical studies and is being used in combination with gemcitabine and carboplatin for treatment in women with triple negative metastatic breast cancer.[27]

5. Signal transduction inhibitors

Along with therapies targeting surface markers overexpressed as EGFR, inhibitors of PARPs have been used, along with chemotherapy based on liposomal doxorubicin (Caelyx®, Myocet®), or nanoliposomal irinotecan (Onivyde®). These treatments are recommended against TNBC (**Figure 2**).[28] Paradoxically, the effectiveness of PARPs activators relies on cancer cells undergoing oxidative stress, increasing DNA damage and depletion of cellular ATP that leads to lysis and cell death (necrosis). However, cancer therapy seeks to inhibit PARPs, because knock-out mice for PARP-1 and -2, showed profound deficiencies in the mechanisms of DNA repair, as well as greater sensitivity to ionizing radiation or alkylating agents.[29] These and others evidence allow us to propose the convenience of inhibiting PARPs as a treatment method for breast cancer. Some examples of PARP inhibitors are iniparib (Sanofi® BSI 201), talazoparib (BMN-673), olaparib (Lynparza® AZD-2281 and TOPARP-A), and niraparib (Tesaro®) (**Figure 1**).[30-34] Rucaparib (AG014699, PF-01367338) and veliparib (ABT-888) have also been used in phase III clinical trials for the treatment of breast cancer and metastatic melanoma.[35,36] Other PARP inhibitors such as CEP- 9722, MK -4827, MK-2206 and NMS-P118 are in phase I trials, some combined with chemotherapy in patients with different types of cancer *in situ* or in metastatic tumors.[37,39] In 2014, Sanofi gave up the use of iniparib after phase III trial results were reported on the "first Word pharma" portal. However, based on previous studies as well as the recent good results obtained from phase II clinical trials in metastatic breast cancer and TNBC, the use of PARP inhibitors including iniparib is being recommended as first-line treatment of these cancer types. On the other hand, inhibitors targeting Src proto-oncogene family could also be adopted for breast cancer therapy.[40] These drugs inhibit the signal transduction pathway that promotes critical processes for the development and progression of cancer such as proliferation, adhesion, invasion, migration, and tumorigenesis of cancer cells. For example, dasatinib (Sprycel® or BMS-354825), a multi-target inhibitor approved for first-line use in patients with chronic myeloid leukemia, was developed since 2006 and was authorized for use by the FDA in 2013. This drug inhibits several tyrosine kinases, including Bcr-Abl, Src, c-Kit, and the Eph receptor, but has no effect on EGFR.[41] Saracatinib (AZD-0530) is an experimental inhibitor of Src, that acts as a dual inhibitor of kinases: Src and Bcr-Abl. However, and despite several studies demonstrating its ineffectiveness against cancer, recently (2018) published articles still promote its use in cancer.[42] We previously mentioned several components participate in the development of TNCB and one of them is the over-expression of c-Abl, a tyrosine kinase that phosphorylates and activates geminin. While in the cytoplasm, c-Abl protein plays an important role in differentiation, proliferation and cell migration. Contrastingly, its translocation to the nucleus promotes apoptosis, damaged DNA repair and chromatin dynamics regulation by histones phosphorylation. In a study where 800 samples of TNBC were analyzed, overexpression of germinin was found in 90% of the cases and most of them were negative to c-Abl in the nucleus. When the activity of c-Abl was inhibited by imatinib (Gleevec®) or nilotinib (Tasigna®), the phosphorylation of geminin in the Y150 residue was prevented, inactivating it and converting the overexpressed geminin into an inducer of apoptosis.[43] Lapatinib (Tykerb®) is a tyrosine kinase inhibitor in HER-1/EGFR/ERBB1 and HER-2/ERBB2, and was authorized to be used in combination with capecitabine (Xeloda®); very good results in the treatment of metastatic breast cancer, and particularly in the treatment for ER/EGFR/HER-2 positive breast tumors that did not respond to trastuzumab, anthracyclines or taxanes.[45] Currently, immunotherapy based on antibodies is being incorporated into almost all cancer treatments. However, a combination therapy of an inhibitory mAb of any of several overexpressed markers, a PARP inhibitor such as olaparib (Lynparza®) and liposomal chemotherapy, appears to be one of the most promising combinations in the treatment against cancer (**Figure 2**).[45] For that, we propose that after surgery, therapy for mammary tumors *in situ* or metastatic should include: i) at least one PARP inhibitor (avoiding repair of DNA damage) or an Src inhibitor, ii) the use of the chemotherapy encapsulated in liposomes (i. e CAELYX®),. The application of CAELYX®, plus spores of *Clostridium* (spores of *C. novyi-NT*) in the site of the tumor or metastasis, will aid *in situ* release.[46,47] iii) Additionally,

the use of antibodies directed to target molecules that promote cell cytotoxicity and blockade in the proliferation of cancer cells together with a specific inhibitor of overexpressed receptors. Also, it is recommendable to verify the production/expression of TNF-α, IFN-γ, and IL-2. This triple approach in the treatment of breast tumors (**Figure 2**) would be a better option compared to first-line treatments with EGFR inhibitors alone or with standard chemotherapy, that have ineffective response rates. Recently, in the treatment of breast cancer and other types of tumors, treatment with nivolumab-ipilimumab (anti-PD-1 and anti-CTLA-4) has been started as an immunotherapy basis.[48,49]

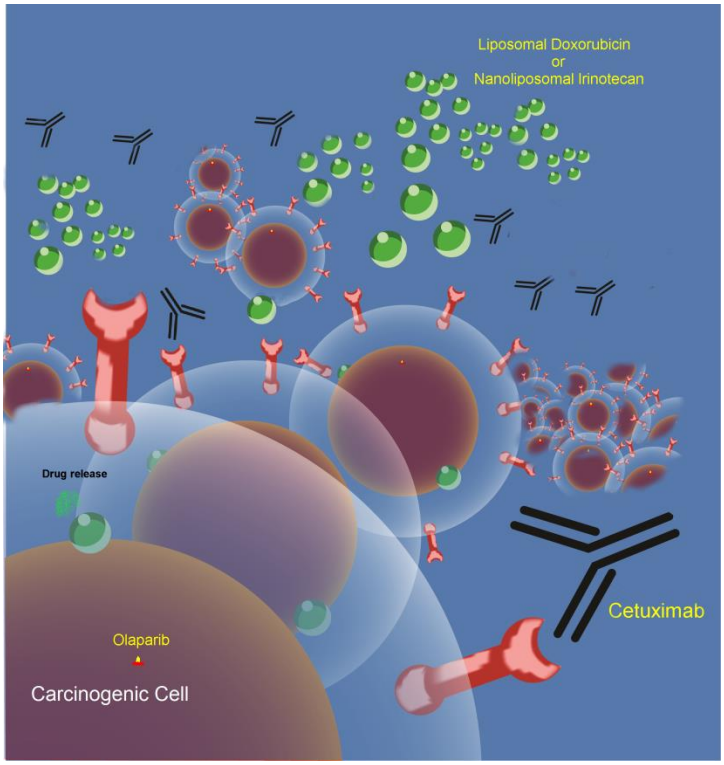

Figure 2. First-line treatment for TNBC. In personalized medicine, this treatment could be very effective since the administration of doxorubicin or irinotecan both encapsulated in liposomes (green balls) to help their delivery into the site of action and diminish their secondary effects on other tissues, which helps to avoid the patient worsening. In the larger cell, the delivery of doxorubicin (or irinotecan) can be observed. In addition to antibodies against EGFR, such as Cetuximab, a PARP-1 inhibitor, such as Olaparib, could be added to avoid cells repairing the DNA damage. The activity of the PARP-1 inhibitor potentiates the chemotherapy.

6. Reactivation of the immune response in combination with antibodies

Despite the therapeutic resources already mentioned, there are still several difficulties in the use of targeted therapies. One of the main problems is adverse effects, secondary reactions and interactions with other therapies, occasionally with altered responses. Still, breast cancer therapy with a combination of liposome-encapsulated chemotherapy, inhibitors and blocking antibodies has given a variety of responses.[50] The promising possibilities of immunotherapy in lung cancer have promoted treatment development for other cancer types. The main objective of immunotherapy is to recognize and eradicate tumors by restoring the immune capacity of the host. One interesting approach is the activation of macrophages (MΦs) with the application of retinoic acid and stimulation of NK cells with IL-15, IL-22 and IL-23 and cytotoxic T cells (CTLs) stimulated with IL-2, TNF-α, IFN-γ, plus the presentation of the antigen. One further step in cancer therapy could be depletion or maintenance of the Treg/Th17 cells balance.[51,52] So, an approach that we propose to boost the anticancer response of the immune system is to "tag" cancer cells with specific antibodies, activate both the antigen presentation and lymphocytes with cytokines or IFN-α, TNF-α, IL-2, IFN-γ and IL-12 (Th1). To minimize the promoted attack, if not physiologically stopped, the IL-10, TGF-β and autologous regulatory T cells (Treg) would be applied to restore equilibrium (**Figure 3B**).[53] In an experimental proposal, autologous $CD4^+$ T cells could be robustly polarized *in vitro* towards Th1 and Th17 subtypes, through cytokines and chemokines, to subsequently identify their activation with molecular adhesion profiles and surface markers, which will open the possibility of detecting effector function *in vitro* and then *in vivo* to treat cancer. Th1 response is generally considered as the main source of tumor rejection, but there is also evidence that Th17-polarized cells mediate the destruction of advanced B16 melanoma.[54] The Th1 therapeutic effect critically depends on the production of IFN-γ, while apparently; depletion of IL-17A and IL-23 has little impact. Visibly,

the appropriate *in vitro* polarization of the effector $CD4^+$ T cells could be decisive for the elimination of the tumor; design of preclinical and clinical trials should consider this therapy based on *in vitro* stimulation of the response and posterior transferring of these immune cells, capable of recognizing the tumor *in vivo*, back to the original tumor (**Figure 3 A**).

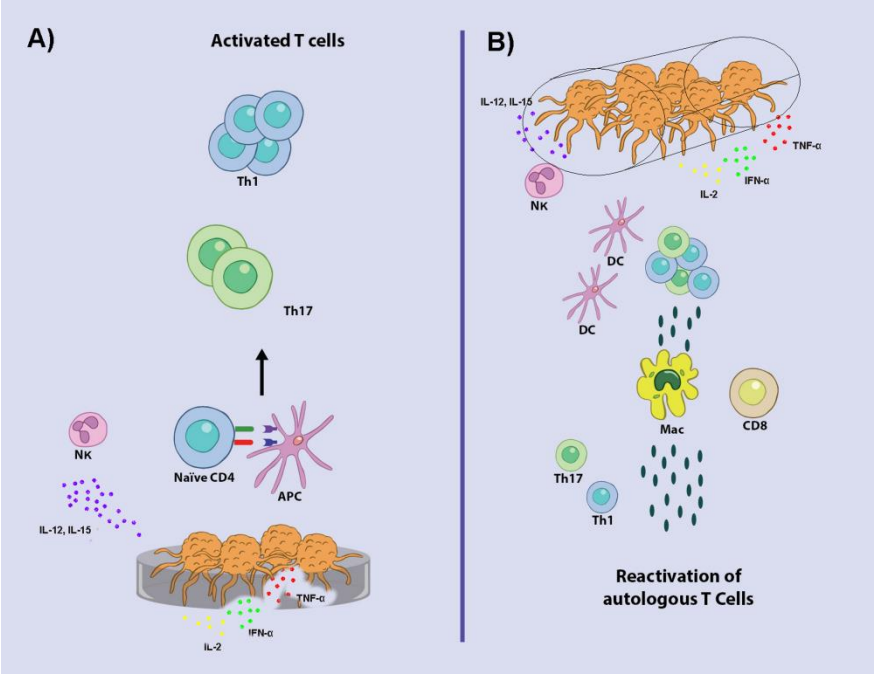

Figure 3

Figure 3. Extracorporal activation of autologous cells to stimulate Th1 and NK responses. A) Several lineages of bystander immune cells are obtained from the cancer patient to be activated. T cells are challenged with chimeric antigens. Further, the experimental in vitro boost of tumoral cells with NK cells and T cells costimulated with interleukin accordingly to the lineage for the maturation and differentiation of immune cells. Later, theoretically stimulated cells are reincorporated to attack the tumor. B) The same tumoral piece can be embedded into a no-degradable synthetic polymeric tube with hexagonal holes. This encapsulated tumoral piece is going to be embedded with Th1 cytokines and chemokines: IL-2, IFN-alpha or -gamma, and TNF-alpha to activate the cell-mediated cytotoxicity, and the NK cells with IL-12, IL-15, and IL-18; for at the last reincorporate the encapsulated piece. Monitoring of maturation of response and the new immune awakening should be screened with the attack to other sites with free tumor.

7. Immunotherapy control point blocking in cancer with PD-1 and CTLA-4 blocking antibodies.

In cancer, T cells are repressed and cannot respond against the tumor through the interaction between T cell receptors: PD-1 and its corresponding ligands (PD-L1/PD-L2 or B7.H1/B7.DC) and CTLA-4 (CD152) and its ligands CD80 (B7-1) and CD86 (B7-2) present in the cancer cell. Recent treatments with nivolumab (PD-1 target), ipilimumab (CTLA-4 target), durvalumab (PD-L1 target) and tremelimumab (CTLA-4 target) used in breast tumors HER-2 negative and TNBC reported that they respond to blocking therapy at the immune control point, due to their high immunogenicity. Atezolizumab (Tecentriq®), a humanized monoclonal antibody of the isotype IgG1 against PD-1, and pembrolizumab (Keytruda®), and which act by blocking PD-1, are also available but they should not be used in immunosuppressed or treated patients with corticosteroid (see **Figure 4A and B**).[55] Recently, Polk et al. conducted a meta-research of the studies for breast cancer treatments until 2017; it highlights the results obtained with chemotherapy, radiotherapy and targeted immunotherapy with HuMab against the PD-1 and CTLA-4 receptors in T cells (durvalumab-tremelimumab and nivolumab-ipilimumab) and other combinations. Other studies have begun the evaluation of the combination of durvalumab and tremelimumab antibodies in breast cancer in early stages, as the treatment in MBC and TNBC has demonstrated good results.[56, 57] Other anti-PD-L1 antibodies for the treatment of breast cancer such as avelumab (Bavencio®) are also being studied, and it must be combined with surgery, chemotherapy, and radiotherapy.[58] Until now, targeted therapy anti-PD-1 and anti-PD-L1 has made great strides in the treatment of breast cancer. Simultaneously, it is also possible to inhibit CTLA-4 with ipilimumab (Yervoy®), a mAb that inhibits its activation, which makes cytotoxic T lymphocytes capable of recognizing and destroying cancer cells. Both nivolumab and ipilimumab are being

tested and their clinical results have been promising in the treatment of several cancers (**Figures 1** and **4**).[59] Clearly, the use of these checkpoint inhibitors has shown great therapeutic potential in several clinical trials, which is why they have been proposed for the first-line treatment of breast cancer. The use of this type of inhibitors is generating great interest in the field of immunotherapy. Nivolumab and ipilimumab are the most used and promising antibodies; Applied in a cocktail for cancer without mutation in the proto-oncogene B-RAF (V600 BRAF) and for metastatic breast cancer. In addition to the combination that has been tested with vemurafenib and cobimetinib (Cotelli®), which is a reversible inhibitor, selective allosteric blocking, the MAPK pathway specifically directed to an extracellular signal regulating protein kinase (MEK) 1 and MEK 2, which causes an inhibition of ERK1/2 phosphorylation.[60] Currently, available agents include the ALK inhibitors certinib and crizotinib and the EGFR inhibitors afatinib, erlotinib, and gefitinib. Four oral targeted therapies are used in the treatment of solid tumor associated with the B-Raf proto-oncogene (BRAF): cobimetinib, dabrafenib, trametinib, and vemurafenib, oral agents for treatment.[61]

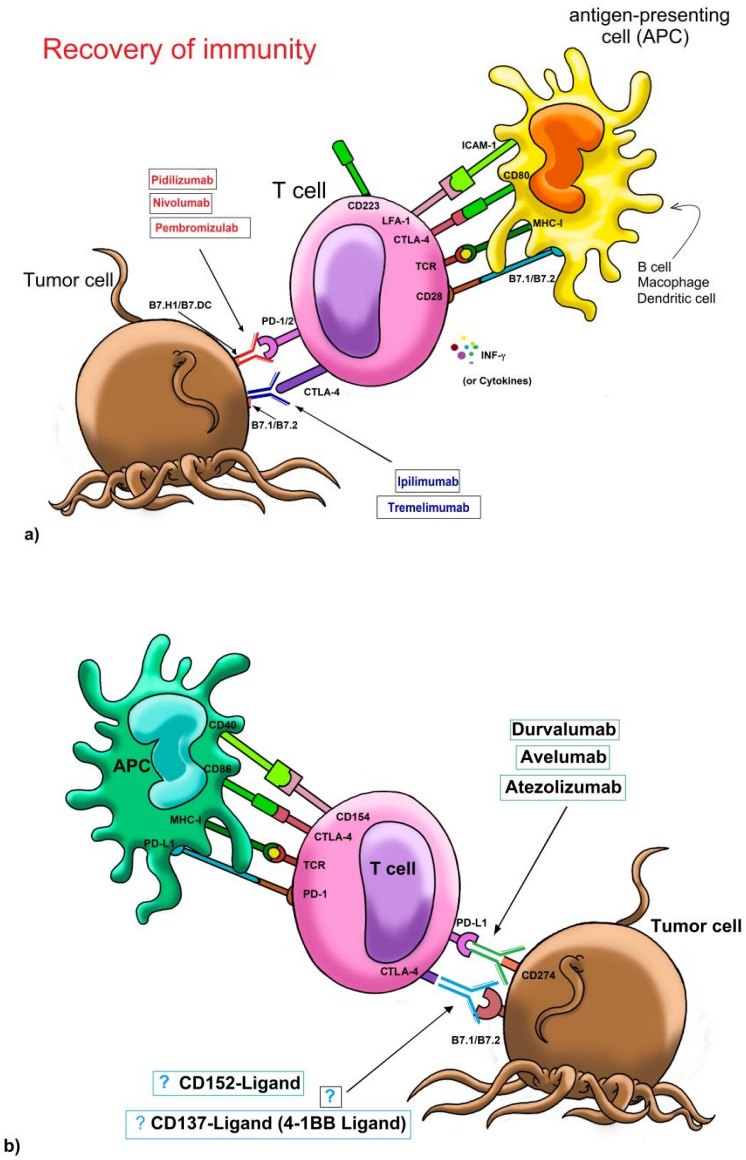

Figure 4. a) Restoring the immune capacity. Binding of PD-1 with its ligand B7.H1 and B7.DC delivers inhibitory signals to T lymphocytes, this lead to anergy and dysfunction of cells. Both activation mechanisms of T cells by antigen presenting cells (APC), and blocking by mAb targeted to PD-1/2 (red) and mAb directed to CTLA-4 (blue) will restore the ability of T lymphocytes for destroying cancerous cells, especially if accompanied by the action of IFN-gamma and other cytokines. b) The PD-L1 interaction of cancerous cells favors the conversion of bystander T cells and the decrease of cytokine secretion. The mAb anti-PD-L1 (green) binding to tumor cells avoid the inactivation of T cells; and further, can function as a marker for the attack to the membrane by the MAC, CTL-mediated death by perforin and granzyme, or NK CD16 cells with its Fc receptor that recognizes the antibodies bound to the cell. On the other side, there are not antibodies targeted to ligands of CTLA-4 (CD152 or CD137).

8. Cytokines and immunoregulation

In the early days of immunotherapy against cancer, some cytokines that regulate the Th1 response, (IFN-γ, IL-2) were used together with the inhibitors nivolumab and ipilimumab. These cytokines can promote a better antitumor response after activating the T cells since the activated cells are able to recognize tumor antigens, while these antibodies would release the brake imposed on the reactive T cells.[62] In experimental studies *in vivo* and *in vitro*, the use of these therapies has been favorable with the administration of IFN-α or IFN-γ and exogenous IL-2 (to activate the antitumor T cells) and the presence of regulatory T cells (Treg) or helper T cells 17 (Th17). Results of retrospective studies with patients who were treated with high doses of cytokines were not as encouraging; still, it is possible that the combined use of cytokines and inhibitors will aid reduce the damages of advanced breast cancer (stages III and IV) and decrease recurrences. Furthermore, these substances can be administered in combination with stage IV breast cancer chemotherapy. Although side effects with the use of cytokines may be a limiting factor, careful management by an immunotherapy medical specialist can significantly reduce the risks.[63,64]

9. NK activation

NK cells are the main cellular effectors of the innate immune system, which mediate the lack of self-recognition, lysis of a marked target and are also a potent and early source of cytokines and chemokines, which does not require prior exposure to antigens. In healthy individuals 90% of NK cells are in peripheral blood (PB) and are mature and cytotoxic, characterized by expressing $CD16^{bright}$ and $CD56^{dim}$, while the remaining 10% of NK cells is a subset of immature cells, which produce cytokines and express $CD56^{bright}$ $CD16^{dim}$ or $CD16^{-}$ and $CD25^{+}$.[65] The activity of NK is regulated by inhibition or stimulation of signals upon contact with tumor cells, where the prevalence of stimulating signals or the absence of inhibitors leads to triggering the lytic effect. That is, if healthy cells do not express high levels of HLA class I, but do not express activating ligands, tolerance will be induced in NK cells.[66] On the other hand, tumor cells, in general, may show a reduction in the expression of HLA class I and at the same time an increase in the activation of ligands such as NKp30 or NKG2D, which facilitate the attack of NK cells to the tumor.[67] During the last decade, it was established that NK cells are activated by IL-12, IL-15, and IL-18, but the NK lineage is sufficiently exclusive since it is not easy to obtain functional NK cells. Colucci et al. proposed three stages in the development of NK cells in vitro.[68] Under these conditions, NK cells show cytotoxicity and are producers of proinflammatory cytokines such as IFN-γ, TNF-α, IL-13, IL-10, and GM-CSF, when there is contact with tumor cells susceptible to be recognized (**Figure 3 A**). However, in most cancer patients, NK cell populations are depleted or almost eliminated or non-existent, resulting in low control of tumor growth. It should be mentioned that the recognition of the tumor by NK cells can be carried out through an activation signal or through the inhibition of the receptors on the surface of the cell, either measured by the MHC, by their KIR receptors; or they can be activated through an event independently of this recognition, and known as CIK (killer cells induced by cytokines). Therefore, we believe that the use of cytokines, to activate autologous NK cells *in vitro*, and then to be perfused back into the patient, so that they can respond against cancer cells, could be excellent immunotherapy (**Figure 3A and B**).[69]

10. Conclusion

The current standard treatment for different types of breast cancer is still chemotherapy since they are highly sensitive. However, patients have high relapse rates. Today the therapeutic target with mAbs that block both PD-1 and PD-L1 and CTLA-4 is being supported by several novel studies. In addition, several studies have reported that the TNBC and the BC hormone receptor have a very high level of genomic instability and high rates of genetic mutations, which may involve the probable generation of more neoantigens and increased immunogenicity. In addition, several inhibitors of signaling pathways (MAPKs) are being studied, as well as the application in the treatment of drugs that inhibit PARP-1/2 cause multiple breaks in double strands and in tumors with BRCA1, BRCA2 present in some tumors of BC. Preliminary data from the clinical trials showed promising results for patients with advanced stage / metastatic TNBC and with possibilities of application in other early stages. Some tests in patients responded to the treatment having a favorable prognosis and frequently showed a significant increase in overall survival. Even so, the objective response rate was relatively low. The design of several mAbs directed to tumor antigens have proven successful in the treatment of various malignancies and are now widely used in the clinic, such as: Trastuzumab, Pertuzumab, Bevacizumab, Ranibizumab, Aflibercept, Cetuximab, Panitumumab, and others. However, the current mAbs directed to the immunological control point are playing a preponderant role in the immunological awakening. In addition, tyrosine kinase inhibitor (TKI) tests have shown that, despite the preservation of tyrosine kinase domains, tyrphostins can be designed and synthesized that discriminate even between closely related tyrosine kinase proteins, such as EGFR, and its close relationship HER2 and thus can be used in breast cancer that expresses it. As well as the use of inhibitors of AKT, Scr and other downstream signal molecules for the activation of genes for growth, differentiation, and proliferation. Therefore, it is necessary to find strategies to improve the response to this therapy in BC and to transform those that do not respond.

We know that through the immunotherapy with mAb the expected response is not always present, either due to the

activation of the response only in some places or without presenting a good response through the antibodies. But we also need to keep in mind that there are zones of immune response development and other areas without immune activity in tumors. In addition, immunotherapy with antibodies apparently does not always activate the immune system efficiently. Consequently, in some cases, the evolution of the therapy does not improve the prognosis and only a partial resolution of the problem is achieved. In the worst scenarios, even with several types of treatment, only a few more years of life are provided to the patient and in some cases, they produce severe adverse reactions. An autoimmune response is a frequent complication that, besides, prevents the adequate identification of the tumor. This event raises several questions: Why do tumors escape immune surveillance? Is tolerance due to the early presence of cancer cells, evading, therefore, immune recognition of the self and nonself? Lack of response is due to molecules that fulfill their role in an abnormal way? Does the immunoediting happen in all cancers? The processes carried out by the immune system to recognize and eliminate a tumor are explained by immunoediting that comprises three stages: elimination, equilibrium, and escape. If in the elimination phase the tumor is not controlled, it enters a stage of equilibrium, escapes and will be allowed. There is a possibility that the recognition of antigens does not occur, there are T anergic cells or the activation of cytokines is not carried out. Taking all this into account, our proposed immunotherapy against tumors includes the reactivation of the immune response through a new screening and activation, using activated T cells and external cytokines, which may be re-implanted in the patient to establish an effective and lasting recognition towards the tumor.

This proposal might be validated as a common treatment against cancer, since, theoretically, the type of cancer in question does not matter because it is based on reactivation of the immune system for the recognition of new variants and the activation of the immune antitumor response. Briefly, our first proposal would be extracting activated or virgin T lymphocytes and tumor cells from the patient, and co-cultivate them in the presence of cytokines that activate the cytotoxic response: i.e. IL-2, INF-α and γ, and TNF-α. Additionally, macrophages or dendritic cells should be obtained; they are to be activated in a classical way (M1) to be lately exposed to cancer cells for them to carry out the antigen processing. An additional possibility is that the excised tumors of patients are confronted with NK cells and exogenous cytokines IL-15, IL-18, IL-22, IL-23, to reactivate the immune response and establish specific tumor recognition. As a result of all these interactions, T cells and antigen presenting cells already activated could be re-implanted into the patient, to raise an efficient and sustained antitumor immune response (**Figure 3A**).

A second immunotherapy proposal consists of tumor extirpation, to infiltrate it later with Th1 and IL-15 cytokines, IL-18 and exogenous IFN-α, and, finally, re-implant the tumor into the patient, in a subcutaneous region closer to some lymph nodes. It must be noted that this implant should be covered in a nanoparticulate network to prevent the escape of tumor cells (**Figure 3B**). As a result, a reactivation of the local immune response mediated by T cells, APC (antigen presenting cells) and NK cells is expected. Through the active migration of immune response cells to the implanted tumor, the stimulated area might be monitored. The immunotherapy success will only be achieved through a deep understanding of the T cells and NKs activation mechanisms, and delicate handling of cytokines, reaching adequate cytotoxic T cells reprogramming. All this additionally depends on the uptake and presentation of tumor antigens by macrophages and dendritic cells, coupled with the expression of various co-stimulatory molecules and cytokines of the antitumor response. Once the antitumor immune response is established, it can be kept under control by regulatory mechanisms, such as immune control points mediated by PD-1 and CTLA-4, as well as other types of immunosuppressive cells such as Treg and TH17. With the recent advances in antitumor immunology and the development of new inhibitors of target molecules, the implementation of multi-therapy against cancer seems to be the most promising option in years to come. This goal will only be reached with further research, increasing the number of clinical studies and obtaining the experience that will allow the future achievement of an effective therapy against one of the diseases that cause many deaths in the world.

11. Funding Information

This study was supported by Instituto Nacional de Enfermedades Respiratorias “Ismael Cosio Villegas” Mexico City.

12. Conflict Of Interest

None declared.

13. Authors’ Contributions

Each and every one contributed equally. Galván-Barrera; Conceptualization original draft and writing. Santiago; Orthography and images, Terán; Review and editing.

14. Acknowledgments

The authors thank Ph.D. Betinna Sommers Cervantes.

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