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New pathways in immune stimulation: targeting OX40

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ABSTRACT

Immune checkpoint blockers (ICB) reinvigorate the immune system by removing the molecular brakes responsible for the scarce activity of immune phenotypes against malignant cells. After having proven their remarkable role as monotherapy, combinations of anti-Programmed cell death 1 (PD-1)/Programmed death-ligand 1 (PD-L1) agents with cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) antibodies, chemotherapy and/or anti-angiogenic compounds provide unprecedented results and durable responses across a variety of tumour types. Nevertheless, the main drawbacks of ICB are represented by primary and acquired resistance, translating into disease progression, as well as by immune-related toxicities. In this sense, novel strategies to foster the immune system through its direct stimulation are being tested in order to provide additional clinical improvements in patients with cancer. In this scenario, the co-stimulatory molecule OX40 (CD134) belongs to the next generation of immune therapeutic targets. Preliminary results of early clinical trials evaluating OX40 stimulation by means of different agents are encouraging. Here we review the rationale of OX40 targeting, highlighting the combination of OX40-directed therapies with different anticancer agents as a potential strategy to foster the immune system against malignant phenotypes.

INTRODUCTION

A dramatic paradigm shift in cancer immunotherapy came from the demonstration that drugs targeting immune checkpoint signalling are able to restore immune anticancer activity reinforcing the biological and clinical significance of immune system/tumour interactions. The treatment strategies involving immune checkpoint blockers (ICB, ie, anti-CTLA-4 and anti-Programmed cell death 1 (PD-1)/Programmed death-ligand 1 (PD-L1) agents) are standard of care in several metastatic settings and have shown their role in earlier disease stages and adjuvant setting, with particular regard to melanoma and non-small cell lung cancer.[1–3]

However, a proportion of the patients does not benefit from ICB, experiencing primary resistance. Moreover, besides their prolonged activity in responding cases, their efficacy is limited by the onset of acquired resistance, turning out in clinical progression. Among the resistance mechanisms, there are tumour-intrinsic pathways leading to diminished infiltration and function of immune cells in tumour microenvironment (TME): (i) genomic defects in interferon-gama (IFN-γ) signalling, like mutations occurring in *JAK1/2*,[4 5] (ii) expression of T-cell inhibitory surface ligands (including PD-L1) by the tumour, (iii) altered tumour antigen presentation,[6] (iv) signalling through Wnt/β-catenin pathway,[7] (v) Phosphatase and TENsin homolog (PTEN) loss,[8] and (vi) induction of indoleamine 2,3-dioxygenase.[9] Regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs) and tumour-associated macrophages (TAMs) release factors (interleukin 4 (IL-4), IL-10, transforming growth factor beta, vascular endothelial growth factor and arginase) in TME that suppress immune cells implicated in anti-tumoural response.[10] To prevent and to overcome the onset of resistance, new strategies envisaging combination therapies with chemotherapy, anti-angiogenic treatments, radiotherapy and new immunomodulatory agents are promising.

The signalling induced by antigenic MHC/peptide interaction with T-cell receptor is a prerequisite to T cell activation, but insufficient to initiate T cell responses by itself. Further signalling by co-stimulatory molecules is crucial to optimal priming, expansion and differentiation of T-cells. These molecules are primarily classified into two groups: immunoglobulin superfamily (IgSF) and tumour necrosis factor receptor superfamily (TNFRSF).[11 12] IgSF includes CD28, inducible co-stimulator (ICOS) and CD226. TNFRSF is composed of CD27, OX40 (CD134) and its ligand OX40L (CD252), 4-1BB (CD137), glucocorticoid-induced tumour necrosis factor receptor (TNFR)-related protein, death receptor 3, CD40 and CD30. Differently from standard ICB that blocks surface receptors in tumour and T cells that are responsible for inhibition of anti-tumoural immune

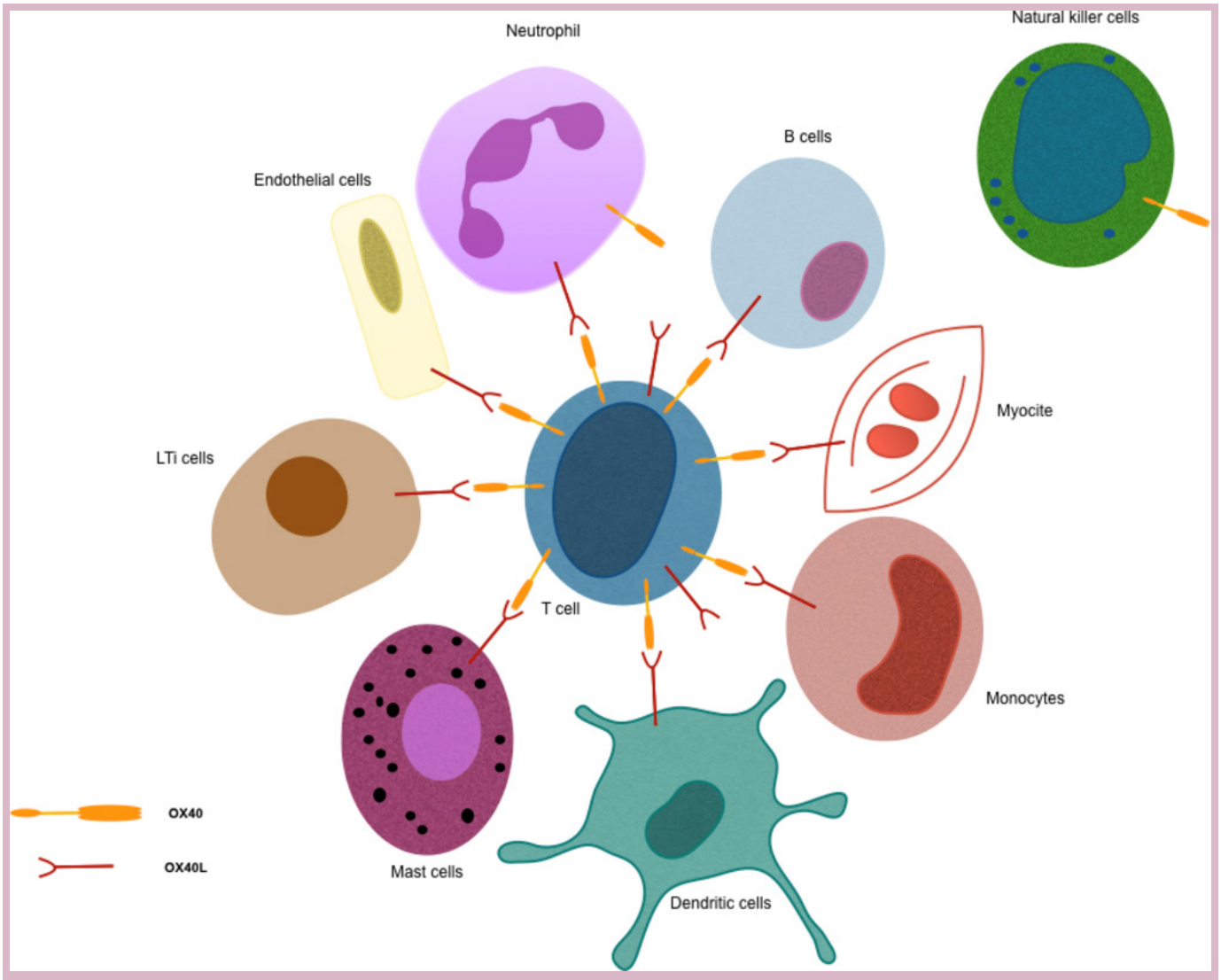

Figure 1 Human cells expressing OX40 and/or OX40L.

response, drugs targeting OX40 act by direct activation and modulation of immune response.

Altogether, targeted therapies to these co-stimulatory molecules combined to standard ICB are an interesting option to overcome primary resistance by enhancing immune response.[13] Here we will review the rationale that has supported the development of clinical trials with drugs targeting OX40 and we will discuss the current understanding of the mechanism of action of compounds designed to potentiate the immune system.

MOLECULAR CHARACTERISTICS

OX40, a type 1 transmembrane glycoprotein, is predominantly expressed by T cells (constitutively by regulatory T phenotypes and, after activation, by effector T cells). Figure 1 shows human cells expressing OX40 and OX40L and its potential interactions, allowing migration of activated T-cells into tissues following inflammatory signals.[11 12 14–16] OX40 has a cytoplasmic tail that binds to molecules implicated in signal transduction pathways, namely TNFR-associated factor-2, -3, and -5,[17 18] mediating nuclear factor-kappa B (NF-kB) activation.

IMMUNE RESPONSE MEDIATED BY OX40 SIGNALLING

T-cell activation

OX40 induces expression of proteins with anti-apoptotic (Bcl-2, Bcl-xl and Bfl-1) and cell-cycle progression (Survivin) properties.[19 20] OX40 counterbalance the inhibition of immune cells (including T lymphocytes CD4+and CD8+, NK cells and B lymphocytes) while directly stimulating effector T cells (figure 2).

Depletion of Treg cells

For anti-CTLA-4 therapy, there is evidence of a selective depletion of Treg cells in TME via Fcγ receptor-expressing macrophages, suggesting that OX40-directed antibodies can also deplete OX40+Tregs in TME without reducing effector T cells expressing the receptor.[21] Zhang *et al* showed that OX40 co-stimulation leads to inhibition of *FOXP3* gene expression, crucial to Treg differentiation,

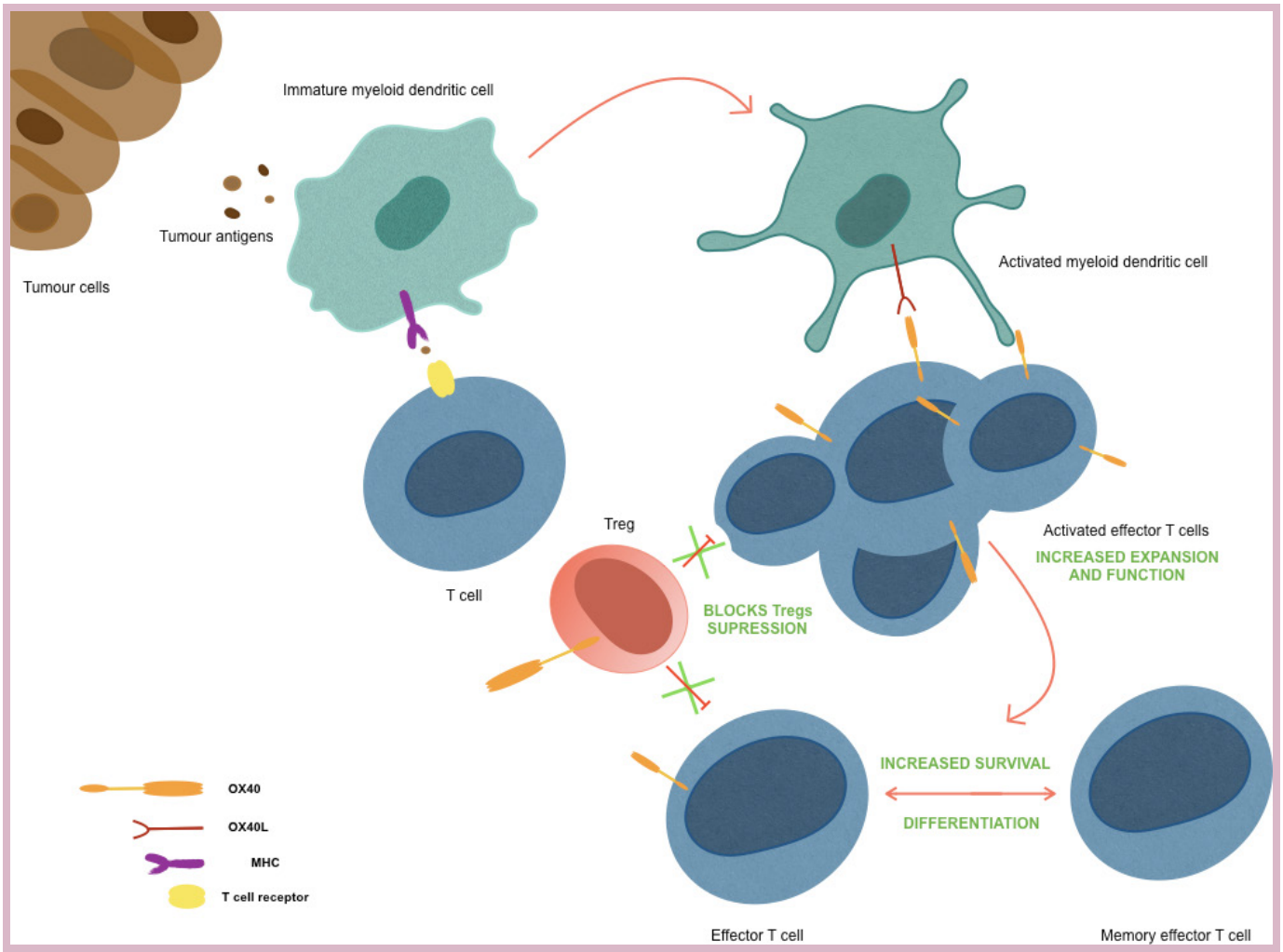

Figure 2 Mechanism of action of OX40.

by two independent mechanisms[1]: enhancing the expression of the activator protein 1 transcription factors *BATF, BATF3* and[2] activating AKT- mammalian target of rapamycin (mTOR) pathway.[22] There is evidence in preclinical models of reduction in IL-10 production by tumour-infiltrating Treg cells after treatment with anti-OX40 monoclonal antibody (mAb), allowing dendritic cells (DC) maturation, probably by downregulation of transcription factor interferon regulatory factor 1 mRNA expression.[23] So, it creates a permissive immune status and leads to myeloid cell accumulation and development of innate and adaptive immunity, important steps to anti-tumoural effect of anti-OX40.[24 25]

Other immune pathways

Whether OX40 influences B cell response is controversial. Nevertheless, initial data suggest that, although it is not crucial for generating humoral response, OX40 activates ICOS pathway and can favour Th2 response by stimulating a profile of high immunoglobulin-producing cells.[26–29] Expressed by DC, OX40L signalling via OX40 T-cell plays a role in antigen-presenting cell (APC) activation.[30 31]

OX40 expression in tumour immune microenvironment

In a preclinical study conducted by Marabelle *et al*, the analysis of tumour tissues in B cell lymphoma line-bearing mouse model and humans with mantle cell and follicular lymphomas showed a high expression of OX40 and CTLA-4 on the surface of tumour-specific Tregs (CD4+Foxp3+),[32] with higher levels than lymphoid tissues. This strengthened the idea of using these drugs as targets of lymphocytes in TME. Burocchi *et al* found higher levels of OX40-expressing Tregs in murine colon carcinoma CT26 than in dLNs.[23]

ROLE OF OX40 AS A BIOMARKER

Ramser *et al* analysed the positivity for OX40+infiltrating immune cells and tumour tissue from biopsies of primary and recurrent stages III and IV ovarian cancer (OC) in humans.[33] Chemosensitivity was associated with high expression of OX40 on immune cells for primary OC and on tumour cells in recurrent OC; the patients who were OX40 negative in immune and tumour cells had the worse recurrence-free survival. In primary colon cancer, the higher expression of OX40 in tumour infiltrating

lymphocytes was significantly associated with better survival, with a difference of 11 months between high and low OX40 expression.[34]

Although relying on a small number of patients, the study by Martins and colleagues showed that patients with gastric cancer (GC) had higher levels of T cells, monocytes and neutrophils with OX40 expression in peripheral blood when compared with healthy controls. Moreover, the percentage of OX40+T cells resulted in reduced more advanced stages, with a median of 3.0% in stages I–II and 1.4% in stages III–IV GC.[35] In a cohort of 20 patients with advanced GC, the expression of OX40 on CD4+/CD8+T cells prior to Nivolumab therapy positively correlated with progression-free survival.[36] Among cutaneous melanoma patients, the expression of OX40 in sentinel lymph node T cells inversely correlated with poor prognostic features such as tumour size, presence of ulceration and nodal infiltration.[37]

DEVELOPMENT OF DRUGS TARGETING OX40

Given the biological rationale to use co-stimulatory receptors as target therapy for enhancing immune response against tumours and based on in vitro results, many drugs that stimulate OX40 signalling have been developed. OX40 signalling can be triggered by OX40-specific agonistic antibodies, OX40L-Fc fusion proteins, transfection of DC with OX40L mRNA and tumour cells engineered to express OX40L on the surface.[10 38 39] Furthermore, the development of a single antibody targeting both OX40 as a T cell co-stimulatory receptor and CTLA-4 as an ICB is ongoing.[40] Table 1 shows drugs tested in in vitro studies either in human clinical trials and its technology.

Table 1 OX40-targeted drugs

Type	Drug
Humanised IgG1 agonist mAb	ABBV-368 GSK3174998 MEDI0562 MOXR0916 (vonlerolizumab)
Fully human IgG1 agonist mAb	INCAGN01949 IBI101 BMS-986178
Fully human IgG2 agonist Ab	PF-04518600
Murine IgG1 agonist mAb	MEDI6469P 9B12
Human IgG1 CTLA-4 × OX40 bispecific Ab	ATOR-1015
Lipid nanoparticle encapsulating mRNAs encoding human OX40L, IL-23 and IL-36γ	mRNA-2752
Human OX40L IgG4P Fc fusion protein	MEDI6383
Dual-sided Fc fusion protein PD1-Fc-OX40L	SL-279252

Ab, antibodies; IL, interleukin; mAb, monoclonal antibodies.

Anti-tumoural activity of OX40 in animal models

The modulation of immune cells and anti-tumour activity of agents targeting OX40 has been shown in several preclinical cancer models. In a B cell lymphoma line-bearing mouse model, a TLR9 agonist, which stimulates APC, were administrated intratumourally in combination with OX40 murine mAb and/or anti-CTLA4, drugs that can modulate Tregs in TME.[32] The intratumoural administration of TLR9 agonist with either OX40 murine mAb or anti-CTL4 was effective in eradicating most of systemic and central nervous system (CNS) metastases and reducing tumour-specific Tregs in injected site, even with low doses than in systemic therapy. These results were more impressive with the combination of the three drugs, with depletion of tumour-specific Tregs and cured most of the mice. In this study, the intratumoural injection generated complete and prolonged responses when compared with systemic infusion and seemed to improve immunologic memory, since mice treated locally did not relapse and were resistant to the development of CNS metastases after a new infusion of lymphoma cell line in the brain.

Oberst *et al* showed that MEDI6383, the human OX40L IgG4P Fc fusion protein, induced activation of T cells in vitro and in vivo models and overcome suppression mediated by Tregs. Its anti-tumoural efficacy was dependent on T cells in mouse models injected with A375 melanoma cells[41] and has been tested in phase 1 trials enrolling advanced malignancy patients (table 2, see section 6). In a murine sarcoma model (MCA205), Moran and colleagues showed that anti-OX40 mAb treatment increased of T cells with strong T cell receptor signalling in the TME and a smaller increase in CD8 +T cells in tumour-draining lymph node (dLN). When used in combination to adoptive T cell therapy, anti-OX40 mAb improved cure rates from 9% to 70%, with greater tumour regressions and longer survival in this MCA205 tumour-bearing mice.[13] Data generated by Weinberg and colleagues show that OX40 signalling is associated with enhanced specific anti-tumoural immune response.[42 43] In mice bearing a colon cancer model (CT26), treatment with murine OX40L:Ig (mOX40L:Ig) prolonged tumour-free survival (cured mice) and these mice resisted to a second CT26 inoculation, remaining tumour free. However, they had to be sacrificed when challenged to a renal cell origin tumour because of tumour burden.[42]

Combinations of agents targeting OX40 with other therapies are promising and under evaluation. In a preclinical model, combined therapy with anti-OX40 and anti-CTLA-4 resulted in significant increase in proliferation and activity of CD4 +and CD8+T cells that was translated into better outcomes compared with anti-OX40 monotherapy.[44] The upregulation of PD-L1 on TAMs and macrophages and of PD-1 on T cells induced by OX40 targeted therapy can explain the resistance.[45] When given

Table 2 Ongoing clinical trials targeting OX40

Monotherapy

Population	OX40 target	Phase	NCT number	Endpoints
Advanced solid tumours	MEDI0562	1	02318394	Safety and DLTs
	ATOR-1015	1	03782467	Safety
	INCAGN01949	1/2	02923349	Safety
	ABBV-368	1	03071757	Safety, pharmacokinetics and preliminary efficacy
Advanced malignancies	SL-279252	1	03894618	Safety and DLTs
CRC	MEDI6469	1	02559024	Safety
HNSCC	MEDI6469	1	02274155	Safety
HNSCC or melanoma	MEDI0562	1	03336606	Activation of immune response

HNSCC, head and neck squamous cell carcinoma.

Combination therapy

Population	OX40 target	Combination therapy	Phase	NCT number	Endpoints
Advanced solid tumours	PF-04518600	Avelumab (anti-PD-L1) Utomilumab (4-1BB agonist mAb) PD 0360324 (anti-CSF1 mAb)	2	02554812	DLTs and ORR
	MEDI6383	Durvalumab (anti-PD-L1)	1	02221960	Safety
	PF-04518600	Utomilumab (4-1BB agonist mAb)	1	02315066	Safety and DLTs
	GSK3174998	GSK1795091(TLR4 agonist) GSK3359609 (anti-ICOS agonist) Pembrolizumab (anti-PD-1)	1	03447314	Safety and DLTs
	MEDI0562	Durvalumab (anti-PD-L1) Tremelimumab (anti-CTLA-4)	1	02705482	Safety and DLTs
	MOXR0916	Atezolizumab (anti-PD-L1)	1b	02410512	Safety and DLTs
	GSK3174998	Pembrolizumab (anti-PD-1)	1	02528357	Safety and DLTs
	IBI101	Sintilimab (anti-PD-1)	1	03758001	Safety
	INCAGN01949	Nivolumab (anti-PD-1) Ipilimumab (anti-CTLA-4)	1/2	03241173	Safety and ORR
	BMS-986178	Nivolumab (anti-PD-1) Ipilimumab (anti-CTLA-4)	1/2a	02737475	Safety
	BMS-986178	TLR9 Agonist SD-101	1	03831295	Safety
Advanced malignancies	MEDI6469	Durvalumab(anti-PD-L1) Tremelimumab (anti-CTLA-4) Rituximab (anti-CD20)	1b/2	02205333	Safety and DLTs
	Anti-OX40	Biological vaccines tetanus toxoid and KLH	1	01644968	DLTs
	mRNA-2752	Durvalumab(anti-PD-L1) Tremelimumab (anti-CTLA-4)	1	03739931	Safety and DLTs
Lymphomas	PF-04518600	Utomilumab (4-1BB agonist mAb) Rituximab (anti-CD20) Avelumab (anti-PD-L1)	1	03636503	Recommended phase 2 dosing and complete response rate
	BMS-986178	TLR9 agonist SD-101 Radiotherapy	1	03410901	DLTs
AML	PF-04518600	Avelumab (anti-PD-L1) Azacitidine Venetoclax (anti-Bcl-2) Gemtuzumab ozogamicin (recombinant humanised IgG4 kappa Ab conjugated with calicheamicin derivative)	1/2	03390296	Safety and composite complete response

Continued

Table 2 Continued

Combination therapy					
Population	**OX40 target**	**Combination therapy**	**Phase**	**NCT number**	**Endpoints**
RCC	PF-04518600	Axitinib	2	03092856	PFS
CRPC	MEDI6469	Cyclophosphamide Radiotherapy	1b	01303705	MTD
BC	MEDI6469	Radiotherapy	1/2	01862900	Safety and MTD
Ovarian, fallopian tube or peritoneal cancers	MEDI0562	Durvalumab(anti-PD-L1) Tremelimumab (anti-CTLA-4) Oleclumab (anti-CD73)	2	03267589	Disease control rate
Urothelial carcinoma	MOXR0916	Atezolizumab (anti-PD-L1)	2	03029832	PFS and overall survival

AML, acute myeloid leukaemia; BC, breast cancer; CRC, colorectal cancer; CRPC, castration-resistant prostate cancer;DLTs, dose limiting toxicities; ICOS, inducible co-stimulator; mAb, monoclonal antibodies; MTD, maximum tolerated dose; NCT, ClinicalTrials.gov identifier; ORR, objective response rate; PD-1, Programmed cell death 1 ; PD-L1, Programmed death-ligand 1; PFS, progression-free survival; RCC, renal cell carcinoma.

in combination anti-PD-1 and/or anti-PD-L1, anti-OX40 significantly increased the expansion and effector properties of differentiated T cells in the dLN and tumour itself, with an increase in CD8+/Treg ratio, that was translated in rapid tumour shrinkage and durable responses.[45 46] In another murine model, the combined therapy anti-OX40 and a drug targeting CD73 (responsible for immunosuppression and pro-angiogenesis in TME),[47] resulted in longer survival, increased immune response and tumour response than controls. Better ascites fluid control was obtained when compared with anti-PD-1 and anti-OX40 combination.[48] ATOR-1015 administration resulted in prolonged survival, tumour shrinkage and complete response rates when compared with anti-OX40 or anti-CTLA-4 monotherapy in a murine model of bladder cancer, by enhancing CD8+T cells infiltrate and reducing Treg in TME.[40] In this study, ATOR-1015 improved the outcomes also when combined to anti-PD1 therapy: all bladder cancer-harbouring mice were cured and colon carcinoma models experienced tumour shrinkage and longer survival.

Depending on the pathway of immune stimulation, the timing of differential drug administration in combinatorial strategies can be crucial. Shrimali *et al* showed that, in a murine model injected with TC-1 tumour cells (mouse lung epithelial cells cotransformed by human papillomavirus strain 16 early proteins 6 and 7 and activated *RAS* oncogene), simultaneous administration of OX40 costimulation and anti-PD-1 had a negative effect on OX40-directed drug, reducing survival and tumour inhibition.[49] They showed that simultaneous infusion lead to apoptosis of antigen-specific T cells, reducing TME-infiltrating CD8+. Although increased CD8+T cells apoptosis was not seen with sequential administration of anti-PD-1 (delay of 7 days), combined therapy did not have negative or additive effects to anti-OX40. Using a different tumour model, Messenheimer *et al* also showed a diminished efficacy of OX40 costimulation when simultaneously administrated with anti-PD-1. Simultaneous infusion increased acute cytokine release (TNF-α, IFN-γ, IL-4, IL-10) and expression of inhibitory markers (eg, CTLA-4 and TIM-3) by tumour-infiltranting CD4+ and CD8+T cells in mammary tumour-bearing mices.[50] In this study, sequential treatment of OX40-targeted followed anti-PD-1 or anti-PD-L1 (delay of 6 days) resulted in better outcomes (tumour control and survival) with reduced T-cell exhaustion. Administration of anti-PD-1 with delayed OX40 did not improve outcomes.

TARGETING OX40 IN CLINICAL TRIALS

Preliminary data about the utilisation of OX40-targeting drugs in humans come from initial trials, which most included advanced and pretreated tumours. Whether these trials included patients that had progressed to ICB and the type of resistance (primary or acquired) is not specified. Initial data from clinical trials evaluating OX40-directed therapy in advanced tumours showed satisfactory safety profiles and signs of clinical activity. The phase 1 clinical trial published by Curti *et al* showed a good tolerance for 9B12, with grades 1 and 2 lymphopenia, fatigue, fever/chills, and rashes and a transient lymphopenia as the only grade 3 and 4 toxicity.[51] Stable disease (SD) was the best response for 20% of the 30 patients with metastatic solid tumours refractory to conventional therapy. During an observation period of 57 days, there was a significant increase of proliferation markers in lymphocytes and activation of CD8+T cells in patients treated with 9B12 compared with controls.[51] Among 48 patients with solid tumours (melanoma, hepatocellular carcinoma, head and neck squamous cell and renal cell carcinoma) treated with PF-8600 in a phase 1 study, grade 1–2 fatigue, nausea and vomiting were the most common adverse events (AE). Out of the 48 treated patients, 4% and 52% experienced, respectively, partial response (PR) and SD as

best response, but there is no published data about duration of response.[52] In a phase 1 dose-escalation study of MEDI0562, which included 55 patients with advanced solid tumours, the treatment was well tolerated and showed clinical activity. AE were mostly grade 1 or 2, including fatigue in 31% of patients and infusion reactions in 15%. Fever occurred in 4% of the patients and was the most common grade 3 event. Two patients (3,6%) carriers of head and neck squamous cell carcinoma (HNSCC) and bladder cancer experienced PR, with an overall survival of 13.8 and 10.2+months, respectively. SD was observed in 22 patients (44%) with a duration of response lasting more than 3 months in 20 of these patients.[53] Preliminary data from a phase 1 dose-escalation trial sustain the safety of OX40-targeted mABs. ABBV-368 was well tolerated when given as monotherapy for patients with advanced or metastatic tumours and, though further evaluation is ongoing, they observed initial tumour activity.[54]

In the neoadjuvant setting, the results from a phase 1b clinical trial conducted by Bell and colleagues showed that MEDI6469 induced proliferation and activity of T cells in the TME in 17 treatment-naïve patients with resectable HNSCC (stage III to IVA). In this trial, treatment was well tolerated, without grade 3 or 4 AE, and did not delay curative surgery.[55]

Published data from phase 1 trials showed that the safety profile seems to be maintained even in combination strategies. In a phase 1 trial that included patients with advanced solid tumours, Infante *et al* showed a safety tolerance profile with vonlerolizumab combined with atezolizumab and evidence of PD-L1 induction and immune activation in tumour paired biopsies.[56] In the same population, another ongoing phase 1 trial of GSK3174998 administered as monotherapy or combined with pembrolizumab (anti-PD-1 Ab) showed no dose-limiting toxicities.[57] The combination of agonistic mAb against OX40 (PF-8600) and 4-1BB (utomilumab) increased the expression of markers and genes related to immune activation in paired biopsies after 6 weeks of treatment when compared with baseline.[58] Data about efficacy are not yet published.

CONCLUSIVE REMARKS

Currently, the biggest lesson from clinical trials using OX40-targeted drugs is its safety when used as monotherapy or combined with ICB. Although OX40-targeted therapy showed impressive results in tumour bearing mice, preliminary clinical data show that its efficacy as monotherapy in humans is modest. OX40 costimulation is a promising strategy when used in combination with immunotherapies targeting inhibitory receptors such as anti-PD-1 and anti-PD-L1. It would be an interesting strategy for tumours benefiting from these treatments, in advanced or localised settings. Following biological rationale and preclinical data, OX40-targeted drugs should be administered as sequential treatment followed by anti-PD-1 or anti-PD-L1. It is important to test combination approaches, evaluating timing and sequential strategies. More studies should be performed in order to find some predictors of response, better comprehension of resistance mechanism and immunological dynamics in order to trigger immune activation and enhance clinical activity of OX40-targeted drugs against tumours, especially in combination strategies.

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