



MINIREVIEW

Open Access



Relevance of tumor-infiltrating lymphocytes in breast cancer

Sathana Dushyanthen^{1†}, Paul A. Beavis^{1†}, Peter Savas¹, Zhi Ling Teo¹, Chenhao Zhou¹, Mariam Mansour¹, Phillip K. Darcy^{1,2} and Sherene Loi^{1,2,3*}

Abstract

While breast cancer has not been considered a cancer amenable to immunotherapeutic approaches, recent studies have demonstrated evidence of significant immune cell infiltration via tumor-infiltrating lymphocytes in a subset of patient tumors. In this review we present the current evidence highlighting the clinical relevance and utility of tumor-infiltrating lymphocytes in breast cancer. Retrospective and prospective studies have shown that the presence of tumor-infiltrating lymphocytes is a prognostic marker for higher responses to neoadjuvant chemotherapy and better survival, particularly in triple negative and HER2-positive early breast cancer. Further work is required to determine the immune subsets important in this response and to discover ways of encouraging immune infiltrate in tumor-infiltrating lymphocytes-negative patients.

Keywords: Breast cancer, HER2, immunity, immunotherapy, NAC, neoadjuvant chemotherapy, TILs, TNBC, triple negative breast cancer, tumor-infiltrating lymphocytes

Introduction

In cancer, neoplastic transformation alters the structure of tissues and induces immune responses leading to the elimination of developing tumors. However, incomplete elimination of transformed cells results in escape from immune control. This process is known as cancer immunoediting and is supported by a large body of experimental data and clinical evidence showing that the intact immune system can prevent and control cancer through the generation of effective tumor-specific immune responses [1, 2]. Immunoediting describes the process of malignant progression on the basis of tumor and immune cell interactions in three phases: (1) elimination, where cancerous cells are eliminated following immunosurveillance; (2) equilibrium, where transformed cells are held in control but are not eliminated by the immune system; and (3) escape, where tumor cell modifications shape disease progression [1, 2]. In general, a patient will present once the tumor has 'evolved' to escape immunosurveillance

and, accordingly, a subset of patients with breast cancer present clear evidence of immune suppression and aggressive disease progression, potentially driven by mechanisms of tumor tolerance [3, 4]. In the elimination phase, the innate and adaptive immune system coordinate to detect and destroy cancer cells before clinical presentation. At this stage the balance is towards antitumor immunity stimulated by natural killer (NK) cells, NK-T cells, T cells, and increased pro-immune factors in the tumor microenvironment [2]. In equilibrium, there is a balance between antitumor and tumor-promoting factors, thus maintaining the tumor in a functionally dormant state [2]. Well-documented escape mechanisms of breast cancer cells include decreased immune recognition through reduced expression of major histocompatibility complex class I (MHC I) and/or co-stimulatory molecules and increased expression of immunosuppressive factors. This results in reduced clearance (lysis) via CD8⁺ cytotoxic T lymphocytes (CTLs) [3, 4]. The mechanisms underlying these processes have previously been reviewed in detail in several papers [1, 2, 5–10].

Several studies have indicated that in addition to T cells, macrophages, NK cells, and dendritic cells (DCs) also infiltrate tumor tissue in varying capacities [1, 2, 8, 10]. It is known that CD4⁺ T helper 1 (Th1) cells, CD8⁺

* Correspondence: sherene.loi@petermac.org

[†]Equal contributors

¹Division of Research and Cancer Medicine, Peter MacCallum Cancer Centre, East Melbourne, VIC, Australia

²Sir Peter MacCallum Department of Oncology, The University of Melbourne, Parkville, VIC, Australia

Full list of author information is available at the end of the article

cytotoxic T cells, NK cells, M1 macrophages, and DCs are protective against tumor growth [11]. Conversely, CD4⁺ forkhead box P3 (FOXP3⁺), CD4⁺ Th2 cells, M2 macrophages, and myeloid-derived suppressor cells (MDSCs) promote tumor growth [11]. These subsets interact in numerous ways; some of these mechanisms of interaction are shown in Fig. 1. Accordingly, tumor

cells are able to suppress tumor-infiltrating lymphocytes (TILs) through multiple mechanisms either through direct suppression of antitumor immune cells or recruitment and reactivation of immunosuppressive subsets. One such mechanism is the expression of PD-L1 on tumor cells, which interacts with PD-1⁺ CD8⁺ T cells and induces subsequent anergy/apoptosis, leading to inactivation or

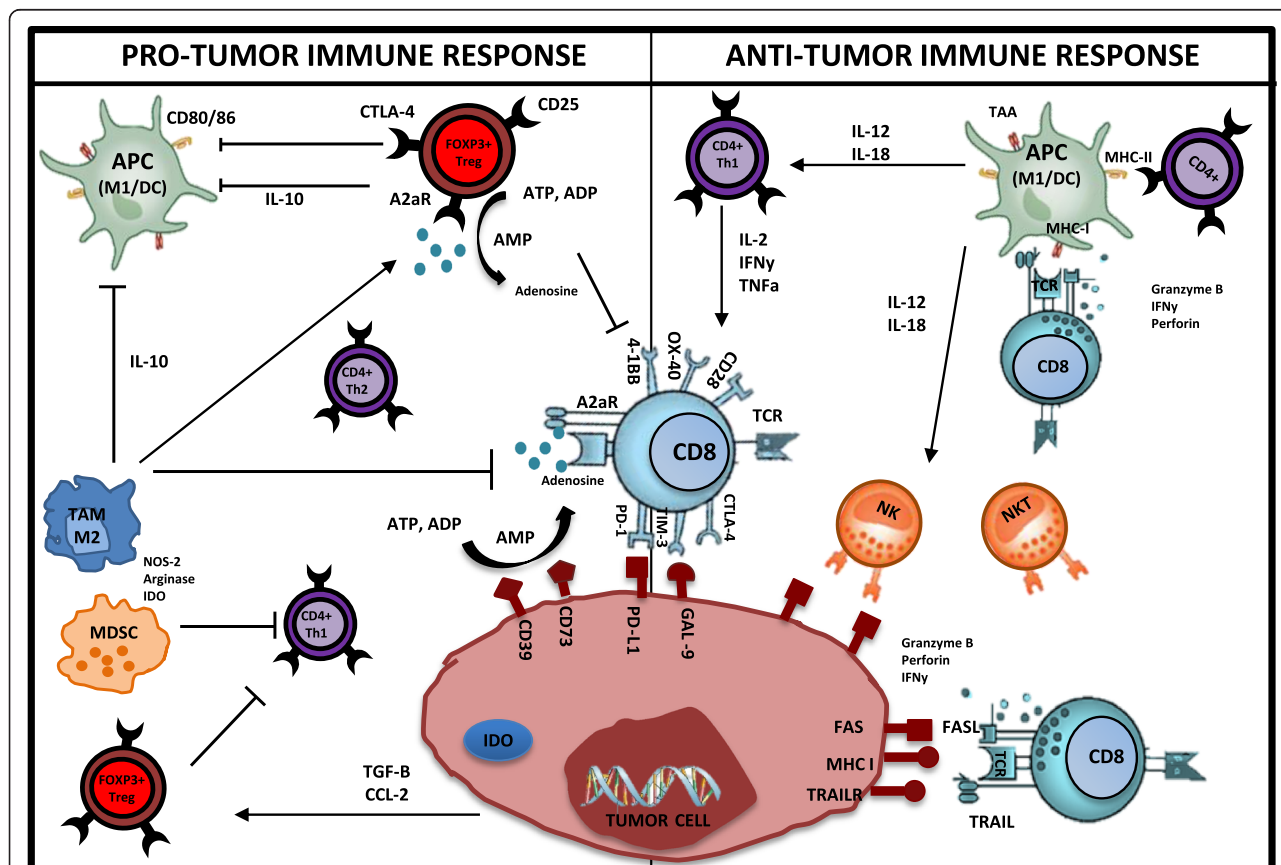


Fig. 1 Interactions between the immune microenvironment and tumor cells in breast cancer. The antitumor immune response is dependent upon CD4⁺ (Th1) IFN γ production, which in turn mediates the expansion, differentiation, and activation of tumor-specific CD8⁺. CD8⁺ cytotoxic T cells induce cell lysis via recognition of specific TAAs such as MHC, FAS, and TRAILR on the surface of cancer cells/APCs. Similarly, CD4⁺ T cells are able to recognize MHC II on APCs. As a result of this complex formation (TCR-MHC/Peptide), high levels of granzymes, IFN γ , and perforin are released from CTLs, resulting in granule exocytosis and tumor cell death via apoptosis. NK and NKT cells with the help of APCs (DCs/M1) and CD4⁺Th1 are able to recognize and eliminate tumor cells. In the pro-tumor environment, CTLA-4, TIM-3, and PD-1 deliver inhibitory signals as a result of T-cell exhaustion/anergy caused by prolonged activation. CTLA-4 negatively regulates T-cell activation during the ‘priming’ phase of T-cell response. PD-1 expressed on T cells in the effector phase of T-cell response binds to its ligand PD-L1, expressed within the tumor microenvironment. This results in inhibition of T-cell activity (apoptosis). FOXP3⁺ Treg cells play a critical role during the selection of high-avidity CD8⁺ T cells, reducing their functionality. Tregs also have inhibitory action on APCs, CD8⁺ T cells, NKs, and CD4⁺ Th1 T cells. Both Tregs and tumor cells produce adenosine, which has inhibitory effects on T cells. Tumor cells can secrete cytokines and chemokines (e.g., TGF- β , CCL2) that recruit and stimulate suppressive cells such as Tregs, MDSCs, and M2 macrophages. M2 macrophages and MDSCs inhibit T-cell responses through nutrient sequestration via arginase, ROS, and NOS generation, as well as interference with trafficking into the tumor site. The upregulation of immunosuppressive enzymes such as IDO and arginase catabolizes essential nutrients required for effector cell activation. Furthermore, tumor cells downregulate MHC molecules, lose expression of antigenic molecules, and upregulate inhibitory molecules such as PD-L1, causing immune recognition to be inhibited and thus allowing immune escape and cancer progression. This figure was made exclusively for this manuscript. *A2aR* A2A adenosine receptor, *ADP* adenosine diphosphate, *AMP* adenosine monophosphate, *APC* antigen-presenting cell, *ATP* adenosine triphosphate, *CCL-2* chemokine ligand-2, *CTL* cytotoxic T lymphocyte, *CTLA-4* cytotoxic t lymphocyte-associated protein, *DC* dendritic cell, *FAS* fatty-acid synthase, *GAL-9* galectin-9, *IDO* indolamine 2,3-dioxygenase, *IFN γ* interferon gamma, *IL* interleukin, *M1/M2 TAM* tumor-associated macrophage, *MDSC* myeloid-derived suppressor cell, *MHC* major histocompatibility complex, *NK* natural killer, *NKT* natural killer T cell, *NOS* nitric oxide synthase, *PD-1* programmed death, *ROS* reactive oxygen species, *TAA* tumor-associated antigen, *TCR* T-cell receptor, *TGF- β* transforming growth factor beta, *TNF α* tumor necrosis factor alpha, *TRAIL* TNF-related apoptosis-inducing ligand, *Treg* T regulatory cell

exhaustion of TILs in the tumor microenvironment. This process leads to diminishing host antitumor immune responses [12]. Checkpoint inhibitors such as CTLA-4 and PD-1 trigger inhibitory pathways which dampen T-cell activity when bound to their ligands (CD80/CD86 and PD-L1/PD-L2) [13]. Both PD-1 and CTLA-4 blockade have proven to be very effective in preclinical animal models of melanoma and some breast cancer models [14–17]. Interestingly, an increasing number of studies are revealing positive outcomes in the clinical setting to checkpoint blockade of PD-1/PD-L1 and CTLA-4 [18–20]. Other targets that are of great interest in the clinical setting are the emerging T cell Ig and mucin domain (TIM) superfamily and lymphocyte activation gene 3 (LAG-3), given that they have both been associated with the inhibition of lymphocyte activity as well as induction of lymphocyte energy [11, 12]. Additionally, several other immunosuppressive factors and inhibitory metabolites, such as adenosine [21–25], FOXP3⁺ regulatory T cells (Tregs) [26], indoleamine 2,3-dioxygenase (IDO) [27–30], arginase [31–33], and MDSCs [34–36], have been implicated in cancer-mediated immunosuppression, where targeting of these pathways has been shown to enhance antitumor immunity *in vivo*.

Preclinical evidence for the role of the immune system in cancer and in response to chemotherapy and radiotherapy

Chemotherapy and radiotherapy are frontline management options for breast cancer and the underlying immunogenic component of these agents make them attractive candidates for neoadjuvant therapy. Preclinical studies of chemotherapy and radiotherapy have revealed the unexpected ability of the immune system to contribute to the success of treatment. There is an abundance of experimental and, more recently, clinical evidence [37–41] showing that chemotherapies are more efficient if they successfully re-activate immune surveillance through the elimination of immunosuppressive cells or through the promotion of danger signals released by the death of tumor cells, hence triggering a long-term immune response against residual tumor cells [42, 43]. Several findings have delineated a sequential series of events driving immunogenic tumor cell death that results in the activation of innate and adaptive immune responses [44]. During immunogenic cell death, dying tumor cells release danger signals such as adenosine triphosphate (ATP) and high-mobility group protein B1 (HMGB1), which act to prime an antitumor immune response [45, 46]. In landmark studies by Zitvogel and colleagues, anthracycline therapy was less effective in mice deficient in P2X7 receptors, NLRP3, and caspase-1, while oxaliplatin was dependent on HMGB1-mediated activation of toll-like receptors (TLRs). The activation of antigen-presenting cells such as DCs by ATP (P2X7)

or HMGB1 (TLR4) leads to enhanced production of IL-1 β , and consequently activation of both innate and adaptive immune responses [23]. Interestingly, the observation of enhanced antitumor immunity following treatment extends to various chemotherapies, including gemcitabine [47–49], cyclophosphamide [50, 51], paclitaxel [52], and doxorubicin [53–55]. Induction of tumor cell death by chemotherapy (e.g., gemcitabine) also enhances tumor antigen cross-presentation [56]. Furthermore, certain chemotherapies have direct effects on immune cells themselves. For example, low dose cyclophosphamide selectively reduces the number of immunosuppressive Treg cells while sparing immune effector cells [51]. These findings are summarized in Table 1. Many preclinical studies have described immune-enhancing activity in response to chemotherapies [57]. However, it is evident that not all chemotherapies induce immunogenic cell death. As such, it is apparent that combinations with specific immune-inducing or immune-targeted inhibitors are necessary to promote tumor regression and immunogenic cell death in these cases. Thus, despite initial theories suggesting that breast cancer is not an immunogenic disease, recent studies have confirmed and consolidated understanding of the underlying immunological component in breast cancer, thus revealing promising targets and novel therapies for treatment of this disease.

Observations on the prognostic value of TILs in breast cancer

The importance of the immune system in breast cancer is increasingly being recognized, owing to the observation by several groups that the presence of TILs is a prognostic indicator for higher rates of pathological complete responses (pCRs) to neoadjuvant chemotherapy (NAC) [37, 58–60]. Notably, Denkert and colleagues [41] were able to first show in a large-scale analysis of 1058 patients' biopsies that TIL⁺ tumors achieved a pCR rate of 40–42 % following NAC, whereas TIL⁻ tumors achieved a pCR of only 3–7 %. This distinction was based upon comparing lymphocyte predominant breast cancer (LPBC; defined as ≥ 60 % TILs in either the intratumoral or stromal compartment) to the rest of the population. Interestingly, this analysis revealed that TILs within either the stromal or intratumoral compartment was of value as a prognostic indicator. Subsequently, this has also been shown in another dataset of 580 triple negative breast cancer (TNBC) and HER2-positive breast cancers (59.9 % in LPBC vs 33.8 % in non-LPBC) [41] as well as in a meta-analysis of 996 patients where an immune gene module indicative of a Th1 response was prognostic of pCR in all cancer subtypes [61]. The protective role of a Th1 response was also reported by an independent group who found that the infiltration of T-bet⁺ lymphocytes correlated with a favorable prognosis [62]. TILs have

Table 1 Effects of chemotherapy on tumor-infiltrating lymphocytes

Drug	Target	Immunological effects and model used	Reference
Gemcitabine	Nucleoside analogue, prevents DNA replication	Reduction in the number of MDSCs	[126]
		HER2/neu model; breast cancer	[127, 128]
		Mesothelioma and lung cancer models	[48]
		Reduction in MDSCs and Tregs when given with cyclophosphamide; CT26 colon carcinoma	[49]
Cyclophosphamide	DNA alkylation, cross-links DNA	Reduction of Tregs in patients with pancreatic cancer	
		Induction of immunogenic cell death, IFN-I mediated activation of dendritic cells; EG7 thymoma, B16F10 melanoma	[129]
		Selective depletion of Tregs in MMTV-neu mice (breast cancer)	[88]
		Selective depletion of Tregs in colon carcinoma model	[42]
Paclitaxel	Inhibition of mitosis through tubulin targeting	Selective depletion of Tregs in melanoma model	[130]
		Reduction in MDSC frequency and suppressive activity	[131]
		Reduction in Treg numbers and suppressive activity	[52, 132]
Anthracyclines	Multiple mechanisms	Reduction in MDSC frequency and suppressive activity	[131]
		Induction of immunogenic cell death; thymoma model, CT26 colon carcinoma	[45]
		Differentiation of CD11b ⁺ LY6C ⁺ APCs; MCA205 fibrosarcoma	[133]
Oxaliplatin	Cross-links DNA	Elimination of MDSCs; 4 T1 and EMT6 breast tumor cell lines	[134]
		Induction of immunogenic cell death resulting in the activation of myeloid cells; thymoma/colon cancer model	[45, 135]
Cisplatin	Cross-links DNA	Induce the accumulation of CD11c ⁺ inflammatory dendritic cells; lung/colon carcinoma models	[136]
Docetaxel	Inhibition of mitosis through tubulin targeting	Reduction in MDSC frequency and suppressive activity; B16 melanoma model	[116]
5-aza-2'-deoxycytidine	DNA methyltransferase inhibition	Increased antigen presentation by tumor cells; 4 T1 breast cancer model	[137]
		Reduction in MDSCs and suppressive function; lung/prostate carcinoma	[138]

APCs antigen-presenting cell, MDSC myeloid-derived suppressor cells, Tregs regulatory T cells

been reported to predict pCR in a prospective study of HER2-negative breast cancers in patients treated with NAC, confirming the prognostic value of TILs in the response to chemotherapy [63].

From a clinical perspective, the observation suggests that higher levels of TILs are associated with higher responses to chemotherapy, seemingly independent of oestrogen receptor (ER), progesterone receptor (PR), and HER2. There is in fact no biologically appropriate TILs cut-point as all studies have shown that the TIL marker is prognostic on a continuous scale: each 1 % or decile increment is associated with a further increase in the rate of pCR. The biological reasons for this observation of chemosensitivity remain poorly understood. Perhaps pre-existing immune antitumor responses are better placed to be able to clear tumor cells after chemotherapy has depleted local immunosuppression or Treg cells. An increase in TILs in the breast cancer post-NAC is also associated with improved outcomes [64]. Notably, however, although TILs correlate with pCR to NAC in all breast cancer subtypes, the correlation between TILs at diagnosis and disease-free or overall survival was only significant in

TNBC and HER2-positive breast cancers, although the reasons for this are not fully understood [39, 64–66]. The studies indicating a positive relationship between TILs and responses to NAC are summarized in Table 2. TILs are important in the efficacy of trastuzumab [65, 67, 68] and, moreover, trastuzumab treatment results in the activation or recruitment of multiple immune cell lineages and increases the susceptibility of tumor cells to antibody dependent cytotoxicity (ADCC) [67, 69] (reviewed by [70]). A study looking at TILs at baseline and correlation with both pCR and disease-free endpoints highlights that high levels of TILs at diagnosis bodes for an improved outcome regardless of attainment of pCR [68]. Those with low levels of TILs and residual tumor at surgery post-NAC and anti-HER2 therapy had the worst outcomes of all, suggesting that it is this group that needs more effective antitumor and immune-enhancing strategies. The fact that TILs are highly associated with responses to chemotherapy and trastuzumab suggests that the presence of TILs, both pre-NAC and post-NAC, should be taken into account for patient treatment decisions—a possible schema for this is shown in Fig. 2. The potential synergistic

Table 2 A summary of the studies investigating a correlation between the extent of TIL infiltrate and responses to neoadjuvant/adjvant chemotherapy in breast cancer

Breast cancer subtype (Number of patients)	TILs predictive of pCR?	TILs predictive of disease-free or overall survival?	Reference	Chemotherapy used
All patients (56)	Yes	ND	[60]	Anthracycline/taxane or epirubicin, cyclophosphamide, and capecitabine
All patients (73)	Yes	ND	[139]	Anthracycline/taxane based
ER ⁺ /PR ⁺ (659)	Yes	ND	[59]	Anthracycline/cyclophosphamide/taxane
ER ⁻ PR ⁻ (266)	Yes	ND		
All patients (1334)		Yes (total or distant stromal CD8 ⁺)	[75]	Cyclophosphamide/methotrexate/fluorouracil
ER ⁺ (911)		No (intratumoral or adjacent stroma CD8 ⁺)		
ER ⁻ (485)		No (total CD8 ⁺)		
HER2 ⁺ (169)		Yes (total CD8 ⁺)		
HER2 ⁻ (1106)		No (total CD8 ⁺)		
		Yes (total CD8 ⁺)		
ER ⁻ (268)	Yes	Yes	[38]	Fluorouracil/epirubicin/cyclophosphamide or docetaxel and docetaxel plus epirubicin
One cohort of 113, one of 255				
All patients (3403)		No	[140]	Adjuvant systemic therapy
ER ⁻ (927)		Yes		
ER ⁺ (2456)		No		
HER2 ⁺ (216)		No		
TNBC (535)		Yes		
All patients (180)	Yes	ND	[37]	Anthracycline/cyclophosphamide or cyclophosphamide/epirubicin/5-fluorouracil
TNBC (82)	Yes	ND		
HER2 ⁺ ER ⁻ PR ⁻ (42)	No	ND		
HER2 ⁻ ER ⁺ /PR ⁺ (46)	No	ND		
All patients (845)	Yes	ND	[61]	Meta-analysis: anthracycline with or without taxane-based NAC
ER ⁻ HER2 ⁻	Yes	ND		
HER2 ⁺ (116)	Yes	ND		
ER ⁺ Her2 ⁻	Yes	ND		
All patients (68)	Yes	ND	[141]	Anthracycline and/or taxane-based treatment
All patients (180)	Yes	ND	[71]	Paclitaxel then fluorouracil/epirubicin/ cyclophosphamide
HER2 ⁻ (313)	Yes	ND	[63]	Anthracycline/taxane
All patients (2009)	ND	No	[39]	Doxorubicin followed by three cycles of cyclophosphamide/methotrexate/fluorouracil
ER ⁻ /HER2 ⁻ (1079)	ND	No		
HER2 ⁺ (297)	ND	No		
TNBC (256)	ND	Yes		
All patients (153)	Yes	ND	[72]	Anthracycline and/or taxane-based treatment
TNBC (38)	Yes			
Non-TNBC (115)	Yes (If CD8 ⁺ component analyzed), No if CD4 ⁺ analyzed			
All patients (175)	Yes	ND	[73]	Anthracycline and/or taxane-based treatment or herceptin+NAC
All patients (12439)	ND	ND	[142]	Cyclophosphamide/methotrexate/fluorouracil or epirubicin plus fluorouracil

Table 2 A summary of the studies investigating a correlation between the extent of TIL infiltrate and responses to neoadjuvant/adjvant chemotherapy in breast cancer (*Continued*)

ER ⁻ (3591)	ND	Yes		
ER ⁺ (8775)	ND	No		
ER ⁺ HER2 ⁺ (772)	ND	Yes		
All patients (934)	ND	No	[65]	Docetaxel or vinorelbine, followed by three cycles of fluorouracil/epirubicin/cyclophosphamide
ER ⁺ HER2 ⁻ (591)	ND	No		
HER2 ⁺ (209)	ND	No		
TNBC (134)	ND	Yes		
TNBC (278)	Yes	Yes	[64]	Anthracycline-based neoadjuvant or anthracycline/taxane
TNBC (47)	Yes	ND	[143]	Panitumumab plus anthracycline/taxane-based chemotherapy
TNBC (481)	ND	Yes	[66]	Doxorubicin plus cyclophosphamide and taxol/docetaxol
All patients (580)	Yes	ND	[41]	Anthracycline/taxane with or without carboplatin
TNBC (314)	Yes	ND		
HER2 ⁺ (266)	Yes			

ER estrogen receptor, HER2 human epidermal growth factor receptor 2, NAC neoadjuvant chemotherapy, ND not determined, PR progesterone receptor, TNBC triple negative breast cancer

immune effects between chemotherapy and immunotherapies also suggest that combination approaches in the neoadjuvant setting, prior to surgery, may also be beneficial.

Recently, efforts have been made to sub-divide the immune infiltrate into lineage subsets to determine the prognostic value of each immune cell type. The presence of CD8⁺ cells in the tumor infiltrate prior to the onset of NAC predicted pCR in several studies [41, 71–75]. The presence of Tregs prior to NAC has also been shown to be a prognostic indicator of pCR [71–73, 76]. Although this seems somewhat counterintuitive given the suppressive role of Tregs, it is probably because FOXP3⁺ infiltrate is also significantly associated with CD8⁺ infiltrate. More informative is the observation that the presence of Tregs following NAC has a significant negative correlation with pCR and disease-free or overall survival [77–79]. Indeed, the ratio of CD8 to Tregs following NAC has been shown to be a strong predictor of clinical responses [78]. The increased CD8 to Treg ratio likely facilitates the acquisition of CD8⁺ T-cell effector functions such as Granzyme B expression, which is elevated post-NAC [78, 80]. In this study they found that the proportion of CD8⁺ lymphocytes remained stable pre-NAC and post-NAC whereas Tregs were significantly reduced in the post-NAC samples. Limited work has been done on the prognostic significance of other immune cell subtypes although the presence of CD4⁺ [72] and CD20⁺ [74] lymphocytes pre-NAC is also associated with pCR. Another study found that the presence of T follicular helper (T_{FH}) cells, which function to attract and promote the formation of memory B cells, was associated with improved responses to chemotherapy in breast cancer. These T_{FH} cells localized to peritumoral

tertiary lymphoid structures, indicating there may be localized orchestration of antitumor immune responses in certain breast cancers [81]. The link between TIL infiltrate and patient outcome has led to heightened interest in utilizing immune-modulating strategies for patients with breast cancer. Other immune subsets of interest include $\gamma\delta$ -T cells and Th17 cells. $\gamma\delta$ -T cells show positive correlations with CD4⁺ FOXP3⁺ T cells, and are associated with poor outcomes and advanced tumor stage, as well as low disease-free and overall survival in patients with breast cancer [82]. However, further work is needed given the apparent antitumor role of $\gamma\delta$ -T cells in some settings [83, 84]. In a small study of 30 patients, Th17 cells negatively correlated with disease stage [85] but further work is needed to evaluate their effect on immune responses to NAC [86]. Accordingly, further work is needed to determine whether these subsets are potential targets for immunotherapy.

Combining conventional treatment with immunotherapy

The strong prognostic significance of TILs in breast cancer opens up important questions for patient management. In particular, it is likely that patients with high TILs post-NAC can benefit from strategies designed to enhance the immune response against the tumor. As discussed above, NAC has the potential to increase the CD8⁺ to Treg ratio, which correlates with the likelihood of pCR [87, 78]. Interestingly, certain chemotherapies, including cyclophosphamide [42, 51, 88], used to treat patients in the cohort studied by Ladoire et al., have been shown to specifically reduce the number of Tregs in preclinical models. Thus, it is worth considering the immunological consequences of

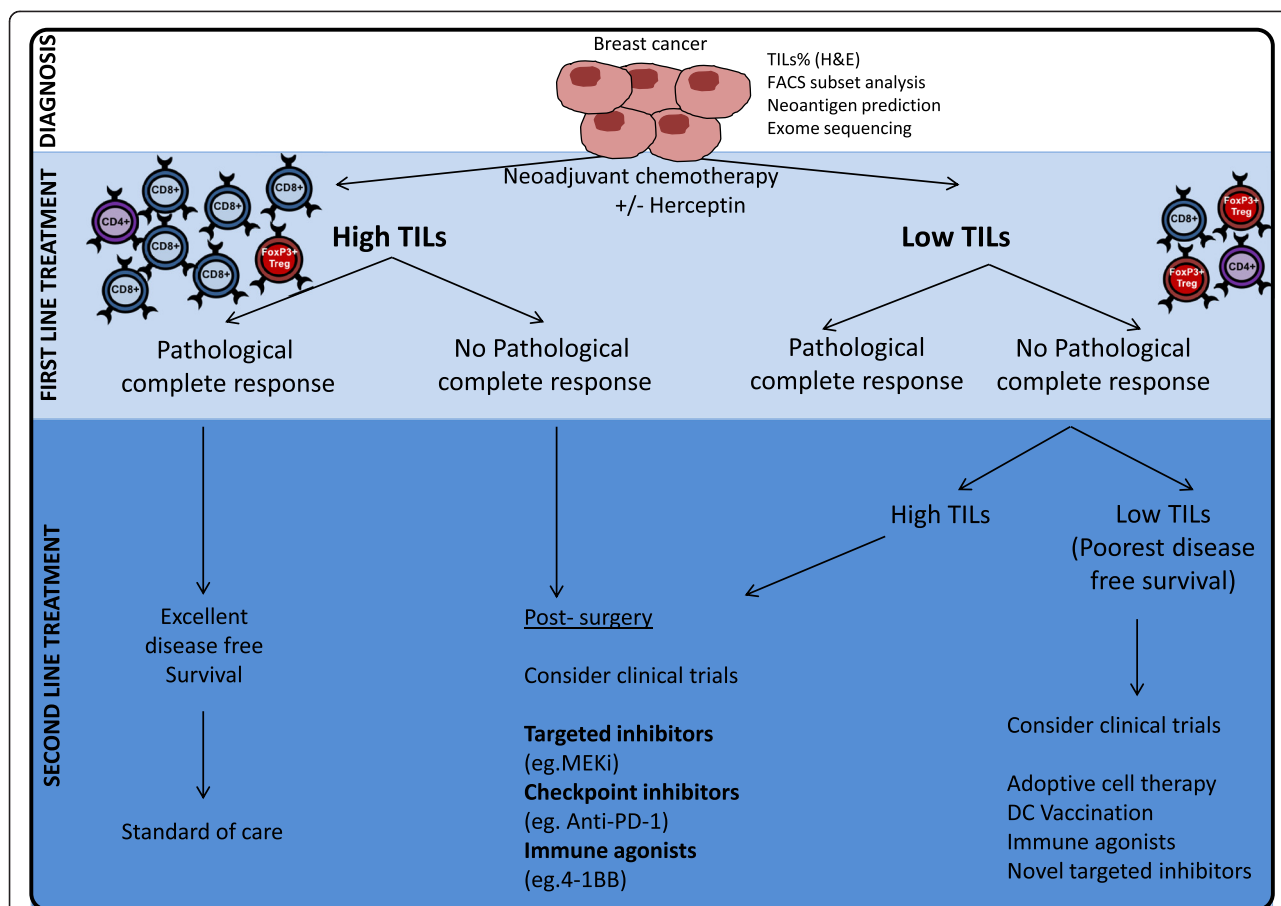


Fig. 2 Using the TIL infiltrate and response to frontline treatments to guide patient management decisions. The presence of tumor-infiltrating lymphocytes (TILs) and response to neoadjuvant chemotherapy (NAC) may be used to guide decisions on second line treatments. Patients with high TILs and exhibiting pathological complete responses to NAC (*far left*) have an excellent prognosis and may not require further intervention other than standard of care. Patients with high TILs at diagnosis but no pathological complete response, or patients with low TILs at diagnosis but high TILs post-NAC, may benefit from immunotherapies, such as checkpoint inhibition (PD-1 blockade), or immune agonists (e.g., 4-1BB). However, patients with little TIL infiltrate either pre-NAC or post-NAC (*far right*) require additional or different treatment strategies to induce an immune response, such as adoptive cellular therapy or vaccination strategies. Targeted inhibitors (e.g., MEK inhibitors) should be considered for all patient groups where appropriate, but the impact of targeted inhibitors on the immune response should be a therapeutic consideration. This figure was made exclusively for this manuscript. DC dendritic cells, FACS fluorescence-activated cell sorting, H&E hematoxylin and eosin staining

any NAC regime. For example, concurrent depletion of Tregs through the use of immunotherapies such as anti-CTLA-4 mAb [89] may enhance the efficacy of NAC in inducing antitumor immune responses. There may also be clinical benefit in depleting other immunosuppressive populations such as MDSCs or tumor-associated macrophages [16]. A histopathological examination of breast cancer tissue pre-NAC and post-NAC by DeNardo et al. revealed that the presence of CD68⁺ macrophages inversely correlated with CD8⁺ TIL following NAC. Furthermore, depletion of this subset enhanced chemotherapy in preclinical models of breast cancer [90]. A recent study showed that a monocyte/DC metagene analysis held similar predictive value to NAC as the T cell/NK cell module [91]. Therefore, further work evaluating this in humans is warranted although the heterogeneity of

this population makes characterization more complex than the T-cell infiltrate.

Although NAC is capable of inducing CD8⁺ infiltrate within the tumor microenvironment, the efficacy of the antitumor immune responses is likely limited by the presence of immunosuppressive networks. For example, a recent retrospective analysis of 94 post-NAC biopsies demonstrated that PD-L1 expression correlates with TIL infiltrate and pCR [40], which is in agreement with studies from other groups evaluating PD-L1 mRNA expression [41, 92]. This is likely because PD-L1 is expressed following IFN γ stimulation and so is a surrogate marker of an immune response. It should be noted, however, that other groups have reported that PD-1 and PD-L1 expression are correlated with poor prognosis in patients with breast cancer [93–95]. Nevertheless, these

studies consistently observed PD-L1 and PD-1 expression in a subset of breast tumors; because PD-L1 expression has previously been shown to be a marker for the efficacy of anti-PD-1 in other tumor types [96], it is possible that PD-L1 expression may be a predictive marker for patients likely to have good responses to a combination of NAC and anti-PD-1. It was also recently reported that expression of B7-H4, another member of the B7 family, is a marker of good prognosis in patients with breast cancer [97]. Further work is needed to evaluate the potential to modulate B7-H4 because it has been reported to inhibit T-cell function [98, 99] and yet B7-H4 expression was reported to limit tumor growth in this study [97]. Immune checkpoint blockade with anti-PD-1 [100] and anti-CTLA-4 [101] has already been shown to enhance the efficacy of chemotherapy and radiotherapy [102] in preclinical models and phase I clinical trials [103], and so there is clear rationale to further evaluate these combinations in patients. Further work will investigate the potential of combining NAC with other immune modulators in breast cancer given that these immunosuppressive pathways appear upregulated in TIL⁺ breast cancers [41].

The success of checkpoint inhibitors is highly dependent on the extent of a pre-existing immune response against the tumor, and the ability of immune-targeted therapies to re-stimulate/activate immune subsets. Because conventional therapies such as chemotherapy and radiotherapy result in the release of tumor-associated antigens (increased antigenicity) from the apoptotic tumor cells, the increased immunogenicity provides a promising target for immunotherapies [103]. These tumor-associated antigens, released from tumor cells after chemotherapy, are taken up by antigen-presenting cells or DCs and used to stimulate downstream effector T cells capable of recognizing and lysing tumor cells [103]. Accordingly, it is evident that subsequent immunotherapy is likely to be a beneficial endeavor, provided that the initial conventional therapy used is able to effectively “prime” tumor cells to express and present foreign tumor antigens. A study conducted by Twyman-Saint Victor et al. demonstrated that radiation is able to enhance the diversity of the T-cell receptor repertoire of intratumoral T cells. This process was found to underpin the synergy of radiotherapy and anti-PD-1/anti-CTLA-4 in patients with melanoma [103]. Notably, high-dose radiotherapy and high-dose chemotherapy can ablate immune function. Therefore, careful consideration must be given to the dosage and the timing of these therapies when combined with immunotherapy.

Combining targeted inhibitors with immunotherapy

While immunotherapy is an attractive adjuvant therapy for patients presenting with high TIL pre-NAC or post-NAC, an alternative approach is required for the treatment of

patients with poorly immunogenic cancer types and low-level TILs at diagnosis. Liu et al. recently used preclinical models of melanoma to investigate the immunogenic function of the MAPK pathway inhibitors trametinib and dabrafenib in combination with immunomodulatory antibodies, including PD-L1/PD-1 and CTLA-4. They reported the potential for synergy between these therapies [104]. The combination of trametinib with anti-PD-1 increased tumor-infiltrating CD8⁺ T cells in CT26 mouse colon carcinoma tumors, as well as downregulating immunosuppressive factors, upregulating HLA molecules, and increasing immune responses in the tumors *in vivo* [104]. Dabrafenib had no suppressive action on the function of CD4⁺ and CD8⁺ T cells, whereas trametinib had mild partial or transient inhibitory effects on T-cell proliferation *in vitro* [104]. This phenomenon is supported by findings of Callahan et al. demonstrating that RAF inhibitors in BRAF wild-type tumors caused hyperactivation of ERK signaling, thereby enhancing T-cell activation and signaling [105]. While targeted blockade of checkpoint inhibitors has proven to be effective, recent evidence suggests that agonists may also be crucial in TIL stimulation. CD8⁺ T-cell responses require T-cell receptor activation in addition to co-stimulation provided through ligation of tumor necrosis factor receptor family members 4-1BB (CD137) and OX40 (CD134). Studies using the OX40 agonist in combination with either anti-4-1BB, anti-PD-L1, anti-CTLA-4, and immunization in sarcoma, melanoma, hepatoma, and breast cancer models have shown significant survival benefit by boosting the T-cell response [106–109]. One hurdle to the application of immunotherapy in breast cancer is the apparent low immunogenicity of breast cancers, perhaps owing to the low mutation rate of breast cancer when compared to cancers such as melanoma, which show a high response rate to immunotherapy [110–112]. One approach is to use targeted inhibitors designed to induce immunogenicity in tumors. For example, Kim et al. proposed the notion of epigenetic modulation, suggesting that MHC I-related genes are downregulated through epigenetic silencing of tumor cells [16]. They investigated this by combining anti-PD-1 and anti-CTLA-4 antibodies with 5-azacytidine (a DNA methyltransferase inhibitor) and entinostat (a class I histone deacetylase inhibitor). This study revealed complete eradication of tumors in murine 4 T1 (breast) and CT26 (colorectal) models [16]. Similarly, studies investigating DC vaccination in melanoma have shown that combination therapy with PI3K inhibitors has significant pro-inflammatory effects via TLR ligands that support antitumor immunity [113]. The results of these studies suggest that combining targeted inhibitors with immunotherapy may be a promising option for clinical treatment of breast cancer for patients with high-level TILs. However, alternative therapeutic options are still required for patients with low-level TILs. Because patients

with high TILs show increased response rates to NAC, one approach is to use strategies that enhance T-cell trafficking to the tumor site. Denkert et al. reported a correlation between CXCL9/CXCL13 expression and the number of TILs in patients with breast cancer [59]. Therefore, treatments that enhance the expression of these chemokines may enhance the efficacy of NAC. Alternatively, adoptive cell therapy (ACT) utilizing TILs or chimeric antigen receptor T cells has emerged as a promising regimen for the treatment of certain cancers, including melanoma [114–118], with recent reports of long-term remission in some patients [119]. In a study conducted by Koya et al., combined treatment of vemurafenib plus ACT of lymphocytes genetically modified with a T-cell receptor recognizing ovalbumin resulted in superior antitumor responses [120]. While absolute numbers of T cells infiltrating the tumor did not increase with vemurafenib treatment, they found that the combination with ACT increased the functionality of antigen-specific T lymphocytes [120]. The findings of these studies suggest that BRAF inhibitors may be effective in combination with ACT; however, evaluation of ACT in combination with other inhibitors is also necessary. The utility of this approach in breast cancer is yet to be determined. One possibility is the targeting of the HER2 antigen [121, 122]. Ishikawa et al. [123] investigated the effects of ACT in addition to anti-CTLA-4, and were able to show that the combination enhanced antitumor activity. Taken together, there is a large body of evidence demonstrating the potential utility of TIL-associated immunotherapies for breast cancer in the future.

Conclusions

There is now overwhelming data on the prognostic and predictive association of TILs. Determination of TILs is potentially universally available to all patients with breast cancer and efforts are underway to ensure that determination of TILs is standardized and reproducible [124]. If successful, the scene will be set for TILs to enter clinical practice as a biomarker that has the potential for clinical utility and prognostic implications [41].

Because TILs are associated with improved survival endpoints in a continuous fashion, they may be integrated with existing models of risk prediction that inform decisions about adjuvant treatment, such as tumor size and nodal status, and may also serve as a stratification factor in randomized clinical trials. This may be of particular use in trial designs using NAC. pCR in the primary tumor with NAC is a very strong predictor of freedom from recurrence and long-term survival [125]. Of note, even patients without a pCR still have a relatively good outcome if they have high TILs in the primary disease [64, 124]. A composite of pCR and TILs could foreseeably be used to select patients who do not need further therapy because their risk of recurrence is negligible (pCR and

high TILs) or low (pCR but low TILs), or conversely to select those at high risk of recurrence (no pCR, low TILs) who would benefit from trials of novel interventions that may include immunotherapy. Furthermore, using TILs to identify patients at very low risk of recurrence affords the opportunity for trials looking at de-escalation of therapy to avoid unnecessary long-term toxicity or focusing on novel therapeutic combinations for the poor prognostic group.

Competing interests

SL receives research funding from Roche-Genentech, Merck, and Novartis. The other authors declare that they have no competing interests.

Authors' contributions

SD, PAB, PS, PKD, and SL contributed to the design, drafting, editing, and revision of the manuscript and preparation of figures. MM, CZ, and ZL revised the manuscript. All authors read and approved the final manuscript.

Authors' information

SL is an Associate Professor and Medical Oncologist specializing in the treatment of breast cancer, and leads the Translational Breast Cancer Genomics and Therapeutics Laboratory at the Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia. In her laboratory, her research team use genomic and immune approaches to understand mechanisms of cancer growth and drug resistance in order to develop new therapeutics for breast cancer patients. PD is an Associate Professor with over 20 years' experience in tumor immunology, and leads the Cancer Immunotherapy laboratory at the Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia. He has extensive experience in mouse models of cancer as well as in testing novel therapeutics that modulate the immune response against tumor cells.

Acknowledgments

SL is supported by Cancer Council Victoria, Australia; the National Health and Medical Research Council (NHMRC) National Breast Cancer Foundation (NBCF) of Australia, and the Breast Cancer Research Foundation (BCRF), NY. PAB was supported by a National Breast Cancer Fellowship (ID# PF-14-008). PKD was supported by an NHMRC Senior Research Fellowship (1041828).

Author details

¹Division of Research and Cancer Medicine, Peter MacCallum Cancer Centre, East Melbourne, VIC, Australia. ²Sir Peter MacCallum Department of Oncology, The University of Melbourne, Parkville, VIC, Australia. ³Peter MacCallum Cancer Centre, East Melbourne, VIC, Australia.

Received: 11 May 2015 Accepted: 22 July 2015

Published online: 24 August 2015

References

- Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*. 2011;331:1565–70.
- Mittal D, Gubin MM, Schreiber RD, Smyth MJ. New insights into cancer immunoediting and its three component phases—elimination, equilibrium and escape. *Curr Opin Immunol*. 2014;27:16–25.
- Andre F, Dieci MV, Dubsky P, Sotiriou C, Curigliano G, Denkert C, et al. Molecular pathways: involvement of immune pathways in the therapeutic response and outcome in breast cancer. *Clin Cancer Res*. 2013;19:28–33.
- Caras I, Grigorescu A, Stavaru C, Radu DL, Mogos I, Szegli G, et al. Evidence for immune defects in breast and lung cancer patients. *Cancer Immunol Immunother*. 2004;53:1146–52.
- Monjazeb AM, Zamora AE, Grossenbacher SK, Mirsoian A, Sckisel GD, Murphy WJ. Immunoediting and antigen loss: overcoming the Achilles heel of immunotherapy with antigen non-specific therapies. *Front Oncol*. 2013;3:197.
- Enderling H, Hlatky L, Hahnfeldt P. Immunoediting: evidence of the multifaceted role of the immune system in self-metastatic tumor growth. *Theor Biol Med Model*. 2012;9:31.
- Vesely MD, Schreiber RD. Cancer immunoediting: antigens, mechanisms, and implications to cancer immunotherapy. *Ann N Y Acad Sci*. 2013;1284:1–5.

8. Quezada SA, Peggs KS, Simpson TR, Allison JP. Shifting the equilibrium in cancer immunoediting: from tumor tolerance to eradication. *Immunol Rev.* 2011;241:104–18.
9. Dunn GP, Bruce AT, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol.* 2002;3:991–8.
10. O'Sullivan T, Saddawi-Konefka R, Vermi W, Koebel CM, Arthur C, White JM, et al. Cancer immunoediting by the innate immune system in the absence of adaptive immunity. *J Exp Med.* 2012;209:1869–82.
11. Emens LA. Breast cancer immunobiology driving immunotherapy: vaccines and immune checkpoint blockade. *Expert Rev Anticancer Ther.* 2012;12:1597–611.
12. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer.* 2012;12:252–64.
13. Wolchok JD, Chan TA. Cancer: antitumor immunity gets a boost. *Nature.* 2014;515:496–8.
14. van Elsas A, Hurwitz AA, Allison JP. Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. *J Exp Med.* 1999;190:355–66.
15. Fan X, Quezada SA, Sepulveda MA, Sharma P, Allison JP. Engagement of the ICOS pathway markedly enhances efficacy of CTLA-4 blockade in cancer immunotherapy. *J Exp Med.* 2014;211:715–25.
16. Kim K, Skora AD, Li Z, Liu Q, Tam AJ, Blosser RL, et al. Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. *Proc Natl Acad Sci U S A.* 2014;111:11774–9.
17. Pentcheva-Hoang T, Simpson TR, Montalvo-Ortiz W, Allison JP. Cytotoxic T lymphocyte antigen-4 blockade enhances antitumor immunity by stimulating melanoma-specific T-cell motility. *Cancer Immunol Res.* 2014;2:970–80.
18. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol.* 2010;28:3167–75.
19. Wolchok JD, Neyns B, Linette G, Negrier S, Lutzky J, Thomas L, et al. Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. *Lancet Oncol.* 2010;11:155–64.
20. Postow MA, Callahan MK, Wolchok JD. Immune checkpoint blockade in cancer therapy. *J Clin Oncol.* 2015;33:1974–198.
21. Beavis PA, Divisekera U, Paget C, Chow MT, John LB, Devaud C, et al. Blockade of A2A receptors potently suppresses the metastasis of CD73+ tumors. *Proc Natl Acad Sci U S A.* 2013;110:14711–6.
22. Beavis PA, Milenkovski N, Henderson MA, John LB, Allard B, Loi S, et al. Adenosine receptor 2A blockade increases the efficacy of anti-PD-1 through enhanced antitumor t-cell responses. *Cancer Immunol Res.* 2015;3:506–17.
23. Beavis PA, Stagg J, Darcy PK, Smyth MJ. CD73: a potent suppressor of antitumor immune responses. *Trends Immunol.* 2012;33:231–7.
24. Waickman AT, Alme A, Senaldi L, Zarek PE, Horton M, Powell JD. Enhancement of tumor immunotherapy by deletion of the A2A adenosine receptor. *Cancer Immunol Immunother.* 2012;61:917–26.
25. Ohta A, Gorelik E, Prasad SJ, Ronchese F, Lukashov D, Wong MK, et al. A2A adenosine receptor protects tumors from antitumor T cells. *Proc Natl Acad Sci U S A.* 2006;103:13132–7.
26. Nishikawa H, Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Curr Opin Immunol.* 2014;27:1–7.
27. Zheng X, Koropatnick J, Chen D, Velenosi T, Ling H, Zhang X, et al. Silencing IDO in dendritic cells: a novel approach to enhance cancer immunotherapy in a murine breast cancer model. *Int J Cancer.* 2013;132:967–77.
28. Holmgaard RB, Zamarin D, Munn DH, Wolchok JD, Allison JP. Indoleamine 2,3-dioxygenase is a critical resistance mechanism in antitumor T cell immunotherapy targeting CTLA-4. *J Exp Med.* 2013;210:1389–402.
29. Holtzhausen A, Zhao F, Evans KS, Hanks BA. Early carcinogenesis involves the establishment of immune privilege via intrinsic and extrinsic regulation of indoleamine 2,3-dioxygenase-1: translational implications in cancer immunotherapy. *Front Immunol.* 2014;5:438.
30. Platten M, von Knebel DN, Oezen I, Wick W, Ochs K. Cancer immunotherapy by targeting IDO1/TDO and their downstream effectors. *Front Immunol.* 2014;5:673.
31. Wang Z, Shi X, Li Y, Fan J, Zeng X, Xian Z, et al. Blocking autophagy enhanced cytotoxicity induced by recombinant human arginase in triple-negative breast cancer cells. *Cell Death Dis.* 2014;5:e1563.
32. Bedoya AM, Tate DJ, Baena A, Cordoba CM, Borrero M, Pareja R, et al. Immunosuppression in cervical cancer with special reference to arginase activity. *Gynecol Oncol.* 2014;135:74–80.
33. Vasquez-Dunddel D, Pan F, Zeng Q, Gorbounov M, Albesiano E, Fu J, et al. STAT3 regulates arginase-1 in myeloid-derived suppressor cells from cancer patients. *J Clin Invest.* 2013;123:1580–9.
34. Ghansah T. A novel strategy for modulation of MDSC to enhance cancer immunotherapy. *Oncoimmunology.* 2012;1:984–5.
35. Forghani P, Khorramizadeh MR, Waller EK. Silibinin inhibits accumulation of myeloid-derived suppressor cells and tumor growth of murine breast cancer. *Cancer Med.* 2014;3:215–24.
36. Markowitz J, Wesolowski R, Papenfuss T, Brooks TR, Carson 3rd WE. Myeloid-derived suppressor cells in breast cancer. *Breast Cancer Res Treat.* 2013;140:13–21.
37. Ono M, Tsuda H, Shimizu C, Yamamoto S, Shibata T, Yamamoto H, et al. Tumor-infiltrating lymphocytes are correlated with response to neoadjuvant chemotherapy in triple-negative breast cancer. *Breast Cancer Res Treat.* 2012;132:793–805.
38. West NR, Milne K, Truong PT, Macpherson N, Nelson BH, Watson PH. Tumor-infiltrating lymphocytes predict response to anthracycline-based chemotherapy in estrogen receptor-negative breast cancer. *Breast Cancer Res.* 2011;13:R126.
39. Loi S, Sirtaine N, Piette F, Salgado R, Viale G, Van Eenoo F, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02–98. *J Clin Oncol.* 2013;31:860–7.
40. Wimberly H, Brown JR, Schalper KA, Haack H, Silver MR, Nixon C, et al. PD-L1 expression correlates with tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy in breast cancer. *Cancer Immunol Res.* 2014;3:326–32.
41. Denkert C, von Minckwitz G, Brase JC, Sinn BV, Gade S, Kronenwett R, et al. Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. *J Clin Oncol.* 2015;33:983–91.
42. Ghiringhelli F, Larmonier N, Schmitt E, Parcellier A, Cathelin D, Garrido C, et al. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol.* 2004;34:336–44.
43. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. *Nat Rev Immunol.* 2008;8:59–73.
44. Ghiringhelli F, Apetoh L. The interplay between the immune system and chemotherapy: emerging methods for optimizing therapy. *Expert Rev Clin Immunol.* 2014;10:19–30.
45. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, et al. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat Med.* 2009;15:1170–8.
46. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med.* 2007;13:1050–9.
47. Nowak AK, Robinson BW, Lake RA. Gemcitabine exerts a selective effect on the humoral immune response: implications for combination chemo-immunotherapy. *Cancer Res.* 2002;62:2353–8.
48. Tongu M, Harashima N, Monma H, Inao T, Yamada T, Kawauchi H, et al. Metronomic chemotherapy with low-dose cyclophosphamide plus gemcitabine can induce anti-tumor T cell immunity in vivo. *Cancer Immunol Immunother.* 2013;62:383–91.
49. Homma Y, Taniguchi K, Nakazawa M, Matsuyama R, Mori R, Takeda K, et al. Changes in the immune cell population and cell proliferation in peripheral blood after gemcitabine-based chemotherapy for pancreatic cancer. *Clin Transl Oncol.* 2014;16:330–5.
50. Mozaffari F, Lindemalm C, Choudhury A, Granstam-Bjorneklett H, Lekander M, Nilsson B, et al. Systemic immune effects of adjuvant chemotherapy with 5-fluorouracil, epirubicin and cyclophosphamide and/or radiotherapy in breast cancer: a longitudinal study. *Cancer Immunol Immunother.* 2009;58:111–20.
51. Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood.* 2005;105:2862–8.
52. Zhu Y, Liu N, Xiong SD, Zheng YJ, Chu YW. CD4+Foxp3+ regulatory T-cell impairment by paclitaxel is independent of toll-like receptor 4. *Scand J Immunol.* 2011;73:301–8.

53. Machiels JP, Reilly RT, Emens LA, Ercolini AM, Lei RY, Weintraub D, et al. Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. *Cancer Res.* 2001;61:3689–97.
54. Park JY, Jang MJ, Chung YH, Kim KY, Kim SS, Lee WB, et al. Doxorubicin enhances CD4(+) T-cell immune responses by inducing expression of CD40 ligand and 4-1BB. *Int Immunopharmacol.* 2009;9:1530–9.
55. Hannesdottir L, Tymoszek P, Parajuli N, Wasmer MH, Philipp S, Daschil N, et al. Lapatinib and doxorubicin enhance the Stat1-dependent antitumor immune response. *Eur J Immunol.* 2013;43:2718–29.
56. Nowak AK, Lake RA, Marzo AL, Scott B, Heath WR, Collins EJ, et al. Induction of tumor cell apoptosis *in vivo* increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. *J Immunol.* 2003;170:4905–13.
57. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. *Annu Rev Immunol.* 2013;31:51–72.
58. Menard S, Tomasic G, Casalini P, Balsari A, Pionetti S, Cascinelli N, et al. Lymphoid infiltration as a prognostic variable for early-onset breast carcinomas. *Clin Cancer Res.* 1997;3:817–9.
59. Denkert C, Loibl S, Noske A, Roller M, Muller BM, Komor M, et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. *J Clin Oncol.* 2010;28:105–13.
60. Ladoire S, Arnould L, Apetoh L, Coudert B, Martin F, Chauffert B, et al. Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. *Clin Cancer Res.* 2008;14:2413–20.
61. Ignatiadis M, Singhal SK, Desmedt C, Haibe-Kains B, Criscitiello C, Andre F, et al. Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: a pooled analysis. *J Clin Oncol.* 2012;30:1996–2004.
62. Yu H, Yang J, Jiao S, Li Y, Zhang W, Wang J. T-box transcription factor 21 expression in breast cancer and its relationship with prognosis. *Int J Clin Exp Pathol.* 2014;7:6906–13.
63. Issa-Nummer Y, Darb-Esfahani S, Loibl S, Kunz G, Nekjudova V, Schrader I, et al. Prospective validation of immunological infiltrate for prediction of response to neoadjuvant chemotherapy in HER2-negative breast cancer—a substudy of the neoadjuvant GearQuinto trial. *PLoS One.* 2013;8:e79775.
64. Dieci MV, Criscitiello C, Goubar A, Viale G, Conte P, Guarneri V, et al. Prognostic value of tumor-infiltrating lymphocytes on residual disease after primary chemotherapy for triple-negative breast cancer: a retrospective multicenter study. *Ann Oncol.* 2014;25:611–8.
65. Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, et al. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. *Ann Oncol.* 2014;25:1544–50.
66. Adams S, Gray RJ, Demaria S, Goldstein L, Perez EA, Shulman LN, et al. Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. *J Clin Oncol.* 2014;32:2959–66.
67. Gennari R, Menard S, Fagnoni F, Ponchio L, Scelsi M, Tagliabue E, et al. Pilot study of the mechanism of action of preoperative trastuzumab in patients with primary operable breast tumors overexpressing HER2. *Clin Cancer Res.* 2004;10:5650–5.
68. Salgado R, Denkert C, Campbell C, Savas P, Nucifero P, Aura C, et al. Tumor-infiltrating lymphocytes and associations with pathological complete response and event-free survival in HER2-positive early stage breast cancer treated with lapatinib and trastuzumab. *JAMA Oncol.* 2015;1:448–54.
69. Arnould L, Gelly M, Penault-Llorca F, Benoit L, Bonnetain F, Migeon C, et al. Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? *Br J Cancer.* 2006;94:259–67.
70. Bianchini G, Gianni L. The immune system and response to HER2-targeted treatment in breast cancer. *Lancet Oncol.* 2014;15:e58–68.
71. Oda N, Shimazu K, Naoi Y, Morimoto K, Shimomura A, Shimoda M, et al. Intratumoral regulatory T cells as an independent predictive factor for pathological complete response to neoadjuvant paclitaxel followed by 5-FU/epirubicin/cyclophosphamide in breast cancer patients. *Breast Cancer Res Treat.* 2012;136:107–16.
72. Seo AN, Lee HJ, Kim EJ, Kim HJ, Jang MH, Lee HE, et al. Tumour-infiltrating CD8+ lymphocytes as an independent predictive factor for pathological complete response to primary systemic therapy in breast cancer. *Br J Cancer.* 2013;109:2705–13.
73. Lee HJ, Seo JY, Ahn JH, Ahn SH, Gong G. Tumor-associated lymphocytes predict response to neoadjuvant chemotherapy in breast cancer patients. *J Breast Cancer.* 2013;16:32–9.
74. Brown JR, Wimberly H, Lannin DR, Nixon C, Rimm DL, Bossuyt V. Multiplexed quantitative analysis of CD3, CD8, and CD20 predicts response to neoadjuvant chemotherapy in breast cancer. *Clin Cancer Res.* 2014;20:5995–6005.
75. Mahmoud S, Paish E, Powe D, Macmillan R, Grainge M, Lee A, et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J Clin Oncol.* 2011;29:1949–55.
76. West NR, Kost SE, Martin SD, Milne K, Deleeuw RJ, Nelson BH, et al. Tumour-infiltrating FOXP3(+) lymphocytes are associated with cytotoxic immune responses and good clinical outcome in oestrogen receptor-negative breast cancer. *Br J Cancer.* 2013;108:155–62.
77. Aruga T, Suzuki E, Saji S, Horiguchi S, Horiguchi K, Sekine S, et al. A low number of tumor-infiltrating FOXP3-positive cells during primary systemic chemotherapy correlates with favorable anti-tumor response in patients with breast cancer. *Oncol Rep.* 2009;22:273–8.
78. Ladoire S, Mignot G, Dabakuyo S, Arnould L, Apetoh L, Rebe C, et al. *In situ* immune response after neoadjuvant chemotherapy for breast cancer predicts survival. *J Pathol.* 2011;224:389–400.
79. Liu F, Li Y, Ren M, Zhang X, Guo X, Lang R, et al. Peritumoral FOXP3(+) regulatory T cell is sensitive to chemotherapy while intratumoral FOXP3(+) regulatory T cell is prognostic predictor of breast cancer patients. *Breast Cancer Res Treat.* 2012;135:459–67.
80. Ruffell B, Au A, Rugo HS, Esserman LJ, Hwang ES, Coussens LM. Leukocyte composition of human breast cancer. *Proc Natl Acad Sci U S A.* 2012;109:2796–801.
81. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, et al. CD4(+) follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest.* 2013;123:2873–92.
82. Ma C, Zhang Q, Ye J, Wang F, Zhang Y, Wevers E, et al. Tumor-infiltrating gammadelta T lymphocytes predict clinical outcome in human breast cancer. *J Immunol.* 2012;189:5029–36.
83. Mattarollo SR, Kenna T, Nieda M, Nicol AJ. Chemotherapy and zoledronate sensitize solid tumour cells to Vgamma9delta2 T cell cytotoxicity. *Cancer Immunol Immunother.* 2007;56:1285–97.
84. Kobayashi H, Tanaka Y, Yagi J, Osaka Y, Nakazawa H, Uchiyama T, et al. Safety profile and anti-tumor effects of adoptive immunotherapy using gamma-delta T cells against advanced renal cell carcinoma: a pilot study. *Cancer Immunol Immunother.* 2007;56:469–76.
85. Yang L, Qi Y, Hu J, Tang L, Zhao S, Shan B. Expression of Th17 cells in breast cancer tissue and its association with clinical parameters. *Cell Biochem Biophys.* 2012;62:153–9.
86. Verma C, Eremin JM, Robins A, Bennett AJ, Cowley GP, El-Sheemy MA, et al. Abnormal T regulatory cells (Tregs: FOXP3+, CTLA-4+), myeloid-derived suppressor cells (MDSCs: monocytic, granulocytic) and polarised T helper cell profiles (Th1, Th2, Th17) in women with large and locally advanced breast cancers undergoing neoadjuvant chemotherapy (NAC) and surgery: failure of abolition of abnormal treg profile with treatment and correlation of treg levels with pathological response to NAC. *J Transl Med.* 2013;11:16.
87. Demaria S, Volm MD, Shapiro RL, Yee HT, Oratz R, Formenti SC, et al. Development of tumor-infiltrating lymphocytes in breast cancer after neoadjuvant paclitaxel chemotherapy. *Clin Cancer Res.* 2001;7:3025–30.
88. Ercolini AM, Ladle BH, Manning EA, Pfannenstiel LW, Armstrong TD, Machiels JP, et al. Recruitment of latent pools of high-avidity CD8(+) T cells to the antitumor immune response. *J Exp Med.* 2005;201:1591–602.
89. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J Exp Med.* 2013;210:1695–710.
90. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov.* 2011;1:54–67.
91. Alistar A, Chou J, Nagalla S, Black M, D'Agostino R, Miller L. Dual roles for immune metagenes in breast cancer prognosis and therapy prediction. *Genome Med.* 2014;6:80.
92. Schalper KA, Velcheti V, Carvajal D, Wimberly H, Brown J, Pusztai L, et al. *In situ* tumor PD-L1 mRNA expression is associated with increased TILs and better outcome in breast carcinomas. *Clin Cancer Res.* 2014;20:2773–82.
93. Muenst S, Schaeferli AR, Gao F, Daster S, Trella E, Droeser RA, et al. Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer. *Breast Cancer Res Treat.* 2014;146:15–24.

94. Muenst S, Soysal SD, Gao F, Obermann EC, Oertli D, Gillanders WE. The presence of programmed death 1 (PD-1)-positive tumor-infiltrating lymphocytes is associated with poor prognosis in human breast cancer. *Breast Cancer Res Treat*. 2013;139:667–76.
95. Sun S, Fei X, Mao Y, Wang X, Garfield DH, Huang O, et al. PD-1(+)-immune cell infiltration inversely correlates with survival of operable breast cancer patients. *Cancer Immunol Immunother*. 2014;63:395–406.
96. Taube JM, Klein AP, Brahmer JR, Xu H, Pan X, Kim JH, et al. Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. *Clin Cancer Res*. 2014;20:5064.
97. Rahbar R, Lin A, Ghazarian M, Yau HL, Paramathas S, Lang PA, et al. B7-h4 expression by nonhematopoietic cells in the tumor microenvironment promotes antitumor immunity. *Cancer Immunol Res*. 2015;3:184–95.
98. Sica GL, Choi IH, Zhu G, Tamada K, Wang SD, Tamura H, et al. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. *Immunity*. 2003;18:849–61.
99. Zang X, Loke P, Kim J, Murphy K, Waitz R, Allison JP. B7x: a widely expressed B7 family member that inhibits T cell activation. *Proc Natl Acad Sci U S A*. 2003;100:10388–92.
100. Lu L, Xu X, Zhang B, Zhang R, Ji H, Wang X. Combined PD-1 blockade and GITR triggering induce a potent antitumor immunity in murine cancer models and synergizes with chemotherapeutic drugs. *J Transl Med*. 2014;12:36.
101. Lesterhuis WJ, Salmons J, Nowak AK, Rozali EN, Khong A, Dick IM, et al. Synergistic effect of CTLA-4 blockade and cancer chemotherapy in the induction of anti-tumor immunity. *PLoS One*. 2013;8:e61895.
102. Verbrugge I, Hagekyriakou J, Sharp LL, Galli M, West A, McLaughlin NM, et al. Radiotherapy increases the permissiveness of established mammary tumors to rejection by immunomodulatory antibodies. *Cancer Res*. 2012;72:3163–74.
103. Twyman-Saint Victor C, Rech AJ, Maity A, Rengan R, Pauken KE, Stelekati E, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. *Nature*. 2015;520:373–7.
104. Liu L, Mayes PA, Eastman S, Shi H, Yadavilli S, Zhang T, et al. The BRAF and MEK inhibitors dabrafenib and trametinib: effects on immune function and in combination with immunomodulatory antibodies targeting PD-1, PD-L1, and CTLA-4. *Clin Cancer Res*. 2015;21:1639.
105. Callahan MK, Masters G, Pratilas CA, Ariyan C, Katz J, Kitano S, et al. Paradoxical activation of T cells via augmented ERK signaling mediated by a RAF inhibitor. *Cancer Immunol Res*. 2014;2:70–9.
106. Lee SJ, Myers L, Muralimohan G, Dai J, Qiao Y, Li Z, et al. 4-1BB and OX40 dual costimulation synergistically stimulate primary specific CD8 T cells for robust effector function. *J Immunol*. 2004;173:3002–12.
107. Gray JC, French RR, James S, Al-Shamkhani A, Johnson PW, Glennie MJ. Optimising anti-tumour CD8 T-cell responses using combinations of immunomodulatory antibodies. *Eur J Immunol*. 2008;38:2499–511.
108. Morales-Kastresana A, Sanmamed MF, Rodriguez I, Palazon A, Martinez-Forero I, Labiano S, et al. Combined immunostimulatory monoclonal antibodies extend survival in an aggressive transgenic hepatocellular carcinoma mouse model. *Clin Cancer Res*. 2013;19:6151–62.
109. Cuadros C, Dominguez AL, Lollini PL, Croft M, Mittler RS, Borgstrom P, et al. Vaccination with dendritic cells pulsed with apoptotic tumors in combination with anti-OX40 and anti-4-1BB monoclonal antibodies induces T cell-mediated protective immunity in Her-2/neu transgenic mice. *Int J Cancer*. 2005;116:934–43.
110. Greenman C, Stephens P, Smith R, Dalgleish GL, Hunter C, Bignell G, et al. Patterns of somatic mutation in human cancer genomes. *Nature*. 2007;446:153–8.
111. Krauthammer M, Kong Y, Ha BH, Evans P, Bacchicocchi A, McCusker JP, et al. Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. *Nat Genet*. 2012;44:1006–14.
112. Wood LD, Parsons DW, Jones S, Lin J, Sjoblom T, Leary RJ, et al. The genomic landscapes of human breast and colorectal cancers. *Science*. 2007;318:1108–13.
113. Marshall NA, Galvin KC, Corcoran AM, Boon L, Higgs R, Mills KH. Immunotherapy with PI3K inhibitor and Toll-like receptor agonist induces IFN-gamma+IL-17+ polyfunctional T cells that mediate rejection of murine tumors. *Cancer Res*. 2012;72:581–91.
114. Dudley ME, Gross CA, Somerville RP, Hong Y, Schaub NP, Rosati SF, et al. Randomized selection design trial evaluating CD8+ enriched versus unselected tumor-infiltrating lymphocytes for adoptive cell therapy for patients with melanoma. *J Clin Oncol*. 2013;31:2152–9.
115. Chacon JA, Wu RC, Sukhumalchandra P, Mollndrem JJ, Sarnaik A, Pilon-Thomas S, et al. Co-stimulation through 4-1BB/CD137 improves the expansion and function of CD8(+) melanoma tumor-infiltrating lymphocytes for adoptive T-cell therapy. *PLoS One*. 2013;8:e60031.
116. Kodumudi KN, Weber A, Sarnaik AA, Pilon-Thomas S. Blockade of myeloid-derived suppressor cells after induction of lymphopenia improves adoptive T cell therapy in a murine model of melanoma. *J Immunol*. 2012;189:5147–54.
117. Radvanyi LG, Bernatchez C, Zhang M, Fox PS, Miller P, Chacon J, et al. Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. *Clin Cancer Res*. 2012;18:6758–70.
118. Kershaw MH, Westwood JA, Darcy PK. Gene-engineered T cells for cancer therapy. *Nat Rev Cancer*. 2013;13:525–41.
119. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med*. 2014;371:1507–17.
120. Koya RC, Mok S, Otte N, Blacketer KJ, Comin-Anduix B, Tumei PC, et al. BRAF inhibitor vemurafenib improves the antitumor activity of adoptive cell immunotherapy. *Cancer Res*. 2012;72:3928–37.
121. John LB, Devaud C, Duong CP, Yong CS, Beavis PA, Haynes NM, et al. Anti-PD-1 antibody therapy potentially enhances the eradication of established tumors by gene-modified T cells. *Clin Cancer Res*. 2013;19:5636–46.
122. Ahmed N, Brawley VS, Hegde M, Robertson C, Ghazi A, Gerken C, et al. Human epidermal growth factor receptor 2 (HER2)-specific chimeric antigen receptor-modified T cells for the immunotherapy of HER2-positive sarcoma. *J Clin Oncol*. 2015;33:1688.
123. Ishikawa T, Adachi S, Okayama T, Kokura S, Mizushima K, Doi T, et al. Cytotoxic T lymphocyte-associated antigen 4 inhibition increases the antitumor activity of adoptive T-cell therapy when carried out with naive rather than differentiated T cells. *Oncol Rep*. 2015;33:2545.
124. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruner G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol*. 2015;26:259–71.
125. Cortazar P, Zhang L, Untch M, Mehta K, Costantino JP, Wolmark N, et al. Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. *Lancet*. 2014;384:164–72.
126. Ko HJ, Kim YJ, Kim YS, Chang WS, Ko SY, Chang SY, et al. A combination of chemoimmunotherapies can efficiently break self-tolerance and induce antitumor immunity in a tolerogenic murine tumor model. *Cancer Res*. 2007;67:7477–86.
127. Fridlender ZG, Sun J, Singhal S, Kapoor V, Cheng G, Suzuki E, et al. Chemotherapy delivered after viral immunogene therapy augments antitumor efficacy via multiple immune-mediated mechanisms. *Mol Ther*. 2010;18:1947–59.
128. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin Cancer Res*. 2005;11:6713–21.
129. Schiavoni G, Sistigu A, Valentini M, Mattei F, Sestili P, Spadaro F, et al. Cyclophosphamide synergizes with type I interferons through systemic dendritic cell reactivation and induction of immunogenic tumor apoptosis. *Cancer Res*. 2011;71:768–78.
130. Xu W, Cai J, Li S, Zhang H, Han J, Wen M, et al. Improving the in vivo persistence, distribution and function of cytotoxic T lymphocytes by inhibiting the tumor immunosuppressive microenvironment. *Scand J Immunol*. 2013;78:50–60.
131. Umansky V, Sevko A. Overcoming immunosuppression in the melanoma microenvironment induced by chronic inflammation. *Cancer Immunol Immunother*. 2012;61:275–82.
132. Zhang L, Dermawan K, Jin M, Liu R, Zheng H, Xu L, et al. Differential impairment of regulatory T cells rather than effector T cells by paclitaxel-based chemotherapy. *Clin Immunol*. 2008;129:219–29.
133. Ma Y, Adjemian S, Mattarollo SR, Yamazaki T, Aymeric L, Yang H, et al. Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. *Immunity*. 2013;38:729–41.
134. Alizadeh D, Trad M, Hanke NT, Larmonier CB, Janikashvili N, Bonnotte B, et al. Doxorubicin eliminates myeloid-derived suppressor cells and enhances the efficacy of adoptive T-cell transfer in breast cancer. *Cancer Res*. 2014;74:104–18.

135. Tesniere A, Schlemmer F, Boige V, Kepp O, Martins I, Ghiringhelli F, et al. Immunogenic death of colon cancer cells treated with oxaliplatin. *Oncogene*. 2010;29:482–91.
136. Kang TH, Mao CP, Lee SY, Chen A, Lee JH, Kim TW, et al. Chemotherapy acts as an adjuvant to convert the tumor microenvironment into a highly permissive state for vaccination-induced antitumor immunity. *Cancer Res*. 2013;73:2493–504.
137. Guo ZS, Hong JA, Irvine KR, Chen GA, Spiess PJ, Liu Y, et al. De novo induction of a cancer/testis antigen by 5-aza-2'-deoxycytidine augments adoptive immunotherapy in a murine tumor model. *Cancer Res*. 2006;66:1105–13.
138. Mikyskova R, Indrova M, Vlkova V, Bieblova J, Simova J, Parackova Z, et al. DNA demethylating agent 5-azacytidine inhibits myeloid-derived suppressor cells induced by tumor growth and cyclophosphamide treatment. *J Leukoc Biol*. 2014; in press.
139. Hornychova H, Melichar B, Tomsova M, Mergancova J, Urmínska H, Ryska A. Tumor-infiltrating lymphocytes predict response to neoadjuvant chemotherapy in patients with breast carcinoma. *Cancer Invest*. 2008;26:1024–31.
140. Liu S, Lachapelle J, Leung S, Gao D, Foulkes WD, Nielsen TO. CD8+ lymphocyte infiltration is an independent favorable prognostic indicator in basal-like breast cancer. *Breast Cancer Res*. 2012;14:R48.
141. Yamaguchi R, Tanaka M, Yano A, Tse GM, Yamaguchi M, Koura K, et al. Tumor-infiltrating lymphocytes are important pathologic predictors for neoadjuvant chemotherapy in patients with breast cancer. *Hum Pathol*. 2012;43:1688–94.
142. Ali HR, Provenzano E, Dawson SJ, Blows FM, Liu B, Shah M, et al. Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients. *Ann Oncol*. 2014;25:1536–43.
143. Nabholz JM, Abrial C, Mouret-Reynier MA, Dauplat MM, Weber B, Gligorov J, et al. Multicentric neoadjuvant phase II study of panitumumab combined with an anthracycline/taxane-based chemotherapy in operable triple-negative breast cancer: identification of biologically defined signatures predicting treatment impact. *Ann Oncol*. 2014;25:1570–7.

**Submit your next manuscript to BioMed Central
and take full advantage of:**

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

