Title: Novel immune targets for the treatment of triple-negative breast cancer

Abstract (**197**/200 words)

Introduction. Although immune checkpoint inhibitors (ICIs), especially exploiting the PD1-PD-L1 axis, have recently been granted landmark regulatory approvals in TNBC, they provide benefit only to a subset of TNBC patients. To impact mechanisms of primary and secondary resistance to the anti-tumor immune response, novel targets such as ICOS, LAG3 and TIM3 are currently being explored at preclinical and early-phase clinical levels.

Areas covered. We introduce the landscape of the immune therapeutics investigated in early-phase clinical trials including TNBC patients. For each immune target, predominant expression, function and preclinical rationale are provided. Clinical implications and preliminary available trial results are discussed.

Expert Opinion. Several immune strategies have been investigated in TNBC, including co-inhibitory molecules beyond PD1-PD-L1 axis, co-stimulatory checkpoints, cancer vaccines, adoptive cell transfer, combination therapies, as well as different routes of administration. Most of approaches showed signs of anti-cancer activity and a good safety profile in early-phase clinical trials. Since IO provided benefit only to a small subgroup of TNBC patients so far, identifying predictive biomarkers is a priority to refine patient-selection. Data from ongoing clinical trials, with the gradually improving interpretation of the breast tumor immune environment will hopefully refine the role of new immune targets for the treatment of TNBC.

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Keywords: triple-negative, cancer, breast cancer, target, translational, drug development, drug discovery

Highlights

1. Immunotherapy, through the PD1-PD-L1 blockade, has recently been approved for triple negative breast cancer (TNBC), both in the advanced and in the early setting.
2. New immune targets are currently in development, in order to impact resistance to the anti-tumor immune response, ultimately improving outcomes.
3. Early phase clinical trials of anti-ICOS, anti-LAG3 and anti-TIM3 antibodies described an overall acceptable safety profile as well as initial signs of anti-cancer activity.
4. Several clinical trials are currently ongoing, and their awaited results may help further understanding the optimal way to turn the immune system against TNBC.
5. Improved biomarker-guided enrichment of responders, appropriate trial design, feasibility and cost-effectiveness analyses are required to improve the value of immune-modulating strategies in TNBC.

Review

1. Introduction

Breast cancer (BC) accounts for ~30% of all female cancers and is one of the leading causes of cancer-related deaths worldwide (1). TNBC is defined by the lack of expression of estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2). Although this BC subtype accounts for 11.2% of new BC cases, it disproportionately accounts for most breast cancer-related deaths (2). Such poor prognosis is due to not only TNBC intrinsic aggressiveness, but also related to the historical lack of therapeutic targets and predictive biomarkers (3). In fact, unlike other BC subtypes, for which endocrine and targeted treatments have improved outcomes in different settings, systemic therapy for TNBC has been limited to chemotherapy (CT) in both early and advanced settings.

In the neoadjuvant setting, CT is administered in order to achieve a better surgical outcome, as well as for a prognostic assessment (4, 5). In fact, patients reaching a pathological complete response (pCR) after neoadjuvant chemotherapy (NACT) display a significantly reduced risk of relapse and death in comparison with patients harboring residual disease (6). In this regard, patients with a sub-optimal response to NACT may benefit from additional post-neoadjuvant CT (7). However, ~35% of TNBC patients still relapse, with an expected survival of less than 2 years (8, 9). In the advanced setting, treatment was historically based on subsequent lines of palliative mono-chemotherapy, until the recent introduction two poly ADP ribose polymerase inhibitors (PARPi), of novel antibody-drug conjugates (ADCs) and of immunotherapy (IO) (2, 10-12).

In this regard, in 2018 the Food and Drug Administration (FDA) granted approval to olaparib for germline breast cancer susceptibility protein (BRCA)-mutated (gBRCAm) patients diagnosed with advanced TNBC and pre-treated with chemotherapy (OlympiAD, median progression-free survival, PFS, 7.0 versus 4.2 months in the olaparib and CT arms, respectively; hazard ratio, HR, 0.58, 95% CI: 0.43, 0.80; P = 0.0009). In the early setting, adjuvant therapy with Olaparib for 1 year significantly

extended disease-free survival (DFS) in patients with high-risk early-stage gBRCAm HER2-negative BC (OLYMPIA, 24-months fup, invasive disease-free survival, IDFS, 85.9% for PARPi versus 77.1% for placebo; 3-year distant disease-free survival, 87.5% for Olaparib versus 80.4% with placebo) (13).

Novel antibody-drug conjugates (ADCs) are entering the clinical practice, as well. Sacituzumab govitecan (SG) is composed of an antibody (Ab) targeting the human trophoblast cell-surface antigen 2 (TROP-2) coupled to SN-38 (topoisomerase I inhibitor). The PFS benefit (SG median PFS 5.6 months versus 1.7 months with standard CT, HR 0.41, 95% CI 0.32-0.52; P<0.001) and the OS advantage (SG median OS 12.1 months versus 6.7 months with standard CT, HR 0.48; 95% CI, 0.38-0.59; P < 0.001) observed in refractory TNBC paved the way for the recent implementation of treatment algorithms in advanced BC guidelines (ASCENT) (14).

Finally, TNBC entered the IO era with the addition of immune-checkpoint inhibitors (ICIs) atezolizumab or pembrolizumab to first-line standard CT (15, 16). In brief, programmed Cell Death Ligand 1 (PD-L1) is a trans-membrane protein that serves as co-inhibitory signal of the immune response, mainly by coupling with programmed cell death protein 1 PD-1 (17). The PD-1/PD-L1 pathway plays an important role in cancer cell immune evasion, as co-inhibitory signals can attenuate the host immune response to tumor cells (immune surveillance) (18). By blocking either PD-1 or PD-L1 the co-inhibitory signal is interrupted, restoring an anti-cancer immune response.

In the phase 3 IMpassion130 clinical trial, the addition of the anti-PD-L1 atezolizumab to CT significantly improved PFS (7.5 vs 5 months, hazard ratio, HR, 0.62; 95% CI, 0.49–0.78; P < 0.001) and a trend toward longer overall survival (OS) in PD-L1 positive TNBC patients receiving atezolizumab in addition to first-line nab-paclitaxel was seen (19). A significant improvement in PFS (9.7 vs 5.6 months, HR 0.65, 95% CI 0.49–0.86; P = 0.0012) was achieved also with the addition to anti-PD1 pembrolizumab to first-line CT in patients with PD-L1-positive (combined positive score [CPS] ≥ 10) TNBC (KEYNOTE-355) (15). Based on these data, the combination of CT and an ICI for patients with programmed death-ligand 1 (PD-L1)-positive metastatic TNBC (about 30%-40% of

all TNBC patients) has been recently approved by regulatory authorities in first treatment line (10, 11, 20). in the early setting, the addition of pembrolizumab to neoadjuvant CT produced a significant event-free survival benefit (HR 0.63, CI, 0.48-0.82; P = 0.00031), compared with CT alone, at the median follow-up of 39 months in the phase 2 KEYNOTE-522 clinical trial. As a result, in July 2021 the FDA approved pembrolizumab high-risk, early-stage, TNBC in combination with CT as neoadjuvant treatment, and then continued as a single agent as adjuvant treatment after surgery (12). Considering the relative paucity of therapeutic targets for TNBC that reached the clinical practice, as well as the somehow unsatisfactory response rates obtained with anti-PD1/PD-L1 plus CT, current research is focusing on identifying novel immune targets to further unleash anti-cancer immune responses (17, 18).

In particular, growing interest revolves around the numerous co-inhibitory and co-stimulatory molecules residing in the tumor microenvironment (TME) (**Figure 1**). These include signals acting on the final lymphoid effectors (lymphocytes and natural killer (NK) cells), but also non-lymphoid cells (innate immune system, fibroblasts, endothelium or tumor cells themselves) (21, 22).

In this review, we describe the emergent landscape of the main immune-modulators, specifically investigated in TNBC, with a focus on their predominant target expression, function, preclinical rationale, potential clinical implications and preliminary clinical results (23).

2. The immune landscape of TNBC

TNBC is typically characterized by heterogeneous immune infiltrates, whose characteristics are under investigation (24). However, an immune-suppressive TME is well-known in TNBC and is responsible for promoting disease progression and resistance to systemic treatments (17). On the other hand, the higher tumor mutational burden (TMB) and tumor-infiltrating lymphocyte (TIL) rates found in TNBC compared with hormone receptor (HR)-positive BC provide a remarkable rationale for IO (17, 25). In particular, T follicular helper and B cells seem to play a critical role in facilitating response to ICIs in TMB-high TNBC, in mice (26). Finally, Resident Memory T cells (RMTs) may provide a key to

interpret the complex interplay between existing immune surveillance and response to ICI (27, 28). The interactions between immune cells and TNBC may be further influenced by tumor-related factors, such as p53 loss, that is able to mediate a Wingless-related integration site (WNT)-dependent inflammatory cascades, ultimately favoring metastatization (28, 29). Additionally, BRCA-related TNBCs may harbor increased tumor-infiltration by macrophages (30). Although differences in terms of immune infiltrates have been observed between BRCA1-mutant and BRCA2-mutant TNBC, no differences have been described in terms of clinical response to ICI (28, 31-33).

In summary, current evidence suggests that the immune landscape of TNBC may impact both prognosis and cancer treatment outcomes (34). However, the experiences of IO in TNBC showed that, while few patients derived long-term clinical benefit from ICIs, the majority of patients still experience poor outcomes (24, 32). Therefore, novel agents as well as patient-selection through optimal predictive biomarkers is a priority (35). Indeed, although PD-L1 testing is currently the recommended strategy to candidate patients for treatment with ICIs, it is a rather imperfect predictive biomarker, displaying relevant differences in both testing and guiding drug administration, among different clinical trials (15, 16, 36-41). Moreover, as preclinical and clinical evidence from first clinical trials with ICIs highlighted different mechanisms of immune escape, a rationale for the investigation of novel strategies to overcome primary and secondary resistance to IO is provided (21). As of May 13th 2021, about 69 early-phase clinical trials investigated compounds acting on immune targets in TNBC, as summarized in **Table 1**. At present, clinical investigation is focusing on the metastatic setting, with the main immune targets summarized in **Figure 1**. Interestingly, Toll-like receptor (TLR) and Colony Stimulating Factor 1 (CSF1) agonists, already investigated in the mid-2000s, are experiencing renewed interest, especially in combination with ICIs (21). Other intriguing combinations are emerging based on preclinical data, with combination immunotherapies (e.g., Tumor necrosis factor receptor superfamily, member 4, OX40, and TLR agonists, CD40 agonists with CSF1 receptor antagonists) or with other treatment modalities, namely radiotherapy, CT or Ab-mediated targeted therapy (21, 22).

3. Lymphoid co-inhibitors

Numerous evolutionarily preserved negative regulators of T-cell activation perform as “immune checkpoint” to regulate the immune response (42). So far, CTLA4 and PD-1/PD-L1 are the most relevant examples of T-cell immune checkpoint molecules (42).

3.1 Adenosine pathway

Adenosine signaling has emerged as a key metabolic pathway that regulates tumor immunity (43). For example, by signaling through purinergic receptors on different immune cells, extracellular adenosine triphosphate (ATP) serves as a Danger-Associated Molecular Pattern (DAMP) to promote both innate and adaptive immune responses (43). Conversely, when extracellular ATP is dephosphorylated by ectonucleotidases - mostly CD39 and CD73 - adenosine is produced. Extracellular adenosine is described to inhibit effector cells function and to stabilize immunosuppressive regulatory cells (43). Of note, CD39 and CD37 are widely expressed on cells within the TME, such as infiltrating immune cells, stromal cells, endothelial cells and tumor cells themselves. Hypoxia-inducible factor 1-alpha (HIF1α) and Transforming growth factor beta (TGF-β)-mediated cascades are involved in the upregulation of these ectonucleotidases (21). In fact, hypoxia causes the release of ATP or nicotinamide adenine dinucleotide (NAD) in its oxidized form (NAD+). These metabolic intermediates are a source of bioactive adenosine by means of enzymes expressed in the TME (e.g., ectonucleotidases, alkaline phosphatase, CD38, CD203a) (21). Immune cells, especially T and NK lymphocytes, can sense adenosine through their amine receptors (Cyclic adenosine monophosphate (cAMP)-modulating receptors), highlighting a link between tumor metabolism and immune regulation (21, 44).

CD73 and adenosine A2A receptor (A2AR) are the most investigated targets in TNBC (**Table 1**). Co-inhibition of CD73 and A2AR improves anti-tumor immune responses, in some preclinical models

(45). As well, synergy with anti-PD-1/PD-L1 and anti-CTLA4 therapies is supported by preclinical evidence (21, 46-48). Currently investigated agents in metastatic TNBC include monoclonal Abs that suppress the enzymatic activity of CD73 and/or promote its internalization, namely MEDI9447 (oleclumab) and LY3475070. NIR178, an oral A2AR antagonist that selectively binds and inhibits A2AR, is mainly investigated with an anti-PD-1 agent (NCT03207867) or within triplet strategies (NCT03742349). Inhibition of adenosine production via anti-CD73 monoclonal antibody (mAb) (CPI-006, mupadolimab) is also under investigation, in combination with either the orally active A2AR antagonist ciforadenant (CPI-444) or pembrolizumab in a phase I trial enrolling patients with advanced solid tumors, including TNBC (NCT03454451, **Table 1**). Finally, etrumadenant (AB928), the first dual adenosine receptor antagonist targeting both A2AR and A2BR, is tested in a phase 1 clinical trial in combination with either pegylated liposomal doxorubicin (PLD) or nanoparticle albumin-bound-paclitaxel with or without eganelisib (IPI-549, a potent inhibitor of phosphatidylinositide 3-kinase γ, PI3K-γ, NCT03719326, **Table 1**). At present, the adenosine pathway is not exploited in early TNBC.

3.2 T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT)

TIGIT, CD226 and CD96 are expressed on T cells and NK cells and share the ligands CD112 and CD155, which are expressed on antigen presenting cells (APCs) and other cells, such as tumor cells (49). CD225 can associate with the integrin lymphocyte function-associated antigen 1 (LFA-1), triggering a positive immune signal. TIGIT, CD96, and CD155 contain immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic tails, with which they deliver a co-inhibitory immune signal (21). TIGIT could bind to CD155 (PVR) on dendritic cells (DCs), macrophages, etc. with high affinity, and also to CD112 (PVRL2) with lower affinity (49).

Well-known synergism links TIGIT with PD-1 and transmembrane immunoglobulin and mucin domain 3 (TIM3) (21, 50, 51). To date, two molecules are in clinical development in metastatic

TNBC, alone or in combination with anti-PD-L1 agents and/or chemotherapy (tiragolumab and SEA-TGT). At present, the TIGIT is the only non-PD-1/PD-L1 lymphoid co-inhibitory pathway in development for the early setting of TNBC (NCT04584112, Cohort B, **Table 1**).

3.3 Transmembrane immunoglobulin and mucin domain 3 (TIM-3)

TIM-3 is a transmembrane glycoprotein expressed on CD4+ and CD8+ T cells, myeloid cells (monocytes, macrophages, DCs, mast cells, NK cells) and on various tumor cell types (49). Apparently, TIM-3 has multiple ligands, including the immunosuppressive carbohydrate-binding protein galectin-9, phosphatidylserine, the immune-stimulatory deoxyribonucleic acid (DNA) alarmin high mobility group box 1 (HMGB1) and the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) (21, 52-54). Moreover, not only co-expression of TIM-3 and PD-1 is frequently found in exhausted CD8+ cells in infections and tumors, but TIM-3 also impacts innate immune cells via a TLR-dependent mechanism (55). Of note, TIM-3 blockade synergizes with anti-PD-1 in preclinical models and can worsen bleomycin-induced lung fibrosis in mice (56, 57). TIM-3 expression is relatively uncommon in BC, with a higher frequency described in estrogen receptor-negative tumors (58). Results from the phase I/Ib clinical trial investigating the anti-TIM-3 sabatolimab (MBG453) alone or in combination with the anti-PD-1 spartalizumab, in advanced solid tumors have been released (n = 219) (59). Overall, sabatolimab plus spartalizumab was well tolerated and showed preliminary signs of antitumor activity, with only five BC patients included in the study (59). Five responses were observed in the combination arm, none of which in BC patients (59). INCAGN02390 is another compound targeting TIM-3 that is investigated in a phase 1 clinical trial for metastatic TNBC (NCT03652077, **Table 1**).

3.4 Lymphocyte activation gene-3 (LAG-3)

LAG-3/CD223 belongs to immunoglobulin (Ig) superfamily and comprises transmembrane protein with four extracellular Ig-like domains (49). Its main ligand is major histocompatibility complex (MHC) class II, to which LAG-3 binds with higher affinity than CD4 (21). Nowadays, LAG-3 is known to trigger a more widespread inhibition, interacting with other ligands and it is generally co-expressed with PD-1 on TILs (60, 61). To date, the best characterized ligand is the lectin LSECtin, selectively expressed in the liver and in melanoma cells (21, 62). Although LAG-3 modulates lymphocyte response in self immune tolerance, chronic infections and cancer, its deficiency is harmless alone (60). Thus, the rationale for combination therapy supports the synergy with PD-1 inhibition or with CT (21, 60). In this sense, a phase I clinical trial on 30 metastatic BC patients treated with eftilagimod alpha (IMP321, a recombinant soluble LAG-3 Ig fusion protein) plus paclitaxel showed an improvement in objective response rate (ORR, 50% compared to the 25% rate reported in the historical control group) (63). On this basis, a phase II clinical trial is currently investigating IMP321 in combination with paclitaxel in metastatic hormone-receptor positive BC (NCT02614833). Five TNBC patients were enrolled in a phase I/II clinical trial investigating the anti-LAG3 LAG525 with or without the anti-PD1 spartalizumab in advanced malignances (64). Of those, two patients showed objective responses, with a trend in conversion of immune-cold to immune-activated biomarker profiles on tumor biopsies (64). Another LAG3 early targeting strategy lies in the bispecific mAb tebotelimab (MGD013) exerting the co-targeting of LAG3 and PD1 (65). In a first-in-human phase I clinical trial, 269 patients were enrolled, of which 23 were affected by TNBC (28). Among 41 patients evaluable for disease response in the dose-escalation cohort, 1 confirmed partial response was reported for TNBC. Among selected patients in the dose-expansion cohort, 3 TNBC patients reported partial response, whereas 5 patients showed stable disease (65). Further research efforts are focusing on INCAGN02385, MGD013 (in association with niraparib) and LAG525 (in association with PDR001 and either NIR178 or capmatinib or MCS110 or canakinumab, NCT03742349, **Table 1**).

4. Lymphoid co-stimulators

4.1 OX40

Tumor necrosis factor receptor superfamily, member 4 (TNFRSF4), also known as CD134 and OX40 receptor, is a member of the TNFR superfamily (66). Unlike CD28, it is not constitutively expressed on resting naïve T cells (66). As a secondary co-stimulatory immune checkpoint molecule, OX40 is expressed after 24-72 hours from lymphoid activation; similarly, its ligand, OX40-L, is expressed on APCs upon their activation (66).

OX40 is essential for the trigger and maintenance of a CD4+ immune memory (66, 67). *In vivo*, OX40 plays a crucial role in both Th1-dependent and Th2-dependent immune responses (66, 67). OX40 agonists are relatively effective alone and showed synergism with other immune modulators, radiotherapy, surgery and CT, in preclinical models (21, 68-73).

In TNBC, OX40/OX40-L modulators currently in development involve mRNA-2752 (OX40-L), ABBV-368, INCAGN01949 and PF-04518600 (**Table 1**). Escalating intratumoral doses of mRNA-2752 are being tested either alone or in combination with durvalumab after at least one prior line of therapy for metastatic or locally advanced TNBC in a phase 1 clinical trial (NCT03739931). mRNA-2752 is a lipid nanoparticle encapsulating messenger RNA (mRNA) encoding human OX40-L, interleukin (IL)-23 and IL-36γ (**Figure 2**). ABBV-368 will be investigated either alone or in combination with an anti-PD-1 molecule (ABBV-181) in a phase 1 clinical trial limited to TNBC patients who have exhausted standard treatment for their incurable disease (NCT03071757). Another phase 1 clinical trial will assess ABBV-368 (NCT03893955) as partner drug of the CD40 agonist ABBV-927 in association to CT, in advanced TNBC (**Table 1**).

INCAGN01949 was investigated either alone or in combination with nivolumab or ipilimumab, or both, in a phase 1/2 clinical trial (NCT03241173). PF-04518600, an OX40 agonist mAb, is currently investigated as partner drug with avelumab either alone or with utomilumab, targeting CD137/4-1BB

in a phase 2 clinical trial (JAVELIN Medley, NCT02554812, **Table 1**). Finally, the mAb MEDI6469 has been investigated as second-line treatment in combination with stereotactic radiotherapy in a phase 1/2 clinical trial enrolling metastatic BC patients, irrespective of the molecular subtype (NCT01862900) (21). Although preliminary data with mAb targeting OX40 showed good safety profiles as monotherapy, with moderate activity across different cancer types, specific data about TNBC are awaited.

4.2 Inducible T-cell COStimulator (ICOS)

ICOS/CD278 is a CD28 immunoglobulin superfamily costimulatory molecule that is expressed on activated T cells (74, 75). Besides being expressed on a population of Th1 cytokine producing and tumor antigen-specific effector cells, the absence of ICOS significantly decreases antitumor responses triggered by anti-CTLA4 compounds (76-78). In this sense, ICOS agonist vopratelimab (JTX-2011) was investigated alone or in combination with anti-PD1 nivolumab in the phase I/II ICONIC clinical trial. Of the 164 patients enrolled, 19 individuals harbored a TNBC diagnosis, showing only one partial response and three disease stabilizations (79). At present, drug development focusing on metastatic TNBC is investigating KY1044 either alone or in combination with the anti-PD-L1 atezolizumab in a phase 1/2 clinical trial (NCT03829501, **Table 1**).

5. Non-lymphoid immune targets

Besides lymphoid cells, the TME is inhabited by several other elements that affect cancer cell survival and antitumoral immune responses (24, 75, 80). Immune targets in this group include growth factor receptors, pathogen recognition networks and enzymes that can be exploited for immunomodulation (21).

5.1 Colony Stimulating Factor 1 (CSF-1)

CSF-1 is a cytokine and hematopoietic growth factor able to cause differentiation of hematopoietic stem cells into macrophages or other monocyte-derived cell types. Such cells are also affected by CSF-1 in terms of increased phagocytic and chemotactic activity, as well as enhanced tumor cell cytotoxicity (81). According to preclinical evidence, the efficacy of CTLA4, PD-1/PD-L1 or IDO1 inhibition is significantly increased upon blockade of the CSF-1 pathway (82, 83). Consistently, T cells that secrete CSF1 following PD-1 blockade can induce secondary resistance (21, 84).

Regarding metastatic TNBC, many agents are currently in development, namely PLX-3397, MCS110 (lacnotuzumab) and PD-0360324 (**Table 1**). PLX-3397 is investigated in association with eribulin in a phase 1/2 clinical trial (NCT01596751). The CSF-1-targeting drug MCS110 is currently explored in association with either immune checkpoint blockade or CT in two phase 1 and in a phase 1/2 clinical trials (NCT03742349, NCT02807844 and NCT02435680, **Table 1**). Preliminary results of the phase 1/1b clinical trial that assessed MCS110 with spartalizumab (PDR001) in patients with advanced melanoma, endometrial, pancreatic, or TNBC have been released (85). Fifty patients were enrolled at 6 combination dose levels. Frequent AEs suspected as drug-related were periorbital edema (30%), increased AST (24%), and increased blood creatine phosphokinase (24%), which was the most frequent Grade ≥3 AE suspected as drug-related (6%). Preliminary antitumor activity, especially in the pancreatic cancer cohort, was observed.

PD-0360324 is tested as partner drug with avelumab in the phase 2 clinical trial JAVELIN Medley (NCT02554812, **Table 1**). To date, the anti-CSF1R cabiralizumab is the only agent of this category being investigated in the early setting, in combination with platinum-based CT and nivolumab in a phase 1/2 clinical trial (NCT04331067).

5.2 Indoleamine-2,3-dioxygenase 1 (IDO1)

IDO1 is a cytosolic enzyme that catalyzes the main steps of the catabolism of tryptophan (21, 86). IDO1-mediated immunomodulation is linked to three different paths. First, the inhibition of mammalian target of rapamycin (mTOR) as a consequence of tryptophan depletion; second, the formation of kynurenine, which is thought to promote Tregs activity; third, the direct signaling via ITIM domains (21, 87-90). In addition, IDO1 can be induced by interferon gamma and is able to mediate resistance to anti-CTLA4 blockade (primary or acquired) (21, 91, 92). As for metastatic TNBC, the IDO1 inhibitor Epacadostat was also tested in combination with pembrolizumab and INCAGN01876 (targeting GITR) in a phase 1/2 clinical trial which was prematurely terminated due to emergent data from other studies and unrelated to safety (NCT03277352).

5.3 Transforming growth factor beta (TGF-β)

TGF-β is a multifunctional cytokine belonging to the transforming growth factor superfamily that includes three mammalian isoforms and exerts immunosuppressive functions (TGF-β 1 to 3) (93). The TGF-β superfamily includes endogenous growth inhibiting proteins, mostly produced by stromal cells (94). An increase in expression of TGF-β often correlates with a malignancy, as well as a defect in the cellular growth inhibition in response to TGF-β (93). The multiple effects exerted by TGF-β depend on its several signaling pathways, namely canonical (*Caenorhabditis elegans* Sma genes and the Drosophila Mad, SMAD-mediated) and non-canonical (93). Although preclinical studies with TGF-β targeting agents led to contradictory findings, several compounds have been investigated in metastatic TNBC, namely ABBV-151, galunisertib, BCA101 and Bintrafusp alfa (M7824) (93). ABBV-151, a humanized mAb, inhibits glycoprotein A repetitions predominant (GARP)-TGF-β1, a transmembrane protein containing leucine rich repeats, mainly present on the surface of stimulated Tregs. This compound is currently under investigation either alone or in combination with the anti-PD-1 ABBV-181 (budigalimab) in a phase 1 first-in-human clinical trial for metastatic TNBC progressing on at least one prior systemic treatment line (NCT03821935, **Table 1**). Galunisertib is a

potent TGFβ receptor I (TβRI) investigated in association with paclitaxel in a phase 1 clinical trial for androgen receptor-negative TNBC, irrespective of prior systemic treatment lines (NCT02672475, **Table 1**). BCA101 is a bifunctional mAb targeting both TGF-β and the epidermal growth factor receptor (EGFR). This agent is currently investigated either alone or in combination with pembrolizumab in EGFR-driven TNBC in a phase 1/1b clinical trial (NCT04429542, **Table 1**). Finally, bintrafusp alfa (M7824) is an innovative first-in-class bifunctional fusion protein composed of the extracellular domain of the TGF-βRII receptor (a TGF-β "trap") fused to a human IgG1 mAb blocking PD-L1 (95). Interim results in patients with advanced TNBC treated with bintrafusp alfa in an expansion cohort of an open-label, phase 1 clinical trial for solid tumors (NCT02517398) showed that 33 patients harboring heavily pretreated TNBC (54.5% of patients had ≥4 prior regimens) were enrolled (95). After a median of 3 doses of bintrafusp alfa (range, 1-24), confirmed responses occurred in 3 patients (1 complete response and 2 partial responses (ORR 9.1%, CI 95%, 1.9%-24.3%). Disease control was achieved in a total of 5 patients (15.2%, CI 95%, 5.1%-31.9%). The median progression-free survival (PFS) was 1.3 months (CI 95%, 1.2-1.4 months), and the median overall survival (OS) was 7.8 months (CI 95%, 2.1-12.8 months), with an acceptable safety profile. Bintrafusp alfa is also being investigated in metastatic TNBC either as monotherapy (NCT04489940) or in combination with an inhibitor of mitogen-activated protein kinases 1 and 2 (MEK1/2) pimasertib (NCT04789668), CT (eribulin, NCT03579472), or with the cancer vaccine recombinant Modified Vaccinia Ankara - Bavarian Nordic (MVA-BN)-Brachyury (BrEAsT, NCT04296942), as depicted in **Table 1**.

5.4 Cytokines

Cytokines are small proteins involved in autocrine, paracrine and endocrine signaling, mainly as immunomodulating agents (96). Since a low dose of cytokines monotherapy leads to a poor

therapeutic outcome and higher doses cause AEs as a result of their pleiotropic impact, investigating their functions and interactions in the cancer immune environment is a prominent issue (97).

5.4.1 Chemokines

Chemokines are small cytokines secreted by cells and able to induce chemotaxis in nearby responsive cells. In tumorigenesis, chemokines are able to mediate angiogenesis, metastasis and recruitment of immune cells (96).

The most relevant targets to TNBC drug development are C-X-C Motif Chemokine Receptor 4 (CXCR4), an alpha-chemokine receptor specific for stromal-derived-factor-1 (SDF-1, also called CXCL12), and C-C chemokine receptor type 5 (CCR5), well-known as co-receptors for human immunodeficiency viruses (HIV) (**Table 1**). Both CXCR4 and CCR5 are G protein-coupled receptors. In solid tumors, CXCR4 is thought to modulate survival and metastatization (21). In particular, *in vitro* and *in vivo* data suggested that CXCR4 inhibition reduces CD4+ T cell exhaustion, reverts the suppressive activity of Tregs and M2 macrophages, enhance cytotoxicity and synergize with anti-PD-1/PD-L1 therapies (98).

Although investigation of such agents primarily addressed hematologic malignancies, the combination of CXCR4 inhibitors and anti-PD-1/PD-L1 agents have been tested in solid tumors, as well (99, 100). However, first disappointing results must be considered, like two studies with the fully human IgG4 ulocuplumab terminated due to lack of efficacy (21, 98, 99). Focusing on TNBC, the CXCR4 antagonist balixafortide (POL6326) received the FDA fast track designation in combination with eribulin for metastatic HER2-negative BC, due to the promising activity highlighted in a phase 1 dose-escalation trial (NCT01837095, **Table 1**) (21, 101). Similarly, a phase 1/2 clinical trial is evaluating the combination of carboplatin and leronlimab (PRO-140), which targets CCR5, for CCR5-positive advanced TNBC in the first line treatment setting, after prior anthracyclines and taxanes in the (neo)adjuvant setting (NCT03838367, **Table 1**). On 12 July 2021 a press release

announced the advancement from the phase 1b to the phase 2 of the clinical trial due to promising preliminary results (102).

5.4.2 Interleukins (ILs)

ILs are involved in both acute and chronic inflammatory responses, and they act in response to the stimulation of specific receptors expressed on the cell surface, activating a particular signaling pathway each time (**Figure 2**) (21). Although preclinical studies identified key ILs which promote BC bone metastases (IL-6, IL-8, IL-1, IL-11) and whose inhibition shows potential preclinical therapeutic effects, clinical trials mainly focused on ILs well-known for their potential to generate a local and systemic antitumor immune response in solid tumors, such as IL-2 and IL-12 (103).

Historically, IL-2 was the first molecule of its category to be proposed in the clinic for its anti-cancer effects (97). IL-2 is mainly secreted by activated CD4+ T-cells, CD8+ cells, NK cells, mast cells and DCs (97). Conversely, IL-2 can promote the proliferation of activated CD8+ cells and NK cells, as well as decrease the expression of PD-1 (97). IL-2-based IO has some downsides, such as the increased expression of CTLA-4 and the stimulation of Tregs, both associated with the suppression of the anti-tumor immune response (97).

The IL-2/IL-2R pathway is investigated with NKTR-214 and IRX-2. Bempegaldesleukin (BEMPEG or NKTR-214) is a CD122-preferential IL2 pathway agonist conjugated with multiple releasable chains of polyethylene glycol and is designed to provide sustained signaling through the heterodimeric IL2βγ (CD122/132) receptor pathway (IL2βγR), with limited binding to the IL2αR subunit (104). Preliminary results from a single-arm, phase 1 dose-escalation trial (PIVOT-02, NCT02983045) that evaluated NKTR-214 plus nivolumab in 38 patients with selected IO-naïve advanced solid tumors (BC not included) are available. Three dose-limiting toxicities (DLTs) were reported in 2 of 17 patients during dose escalation, namely hypotension (n = 1), hyperglycemia (n = 1) and metabolic acidosis (n = 1). Total ORR across tumor types and dose cohorts was 59.5% (22/37),

with 7 complete responses (18.9%). Data suggested that NKTR-214 can be successfully combined with ICIs as dual IO (104). On this basis, the PIVOT-02 clinical trial has opened additional cohorts including metastatic breast, urothelial, and colorectal cancers.

IL-12 activity is mainly mediated by stimulation of INFγ production by cytotoxic cells (CD8+ T cells and NK cells) and Th1 cells (22). In return, treatment with IL-12 causes increased cytotoxicity of NK cells by the consequent increase in CD2 and LFA-1 expression, as well as in proliferation of CD56+ NK cells (97). IL-12 also boosts the production of INFγ in cytotoxic CD8+ cells, resulting in enhanced proliferative activity and cytotoxicity (97, 105). As a direct effect of immune regulation, IL12-stimulated CD8+ T-cells can diminish the number of Tregs in the TME by Fas-mediated apoptosis (22). The use of IL-12 in the treatment of cancer has been complicated by relevant side effects observed in the first clinical trials, especially interferon gamma-mediated (IFNγ)-mediated toxicity (21). Hence, more recent clinical trials are focusing on administering IL-12 via plasmids able to regulate its expression (97). Moreover, agents among this category are often administered as intralesional therapies, as depicted in **Table 1**.

As for TNBC, ILs are typically investigated in early-phase clinical trials as combination therapies, mostly in association with anti-PD-1/L1 molecules. The IL-12/IL-12R pathway is investigated with several active compounds, namely DF6002 (monovalent human IL12-constant fragment (Fc) fusion protein), IT-pIL12-EP (intratumoral plasmid IL-12 electroporation) and Tavokinogene Telseplasmid (tavo, pIL 12, an IL-12 plasmid-based gene therapy), in the metastatic setting. Of note, an adenoviral-mediated IL-12 gene therapy is being assessed in the neoadjuvant setting as well, in association with CT, pembrolizumab and the immunomodulatory agent NG-monomethyl-L-arginine acetate (L-NMMA, pan-nitric oxide synthases, NOS, inhibitor) in a phase 2 clinical trial (INTEGRAL, NCT04095689, **Table 1**).

IRX-2 is a cell-derived biologic drug with multiple active cytokine components, mainly Th1 cytokines (i.e., IL2, IL1β, IFNγ), and tumor necrosis factor alpha, TNFα). This compound is currently

investigated in a phase 2 clinical trial in combination with CT and pembrolizumab in the neoadjuvant setting of locally advanced TNBC (NCT04373031, **Table 1**).

Other agents involved in early-phase clinical trials for TNBC are targeting IL-1/IL-1R, IL-6, IL-7, IL-15 and IL-17. Preliminary results are available for a phase 1b/2 clinical trial assessing GX-I7, a long-acting interleukin-7, administered in combination with pembrolizumab in patients with refractory or recurrent metastatic TNBC (NCT03752723, KEYNOTE-899). At the data cutoff of January 30, 2020, GX-I7 and pembrolizumab were administered to 24 patients. Treatment was discontinued in 13 patients (54.2%), mainly due to progression of disease. No DLTs were reported. Confirmed ORR from ongoing patients included one partial response (5.9%), 2 stable disease (11.8%) and 1 durable unconfirmed progression of disease (5.9%). Overall, GX-I7 in combination with pembrolizumab was well tolerated, with no DLTs reported (106). A summary of all the early-phase clinical trials currently investigating interleukins in TNBC is provided in **Table 1**.

6. Non-lymphoid stimulators

6.1. Cluster of differentiation 40 (CD40)

CD40, a TNFR family member, is a costimulatory protein found on cells, including APCs and B cells (107). The binding of its ligand, CD40L (CD154), on Th cells to CD40 activates APCs and triggers a variety of downstream pathways, such as the activation of cytotoxic lymphocytes and NK cells (21). Although agonistic mAbs that promote CD40 signaling are in development as cancer therapeutics, their clinical benefit has been limited (107, 108). Important for drug design, co-engagement of the Fc domain of agonistic CD40 mAbs with the inhibitory Fcγ receptor FcγRIIB has been found to be required for immune activation and to generate greater anti-tumor responses (108).

CD40 agonism has been explored in TNBC, as well. To date, two compounds are under assessment in early phase clinical trials, namely selicrelumab and ABBV-927. The former is investigated in a

phase 1/2 umbrella clinical trial testing several combinations of drugs, including CT, anti-PD-1/PD-L1 agents, antiangiogenic compounds, tocilizumab (anti-IL6), the antibody-drug conjugate (ADC) SG and many others. Specifically, selicrelumab is associated with atezolizumab and bevacizumab as second treatment line, for IO-naive metastatic TNBC (NCT03424005, Morpheus-TNBC). The latter, ABBV-927, is at the center of a phase 1 clinical trial (NCT03893955) involving several partner drugs, such as ABBV-368 (targeting OX40), the anti-PD-1 ABBV-181, nab-paclitaxel or carboplatin. The agents will be administered in both first and later treatment lines in IO-naïve locally advanced or metastatic TNBC. Interestingly, arm 4 (first line) has been specifically designed for PD-L1 negative advanced TNBC (**Table 1**).

6.2. Pathogen Associated Molecular Patterns (PAMP) and damage-associated molecular pattern (DAMP) receptors

PAMP and DAMP receptors recognize diverse structurally conserved molecules derived from microorganisms (97). This category includes TLRs, that are expressed on the membranes of leukocytes, DC, macrophages, NK cells, T cells, B cells and non-immune cells (e.g. epithelial, endothelial cells, fibroblasts) (109). Upon activation, TLRs recruit adaptor proteins within the cytosol of the immune cell to propagate the antigen-induced signal transduction pathway (109). These recruited proteins ultimately lead to the upregulation or suppression of genes that orchestrate inflammatory responses (cytokine production, proliferation, survival) and adaptive immune responses (109). TLRs are also considered an important link between innate and adaptive immunity, mainly due to their presence on DCs.

In the setting of metastatic cancer, TLR7/8/9 are of most importance, since they are expressed on monocytes and DCs, where they induce production of interferon as well as other cytokines able to enhance antigen presentation. In this sense, they are thought to contribute to the transformation of ‘cold’ tumors into ‘hot’ (21). After first safety issues faced during systemic administration of TLR7/8

and 9 agonists in the late 2000s, more recent intratumoral administration in combination with systemic CT and/or ICIs, provided momentum for investigating these agents (97). A few active agents are currently under investigation in metastatic TNBC, namely BDB001 (TLR7/8), NKTR-262 (TLR7/8), CMP-001 (TLR9), Poly- polyinosinic-polycytidylic acid (ICLC, targeting TLR3) and the PAMP-mimetic Imprime PGG (**Table 1**).

BDB001 is an intravenously administered TLR7/8 agonist that activates plasmacytoid and myeloid DCs and has shown to have activity in preclinical studies (97). The compound was first investigated in a phase 1, open label, dose escalation/expansion trial in which BDB001 was administered weekly in patients with advanced solid tumors (BDB001-101, NCT03486301). Preliminary results confirmed a good safety and tolerability. Eleven (30.5%) subjects did not have TRAEs and the majority of AEs were Grade 1 or 2. No grade 4 or 5 AEs were reported. Of 32 subjects evaluable for efficacy, best ORR was constituted by 6% durable partial response, 56% stable disease, 38% progressive disease, for a disease control rate of 62%. Of note, clinical activity favored subjects with tumors that had progressed on prior anti-PD-1/PD-L1 therapy. On this basis, BDB001 is also being evaluated in combination with pembrolizumab (NCT03486301), with atezolizumab (anti-PD-L1, NCT04196530) and also with atezolizumab plus immunogenic radiotherapy (AGADIR, NCT03915678, specifically for anti-PD-1/L1 refractory TNBC, **Table 1**).

TLRs agonists are among the most promising agents evaluated for intratumoral administration, including in TNBC, both in preclinical and clinical setting (110). For example, NKTR-262 is a small-molecule TLR7/8 agonist. Preclinically, the CD122-preferential IL-2 pathway agonist Bempegaldesleukin and NKTR-262 combined innate immune signaling and enhanced antigen presentation, with sustained T-cell activation, resulting in tumor growth inhibition of cancer lesions (97). On this basis, NKTR-262, intratumorally administered, was first investigated in a phase 1/2 clinical trial addressing several locally advanced or metastatic solid tumors, including TNBC (REVEAL, NCT03435640, **Table 1**). The dose-escalation part enrolled 36 patients as of June 15, 2020. Overall, robust TLR7/8 engagement supported the NKTR-262 mechanism of action, while the

low toxicity profile highlighted the benefit of local delivery of NKTR-262, as BEMPEG promoted systemic activation of T and NK cells. One DLT, transient transaminase elevation, was observed at the highest NKTR-262 dose (3.84 mg). The dose-expansion phase is currently ongoing and updated data are awaited (111). CMP-001 is a novel TLR9 agonist that has already been granted Orphan Drug designation and, subsequently, Fast Track designation by the FDA, due to its promising results in combination with nivolumab and ipilimumab for unresectable or metastatic melanoma (112). At present, a phase 2 clinical trial is evaluating the TLR9 agonist CMP-001 with pre-operative stereotactic body radiation therapy (SBRT) in the neoadjuvant setting of TNBC (cT1-2, at least 5 mm, cN0-1), not candidate for NACT (NCT04807192, **Table 1**). The TLR3 agonist Poly-ICLC is administered with a Mucin 1, cell surface associated (MUC-1) peptide vaccine to patients harboring early-stage TNBC in the adjuvant setting, after completion of standard (neo)adjuvant therapy (NCT00986609, **Table 1**). Finally, Imprime PGG is a novel beta glucan derived from *Saccharomyces*, that acts as PAMP in order to stimulate an anti-cancer immune response. Imprime has been investigated combination with pembrolizumab in a phase 2 clinical trial for CT-resistant metastatic TNBC (NCT02981303, **Table 1**). Although preliminary data suggest that Imprime PGG provides added clinical benefit in this subset of patients, further development and updated data for this combination are awaited (113).

7. Novel strategies

7.1 Cancer vaccines

Historically, BC represents the third most studied tumor for cancer vaccination (CV), an active immunotherapy (114). The most common tumor-associated antigens (TAAs) targeted in BC were HER2, Mucin 1, cell surface associated (MUC-1), carcinoembryonic antigen (CEA) and human telomerase reverse transcriptase (hTERT) (114). After almost two decades of poor clinical trial

results, CVs have come back in the spotlight because of some technological advancements, ultimately boosted by coronavirus disease 2019 (COVID-19) pandemic, with neoantigens emerging as the preferred targets for CVs (114-118). For example, PVX-410 is a novel tetra-peptide HLA-A2-restricted vaccine composed of 3 of the 4 antigens most commonly overexpressed in TNBC. A phase Ib multi-center, single arm clinical trial included HLA-A2-positive patients with early TNBC of least 1 cm in size or with positive loco-regional lymph nodes (119). Ten out of 12 patients with complete immune response assessment available showed a PVX-410-specific immune response, that persisted in all patients tested at 6 months (119). Other interesting approaches under investigation involve P10s-PADRE, a peptide-based vaccine; TPIV200, a penta-epitope vaccine, carrying five fragments of the FRα; Galinpepimut-S, a peptide-based vaccine constituted by the four peptide chains of Wilms' tumor gene 1 (WT-1) protein; a CV constituted by the TAA Globohexaosylceramide; a MVA virus engineered to express wild type TP53 transgene; neoantigen gene-based vaccines (e.g., TNBC-MERIT, DNA plasmid-based vaccine STEMVAC) (120).

7.2 Chimeric antigen receptor (CAR)-T cell therapy.

This type of adoptive cell therapy combines the antigen specificity of an Ab with the effector functions of a T cell. Although CAR-T cell therapy has been successful in B-cell malignancies, additional obstacles arise when targeting solid cancers (121). Nevertheless, CAR-T has emerged as a promising IO approach to improve the survival rates of TNBC patients, with the concomitant development of strategies to overcome major challenges in targeting solid tumors (121). In this regard, the recent results of a phase I clinical trial investigating c-Met-CAR-T cells intratumorally injected into patients with TNBC (NCT01837602) showed acceptable tolerability and elicited inflammatory responses within the tumors (122). Other similar early-phase clinical trials are ongoing (NCT04020575, NCT04025216, NCT02706392, NCT04107142) (121).

8. Conclusions

In the last few years IO has been finally included in the treatment arsenal of TNBC, in both the advanced and the early setting. However, the well-known poor immunogenicity of BC compared with other tumor types, has led to research efforts aiming at dissecting the complex immune landscape of TNBC, as well as to a wide range of novel immune-modulatory molecules, in order to overcome primary and secondary immune resistance. Several ongoing clinical trials investigating novel immune targets and compounds will ultimately inform about the potential additional clinical benefit of novel IO combinations, maybe identifying and clarifying the role of predictive biomarkers for optimal patient selection.

Expert Opinion (**777 words**/500-1000 words)

The current clinical role played by IO in TNBC is as part of combination therapies, consisting of anti-PD1-PD-L1 agents plus CT, both in the early and the advanced first-line treatment setting. Anyway, the clinical benefit provided by IO in TNBC is lower compared with other cancer types, with some causing factors yet to be elucidated. For example, PD1-PD-L1 blockade monotherapy typically benefits a minority of patients. Likewise, a small proportion of patients seem to derive benefit from the addition of PD1-PD-L1 blockade to frontline CT (15, 19, 35).

Nevertheless, final OS results from the KEYNOTE-355 clinical trial confirmed that ICIs exploiting the PD1-PD-L1 axis support the administration of IO plus CT as first-line treatment in patients with PD-L1-positive (CPS ≥ 10) metastatic TNBC (OS, 23 months IO-CT versus 16.1 months placebo-CT, median follow-up 44.1 months, ESMO2021). The CT-IO combination treatment reduced the risk of death by 27% (HR, 0.73; 95% CI, 0.55–0.95; p=0.0093), but no benefit was reported for pembrolizumab plus CT over CT alone by applying a cutoff of CPS ≥ 1. In this regard, PD-L1 seems to be an imperfect biomarker for patient selection, a strategy that clearly need refining (123). As stated, optimal assay and cutoff has not been established yet, with different thresholds adopted in

different clinical trials (36). More importantly, since ~60-70% of TNBC lack PD-L1 expression, new approaches are needed to benefit this subgroup of patients, like novel combinatorial strategies as well as new drug classes e.g., PARPi and ADCs (120, 124, 125). In this context, a number of novel immune targets are currently in development for TNBC. Specifically, the therapeutic agents discussed in this review aim at reverting T cell exhaustion, tackling the upregulation of inhibitory checkpoint receptors and, in general, promoting a 'hot' TME.

Although relevant preclinical evidence highlights enhanced anti-cancer immune stimulation with the exploitation of pathways mainly associated to LAG3, TIM3, TIGIT, ICOS and OX40, results from early-phase clinical trials have not shown solid signals of improved patients' outcomes. Similar observations include other strategies like cancer vaccines and adoptive cell transfer, such as TILs and CAR-T cells. Consequently, the identification of the main mechanisms of primary and secondary resistance to IO is a major field of interest. In this regard, not only different tumor histologies show different patterns of response, but also prior medical history, (epi)genetics, the tissue-specific immune environment, the presence and type of neoantigens, as well as sex-based differences are thought to play a role (75, 126). Furthermore, a better characterization of each TME and immunogenicity, with the definition of novel predictive biomarkers, may clarify how to better tailor treatment strategies (127).

As well, ICIs plus CT demonstrated to improve long-term outcomes in the neoadjuvant, i.e., curative, setting (20). On this basis, novel immune targets will be investigated also in the early setting, in order to improve the benefit of PD1-PD-L1 blockade and hopefully increase the rate of cured patients. However, massive efforts are required to tackle the complexity of identifying responders, in order to de-escalate or escalate treatments, depending on the individualized risk of relapse (128). In this perspective, liquid biopsies as well as big data analysis of multi-national cancer datasets, may better dissect the variables influencing TNBC prognosis, improving individualized treatment approaches (128, 129). In this sense, also assuming that patient selection will be perfected in the next future, the addition of many molecular diagnostic assays to each patient work-up will be debated, especially with

regards to feasibility, equitable global access and, in general, the economic impact for patients and healthcare systems. Therefore, despite the high expectations of all the stakeholders involved in drug development (physicians, patients, industry), caution and rigorous data interpretation must be advised in this fast-evolving era.

Importantly, the appropriate trial design to evaluate the therapeutic benefit of many novel immunotherapeutics may distance itself from the traditional phase 3 trial design. In fact, conventional two-arm clinical trials often requires a long period of time for quantification of durable survival and cost (21). In this regard, 'platform' trials, that are clinical trials with a single master protocol in which multiple treatments are evaluated simultaneously, may be a solution (21). However, since the advantages and disadvantages of adaptive clinical trial design can vary according to the clinical setting, their application in a particular field should be clearly justified.

Finally, cost-effectiveness analysis should be an essential tool in drug development of novel immune targeting agents, for several reasons. Combinatorial strategies are expensive, since they associate drugs that are per se high-priced. Moreover, since each drug or drug combinations may benefit a small subgroup of patients, a biomarker-driven tailored medicine approach should take cost-effectiveness and optimization into account during clinical trial design (21, 75).

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Target	Drug	Route	Trial ID	Phase	# of pts	Setting	Patient cohort	Treatment arms
CTLA-4 and PD-1	KN046	iv	NCT03872791	I/II	90	Metastatic	TNBC progressed on at least one prior anthracycline and taxane (monotherapy); treatment naive (combination therapy)	KN046 +/- Nab-paclitaxel
	SI-B003	iv	NCT04606472	I	159	Metastatic	Pretreated TNBC. Other histologies included	SI-B003
	XmAb20717	iv	NCT03517488 (DUET-2)	I	154	Metastatic	Pretreated TNBC. Other histologies included	XmAb20717
CTLA-4	Tremelimumab	iv	NCT03982173° (MATILDA)	II	88	Metastatic	Basket trial including pretreated TNBC	Durvalumab + Tremelimumab
CSF1-R	Cabiralizumab	iv	NCT04331067	I/II	50	Neoadjuvant	Stage II or III TNBC	Paclitaxel + Carboplatin + Nivolumab +/- Cabiralizumab
	PLX-3397	os	NCT01596751^	I/II	67	Metastatic	mBC, at least one prior line of therapy	PLX3397 + Eribulin
M-CSF-1	Lacnotuzumab (MCS110)	iv	NCT02435680^	II	50	Metastatic	TNBC with high TAMs content; first line	Carboplatin + Gemcitabine +/- MCS110
			NCT02807844^	I/II	141	Metastatic	TNBC who did not receive anti-PD-1/L1. Other histologies included	MCS110 + PDR001
IL-15	SO-C101	sc	NCT04234113	I	96	Metastatic	Pretreated TNBC. Other histologies included	SO-C101 +/- Pembrolizumab
IL-12R	DF6002	sc	NCT04423029	I/II	380	Metastatic	Pretreated TNBC (only dose escalation). Other histologies included	DF6002 +/- Nivolumab
IL-12	Tavokinogene Telseplasmid (Tavo)	it	NCT03567720	II	65	Metastatic	At least one prior line in Cohort 1; first line in Cohort 2; accessible cutaneous/subcutaneous disease	Cohort 1: Tavo + Electroporation (EP) + Pembrolizumab; Cohort 2: Tavo + EP + Pembrolizumab + Nab-paclitaxel
	IT-pIL12-EP	it	NCT02531425^	I	10	Metastatic	Refractory TNBC with cutaneous or subcutaneous disease	IT-pIL12-EP + Electroporation
IL-7	GX-I7	im	NCT03752723 (KEYNOTE-899)	I/II	83	Refractory or relapsed	Previous treatment with anthracycline and taxane; no prior immunotherapy	GX-I7 + Pembrolizumab +/- Cyclophosphamide
	NT-I7 (Efineptakin Alfa)	im	NCT04332653 (KEYNOTE A60)	I/II	168	Metastatic	CPI treated TNBC. Other histologies included	NT-I7 + Pembrolizumab
IL-6	Sarilumab	sc	NCT04333706 (EMPOWER)	I/II	50	Adjuvant	Stage I-III TNBC with high-risk residual disease	Sarilumab + Capecitabine
IL-2Rβ (CD122)	NKTR-214	iv	NCT02983045° (PIVOT-02)	I/II	557	Metastatic	First/second/third line in mBC and other histologies	NKTR-214 + Nivolumab +/- Ipilimumab
IL1RAP	CAN04	iv	NCT03267316 (CANFOUR)	I/II	100	Metastatic	TNBC (only dose escalation)	CAN04
Th1 cytokines (IL2, IL1β, IFNγ, and TNFα)	IRX-2	sc	NCT04373031	II	30	Neoadjuvant	Locally advanced TNBC (T1c N1-2 or T2-4 any N)	Pembrolizumab + ACT +/- IRX-2

OX40L, IL-23, IL-36γ	mRNA-2752	it	NCT03739931	I	126	Metastatic	TNBC progressed following at least one prior line of therapy and other histologies	mRNA-2752 +/- Durvalumab
GARP-TGF-β1	ABBV-151	iv	NCT03821935	I	184	Metastatic	TNBC treated with at least one systemic therapy (including taxane). Other histologies included	ABBV-151 +/- ABBV-181 (anti-PD1)
TGF-βR1	Galunisertib	os	NCT02672475°	I	29	Metastatic	Androgen Receptor Negative (AR-) TNBC, any number of prior therapies	Galunisertib + Paclitaxel
CCR5	Leronlimab (PRO 140)	sc	NCT03838367	I/II	48	Metastatic	First line; CCR5+ mTNBC	Leronlimab + Carboplatin
CXCR4	POL6326	iv	NCT01837095^	I	54	Metastatic	TNBC; at least 2 but no more than 3 previous regimens	POL6326 + Eribulin
TLR7/8	BDB001	iv	NCT03915678 (AGADIR)	II	247	Metastatic	anti-PD-1/L1 refractory TNBC. Other histologies included	BDB001 + Atezolizumab + Radiotherapy
TLR9	CMP-001	sc/it	NCT04807192*	II	40	Neoadjuvant	Early stage TNBC (cT1-2, at least 5 mm, cN0-1) not candidate for NACT	SBRT +/- CMP-001
CD11b	GB1275	os	NCT04060342	I/II	242	Metastatic	Pretreated TNBC (only phase 1). Other histologies included	GB1275 +/- Pembrolizumab
CD16	FT516	iv	NCT04551885	I	27	Metastatic	TNBC progressed after at least one line of therapy. Other histologies included	FT516 + Avelumab
CD27	MK-5890	iv	NCT03396445	I	202	Metastatic	Pretreated (arm 1-2) or treatment naive (arm 4) TNBC. NSCLC also included	Arm 1-2: MK-5890 +/- Pembrolizumab; Arm 4: MK-5890 +Pembrolizumab + Nab-paclitaxel
CD38	Daratumumab	iv	NCT03098550°	I/II	120	Metastatic	Advanced pancreatic, NSCLC or TNBC	Daratumumab + Nivolumab
	TAK-573	iv	NCT04157517	I/II	114	Metastatic	Pretreated TNBC (only dose escalation). Other histologies included	TAK-573 + Pembrolizumab
CD73	Oleclumab	iv	NCT03742102 (BEGONIA)	I/II	200	Metastatic	First line TNBC	Durvalumab + Paclitaxel + Oleclumab (Arm 5)
	LY3475070	os	NCT04148937	I	150	Metastatic	Pretreated TNBC. Other histologies included	LY3475070 +/- Pembrolizumab
4-1BB (CD137) and PD-L1	GEN1046	iv	NCT03917381	I/II	512	Metastatic	Pretreated TNBC. Other histologies included	GEN1046 + Docetaxel (in a single expansion cohort)
B7-H3 (CD276)	MGA271	iv	NCT01391143^	I	179	Metastatic	Pretreated TNBC that overexpresses B7-H3. Other histologies included	MGA271
CD3 and 5T4	GEN1044	iv	NCT04424641	I/II	378	Metastatic	Pretreated TNBC. Other histologies included	GEN1044
5T4	Naptumomab Estafenatox	iv	NCT03983954	I	45	Metastatic	Pretreated TNBC. Other histologies included	Naptumomab estafenatox + Durvalumab
GITR	INCAGN01876	iv	NCT03126110°	I/II	145	Metastatic	Pretreated TNBC. Other histologies included	INCAGN01876 + Nivolumab or Ipilimumab or both
PVRIG	COM701	iv	NCT03667716	I	140	Metastatic	TNBC progressed after at least one systemic therapy, including PARPi for BRCA-mutated. Other histologies included	COM701 +/- Nivolumab

TIGIT	SEA-TGT	iv	NCT04254107	I	377	Metastatic	Pretreated TNBC (parts A and B). Other histologies included	SEA-TGT
	Tiragolumab	iv	NCT04584112	I	80	Neoadjuvant (Cohort B)	Stage II or III TNBC	Tiragolumab and Atezolizumab + Nab-paclitaxel + AC +/- Carboplatin
						Metastatic (Cohort A)	First line, PD-L1 positive TNBC	Tiragolumab and Atezolizumab + Nab-paclitaxel
OX40	ABBV-368	iv	NCT03071757°	I	170	Metastatic	Pretreated TNBC, naive to anti-PD1/PD-L1 in combination arm. Other histologies included	ABBV-368 +/- ABBV-181 (anti-PD1)
	INCAGN01949	iv	NCT03241173^	I/II	52	Metastatic	Pretreated TNBC (only phase 1). Other histologies included	INCAGN01949 + Nivolumab or Ipilimumab or both
Siglec15 (S15)	NC318	iv	NCT03665285	I/II	143	Metastatic	Pretreated TNBC, PD-L1 low. Other histologies included	NC318
TIM-3	INCAGN02390	iv	NCT03652077°	I	40	Metastatic	Pretreated TNBC. Other histologies included	INCAGN02390
ICOS	KY1044	iv	NCT03829501	I/II	412	Metastatic	Pretreated TNBC. Other histologies included	KY1044 +/- Atezolizumab
A2AR	NIR178	os	NCT03207867	II	376	Metastatic	PD-L1 negative TNBC. Other histologies included	NIR178 + PDR001
LAG-3	INCAGN02385	iv	NCT03538028^	I	22	Metastatic	Pretreated TNBC. Other histologies included	INCAGN02385
PD-1 and LAG-3	MGD013	iv	NCT04178460	I	164	Metastatic	TNBC with LAG-3 moderate-high expression, after at least 2 prior lines. Other histologies included	MGD013 + Niraparib
IKZF2	DKY709	na	NCT03891953	I	300	Metastatic	Pretreated TNBC naive to anti-PD-1/PD-L1 therapy. Other histologies included	DKY709 +/- PDR001
TGF-β and EGFR	BCA101	iv	NCT04429542	I/Ib	292	Metastatic	Refractory EGFR-driven TNBC. Other histologies included	BCA101 +/- Pembrolizumab
TRAIL-R2 (DR5)	Tigatuzumab	iv	NCT01307891^	II	64	Metastatic	mTNBC, any line	Nab-paclitaxel +/- Tigatuzumab
PAMP	Imprime PGG	iv	NCT02981303^	II	64	Metastatic	mTNBC and melanoma who have failed first line therapy	Imprime PGG + Pembrolizumab
TGF-β and PD-L1	Bintrafusp alfa (M7824)	iv	NCT04789668	I/II	36	Metastatic	TNBC and other cancers with brain metastases	Bintrafusp Alfa + Pimasertib
			NCT04489940	II	29	Metastatic	At least one line of systemic therapy for metastatic disease; HMGA2-expressing TNBC	Bintrafusp alfa
			NCT03579472	I	20	Metastatic	TNBC, up to 5 previous lines, no prior immunotherapy	Bintrafusp alfa + Eribulin
			NCT04296942 (BrEAsT)	I	65	Metastatic	TNBC and HER2-2 + BC; at least one prior therapy	M7824 + BN-Brachyury + TDM1 +/- Entinostat (if HER-2+)
MUC1	MUC1 peptide vaccine	sc	NCT00986609^	I	29	Adjuvant	Stage I-III TNBC who have completed standard adjuvant or neoadjuvant therapy	MUC-1 peptide vaccine + poly ICLC
TLR3	Poly-ICLC	im						
A2AR and A2BR	Etrumadenant (AB928)	os	NCT03719326°	I	214	Metastatic	Pretreated TNBC (no more than 3 prior lines in dose expansion) or ovarian cancer	Etrumadenant + liposomal Doxorubicin +/- IPI-549 Etrumadenant + Nab-paclitaxel

PI3K-gamma	Eganelisib (IPI-549)	os						
CD137 (4-1BB)	Utomilumab (PF-05082566)	iv	NCT02554812° (JAVELIN Medley)	II	620	Metastatic	Pretreated TNBC. Other histologies included	Avelumab + Utomilumab or PF-04518600 or both
OX40	PF-04518600							
M-CSF1	PD-0360324							
TLR7/8	NKTR-262	it	NCT03435640° (REVEAL)	I/II	64	Metastatic	Refractory TNBC. Other histologies included	NKTR-262 with Bempegaldesleukin +/- Nivolumab
CD122 (IL-2Rβ)	Bempegaldesleukin (NKTR-214)	iv						
CD73	NZV930	iv	NCT03549000	I	344	Metastatic	Pretreated TNBC. Other histologies included	NZV930 A +/- PDR001 and /or NIR178
A2AR	NIR178	os						
CD73	CPI-006	iv	NCT03454451	I	378	Metastatic	Pretreated TNBC. Other histologies included	CPI-006 + Ciforadenant or Pembrolizumab
A2AR	Ciforadenant	os						
IL-6	Tocilizumab	iv	NCT03424005 (Morpheus-TNBC)	I/II	280	Metastatic	First line, PD-L1 positive TNBC	Umbrella study including combination of Atezolizumab + Nab-Paclitaxel + Tocilizumab
CD40	Selicrelumab	sc					Second line, immunotherapy-naive TNBC (enrollment is closed)	Umbrella study including combinations of Atezolizumab + Selicrelumab + Bevacizumab
IL-12	IL-12 gene therapy	na	NCT04095689 (INTEGRAL)	II	43	Neoadjuvant	Early-stage TNBC receiving standard of care NACT	NACT + Pembrolizumab + Interleukin-12 Gene Therapy + L-NMMA
NOS	L-NMMA	na						
GITR	INCAGN01876	iv	NCT03277352^	I/II	10	Metastatic	Pretreated TNBC. Other histologies included	INCAGN01876 + Pembrolizumab + Epacadostat
IDO1	Epacadostat	os						
CD40	ABBV-927	iv	NCT03893955	I	150	Metastatic	Arm 1-3: at least one prior therapy (including taxane) and immunotherapy-naïve; Arm 4: first line, PD-L1 negative TNBC	Arm 1: ABBV-927 + Carboplatin + ABBV-368 Arm 2: ABBV-927 + Carboplatin + ABBV-181; Arm 3: ABBV-927 + Carboplatin TNBC; Arm 4: ABBV-927+ Nab-paclitaxel + ABBV-368;
OX40	ABBV-368							
IL-1β	Canakinumab	iv	NCT02900664^	I	289	Metastatic	Pretreated TNBC. Other histologies included	PDR001 + Canakinumab or CJM112 or other agents
IL-17	CJM112							
CD137	Utomilumab	iv	NCT03971409 (InCITe)	II	150	Metastatic	TNBC. Up to 3 prior lines, no more than 1 prior line of CPI	Avelumbab + Binimetinib or PF-04518600 or Utomilumab
OX40	PF-04518600							
LAG-3	LAG525	iv	NCT03742349	I	220	Metastatic	TNBC treated with taxane-based CT in any setting; at least one but no more than 2 prior lines of chemotherapy	PDR001 + LAG525 + NIR178 or Capmatinib or MCS110 or Canakinumab
A2AR	NIR178	os						
CSF-1	MCS110	iv						
IL-1β	Canakinumab	iv						

Table 1. Early-phase clinical trials investigating immune targets in triple negative breast cancer, as of May 13th 2021. A search was conducted on Clinicaltrials.gov with the following keywords: "immunotherapy", "immune target", "immune checkpoint", "breast cancer", "triple negative breast cancer".

Abbreviations: TNBC, triple negative breast cancer; mBC, metastatic breast cancer; sc, subcutaneous; id, intradermal; it, intratumoral; iv, intravenous; CD, cluster of differentiation; IL, Interleukin; CTLA-4, Cytotoxic T-Lymphocyte Antigen 4; PD-1, programmed cell death protein 1; CSF-1, colony-stimulating factor-1; TAM, Tumor-associated macrophages; CPI, checkpoint inhibitor; Th, T helper; IL1RAP, Interleukin 1 Receptor Accessory Protein; TNF, Tumor necrosis factor; IFN-γ, Interferon gamma; ACT, anthracycline cyclophosphamide taxane; TGFβ, Transforming Growth Factor-β; CCR5, chemokine receptor type 5; CXCR4, C-X-C Motif Chemokine Receptor 4; TLR, Toll-Like Receptor; NACT, Neoadjuvant Chemotherapy; NSCLC, non-small-cell lung cancer; GITR, glucocorticoid-induced tumor necrosis factor receptor; PVRIG, PVR Related Immunoglobulin Domain Containing; PARP, poly ADP ribose polymerase; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TIM3, T cell immunoglobulin and mucin domain-containing protein 3; ICOS, Inducible Co-Stimulator; LAG-3, Lymphocyte Activating 3; A2AR, A2BR Adenosine 2A/B receptor; DR5, death receptor 5; TRAIL-R2, tumor necrosis factor-related apoptosis-inducing ligand receptor 2; PAMP, Pathogen-Associated Molecular Pattern; EGFR, epidermal growth factor receptor; PI3K, phosphoinositide-3-kinase; SBRT, stereotactic body radiotherapy; NOS, Nitric oxide synthases; IDO1, Indoleamine-pyrrole 2,3-dioxygenase. °Active, not recruiting; *Not yet recruiting; ^Completed.

Figure Legends:

Figure 1. **Selected novel immunomodulators in early-phase clinical development, grouped by pathway**. An effective anti-cancer immune response is mainly mediated by T-cell activation. Antigen-specific T-cell receptor complex recognizes HLA-loaded epitopes loaded by antigen-presenting cells. Then, a complex balance of co-inhibitory and co-stimulatory signals determines either an immune response or immune suppression/anergy. Co-stimulatory signals include 4–1BB, OX40, GITR, and CD40L, as well as CD28 and ICOS. Co-inhibitory signals involve CTLA-4, PD-1, as well as LAG-3 and TIM-3. Abbreviations: A2AR, Adenosine 2A receptor; TIGIT, T cell immunoglobulin and ITIM domain; TIM-3, T cell immunoglobulin and mucin domain-containing protein 3; LAG-3, Lymphocyte-activation gene 3; IDO1, Indoleamine 2,3-Dioxygenase 1; GITR, Glucocorticoid-Induced TNFR-Related; ICOS, inducible co-stimulator; CSF1, Colony Stimulating Factor 1; CSF1R, Colony Stimulating Factor 1 receptor; TGFβ, Transforming Growth Factor β 1; CXCR4, C-X-C motif chemokine receptor 4; CCR5, C-C Motif Chemokine Receptor 5; PAMP, Pathogen Associated Molecular Pattern; TLR, Toll-Like Receptor; CD, cluster of differentiation. Created with biorender.com.

Figure 2. Structural basis of main families of cytokine receptors involved in anti-cancer immune responses. (1) Interleukin-1 family cytokines are key signaling molecules in both the innate and adaptive immune systems, that mediate inflammation. The basic mechanism of signal initiation is a stepwise process in which an agonist cytokine binds its matched receptor. The cytokine-receptor complex typically recruits a secondary receptor. Intracellularly, the Toll/IL-1 Receptor (TIR) domains of the two receptors are brought into close proximity, initiating an NF-κB signal transduction cascade. (2) The tumor necrosis factor receptor superfamily is a protein superfamily of cytokine receptors characterized by the ability to bind tumor necrosis factors (TNFs) via an extracellular cysteine-rich domain. Most TNF receptors require specific adaptor protein such as TRADD, TRAF, RIP and FADD for downstream signaling. TNF receptors are primarily involved in apoptosis and inflammation, but they can also take part in proliferation, survival, and differentiation. (3) Cytokine receptors can be divided in two main types. Type I cytokine receptors are transmembrane receptors expressed on the surface of cells. These receptors typically share a common amino acid motif (WSXWS) in the extracellular portion adjacent to the cell membrane. Members of the type I cytokine receptor family are involved in ligand/cytokine interaction and others that are involved in signal transduction. Type II cytokine receptors are transmembrane proteins that bind and respond to a select group of cytokines including interferon type I, interferon type II, interferon type III. These receptors are characterized by the lack of a WSXWS motif. Both receptor classes are typically associated with a tyrosine kinase belonging to the Janus kinase (JAK family). Binding of the receptor typically leads to activation of the canonical JAK/STAT signaling pathway. (4) Interleukin-17 receptor (IL-17R) family represents a group of receptors binding proinflammatory cytokine interleukin 17A, produced by T helper 17 cells. IL-17R family consists of 5 members: IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE. A functional IL-17R is a transmembrane receptor complex usually consisting of one IL-17RA, which is a founding member of the family, and a second other family subunit, thus forming a heteromeric receptor. Binding of IL-17A to IL-17 receptor causes important conformational changes that allow binding of adaptors, such as Act1 or TRAF proteins. Then, several signaling pathways are triggered, including NF-κB and MAPKs (mitogen-activated protein kinases). Abbreviations: IL, interleukin; TNF, tumor necrosis factor; MyD88, Myeloid differentiation factor 88; CD, cluster of differentiation; GITR, Glucocorticoid-Induced TNFR-Related; G-CSF, granulocyte Colony Stimulating Factor; GM-CSF, Granulocyte-Macrophage Colony Stimulating Factor; ACT1, activator 1; TRAF6, TNF Receptor Associated Factor 6. Created with biorender.com.