Immunotherapeutic approaches in triple-negative breast cancer: latest research and clinical prospects

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Abstract: Triple-negative breast cancer (TNBC), as defined by the absence of estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 expression, is a challenging disease with the poorest prognosis of all breast cancer subtypes. Importantly, there are currently no known molecular targets for this subgroup of patients. Recent advances in genomics and gene expression profiling have shed new light on the molecule heterogeneity of TNBC. We present an overview of the scientific evidence suggesting that clinical outcome in TNBC is affected by tumor-infiltrating immune cells. We also describe tumor-associated antigens recently identified in TNBC. Finally, we review the current literature on promising immunotherapies for TNBC, including tumor vaccine approaches, immune-checkpoint inhibitors, antagonists of immunosuppressive molecules and adoptive cell therapies. It is our contention that selected patients with TNBC with lymphocytic tumor infiltrates at diagnosis may benefit from immune-based therapies and that these immunotherapies will be most beneficial in combination with cytotoxic drugs that potentiate adaptive anti-tumor immunity.

Keywords: basal like, breast cancer, immunotherapy, T cell

Defining triple-negative breast cancer: the need for international standards

Breast cancer is a heterogeneous disease composed of different subtypes. Clinically, the classification of breast cancer depends on the expression of three biomarkers: estrogen receptors (ERs), progesterone receptors (PRs) and human epidermal growth factor receptor 2 (HER2). Breast cancers that are negative for ER, PR and HER2 are considered as triple-negative breast cancer (TNBC) and represent 15–20% of newly diagnosed cases. While HER2 assessment follows standardized guidelines [Wolff *et al.* 2006], hormone receptor assessment can vary across countries. We believe this lack of uniformity in hormone receptor assessment is a major obstacle to the worldwide research effort on TNBC. Reproducible assessment of triple-negative status would enable better definition of patients with TNBC and greatly improve the quality of research. According to the American Society of Clinical Oncology and the College of American Pathologists, ER and PR status should be considered positive when at least 1% of tumor cells are positive by

immunohistochemistry (IHC) [Hammond *et al.* 2010]. Compliance with these recommendations and guidelines should be the gold standard for identifying patients with TNBC.

Clinical characteristics of TNBC

Compared with other forms of breast cancer, TNBC is associated with a younger age at diagnosis [Bauer *et al.* 2007; Dent *et al.* 2007; Anders *et al.* 2011], African American ethnicity [Bauer *et al.* 2007; Lund *et al.* 2009], advanced stage at diagnosis, increased risk of visceral metastasis and poorer outcome [Perou *et al.* 2000; Nielsen *et al.* 2004; Carey, 2006; Liedtke *et al.* 2008; Dent *et al.* 2009; Canello *et al.* 2010; Metzger *et al.* 2010]. Because TNBC lacks a therapeutic target, patients do not benefit from endocrine therapy or HER2-targeted therapy. Accordingly, TNBC remains an important challenge in today's clinical practice.

Cytotoxic chemotherapy is the only systemic treatment option for patients with TNBC. Although as

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a group TNBC is associated with a poor prognosis, some patients respond well to anthracycline-based chemotherapy, suggesting a significant degree of molecular heterogeneity. Unfortunately, the molecular mechanisms underlying this heterogeneity and its relationship to treatment response are still poorly understood.

Heterogeneity of TNBC

Significant research effort has recently been deployed to better understand the molecular heterogeneity of TNBC with the objective of identifying potential therapeutic targets. One of the first molecular insights into TNBC was the observation that it often presents a basal-like profile. However, not all TNBCs are basal-like [Perou *et al.* 2000]. It has been suggested that approximately 70% of TNBCs present a basal-like gene expression profile [Rakha and Ellis, 2009]. At the histopathological level, TNBC (defined by IHC) and basal-like breast cancers (defined by gene expression) share several characteristics, such as high histologic grade, pushing borders of invasion and stromal lymphocytic infiltrates [Fulford *et al.* 2006; Livasy *et al.* 2007]. Also, both TNBC and basal-like breast cancers show links with *BRCA1*-mutated breast cancers and the *BRCA1* pathway in general. Accordingly, up to 90% of *BRCA1*-associated tumors are TNBC by IHC [Foulkes *et al.* 2003; Arnes *et al.* 2005] and mice deficient in *Brcal* and *p53* in mammary epithelial cells develop tumors that are both triple negative and basal like [Molyneux *et al.* 2010].

Although the majority of TNBCs present a basal-like gene expression profile, some cases lack expression of basal markers, such as epidermal growth factor receptor (EGFR) and cytokeratin (CK) 5/6. Notably, these non-basal TNBCs are associated with better outcomes than core basal-like breast cancers [Cheang *et al.* 2008]. Various studies have proposed the use of surrogate markers detected by IHC to identify core basal-like breast cancers. These include basal CKs (i.e. CK5/6, CK14, and CK17), EGFR and C-kit [Nielsen *et al.* 2004]. Expression of these surrogate markers in TNBC is associated with shorter survival compared with the remaining patients with TNBC [Cheang *et al.* 2008]. However, before using these surrogates as prognostic tools, standardized cutoff levels must be defined.

Gene expression subtypes in TNBC

Recent advances in genomics offer great potential to better understand the heterogeneity of TNBC. Using gene expression profiling from over 3000 cases, Lehmann and colleagues recently described six different TNBC subtypes [Lehmann *et al.* 2011]. These TNBC molecular subtypes included two basal-like, two mesenchymal, a luminal androgen-receptor positive and an immunomodulatory subtype. The two basal-like subtypes were characterized by high levels of genes involved in cell proliferation and DNA damage response. The two mesenchymal subtypes shared common features with previously identified claudin-low breast cancers [Prat *et al.* 2010]. These mesenchymal subtypes showed increased expression of genes involved in cell motility (e.g. Rho pathway), cellular differentiation, and growth pathways [e.g. ALK, transforming growth factor β (TGF β), EGFR, platelet-derived growth factor, G-protein coupled receptor, ERK1/2 signaling and Wnt/ β -catenin]. The androgen-receptor-positive TNBC subtype was associated with genes involved in steroid synthesis, porphyrin metabolism, and androgen/estrogen metabolism. Notably, this subtype is reminiscent of previously described apocrine breast cancers [Farmer *et al.* 2005]. Finally, the immunomodulatory subtype was shown to be characterized by elevated expression of genes involved in T-cell function, immune transcription, interferon (IFN) response and antigen processing. Notably, the immunomodulatory subtype showed overlap with medullary breast cancers, a rare form of TNBC with prominent lymphocytic reaction that is associated with a favorable prognosis [Bertucci *et al.* 2006].

Immune infiltrates and prognosis in breast cancer

The identification of a TNBC subtype characterized by elevated expression of immune genes suggests that some patients may benefit from immune-based therapies. Over recent years, overwhelming data have revealed the importance of tumor-infiltrating lymphocytes (TILs) in controlling the clinical progression of various epithelial cancers [Fridman *et al.* 2012]. In colorectal cancers, for instance, immune-scoring determined by IHC of intratumoral CD8+ T cells has shown superior prognostic power than standard tumor staging (AJCC/UICC-TNM classification) methods [Mlecnik *et al.* 2011]. In breast cancer, two large series, both in newly diagnosed or early

stage breast cancer, support a correlation between immune gene signature and better clinical outcomes [Denkert *et al.* 2010; Mahmoud *et al.* 2011]. Unsupervised gene expression profiling of breast cancer-associated stroma has also revealed a gene signature predictive of good prognosis (>98% 5-year survival) that was enriched for cytotoxic CD8+ T cell genes and natural killer cell activity [Finak *et al.* 2008].

While CD8+ T-cell infiltrates are generally associated with better prognosis, CD4+ T cells, which include T-regulatory cells, and tumor-associated macrophages (TAMs) have been associated with worse outcomes. A recent study assessed whether intratumoral T cells and macrophages correlated with clinical outcome in patients with breast cancer. IHC analysis of tissue microarrays derived from 179 treatment-naïve breast tumors revealed that high levels of macrophages and CD4+ T cells correlated with reduced overall survival (OS), while high levels of CD8+ T cells combined with low levels of macrophages and CD4+ T cells correlated with increased OS [DeNardo *et al.* 2011]. Thus, CD8+ T cells can control human breast cancer, but the presence of immunosuppressive cells, that is, CD4+ T-regulatory cells and macrophages, abrogates this action.

Intratumoral B cells have also been associated with favorable prognosis in breast cancer. In 200 consecutive lymph node negative cases, a B-cell metagene primarily formed by immunoglobulin heavy- and light-chain genes was associated with metastasis-free survival in highly proliferating breast tumors [Schmidt *et al.* 2008]. Interestingly, immunoglobulin κ C as a single marker has recently been shown to have similar predictive and prognostic value in breast cancer as the entire B-cell metagene [Schmidt *et al.* 2012].

Immune signature in TNBC

One of the most important conclusions of recent meta-analyses in breast cancer is that the correlation between lymphocytic infiltrates and clinical outcomes varies across subtypes. Historically, traditional assessments of lymphocytic infiltrates (by haematoxylin and eosin stain) have highlighted some uncertainty. However, recent advances in gene expression profiling now reveals that several immune signatures are associated with favorable outcomes. DeNardo and colleagues performed a meta-analysis of CD68 and CD8 gene expression

in 4000 breast cancer cases, and reported that a CD68^{high}/CD8^{low} immune gene signature correlated with reduced OS for basal or HER2+ breast cancer subtypes, but not for luminal breast cancers [DeNardo *et al.* 2011]. Similarly, a metagene of STAT1 signaling, a surrogate of IFN response, was associated with better outcomes in TNBC and HER2+ breast cancers, but not in luminal cases [Desmedt *et al.* 2008; Ignatiadis *et al.* 2012]. Another independent group identified an immune response prognostic gene module in ER- but not ER+ breast cancers [Teschendorff *et al.* 2007]. Consistent with these findings, a recent study investigated the relationship between TIL at diagnosis and clinical outcome after anthracycline-based chemotherapy and reported significant prognostic association only for ER-/HER2+ and TNBC, but not for luminal breast cancers. Patients with TNBC with at least 50% TILs (10.5% of patients) had a 5-year disease-free survival of 89% compared with 62% ($p = 0.018$, hazard ratio 0.29, 95% confidence interval 0.11–0.81).

Amongst ER- breast cancers, accumulating evidence suggests that basal TNBC may be the most regulated by intratumoral T cells, and thus the most responsive to immunotherapies. Liu and colleagues recently performed a large-scale IHC analysis of CD8 staining on 3400 breast cancer cases representing different subtypes [Liu *et al.* 2012]. Multivariate analysis indicated that amongst all breast cancer subtypes, only core basal TNBC demonstrated a significant correlation between intratumoral CD8 staining and favorable prognosis. The B-cell metagene has also been associated with good outcome in TNBC. A gene expression analysis of 579 cases of TNBC revealed that a ratio of high B-cell and low interleukin (IL)-8 metagenes identified 32% of patients with TNBC with good prognosis [Rody *et al.* 2011]. Taken together, these studies suggest that clinical outcomes in ER- breast cancers, especially TNBC, are particularly influenced by tumor immune responses.

The intrinsic nature of TNBC might help explain its increased propensity to inflammatory responses. For instance, signaling pathways that downregulate hormone receptor and HER2 expression may in return stimulate proinflammatory activity. A recent study identified lactoferrin as a repressor of ER, PR, and HER2 expression in breast cancer cells and a direct stimulator of endothelin 1 [Ha *et al.* 2011]. Alarmins like lactoferrin are proinflammatory molecules that play important intracellular roles and are released

upon tissue damage. When released, lactoferrin induces the production cytokines and chemokines, such as IL-10, IL-4, macrophage inflammatory protein (MIP)-1 α and MIP-2 [Yang *et al.* 2009]. Lactoferrin may thus contribute to a triple-negative phenotype in breast cancer and could represent a novel target to stimulate antitumor immune responses.

Immune responses improves chemotherapy of TNBC

Accumulating data now suggest that certain chemotherapeutic drugs, such as anthracyclines, mediate their anticancer activity not only by direct cytotoxic effects but also through activation of CD8+ T-cell responses. Thus, the correlation between intratumoral immune responses and clinical outcomes in TNBC is potentially related to the role of immune cells in the activity of cytotoxic chemotherapeutics. In mice, chemotherapy with anthracyclines requires priming of IFN γ -producing CD8+ T cells [Ghiringhelli *et al.* 2009]. In humans, correlative studies have reported that high intratumoral levels of IFN γ and CD8+ T cells [Mattarollo *et al.* 2011], or TILs, are associated with better clinical responses to anthracycline-based chemotherapy [Denkert *et al.* 2010]. Consistent with these studies, West and colleagues recently reported that in ER- breast cancer, high lymphocyte gene expression is associated with a remarkably high rate (74%) of pathological complete response to neoadjuvant anthracycline-based chemotherapy [West *et al.* 2011].

The immune-stimulating property of anthracyclines is a three-step process: preapoptotic translocation of calreticulin (CRT) on the tumor cell surface; postapoptotic release of the chromatin-binding protein high-mobility group B1 (HMGB1); and extracellular release of adenosine triphosphate (ATP). CRT, HMGB1, and ATP act in concert to promote tumor antigen presentation by dendritic cells via activation of CD91, Toll-like receptor 4 (TLR), and purinergic P2X7 receptors respectively. Notably, chemotherapy-induced autophagy is essential for the release of ATP and subsequent anticancer immunity. This suggests that patients with TNBC with autophagy-deficient tumor cells might benefit from therapeutic strategies designed to compensate this process.

Because of the suppressive effects of TAMs on antitumor CD8+ T cells, chemosensitivity might also be regulated by the ratio of TAMs to CD8+ T

cells in human breast tumors. In support of this, the CD68/CD8A ratio was found to be a predictive biomarker for therapeutic response to neoadjuvant chemotherapy in women with breast cancer. Interestingly, chemotherapy with paclitaxel was shown to increase TAM infiltration in breast tumors [DeNardo *et al.* 2011]. Indeed, it was shown that breast tumors receiving neoadjuvant chemotherapy have higher levels of TMAs compared with those undergoing primary surgery without preoperative treatment. Using a transgenic mouse model of breast cancer, the authors further demonstrated that TMA depletion significantly improves chemotherapy by enhancing antitumor CD8+ T-cell responses.

Tumor-associated antigens in TNBC

In order to kill tumor cells, CD8+ T cells must recognize specific antigens on tumor cells presented by major histocompatibility complex class I molecules. Hundreds of tumor-associated antigens (TAAs) and respective T-cell epitopes have been identified (see the Cancer Immunity Peptide Database: <http://cancerimmunity.org/peptide/>). TAAs have been classified into five major groups on the basis of their expression pattern: tissue-differentiation antigens that are shared with the tissue of origin; mutation antigens that arise from point mutations in genes expressed ubiquitously; viral antigens that are associated with transforming viruses; antigens derived from nontransformed cells, such as blood vessel cells and fibroblasts; and antigens preferentially expressed in tumors as a result of epigenetic changes, such as cancer-testis (CT) antigens.

Because CT antigens are not shared with normal somatic cells, they represent attractive candidates for cancer immunotherapy. Over 150 CT antigens have been described thus far, and of these, 83 are encoded on the X-chromosome (also known as CT-X antigens). Grigoriadis and colleagues recently reported the analysis of CT-X antigen expression in human breast cancer samples [Grigoriadis *et al.* 2009]. Interestingly, the study revealed higher expression of MAGE-A and NY-ESO-1 in ER- *versus* ER+ breast cancers, and possible coexpression of these CT-X antigens with basal cell markers. Similar findings were subsequently reported by Curigliano and colleagues [Curigliano *et al.* 2011]. Consistent with these finding, IHC analysis of eight CT antigens, including MAGE-A and NY-ESO-1, also demonstrated significantly more frequent expression in ER- *versus* ER+ human

breast cancers [Chen *et al.* 2011]. By IHC, CT antigens were found expressed in 12–24% of ER–tumors *versus* 2–6% in ER+ tumors. In another recent study, Karn and colleagues reported genes differently expressed in human TNBC [Karn *et al.* 2012]. From a total of 133 bimodally expressed genes, 69 correlated with known molecular subtypes (described above). Interestingly, amongst the 64 remaining genes that showed no correlation with known subtypes of TNBC, the most prominent group of genes encoded for CT antigens. Taken together, these studies strongly suggest that CT antigen expression, including MAGE-A and NY-ESO-1, is a common feature of TNBC.

NY-ESO-1 as an immunotherapeutic target in TNBC

The prognostic implications of CT antigen expression in TNBC remain unknown. Nevertheless, CT antigens represent attractive candidates for cancer immunotherapy. Amongst the different CT antigens, NY-ESO-1 is believed to be one of the most immunogenic. NY-ESO-1 expression has been associated with spontaneous antitumor immune responses in patients with cancer [Valmori *et al.* 2000; Theurillat *et al.* 2007]. Ademuyiwa and colleagues recently investigated whether NY-ESO-1 expression in TNBC was also associated with antitumor immune responses [Ademuyiwa *et al.* 2012]. Consistent with previous studies, NY-ESO-1 expression could be detected by IHC in 16% of TNBC compared with only 2% in ER+ tumors. Remarkably, 8 out of 11 (72.7%) patients with NY-ESO-1+ TNBC had detectable antibody responses to NY-ESO-1. Furthermore, patients with NY-ESO-1+ TNBC had higher levels of CD8+ T-cell infiltrates compared with those with NY-ESO-1– TNBC. As highlighted by the authors, ‘these results suggest that the subset of patients with TNBC whose tumors express NY-ESO-1 have particularly high inherent immunogenicity’, and may represent a population most likely to benefit from immunotherapy.

Immunotherapies for TNBC

The genetic and epigenetic alterations in TNBC provide a set of tumor-associated antigens that the immune system can in theory use to distinguish tumor cells from normal cells [Shah *et al.* 2012; Stephens *et al.* 2012]. While evidence suggests that antitumor immunity can control TNBC progression and patient outcome, tumors persist despite being infiltrated with tumor-specific CD8+ T cells. This apparent paradox is at least partly due to the

exhausted nature of tumor-infiltrating T cells and the presence of immunosuppressive factors in the tumor microenvironment. We hereafter present an overview of the most promising immunotherapies that may benefit patients with TNBC (Figure 1). These include vaccination strategies to bolster tumor antigens recognized by immune cells, immunotherapies aimed at overcoming immune exhaustion and therapeutic strategies to block tumor-mediated immunosuppression.

MAGE-A3 and MUC-1 tumor vaccines

Therapeutic tumor vaccines targeting the CT-X antigen MAGE-A3, expressed in breast cancer, lung cancer, and melanoma, are currently being tested in clinical studies. In a phase II trial, GlaxoSmithKline (GSK) reported that patients with non-small cell lung cancer (NSCLC) whose tumors had been removed by surgery experienced 25% fewer recurrences following vaccination against MAGE-A3. GSK now aims to enrol around 2300 patients with NSCLC positive for MAGE-A3 antigen in a phase III trial [Tyagi and Mirakhur, 2009]. The outcome of this phase III clinical trial could be decisive in the development of tumor vaccines targeting MAGE-A3 or other CT-X antigens expressed in TNBC.

Another candidate target of an immunotherapeutic vaccine is the MUC-1 antigen, which is expressed in the vast majority of breast cancers, including TNBC. While both normal and cancerous breast cells express MUC-1, cancer cells express an aberrantly glycosylated form of MUC-1 due to a lack of core 1,3-galactosyltransferase (T-synthase) [Ju and Cummings, 2002]. The presence of circulating antibodies against MUC-1 at the time of cancer diagnosis has been correlated with a favorable disease outcome in patients with breast cancer [Von Mensdorff-Pouilly *et al.* 2000]. Furthermore, CD8+ T cells isolated from patients with breast cancer can recognize epitopes present on MUC-1 [Brossart *et al.* 1999]. Unfortunately, early MUC-1 vaccines have failed to elicit effective antitumor immune responses. A recent preclinical study, however, demonstrated that a glycosylated MUC-1-derived glycopeptide covalently linked to a TLR agonist can elicit potent humoral and cellular immune responses and generate a therapeutic response [Lakshminarayanan *et al.* 2012]. This study highlighted the importance of maintaining conformational elements of aberrant glycosylation of MUC-1 in order to achieve successful vaccination.

Figure 1. Immunotherapeutic opportunities for triple negative breast cancer (TNBC). Compared with other subtypes, the outcome of TNBC has been shown to be particularly influenced by tumor-infiltrating lymphocytes. Therefore, different immunotherapeutic strategies, aiming at blocking or activating specific targets, are currently envisaged. One of the most promising approaches consists of the use of antagonists against inhibitory receptors that become upregulated on antitumor T cells, such as programmed cell death protein 1 (PD-1), T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), cytotoxic T-lymphocyte antigen 4 (CTLA-4), and lymphocyte-activation gene 3 (LAG-3). Antitumor T cells also express the adenosine receptor A2A (ADORA2a), and antagonists of ADORA2a have been shown to enhance antitumor immunity. PD-L1 and IDO (indoleamine 2,3-dioxygenase), expressed on dendritic cells and tumor cells, as well as immunosuppressive cytokines [such as interleukin (IL)-10, IL-23, transforming growth factor β (TGFβ), adenosine, prostaglandin E2 (PGE2), vascular endothelial growth factor A (VEGF-A)] or immunosuppressive enzymes [such as CD73 and arginase I], could also be targeted to stimulate antitumor immune responses. Alternatively, some approaches aim to activate specific immune targets, such as the coreceptors CD137 (41BB) and OX40, expressed on T cells. Finally, recent evidence suggests that specific tumor antigens, such as MUC-1 and NY-ESO-1, may constitute targetable vaccine antigens. The adoptive transfer of chimeric antigen receptor (CAR)-expressing T cells specific for these tumor antigens is also envisaged.

Immune checkpoint blockade

In humans with cancer, antitumor immunity is often ineffective due to the tight regulation associated with the maintenance of immune homeostasis. One of the major limitations is a process known as 'T-cell exhaustion', which results from chronic exposure to antigens and is characterized by the upregulation of inhibitory receptors. These inhibitory receptors serve as immune checkpoints in order to prevent uncontrolled immune reactions. Blocking of one or several of these immune checkpoints with monoclonal antibodies (mAbs) has been shown to rescue otherwise exhausted antitumor T cells, and most importantly, has been associated with objective clinical responses in cancer patients. The first immune-checkpoint inhibitor to be tested in a clinical trial was ipilimumab (Yervoy, Bristol-Myers Squibb), an anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) mAb. CTLA-4 belongs to the immunoglobulin superfamily of receptors, which also includes programmed cell death protein 1 (PD-1), B and T lymphocyte attenuator, T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), and V-domain immunoglobulin suppressor of T cell activation. In 2011, the US Food and Drug Administration approved the use of ipilimumab

in patients with metastatic melanoma, either as initial therapy or after relapse.

Anti-CTLA-4 mAb therapy enhances the antitumor function of CD8+ T cells, increases the ratio of CD8+ T cells to Foxp3+ T regulatory cells, and inhibits the suppressive function of T regulatory cells [Quezada *et al.* 2006]. CTLA-4 blockade has also been shown to expand a subpopulation of tumor-infiltrating CD4+ T cells that express high levels of inducible T-cell costimulator (ICOS) and secrete IFNγ [Chen *et al.* 2009]. These CD4+ ICOS+ T cells might play a role in the therapeutic activity of anti-CTLA-4 mAb therapy, as their frequency correlates with survival in treated patients with melanoma. The major drawback to anti-CTLA-4 mAb therapy is the generation of autoimmune toxicities due to on-target effects. It has been reported that up to 25% of patients treated with ipilimumab developed serious grade 3–4 adverse events [Kähler and Hauschild, 2011], reflecting the importance of CTLA-4 in maintaining immune homeostasis. Unfortunately, toxicity is not always associated with therapeutic benefit. Thus, a major challenge in the use of anti-CTLA-4 mAb is to define favorable clinical settings that strike an optimum balance between tumor immunity and autoimmunity.

PD-1 is another inhibitory coreceptor expressed on activated and exhausted T cells. Its ligand, PD-L1, is often found overexpressed in various types of cancer. Administration of blocking anti-PD-1/anti-PD-L1 mAbs enhance adaptive antitumor immune responses by preventing T-cell exhaustion [Hirano *et al.* 2005]. Anti-PD-1 mAb blocks interactions between PD-1 and its ligands, PD-L1 and PD-L2, whereas anti-PD-L1 mAb blocks interactions between PD-L1 and both PD-1 and CD80. PD-1 is expressed by activated CD4+ and CD8+ T cells, B cells, monocytes and natural killer T cells. It has two ligands, PD-L1 and PD-L2, with distinct expression profiles. Expression of PD-L1 has been shown to be associated with poor prognosis in melanoma and hepatocellular carcinoma [Gao *et al.* 2009; Gadiot *et al.* 2011]. Notably, cytotoxic chemotherapeutics such as paclitaxel, etoposide, and 5-fluorouracil have been shown to upregulate PD-L1 expression on breast cancer cells [Zhang *et al.* 2008].

Two phase I trials recently reported clinical responses with anti-PD-1 or anti-PD-L1 mAb in pretreated patients with diverse tumor types [Brahmer *et al.* 2012; Topalian *et al.* 2012]. Anti-PD-1 mAb therapy was associated with objective responses in 18% of patients with NSCLC (14 of 76 patients), 28% of patients with melanoma (26 of 94 patients), and 27% of patients with renal-cell cancer (9 of 33 patients) [Topalian *et al.* 2012]. Anti-PD-L1 mAb therapy was associated with objective responses in 17% of patients with melanoma (9 of 52), 12% of patients with renal-cell cancer (2 of 17), 10% of patients with NSCLC (5 of 49), and 6% of patients with ovarian cancer (1 of 17) [Brahmer *et al.* 2012]. Notably, anti-PD-1 and anti-PD-L1 mAb therapy caused drug-related grade 3 or 4 adverse events in 14% and 9% of patients respectively. Strikingly, in the context of anti-PD-1 mAb therapy, objective responses occurred only in PD-L1+ tumors (36% response rate) compared with no clinical responses in PD-L1- tumors.

Combination of checkpoint inhibitors

While inhibition of a single immune checkpoint can prolong the survival of patients with cancer, an important question that remains is whether combinatorial checkpoint blockade can be synergistic in promoting anticancer activity. The first combination of immune checkpoint inhibitors to be tested in mice was the combination of anti-CTLA-4 and anti-PD-1 mAbs. Curran and colleagues

demonstrated that blockade of CTLA-4 and PD-1 in mice allows CD8+ and CD4+ T cells to survive in the tumor microenvironment, proliferate, and carry out effector function [Curran *et al.* 2010].

More recently, TIM-3 has been identified as another important inhibitory receptor expressed by exhausted CD8+ T cells. In mouse models of cancer, it was shown that the most dysfunctional tumor-infiltrating CD8+ T cells actually coexpress PD-1 and TIM-3 [Sakuishi *et al.* 2010]. Based on these findings, a direct comparison of the therapeutic activity of anti-CTLA-4, anti-PD-1 and anti-TIM-3 mAbs was made in various mouse models of cancer [Ngiow *et al.* 2011]. It was observed that the combination of anti-PD-1 and anti-TIM-3 mAbs had the most potent anticancer effect against well established experimental and carcinogen-induced tumors. From a molecular point of view, a recent paper by Rangachari and colleagues identified Bat3 as a key regulator of TIM-3 activity on T lymphocytes [Rangachari *et al.* 2012]. Bat3, through its binding to the intracellular tail of TIM-3, prevents TIM-3 mediated cell death or exhaustion in T lymphocytes. Interestingly, the authors demonstrated that Bat3 is highly downregulated in TIM3+ or PD1+ TILs and that this downmodulation is associated with a decreased cytotoxic potential as revealed by a reduced secretion of IFN γ and tumor necrosis factor α (TNF α).

In addition to its inhibitory role on CD8 T cells, TIM-3 has also been reported as a key regulator of nucleic acid mediated antitumor immunity. In a very recent paper, TIM-3 was shown to be upregulated on tumor-associated dendritic cells (TADCs) extracted from both mouse and human tumors [Chiba *et al.* 2012]. The authors identified IL-10, vascular endothelial growth factor A, and arginase I as the main tumor-released immunosuppressive factors responsible for TIM-3 upregulation on TADCs. TIM-3 expression in TADCs was linked to an impaired nucleic acid mediated innate immune response as revealed by a reduced secretion of cytokines such as IFN β or IL-12. Accordingly, it was proven that anti-TIM-3 mAb therapy greatly enhanced the antitumor efficacy of nucleic-acid based adjuvants in a B16F10 mouse melanoma model, and that this synergistic activity depended on IFN β and IL-12 secretion. More importantly, using a CD11c DTR mouse strain (in which CD11c can be depleted upon diphtheria toxin administration), it was demonstrated that TIM-3 expression on

TADCs (and not on CD8 T cells) was the main limit to the triggering of a nucleic acid mediated antitumor immune response. From a mechanistic point of view, TIM-3 limited dendritic cell innate immune response in a HMGB1-dependent fashion, restraining the HMGB1-mediated transport of nucleic acid into endosome and thus limiting the activation of cytosolic sensors responsible for nucleic-acid-mediated immune response. Finally, the authors extend the relevance of their study showing that TIM-3 mAb therapy strongly synergizes with standard chemotherapy in a subcutaneous colon tumor model, which reinforces the rationale for combining 'immunogenic cell death' inducing chemotherapeutic agents with immune checkpoint inhibitors for cancer therapy.

Lymphocyte-activation gene 3 (LAG-3) is another recently identified inhibitory receptor that acts to limit effector T-cell function and augment the suppressive activity of T regulatory cells. Woo and colleagues recently revealed that PD-1 and LAG-3 are extensively coexpressed by tumor-infiltrating T cells in mice, and that combined blockade of PD-1 and LAG-3 provokes potent synergistic antitumor immune responses against mouse models of cancer [Woo *et al.* 2012]. These studies suggest that combined blockade of immune checkpoint inhibitors may represent a promising strategy for cancer immunotherapy.

Agonistic of tumor necrosis factor receptor superfamily

Members of the TNF receptor superfamily also play an important role as regulators of T-cell function [Croft *et al.* 2012]. Activation of these costimulatory receptors may further enhance the generation of tumor-reactive T cells in the context of cancer therapy. Costimulatory receptors of the TNF receptor family are composed of OX40 (CD134), 4-1BB (CD137), CD27, CD30, and herpes virus entry mediator. When activated, each of these receptors can enhance cytokine production and T-cell proliferation in response to T-cell receptor signaling. OX40 and CD137 activation are particularly effective at allowing activated T cells to survive and proliferate in the late phase of immune responses. The administration of agonistic mAbs against OX40 or CD137 has been shown to enhance tumor immunity and induce regression of established mouse models of cancer [Piconese *et al.* 2008; Narazaki *et al.* 2010; Palazón *et al.* 2011]. Taken

together, the use of agonists to costimulatory receptors or antagonists to inhibitory receptors may provide efficient means to rescue or enhance the activity of tumor-reactive T cells.

Blocking the immunosuppressors

Targeting immunosuppression by soluble mediators is another attractive approach for cancer immunotherapy. A plethora of immunosuppressive factors has been associated with tumorigenesis, including TGF β , indoleamine 2,3-dioxygenase (IDO), arginase, prostaglandin E2, and extracellular adenosine [Stagg *et al.* 2007; Stagg and Smyth, 2010]. Determining which immunosuppressive factors are minimally required for maintaining tumor tolerance in a given patient population remains a great challenge. Recent studies in mouse models of cancer and clinical correlative studies suggest that IL-23 may be a key cytokine governing the balance between protumorigenic and anti-tumorigenic immune responses [Langowski *et al.* 2006; Teng *et al.* 2011; Gangemi *et al.* 2012]. In support of this model, mice genetically deficient in IL-23 are significantly protected against a wide range of malignancies and mice treated with a blocking antibody against IL-23 have a decreased risk of tumor formation and a faster elimination of transplanted tumor cells [Teng *et al.* 2011].

Enzymes that metabolize L-arginine (such as arginase I), the tryptophan-catabolizing enzyme IDO, as well as enzymes that regulate extracellular adenosine levels (such as the ecto-nucleotidases CD39 and CD73) also significantly contribute to the inhibition of anticancer immune responses [Stagg *et al.* 2010; Singer *et al.* 2011]. CD73 is at a critical checkpoint in the conversion of immune-activating ATP into immunosuppressive adenosine, making it a potential therapeutic target. Tumors often overexpress CD73 as a consequence of tissue hypoxia or, in the case of breast cancer, consequent to the loss of ER expression. Proof of principle studies have revealed that anti-CD73 mAb therapy can reduce tumor burden and prevent metastasis in mice [Stagg *et al.* 2010, 2011, 2012; Wang *et al.* 2011]. While tumor-derived CD73 is a significant contributor to the generation of adenosine, host CD73 also exacerbates tumorigenesis, highlighted by the reduced susceptibility of CD73-deficient mice against a number of transplantable and spontaneous tumors. Given the promising results of anti-CD73 targeted therapy in mice, future studies aimed at

translating this approach into the clinic are warranted. A combination of these agents with immunogenic cytotoxic agents could also be a reasonable approach to enhance tumor antigen recognition by the host.

Adoptive T-cell therapy

Another promising immunotherapy consists of the adoptive transfer of tumor-specific T cells. Work pioneered by the Rosenberg lab established that autologous TILs can be isolated from primary tumors, expanded *ex vivo*, and adoptively transferred back to patients following lymphodepletion. For patients with metastatic melanoma capable of withstanding treatment, TIL therapy combined with IL-2 is the best available option, with responses rates ranging from 49% to 72% [Rosenberg *et al.* 2011]. TIL therapy, however, has not proven to be readily applicable in most clinical settings, and to date, only patients with melanoma have consistently demonstrated favorable outcomes.

Building up from the successes of TIL therapy in patients with melanoma, the past decade has seen the emergence of a novel form of adoptive cell therapy based on the transfer of genetically engineered T cells expressing a single-chain antibody structure fused to an intracellular T-cell receptor signaling domain called chimeric antigen receptor (CAR) [Jena *et al.* 2010]. The inclusion of CD28 and CD137 costimulatory signaling in the intracellular domain of CAR has substantially improved their therapeutic efficacy. The therapeutic potential of CAR-expressing T cells for treatment of chronic lymphoid leukemia was recently revealed in a clinical study in which infusion of autologous T cells modified to express a CD19-specific CAR induced complete regression of a patient with refractory disease [Porter *et al.* 2011]. In a breast cancer clinical trial, however, T cells genetically engineered to target HER2+ tumors resulted in the death of the patient, presumably due to cross reactivity of CAR T cells with normal cells expressing low levels of HER2 [Morgan *et al.* 2010].

A search for tumor antigens associated with TNBC recently identified mesothelin as a novel immunotherapy target [Tchou *et al.* 2012]. Mesothelin is a cell-surface glycoprotein normally present on mesothelial cells that has previously shown promise as an immunotherapeutic target in

ovarian cancer and mesothelioma. Tchou and colleagues screened 99 primary breast cancer samples by IHC for mesothelin expression [Tchou *et al.* 2012]. While mesothelin was rarely expressed in ER+ or HER2+ breast cancers, 67% (29 out of 43) of patients with TNBC exhibited mesothelin expression in at least 5% of tumor cells, with 19% of patients with TNBC expressing mesothelin in over 50% of tumor cells. In proof-of-concept experiments, the authors also demonstrated specific cytotoxicity of primary mesothelin-positive breast cancer cells by genetically modified T cells expressing a CAR specific for mesothelin. While further validation in larger cohorts is needed, this study suggests that mesothelin might represent a valid and previously unrecognized target for adoptive T-cell therapy of TNBC.

Conclusion

Unlike other molecular subtypes of breast cancers, triple-negative disease does not benefit from targeted therapies and is associated with worse clinical outcomes. As such, TNBC remains a challenge in medical practice. Among the new therapeutic options for TNBC, cellular or molecular immunotherapies appear to be very promising approaches. Their rational has been supported by several studies showing that TNBC outcome is correlated with tumor-immune infiltrate. Building on these results, a variety of different immunotherapeutic strategies are currently being tested in preclinical and clinical studies, with some showing promising therapeutic activity. Among them, the use of immune-checkpoint blocking antibodies, such as anti-PD-1 or anti-PD-L1, is particularly attractive. Importantly, immune-stimulating therapies might act synergistically when combined with chemotherapeutic drugs currently used in the clinic. As we have discussed, other immune-based strategies that might be promising for TNBC include the adoptive transfer of CAR-engineered T cells or the development of tumor vaccines against CT antigens, which appear highly expressed in TNBC.

In conclusion, in our endeavor towards new therapeutic approaches, one should keep in mind that TNBC itself is a highly heterogeneous disease, implying that not all patients will benefit from given immunotherapeutic strategies. The next challenge will be to find the optimal clinical setting for each new treatment.

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Conflict of interest statement

The authors declare no conflicts of interest in preparing this article.

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